IDENTIFICATION AND PURIFICATION OF A BACTERIOCIN SECRETED BY LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL THAI FERMENTED FOOD

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Thesis
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IDENTIFICATION AND PURIFICATION OF A BACTERIOCIN SECRETED BY LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL THAI FERMENTED FOOD

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ABSTRACT

Bacteriocin-producing lactic acid bacteria and purified bacteriocins have the potential for use as bio-preservatives to extend shelf-life or to enhance the safety of foods. Study of bacteriocin–producing strains may also be useful to improve microbial safety in fermented food products. A total of 35,717 colonies were isolated from 30 Thai fermented food products and screened for bacteriocin production using direct plating method. Twenty four bacteriocin-producing strains (WNK1-24) were isolated from fermented meat, fish, fruit and vegetable samples. By using PCR identification, we found that 4 strains produced nisin (WNK4, 21, 23 and 24) and 3 strains (WNK8, 9 and 13) produced Plantaricin W (plw). All other isolates produced unknown bacteriocins. Strain WNK19 isolated from Nham was chosen for further study. This bacteriocin inhibited the growth of food-borne pathogenic bacteria including *Listeria monocytogenes, Enterococcus faecali* and a number of lactic acid bacteria strains; *Lactobacillus sakei*, *Lactobacillus plantarum, Lueconostoc mesenteriodes* and *Pediococcus pentosaceus*. It was heat stable at 120°C for 15 min. This bacteriocin of strain WNK19 was purified to homogeneity by a 3-step purification method: hydrophobic chromatography, cation exchange chromatography and reverse phase high performance liquid chromatography. Purified bacteriocin of strain WNK19 had a molecular mass of 4625.485 Da as determined by matrix assisted laser desorption /ionization- time of flight mass spectrometry. The molecular weight of WNK19 bacteriocin was identical to the molecular weight of Pediocin PA-1 bacteriocin. WNK19 bacteriocin was confirmed to be Pediocin PA-1 by PCR amplification using specific primers from the structural gene of Pediocin PA-1. The amplified fragment of 369 base pairs was sequenced and shown to be 100% identical to the sequence of Pediocin PA-1 structural gene.

KEY WORDS: BACTERICIN / LACTIC ACID BACTERIA / PEDIOCIN / FERMENTED FOOD

99 pp.

การจัดจำแนกและทำให้บริสุทธิ์แบคเทอริโอซินจากแบคทีเรียกลุ่มแลคติกแยกจากผลิตภัณฑ์อาหารหมักพื้นบ้านไทย
(IDENTIFICATION AND PURIFICATION OF A BACTERIOCIN SECRETED BY LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL THAI FERMENTED FOOD)

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บทคัดย่อ

แบคทีเรียกรดแลคติกที่ผลิตแบคเทอริโอซินและแบคเทอริโอซินบริสุทธิ์ สามารถช่วยเพิ่มคุณค่าของอาหารหมักในด้านความปลอดภัยและอายุการเก็บรักษาได้ ดังนั้นการศึกษาเชื้อที่มีคุณสมบัติในการสร้างแบคเทอริโอซินจึงมีประโยชน์ในกระบวนการผลิตอาหารหมัก ทดสอบตัวอย่างการผลิตแบคเทอริโอซินในแบคทีเรีย 35,717 โคโลนี จากอาหารหมัก 30 ตัวอย่าง โดยวิธี Direct Plating พบแบคทีเรียแลคติกจำนวน 24 เชื้อ(WNK 1-24) จากอาหารหมักประเภทผักผลไม้และเนื้อสัตว์ สามารถผลิตแบคเทอริโอซิน จากการตรวจสอบด้วยวิธี PCR พบว่า 4 เชื้อสามารถผลิตสาร Nisin (WNK4, 21, 23 และ24), 3 เชื้อสามารถผลิตสาร Plantaricin W (WNK8, 9 และ3) และอีก 17 เชื้อไม่ทราบว่าผลิตแบคเทอริโอซินชนิดใด จากกลุ่มเชื้อนี้ได้เลือกเชื้อ WNK19 จากแหนมมาศึกษาต่อ เนื่องจากผลิตแบคเทอริโอซินที่ยับยั้งเชื้อแบคทีเรียสายพันธุ์ก่อโรคได้แก่ *Listeria monocytogenes, Enterococcus faecalis* และสายพันธุ์ใกล้เคียงได้แก่ *Lactobacillus sakei*, *Lactobacillus plantarum, Lueconostoc mesenteriodes,*และ *Pediococcus pentosaceus* และสามารถทนความร้อนที่ 121°C นาน 15 นาที จากการแยกแบคเทอริโอซินนี้ให้บริสุทธิ์โดยใช้ 3 ขั้นตอนคือ hydrophobic chromatography; cation exchange chromatography และ reverse phase chromatography พบว่ามวลของแบคเทอริโอซินบริสุทธิ์ที่แยกได้มีขนาดเท่ากับ 4625.485 Da ซึ่งตรวจสอบมวลด้วยเทคนิค MALDI–TOF พบว่ามีน้ำหนักมวลเหมือนกับ Pediocin PA-1 แบคเทอริโอซิน. ตรวจสอบการสร้าง pediocin PA-1ของแบคทีเรียสายพันธุ์ WNK19 โดยวิธีพีซีอาร์ใช้ไพรเมอร์ที่จำเพาะต่อยีนโครงสร้างของ pediocin PA-1 ได้ PCR Product ขนาด 369 bp จากการตรวจสอบลำดับเบสของ PCR Product พบยีนที่สร้างสายเปปไทด์ต้นแบบของแบคเทอริโอซิน Pediocin PA-1 ใน แบคทีเรียสายพันธ์ WNK19

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LIST OF ABBREVIATIONS

Abbreviations used throughout this thesis are listed below.

AU	=	Arbitory unit
bp	=	basepair(s)
°C	=	degree Celsius
cm	=	centimeter(s)
EDTA	=	ethylene diamine tetraacetic acid
Da	=	Dalton
HPLC	=	High Performance Liquid Chromatography
g	=	gram
kb	=	kilo base pair
kDa	=	kilo Dalton
M	=	molar
mA	=	milliampere
MALDI-TOF	=	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
min	=	minute
mg/ml	=	milligram per millilitre
ml	=	millilitre
mM	=	millimolar
MS	=	Mass Spectrometry
PCR	=	polymerase chain reaction
Plw	=	Plantaricin W
rpm	=	round per minute
SDS	=	sodium dodecyl sulphate
SDS-PAGE	=	sodium dodecyl sulphate polyacylamide gel electrophoresis
TEMED	=	*N,N,N',N'*-tetramethylethylethylenediamine
Tris	=	Tris-(hydroxymethyl)-methylamine
Tween20	=	Polyoxyethylene-sorbitan monolaurate

LIST OF ABBREVIATIONS (continued)

U	=	unit
V	=	volt
vol.	=	volume
v/v	=	volume by volume
w/v	=	weight by volume
µg	=	microgram
µg/ µl	=	microgram per microlitre
µl	=	microlitre
µM	=	micromolar
%	=	percent

CHAPTER I
INTRODUCTION

1.1 Bacteriocin

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by the bacterium. They are active against other bacteria, either closely related species (narrow spectrum of antimicrobial activity), or non related strains (broad spectrum of antimicrobial activity). They act as with host defence peptides and are also involved in cell-cell communication. The producers are immune to their own bacteriocin(s) by specific immunity proteins. Between 30–99% of the eubacteria and archaea bacteria make at least one bacteriocin (1-3). Many bacteriocins are produced by food-grade lactic acid bacteria (LAB), and offer the possibility of manipulating food microbial ecosystems in a deliberate fashion - for example, by using bacteriocins to protect food against contamination with, or prevent the growth of pathogenic bacteria. Importantly, lactic acid bacteria are widely used and play an important role in food and feed fermentation and preservation via acid production and other antimicrobial substances. Bacteriocin antimicrobial substances produced by LAB are of special interest to improve quality and hygiene of fermented food products. We are convinced that bacteriocins can be exploited in food in various imaginative and commercially important applications.

1.2 The lactic acid bacteria (LAB)

LAB comprise of Gram positive, low-GC, acid tolerant, non-spore forming, non-motile, rod or cocci in shape catalase-lacking organisms. They can be characterized by their common metabolic pathway and physiological characteristics. These bacteria, usually found in fermented products (milk, meat, beverages, vegetable) produce lactic acid as the major metabolic end product of carbohydrate fermentation (4). There are two main hexose fermentation pathways that are used to classify lactic

aid bacteria. Homolactic lactic acid bacteria can metabolize glucose in the glycolysis (Embden-Meyerhof), to yield pyruvate and lactic acid. Heterofermentative LAB use 6-phosphogluconate/phosphoketolase pathway. The major end products are lactate as in homofermentation and ethanol, acetate and CO_2 in heterofermentation. The genera that comprise LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus, Lactococcus,* and *Streptococcus* as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Teragenococcus, Vagococcus,* and *Weisella*; these belong to the order Lactobacillales. The current genera included in the lactic acid bacteria and their common ecological sources are listed in Table 1 (5).

LAB are found in many ecological niches, including certain foods, in the mouth, and in the gastrointestinal and urogenital tracts of humans and animals. They also are widespread in plants and dominate the fermentation microflora of ensiled cereal and forage crops. The *Streptococci*, except *Streptococcus thermophilus*, are human and animal pathogens, and *Enterococcus* species are enteric lactic acid LAB that often are implicated as opportunistic human pathogens. LAB that are commonly found in food and animal feeds are generally regarded as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human (6, 7).

LAB are renowned for their preservative effect in foods. This mainly is attributed to reduced pH and production of acetic and lactic acids, as acidification inhibits the growth of spoilage organism, and lactic acid and other metabolic products contribute to the organoleptic and textural profile of food. But other inhibitory substances (hydrogen peroxide, diacethyl (8) and bacteriocins) can be involved. Preservation of foods in a sound and safe condition has long been, and remain, an on-going challenge for humans (9).

Table 1 Origins and associations of lactic acid Bacteria (5).

Genus	Origin and common association
Aerococcus	
Carnobacterium	Derived from nonaciduric lactobacilli that grow in meats
Enterococcus	Previously group D Streptococci (enterococci). Intestinal organism; opportunistic pathogen; possible probiotic
Lactobacillus	Widespread intestinal and extraintestinal organism; food fermentations
Lactococcus	Previously group N (lactic) streptococci; starter cultures for most cheeses
Leuconostoc	Meat preservation and spoilage; sugar spoilage
Oenococcus	Malolactic fermentation of wine
Pediococcus	Tradition genus involved in meat fermentation, beer spoilage
Streptococcus	Mainly pathogens, except *S. thermophilus* that is used in cheese and yogurt making
Tetragenoccus	Previously *Pediococcus*; used in production of soy sauce
Vagococcus	Motile “steptococci”
Weissella	New genus including species of *Lactobacillus* and *Leuconostoc*. Associated with meats and and other foods

1.3 Role of lactic acid bacteria in fermentation

Fermentation is defined as an energy yielding process whereby organic molecules serve as both electron donors and electron acceptors. The molecule being metabolized does not have all its potential energy extracted from it. In other words, it is not completely oxidized. Hence lactic acid bacteria are widely used as a low cost method for food preservation by fermentation and generally no/or little heat is required during the fermentation.

LAB play an essential role in food fermentations given that a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat and vegetable products. Their growth lowers both the carbohydrate content of the foods that they ferment and the pH due to lactic acid production. It is this acidification process which is one of the most desirable side-effects of their growth. The pH may drop to as low as 4.0, low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolonged shelf life. This is due to competition for nutrients and the presence of inhibitors produced by the bacteriocins and the change in the organoleptic properties of the foods. A benefit of the fermentation process is preservation of the foods for later consumption. Many lactic acid bacteria produce bacteriocins which have inhibitory action against other microorganisms. However, the spectrums of antimicrobial activity of most bacteriocins are rather narrow and they may not inhibit all the variety of microorganisms found in foods. Cells of some lactic acid bacteria produce inhibitory levels of hydrogen peroxide at low temperature without producing acid. Opportunities exist for the addition of cells of these bacteria to foods thus exerting inhibitory action toward other microorganisms. Fermentation is conducted at around 45-50°C. This high temperature has the advantage of effectively preventing infection of the medium with unwanted micro-organisms (10, 11)

Lactic acid bacteria in particular produce a wide variety of antimicrobial substances including organic acids (lactic, acetic), hydrogen peroxide, diacetyl, and bacteriocins, which have potential to inhibit a variety of other microorganisms. The extensive interest in bacteriocins of lactic acid bacteria and the potential commercial value of these antimicrobials in food preservation and other biological applications have been well documented (12). LAB have been recognized as a potential source of

biopreservative for foods. The bacteriocin nisin has a long history of food use in at least 48 different countries and was awarded FDA approval in 1988 (U.S. Government Federal Register). More recently, biomedical applications have been proposed which include its potential to treat mastitis infections in cows, and replacement of pathogenic *Streptococcus* in mouth of humans (13). In general, the potential applications of specific bacteriocins can be predicted by their properties, having, host range, pH and heat stability.

1.4 Bacteriocins of lactic acid bacteria.

A large number of bacteriocins produced by LAB have been identified and characterized. LAB-bacteriocins comprise a heterogeneous group of physicochemically diverse ribosomally-synthesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria. Bacteriocins are divided into four classes (13): the lantibiotics (Class I), low molecular weight heat-stable posttranslationally unmodified modified peptides that contain unusual amino acids such as lanthionine and subdivided into type A linear peptides and type B globular peptides bacteriocin; antilisterial group (Class II), further subdivided into pediocin-like bacteriocins (subclass IIa), two-peptide bacteriocins (subclass IIb), and thiol-inactivated peptide bacteriocins (subclass IIc); and large (>30 kDa) heat-labile non-lantibiotics (Class III) (Table 2). Most bacteriocins characterized to date belong to class I and Class II bacteriocins (14-16). This is summarized in Table 2.

1.4.1 Classification of LAB bacteriocin.

1.4.1.1 Class I bacteriocins: Lantibiotics

The lantibiotic bacteriocins (lanthionine-containing antibiotics) are a large family of bacteriocins. This group contains small peptides of between 9-37 amino acid residues (molecular mass of about 1959 to 4635 Da).

Table 2 Classification of LAB bacteriocins (13).

Main Category	Characteristics	Subcategory	Examples
Class I	Lantibiotics Ribosomally produced peptides that undergo extensive post-translational modification, small peptides containing lanthionine and β-methyl lanthionine.	Type A (elongated molecules, one or two peptide lantibitics) Type B (globular molecules)	Nisin A Plantaricin W Lacticin 3147 Mersacidin
Class II	Nonmodified heat-stable bacteriocins. Ribosomally synthesized as inactive prepeptides that get activated by post-translational cleavage of the N-terminal leader peptide	Subclass IIa (antilisterial pediocin like bacteriocins) peptides that contain YGNGV amino acid motif near their N termini Subclass IIb (two peptide bacteriocins) Subclass IIc (Thiol-activated require reduced cystein for activity)	Pediocin PA1, Leucocin A Enterocin P Plantaricin EF Plantaricin JK
Class III	Protein bacteriocins with molecular masses of <30 kDa		Helveticin J Millericin B
Class IV	Complex bacteriocin carrying lipid or carbohydrate moieties		

Their precursors are biosynthesized ribosomally, and usually containing unusual amino acids, such as lanthionines, present in their structures that result form post-translational modifications and proteolytic processing of the gene-encoded precursor peptides (Figure 1). In the peptides chains, serines, theronines or cysteins residues are modified to from lanthionine or β-methyllanthionine residues (17-19). These unusual residues form covalent bridges between amino acids, which result in internal 'rings' and gives lantibiotics their characteristic structural feature. The biological significance of lanthionine and D-alanine residues has been proposed that they contribute to enhanced stability under extreme temperature and oxidizing conditions, to increased tolerance to acids, to resistance to proteolytic enzymes and to antimicrobial activity (20, 21).

The lantibiotics can be divided on the basis of their structure and mode of action into two groups, type A and B lantibiotics, which comprise peptides with straight-chain and globular structures respectively. In general, type A, straight-chain or the elongated amphiphilic cationic lantibiotics (for example, nisin, one peptide lantibiotic and plantaricin W (plw), two peptide lantibiotic bacteriocin) are active through the pore formation of target cells , leading to dissipation of membrane potential and efflux of small metabolites from sensitive cells. In type B, the globular lantibiotics (for example, mersacidin) were originally defined as those lantibiotics that act through enzyme inhibition of cell wall biosynthesis of a sensitive cell. However, it has now been established that nisin possesses both mechanisms of action. Although a precise mechanism of action has not been elucidated for every lantibiotic, it is apparent that a docking molecule or target is involved, lipid II, which is a membrane-anchored cell-wall precursor that is essential for cell-wall biosynthesis (17).

The binding of nisin to lipid II facilitates a dual mechanism of action involving the prevention of peptidoglycan synthesis and pore formation. Nisin is the most well known and studied lantibiotic bacteriocin because of its potent bactericidal activity and nisin has been used as a natural preservative in the food product in more than 48 countries (6). It produced by some strains of *Lactococcus lactis*, and has 34 amino acids in length and has an overall positive charge with a molecular mass of 3353 Da. Nisin seems to have a modular structure in that several N-terminally located residues,

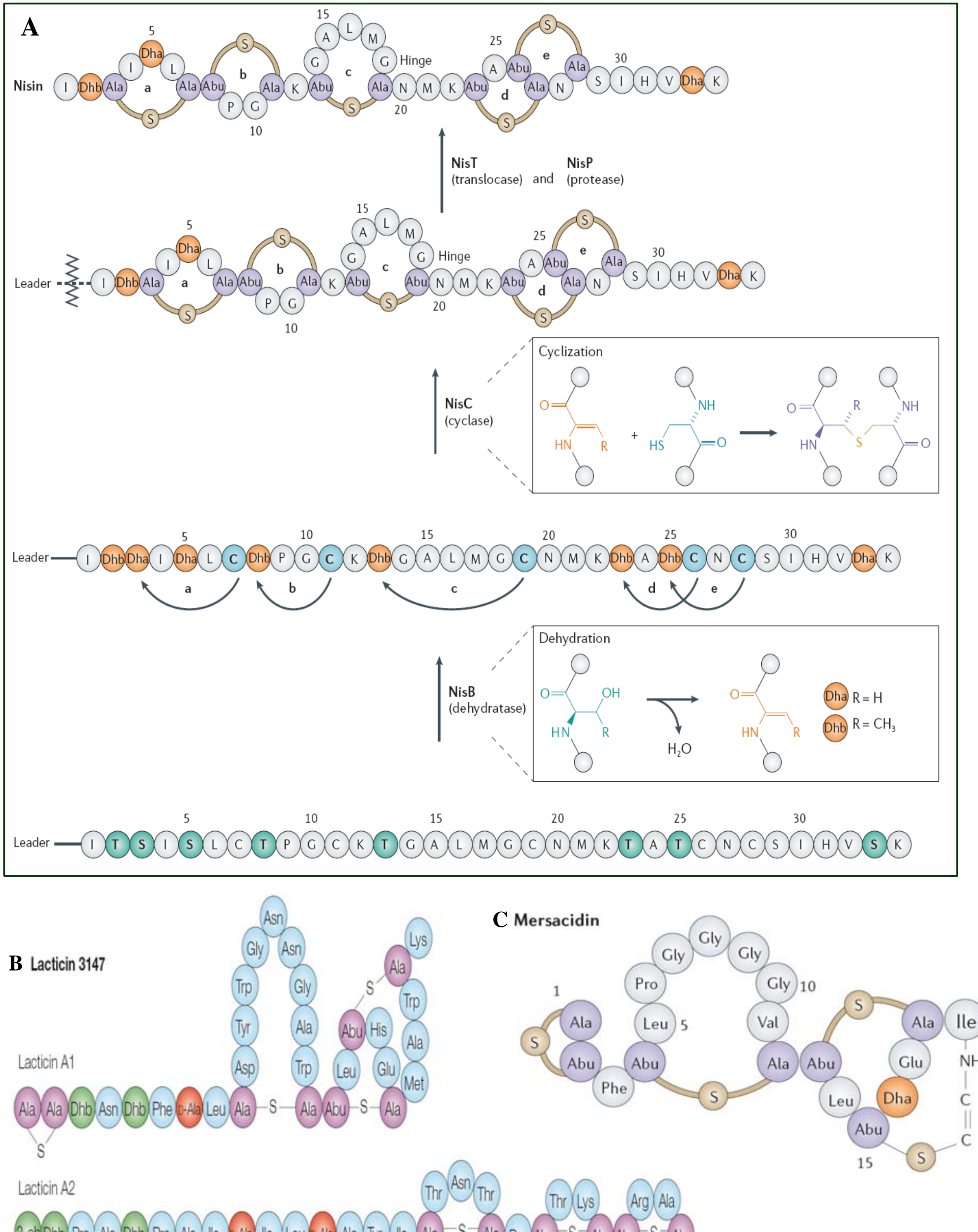

Figure 1 Post-translational modification in nisin (A). The mature peptide containing 5 lantionine rings. Two peptides class I lantibiotics: lacticin 3147(B). the globular molecular class I lantibiotics : Mersacidin(C). Adapted from Cotter PD,Hill C and Ross RP 2005 and Breukink E and de Kruijff B 2006 (3,17).

which are also present in other nisin-like, epidermin-like and streptin-like lantibiotics, have been shown. Like all other lantibiotics, nisin is produced in an inactive precursor form containing a leader peptide that directs it to the modification and transport machinery (Table 3) (15). The modifications are performed in two steps. First, dehydration of the serines and threonines by NisB produces didehydroalanines (Dha) and didehydrobutyrines (Dhb) respectively. Next, NisC catalyses the cyclization of the didehydro amino acids with cysteine residues that are located downstream (towards the carboxy terminus), which forms the lanthionine rings (Ala-S-Ala = lanthionine; Abu-S-Ala = methyl-lanthionine). During the dehydration and cyclization steps the stereochemistry of the serine (or threonine) residue is changed from the L- to the D-stereoisomer. Only when the modifications are complete there peptide exported (by NisT) from the cell and the precursor peptide cleaved (by NisP), rendering nisin with five lanthionine rings (17).

In this group of lantibiotic bacteriocins, a two-peptide system is required for full activity. These peptides are highly modified and contain many cysteine, serine, and threonine residues, which are required for the formation of lanthionine bridges. They are also ribosomally produced as prepeptides which are enzymatically modified followed by removal of the leader to generate the active species. Two-peptide lantibiotics that have been reported are cytolysin, lacticin 3147, plantaricinW and staphylococcin C55 (13).

Most of the class I bacteriocins have a very broad inhibitory spectrum. They not only inhibit closely related bacteria, such as species from the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus,* but also inhibit many less closely related gram-positive bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus, Bacillus cereus,* and *Clostridium botulinum.* Several bacteriocins in this class, such as nisin, prevent outgrowth of spores of *B.cereus* and *C. botulinum* (Table 4) (22). These properties of broad spectrum and heat stability have allowed nisin to be used as biopreservative in many fermented products.

Table 3. Amino acid sequence of some LAB pre-probacteriocins(15).

LAB-preprobacteriocins	N-terminal Extension[a] (−30 −20 −10 −1)	Probacteriocin[b] (+1 +10 +20 +30 +40 +50)
Class I (lantibiotics)		↓
Nisin A	MSTKDFNLDLVSVSKKDSGAS P R	I TSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Nisin Z	MSTKDFNLDLVSVSKKDSGAS P R	I TSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Salivaricin A	MKNSKDILNNAIEEVSEKELMEVA G G	K RGSGWIATITDDCPNSVFVCC
Lacticin 481	MKEQNSFNLLQEVTESELDLIL G A	K GGSGVIHTISHECNMNSWQFVFTCCS
Class II		
Pediocin PA-1	MKKIEKLTEKEMANII G G	K YYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC
Leucocin A-UAL187	MMNMKPTESYEQLDNSALEQVV G G	K YYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
Mesentericin Y105	MTNMKSVEAYQQLDNQNLKKVV G G	K YYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW
Sakacin A	MNNVKELSMTELQTIT G G	A RSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM
Sakacin P	MEKFIELSLKEVTAIT G G	K YYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK
Acidocin A	MISMISSHQKTLTDKELALIS G G	K TYYGTNGVHCTKKSLWGKVRLKNVIPGTLCRKQSLPIKQDLKILLGWATGAFGKTFH
Carnobacteriocin BM1	MKSVKELNKKEMQQIN G G	A ISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH
Carnobacteriocin B2	MNSVKELNVKEMKQLH G G	V NYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
Enterocin A	MKHLKILSIKETQLIY G G	T THSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC
Lactococcin Gα	MKELSEKELRECV G G	G TWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH
Lactococcin Gβ	MKNNNNFFKGMEIIEDQELVSIT G G	K KWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI
Lactococcin M	MKNQLNFEILSDEELQGIN G G	I RGTGKGLAAAMVSGAAMGGAIGAFGGPVGAIMGAWGGAVGGAMKYSI
Lactococcin N	MKKDEANTFKEYSSSFAIVTDEELENIN G S	G SIWGAIAGGAVKGAIAASWTGNPVGIGMSALGGAVLGGVTYARPVH
Lactococcin A	MKNQLNFNIVSDEELSEAN G G	K LTFIQSTAAGDLYYNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGLHH
Carnobacteriocin A	MNNVKELSIKEMQQVT G G	D QMSDGVNYGKGSSLSKGGAKCGLGIVGGLATIPSGPLGWLAGAAGVINSCMK
Enterocin B	MQNVKELSTKEMKQII G G	E NDHRMPNELNRPNNLSKGGAKCGAAIAGGLFGIPKGPLAWAAGLANVYSKCN
Lactococcin B	MKNQLNFNIVSDEELAEVN G G	S LQYVMSAGPYTWYKDTRTGKTICKQTIDTASYTFGVMAEGWGKTFH
Enterocin P[c]	MRKKLFSLALIGIFGLVVTNFGTK VDA	A TRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
Bacteriocin 31[c]	MKKKLVICGIIGIGFTALGTN VEA	A TYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR
Divergicin A[c]	MKKQILKGLVIVVCLSGATFFSTPQQ ASA	A APKITQKQKNCVNGQLGGMLAGALGGPGGVVLGGIGGAIAGGCFN
Acidocin B[c]	MVTVKYGRNLGLSKVELFAIWAVLVVALLL ATA	N IYWIADQFGIHLATGTARKLLDAVASGASLGTAFAAILGVTLPAWALAAAGALGATAA

[a]Vertical arrows indicate the processing sites of the preprobacteriocins.

[b]The pediocin-like consensus sequence and the cysteine residues are underlined. Numbers indicate the position of the amino acid residue respect to the processing site.

[c]*Sec*-dependent bacteriocins.

Table 4 Activity spectra of some Class I and Class II bacteriocins (22)

Bacteriocins	Strain	Activity spectra
Class I lactocin S	*Lactobacillus sake* L45	Active against different species of *Enterococcus* (1/1), *Lactobacillus* (8/8), *Lactococcus* (1/3), *Leuconostoc*(1/1), *Pediococcus* (3/3), *L.monocytogenes* (5/5), *L. innocua* (1/1), *Staphylococcus* (6/6), *Bacillus cereus* (1/1), and *Clostridium spp.* (5/5).
nisin	*Lactococcus lactis* subsp. lactis	Active against different species of *Enterococcus* (2/2), *Lactobacillus* (9/9), *Lactococcus* (5/5), *Leuconostoc* (1/1), *Pediococcus* (4/4), *L.monocytogenes* (14/14), *L. innocua* (2/2), *Listeria grayi* (1/1), *Listeria ivanovii* (1/1), *Listeria murrayi* (1/1), *Listeria seeligeri* (1/1), *Listeria welchimeri* (1/1), and *Staphylococcus spp.* (7/7). Prevents outgrowth of *Bacillus spp.* and *Clostridium spp.* and bactericidal to their vegetative cells.
Class II enterocin A	*Enterococcus faecium* CTC492	Active against different species of *Enterococcus* (4/4), *Lactobacillus* (2/2), *Pediococcus* (2/2), *L. monocytogenes* (4/4), and *L. innocua* (2/2)
sakacin P	*Lactobacillus sake* LB674	Active against different species of *Enterococcus* (7/8), *Lactobacillus* (3/7), *Pediococcus* (2/4), *L.monocytogenes* (5/5), *L. innocua* (3/3), *and L. ivanovi (*1/1). Not active against *Lactococcus* (0/1) and *Leuconostoc* (0/3).
pediocin PA-1	*Pediococcus acidilactici* PAC 1.0	Active against different species of *Carnobacterium* (3/3), *Enterococcus* (2/3), *Lactobacillus* (23/31), *Lactococcus* (1/14), *Leuconostoc* (3/4), *Pediococcus* (8/11), *L.monocytogenes* (12/12), *L.innocua* (2/2), *L.ivanovii* (1/1), *Staphylococcus spp.* (2/6) and *Clostridium spp.*(4/17).

*the numbers inside the bracket represent number of strains inhibited/number of strains tested.

1.4.1.2 Class II bacteriocins: non-lanthionine

Class II bacteriocins are the most abundant and thoroughly studied bacteriocins. They have a common feature in that they are non-lanthionine containing small (<10 kDa) heat-stable peptides. They are not subject to extensive post-translational modification with the exception of cysteine residues establishing disulfide bridges and oxidized-methionines (23). They are ribosomally synthesized as inactive prepeptides that are cleaved at the N-terminal leader peptide, generally at a double glycine (-2, -1) residue, to release mature peptides (Table 3). The mature bacteriocins are generally cationic, amphiphilic, relatively hydrophobic peptides with little or no cysteine present. The environments in which such peptides are found can strongly dictate their secondary structures. In cases that have been examined, cationic peptides from various sources usually exist as random coils under aqueous conditions, but can self-associate under certain conditions. These nonlantibiotic bacteriocins are further subdivided into the 3 subgroups (24, 25). Examples of class II bacteriocins are listed in Table 3.

Subclass IIa bacteriocins from the largest subgroup that is active to *Listeria*. These peptides are characterized by the presence of conserved motif, the YGNGV box, which is usually located within the N-terminus of the mature peptide.

Subclass IIb bacteriocins are two-peptide bacteriocins that require both peptides for optimum inhibitory activity. The two structural genes of the two active peptides are in the same operon.

Subclass IIc bacteriocins are referred as thiol-activated peptides, which require a reduced cysteine for activity.

These class II, compared to class I bacteriocins, have comparatively narrow activity spectra bacteriocin and only inhibit closely related Gram-positive bacteria. In general, members of the genera *Listeria, Enterococcus, Lactobacillus,* and *Pediococcus* are sensitive to class II bacteriocin. Pediocin PA-1, class IIa bacteriocin, has a fairly broad inhibitory spectra strongly active against *Listeria and* can inhibit

some less closely related gram-positive bacteria, such as *Staphylococcus aureus.* (Table 4) (22).

1.4.1.3 Class III bacteriocins: (Large protein bacteriocins)

The high molecular weight bacteriocins with molecular masses of 30 kDa have been identified within the genus *Lactobacillus* and, *Enterococcus*. These bacteriocins, in contrast to Class I and II bacteriocins, are inactivated upon heat treatment (e.g., 60–100 ºC for 10–15 min). They have a domain-type structure, in which different domains have functions for receptor binding and bacteriocidal activity. Only four LAB bacteriolysins have been genetically characterized so far. Their mechanism of action is distinct from that of bacteriocins as they function through the lysis of sensitive cells by catalyzing cell-wall hydrolysis (Figure 2). These proteins are modular in structure and have a catalytic domain at the N-terminus that shows homology to endopeptidases, and a C-terminus that probably represents the target recognition site (26, 27).

1.4.2 Mode of action

Most bacteriocins show a bacteriocidal mode of action against sensitive microorganisms (Figure 2). Bacteriocidal activity of bacteriocins may be accompanied by lysis of sensitive cells (bacteriolytic bacteriocins) (28), as is the case for nisin A. Binding of these bacteriocins to the cell wall components such as lipid II of sensitive bacteria, creates pores in the membrane and leads to release of intracellular content (H^+, Mg^{2+}, NaCl, amino acid and other small molecule in cytosol) (17, 18). Although the formation of pore is a general feature, the size, stability and conductivity of these pores differs considerably from bacteriocin to bacteriocin. Bacteriocins exert their bactericidal mode of action by destabilization and permeabilization of sensitive cell membranes. The primary mechanism of action of bacteriocins consists of pore formation complexes or ionic channels in the cytoplasmic membrane of sensitive cells. Breukink E and de Kruijff B (17) has recently shown that nisin uses lipid II as a 'docking molecule' to form pores in a sensitive cell. In this mechanism, which requires micromolar concentrations of nisin

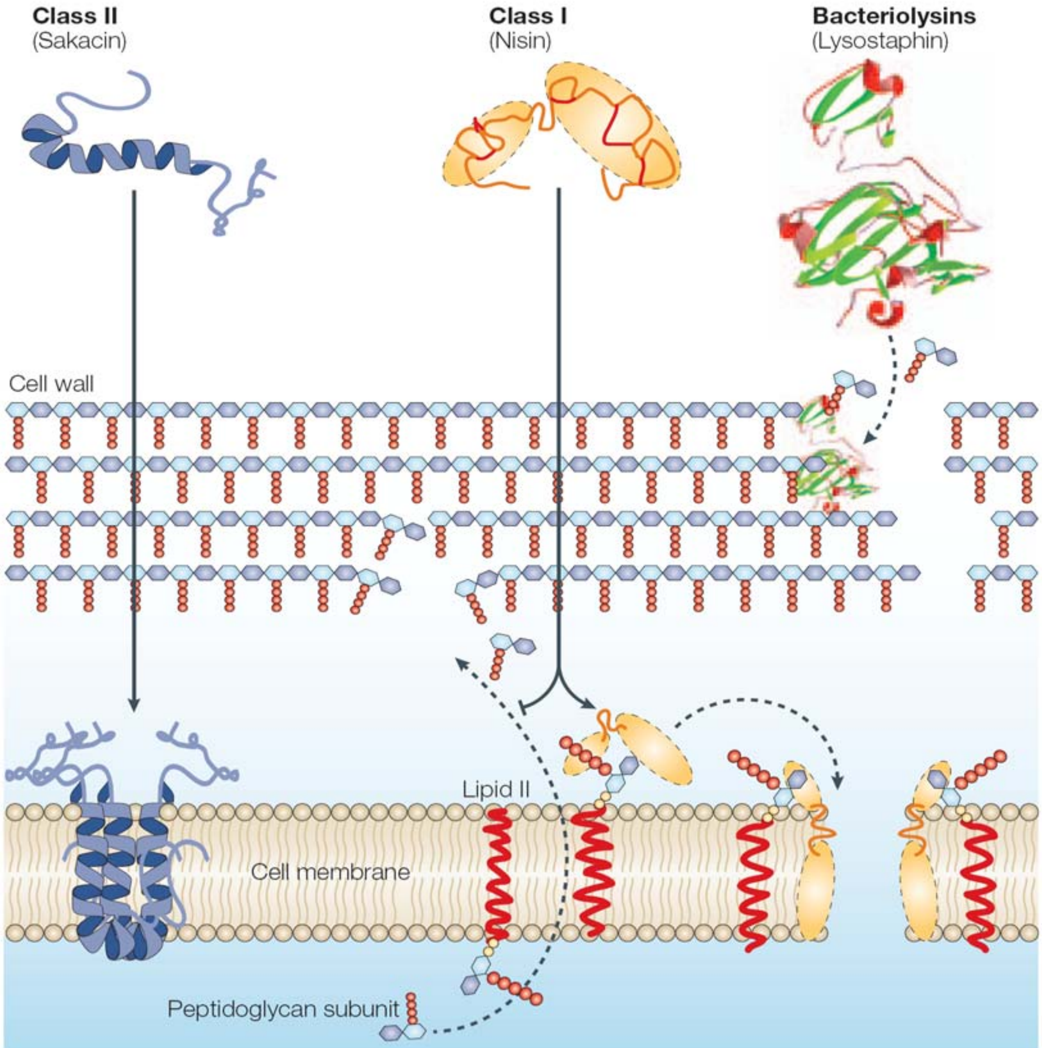

Figure 2 Mode of action of lactic acid bacteria's bacteriocins.

Lactic acid bacteria (LAB) bacteriocins can be grouped on the basis of structure, but also on the basis of mode of action. Some members of the class I (or lantibiotic) bacteriocins, such as nisin, have been shown to have a dual mode of action. They can bind to lipid II, and prevent correct cell wall synthesis, leading to cell death. Nisin use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation. The class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation. Large bacteriolytic proteins (class III bacteriocins), such as lysostaphin, can function directly on the cell wall of Gram-positive targets, leading to death and lysis of the target cell (3).

and the presence of anionic lipids in the target membrane of sensitive cell, nisin binds to the anionic lipids, and then inserts between the phospholipids head groups. The accumulation of nisin in the outer lipid monolayer of the target membrane drives aggregation of nisin monomers, which is followed by formation of short-lived pore-like structures.

Relaxation of the pore-like structure can lead to translocation of the peptides across the lipid bilayer. However, studies on the effect of pediocin PA-1 on liposomes from *Listeria monocytogenes* and sensitive cells of this bacterium show pediocin binding to cytoplasmic membrane, insertion of bacteriocin molecules in the membranes, and formation of the poration complex. This process finally leads to cell death that may occur with or without cell lysis, probably depending on concomitant activation of the cell autolysins. Initially, pediocin-sensitive cells adsorb the bacteriocin and the specificity of pediocin PA-1 is at least not enterely dependent on the presence of specific receptor sites on sensitive cells, and that pediocin may have a high surface binding capacity responsible for the lethal binding to sensitive bacteria. Bhunia *et al* (29) reported that the bacteriocin does not bind to the cell surface of Gram-negative bacteria. Although the presence of lipoteichoic acid in the cell wall of Gram-positive bacteria and its absence in Gram-negative bacteria could explain this differential adsorption ability, it does not provide any explanation for the existance of sensitive and insensitive Gram-positive bacteria. These authors also described how pediocin PA-1 treated sensitive cells lost intracellular K^+ ions. The proton motive force (PMF) constitutes a key parameter in the understanding of the mode of action of LAB bacteriocins because it is required for many of the cells energy-dependent metabolic processes. They hypothesized that PMF collapse leads to growth inhibition and death because of the low intracellular ATP level and the inability to carry out active transport of nutrients and to maintain sufficient concentrations of cofactors, such as K^+ and Mg^{2+}.

In recently, an alternative mechanism of mode of action from lantibiotics bacteriocin has been proposed. The binding of lantibiotics to lipid II disrupts cell wall formation during cell division and bacteria are killed. The death of cells might be due to lysis of the cell. The fact that bacteriocin peptides are short in length and cannot

span a bacterial bilayer to form pores, and thus the pore formation mechanism may be incorrect. Cell lysis may be the mode of bactericidal activity (30).

Most LAB-bacteriocins are antimicrobial peptides ribosomally synthesized as biologically inactive precursors or prepropeptides (preprobacteriocins) containing a C-terminal propeptidic domain (probacteriocin) and an N-terminal extension playing an important role in recognition and processing of the preprobacteriocins and in determining the mechanism involved in the transport of the corresponding probacteriocin to the extracellular medium. In addition another important feature of the N-terminal extension is that it maintains intracellularly located bacteriocins biologically inactive and, therefore, protects the bacteriocin producer against the toxic effects of these peptides while inside the cell. And bacteriocin producer can protect itself by specific mechanisms involving immunity proteins.

1.4.3 Producer self-protection (immunity)

Bacteriocin producing LAB exhibit a specific mechanism (immunity) which offers self-protection against the toxicity of their own bacteriocins. This mechanism of producer cell self-protection is important, but is poorly understood. This mechanism mainly depends on a bacteriocin-specific immunity protein. In addition, the immunity may be mediated by intracellular proteases that inactivate bacteriocins in the producer cells. The putative immunity proteins identified so far are fairly small peptides, which usually consist of 51 to 154 amino acid residues, have high isoelectric point (pI) values (between 7 and 10) and contain putative transmembrane α-helices (15).

During the last few years, a number of putative immunity proteins have been identified and several models of mechanism of bacteriocin-producer self-protection have been proposed. One mechanism proposed is that immunity proteins interact with the bacteriocin molecules freely located in the extracellular medium, resulting in inhibiting their adsorption to the cell, subsequently pore formation (15); however, studies of lactococcin A (LciA) immunity proteins inhibiting lead to an abandonment of this hypothesis as immunity proteins do not act from outside the cells. Recently, there has been a report on another mechanism of this immunity protein, Lactococcin A (31). The immunity protein LciA binds directly to bacteriocin-receptor complex

resulting in blocking the subsequent steps leading to membrane permeabilization and cell death.

1.4.4 Application of bacteriocin

1.4.4.1 Food application.

Food processors face a major challenge in an environment in which consumers demand safe foods with a long shelf-life, but also express a preference for minimally processed products that do not contain chemical preservatives. Bacteriocins are an attractive option that could provide at least part of the solution. They are produced by food-grade organisms, are usually heat stable and can inhibit many of the pathogenic and spoilage organisms that cause problems in minimally processed foodstuffs. However, at present, only nisin and pediocin PA-1 have found widespread use in food. The form of nisin used most widely in food is Nisaplin (Danisco), which is a preparation that contains 2.5% nisin with NaCl (77.5%) and non-fat dried milk (12% protein and 6% carbohydrate). The use of pediocin PA1 for food biopreservation has also been commercially exploited in the form of ALTA 2431 (Quest), which is based on LAB fermentates generated from a pediocin PA1-producing strain of *Pediococcus acidilactici*. Its use is covered by several US and European patents. Bacteriocins have been shown to have potential in the biopreservation of meat, dairy products, canned food, fish, alcoholic beverages, salads, egg products, high-moisture bakery products, and fermented vegetables, either alone, in combination with other methods of preservation, or through their incorporation into packaging film/food surfaces. The use of bacteriocins in food application are shown in the Table 5 and 6 (7, 32-34).

1.4.4.2 Clinical application.

Many studyies of bacteriocins in food application have been reported but potential clinical application of bacteriocin also under investigation. In particular, the

Table 5 Use of bacteriocinogenic protective cultures to control Listeria monocytogenes in meat products (22)

Meat products	Protective culture
Meats	
Minced meat and comminuted cured raw pork filled into casings	*Lactobacillus sake* Lb 706
Vacuum packaged	
Minimally heat-treated beef cubes	*Lactobacillus bavaricus* MN
wieners	*Pediococcus acidilactici* JBL 1095
frankfurters	*Pediococcus acidilactici* JD1-23
Fermented	
dry fermented sausage	*Staphylococcus xylosus* DD-34, *Pediococcus acidilactici* PA-2, and *Lactobacillus bavaricus* MI-401
dry fermented sausages	*Lactobacillus sake* CTC494
chicken summer sausages	*Pediococcus acidilactici*
salami	*Lactobacillus plantarum* MCS
dry fermented sausage	*Pediococcus acidilactici* PAC 1.0
turkey summer sausage	*Pediococcus acidilactici* JBL 1095
Modified atmosphere packaged	
Brazilian sausage	*Lactobacillus sake* 2a

Table 6 Application of bacteriocin to enhance food safety (22).

Bacteriocins	Inactivation effects
In combination with heat	
Nisin	Nisin (1000 IU/g) enhances inactivation of Listeria monocytogenes in lobster by mild heat (60 or 65 °C).
Nisin	Nisin (500 to 2500 IU/ml) enhances inactivation of Salmonella Enteritidis by mild heat (55 °C by mild heat (55 °C)
In combination with antimicrobials	
Nisin	The combined use of potassium sorbate The combined use of potassium sorbate (0.3%) and nisin (400 IU/ml) nhibited the growth of L. monocytogenes.
Pediocin	pediocin PA-1 Synergistic effects between sodium diacetate (0.3 and 0.5%) and pediocin (5000 AU/ml) against L. onocytogenes.
In combination with other bacteriocins	
	pediocin PA-1When used with nisin, lacticin 481, or lactacin F, produced synergistic effects.

elucidation of the precise mechanism of action of some lantibiotics and their activity against multidrug-resistant pathogens by a novel mechanism makes them an attractive option as possible therapeutic agents. The broad-spectrum lantibiotics could theoretically be of use against any clinical Gram-positive human or animal pathogen (35-38). For example, the two-peptide lantibiotic lacticin 3147 has *in vitro* activity against *Staphylococcus ureus* (including methicillin-resistant *S. aureus*), *Enterococcus* , *Streptococci* (*S. pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Streptococcus mutans*), *Clostridium botulinum*, and *Propionibacterium acnes*. Initial *in vivo* trials with animal models have demonstrated the success of lantibiotics in treating infections caused by *S. pneumoniae*, and in preventing tooth decay and gingivitis. The use of nisin for human clinical applications has been licensed to Biosynexus Incorporated by Nutrition 21 and ImmuCell Corporation has licensed the use of the anti-mastitic nisin-containing product Mast Out to Pfizer Animal Health. A strain that produces the lantibiotic mutacin is entering Phase I clinical trials in the US with a view to replacement therapy, and the dietary supplement BLIS K12 throat guard, which contains a *Streptococcus salivarius* that produces two lantibiotics salivaricin A2 and B, is sold in New Zealand as an inhibitor of the bacteria responsible for bad breath.

1.5 The pediocins

Many LAB bacteriocins have been identified in the recently years but only nisin have been examined extensively in application studies in food systems. Considerable research has focused on the application of molecular technologies to study lactic acid bacteria, an economically significant group of organisms used to prepare and preserve foods. There have been reports describing *Pediococcus* that harbor plasmids, including plasmids encoding pediocins. Many *Pediococcus* spp. also produce pediocins that exhibit antilisterial activity. The efficacy of biopreservatives (i.e., pediocins and *pediococci*) for controlling *L. monocytogenes* has been demonstrated with fresh, processed, and fermented meats as well as refrigerated dairy-based products. *Pediococci* reside on plants, in plant products and can also be isolated from

humans. *Pediococci* are also a concern in the spoilage of alcohol beverage. But their occurrence in raw milk is not considered a problem. *Pediococci* have been isolated from raw sausages, rumen of cows and turkey feces. The bacteriocins of *Pediococcus* sp are of interest for use in food industry as a starter culture or as a food preservative in many kind of food products (16,22).

1.5.1 Genus *Pediococcus*

Pediococcus is a genus of Gram-positive lactic acid bacteria, placed within the family of Lactobacillaceae. They usually occur in pairs or tetrads, and are the only lactic acid cocci in shape that divide along two planes of symmetry. They are purely homofermentative. *Pediococcus* bacteria are usually considered contaminants of beer and wine although certain *Pediococcus* isolates produce diacetyl, which gives a buttery or butterscotch aroma to some wines (such as Chardonnay) and a few styles of beer. *Pediococcus* species are often used in silage inoculants too. *Pediococcus pentosaceous* and *Pediococcus acidilactici* grow equally well aerobically or anaerobically. The typical difference between these two species used as commercially meat starter cultures is that *P. acidilactici* can grow at 50 °C while *P. pentosaceus* cannot. In the laboratory this distinction is not always clear cut. Several species and strains of *Pediococci* that are used as starter cultures in fermentation of meat, sausage products, vegetables and cheddar cheese have been reported (6).

1.5.2 Bacteriocins in *Pediococci*

Some bacteriocins have been characterized from *Pediococcus acidilactici* and *Pediococcus pentosaceus* for effective use in foods. Pediocin AcH, Pediocin PA-1, Pediocin JD, Pediocin 347 and Mesentericin 5 produced by different strains of *Pediococcus acidilactici* were purified, and later demonstrated to be identical. Characteristics common to these bacteriocins are that their genetic determinants appear to be plasmid-borne and the bacteriocins are active against a broad spectrum of Gram-positive bacteria, many of which are associated with food spoilage and food related health hazards. The ability of these bacteriocins to inhibit many food borne pathogens, including *Listeria monocytogenes*, make them attractive as potential food preservation agents and also active against different species of *Carnobacterium,*

Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Staphylococcus* spp., *Bacillus cereus*, and *Clostridium* spp. Another strain of *Pediococcus* examined for bacteriocin activity is *P. acidilactici* PO2. This strain is used in the Wisconsin Process for bacon. In this process bacon is manufactured with 40 – 80 ppm sodium nitrate, *P. acidilactici* and 0.7 % sucrose. It has a shelf life and sensory characteristics similar to conventional bacon made with 120 ppm sodium nitrate. It is equally active for antibutulinal protection, while having markedly reduced levels of nitrosamines. Bacteriocin activity in the *P. acidilactici* PO2 is associated with plasmid of approximately 5.5 MDa and is active against *Listeria* as the typical class IIa bacteriocins. Pediocin A has been isolated from *Pediococcus pentosaceus* FBB61 originally isolated in 1953 from cucumber fermentation (39). The inhibitory activity of this strain was observed by pure culture fermentation of cucumbers and was further investigated by Daeschel MA and Klaenhammer TR (39, 40) linked the bacteriocin production and immunity phenotype to a 13.6 MDa plasmid. *P. pentosaceus* FBB61 is inhibitory to several strains of *L. monocytogenes* as other pediocin-like peptides and is also active against many species of *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Staphylococcus, Enterococcus, Listeria and Clostridium* (39, 40). The reported of pediocin ISK-1 has been characterized by Kimura *et al* (41) which is product of *Pediococcus* sp. ISK-1. The molecular weight of this bacteriocin is 2960 and it contains three lanthionine or-methyl lanthionine residues with broad spectrum of antimicrobial activity, Characteristics of a lantibiotic group such as nisin. This is the first report dealing with lantibiotic among *Pediococcus* to date.

1.5.3 Pediocins in food applications

The application of pediocin bacteriocins to foods is to provide protection against the growth of spoilage or pathogenic organisms. The combination of bacteriocins with other sub-lethal treatments has provided effective antimicrobial hurdles in food systems. For example, the combination of pediocin with sodium diacetate and sodium lactate had a greater inhibitory effect on the growth of *Listeria monocytogenes* on beef frankfurters stored at 4°C for 3 weeks than did pediocin alone. Moreover, a combination of ALTA 2341 (containing pediocin PA-1) and sodium

lactate extended the lag phase of *Listeria monocytogenes* on the surface of cooked chicken. Pediocin enhances the antilisterial activity in a meat model system. Pediocin PA-1 producing LAB is the most studied and used as starter cultures for many years. Pediocin PA-1 has been generated since 1992 and has greatly facilitated research on this bacteriocin. The studies involving application of pediocin PA-1 in food substrates will also be presented in following sections (6).

Meat and Meat Products

Pediocin PA-1 (1,400 AU/ml) has been added to beef meat inoculated with food-spoilage strains of *Clostridium laramiae*, *Lactobacillus* spp., and *Leuconostoc*. Subsequently, the meat samples were vacuum-packaged and stored in refrigeration. Under these conditions, the treated samples remain unaltered after storage for 12 weeks and the population levels reached by the spoilage bacteria are significantly lower in the samples containing the bacteriocin than in the respective control samples. Simultaneously, another study evaluated the ability of pediocin PA-1 (500 to 5000 AU/ml) to prevent the adhesion of *L. monocytogenes* to irradiated beef meat and to decontaminate meat inoculated with 10^2 to 10^4 cfu g^{-1}. Residual bacteriocin activity was detected on the meat surface for at least 28 days at 5°C. They employed the pediocin-producing strain *P. acidilactici* JD1-23 as starter culture for the elaboration of meat products and found that it provides a reduction in added *L. monocytogenes* (10^6 cfu g–1) during summer sausage production (42).

They examined the effectiveness of pediocin PA-1 in reducing the population of three *Listeria* strains in sterile ground beef, sausage mix, Cottage cheese, ice cream, and reconstituted dry milk and observed that the bacteriocin action is immediate and is not affected by the food system tested. In addition, these authors showed that the activity is concentration and strain dependent. The listericidal activity of pediocin PA-1 has also been evaluated in slurries of beef muscle tissue, beef tallow, nonfat dry milk, and butterfat inoculated with a mixture of two *L. monocytogenes* strains. For all slurries tested, the greatest decrease in *Listeria* occurs within 1.5 min of pediocin addition. In the same study, pediocin was also encapsulated within phosphatidyl-choline-based liposomes before addition to the slurries or unencapsulated in slurries containing the emulsifier Tween 80. In both cases, the pediocin activity recovered

from the slurries is notably higher than that obtained after adding free pediocin PA-1 (22, 42).

They bound pediocin PA-1 to heat killed producer cells by adjusting the pH of the medium to 6.0 and the preparation was added to irradiation-sterilized raw chicken breast meat. The results suggested that pediocin-treated raw chicken exhibits antilisterial activity both before and after cooking, and therefore may provide protection to consumers against bacterial post processing recontaminations and/or undercooking. Application of pediocin to food packaging films may constitute an alternative approach to control *L. monocytogenes* in meats and poultry. They obtained a pediocin PA-1 extract from milk-based media and prepared antilisterial cellulose casings by internal coating with the pediocin (7.75 µg cm^{-2}). The coated bags completely inhibit growth of inoculated *L. monocytogenes* through 12 weeks storage at 4ºC (22,42).

Milk and Dairy Products

Inhibition of *L. monocytogenes* has been demonstrated in several dairy systems, including dressed Cottage cheese, half-and-half cream, and cheese sauce. In all these food systems, there is a rapid decrease in viable counts of *L. monocytogenes* in the presence of pediocinPA-1 powder over the pH range 5.5 to 7.0 and at both 4 and 32°C. Antilisterial activity of pediocin PA-1 in dairy products has been reported despite the fact that pediococci are poorly adapted to dairy substrates, it has been reported that different pediocin-producing *P. acidilactici* strains may contribute to *L. monocytogenes* control in milk when a very high inoculums (10^9 cfu ml^{-1}) is used (42). In contrast, the pediocin producer *Lb. plantarum* WHE 92 was originally isolated from a smear-surface soft cheese and therefore, it must be well-adapted to this dairy environment. In fact, the presence of *L. monocytogenes* in Munster cheese could be prevented by spraying a cell suspension of *Lb plantarum* WHE 92 on the cheese surface at the beginning of the ripening period. Although *L. monocytogenes* was sometimes detected at low levels (<50 cfu g–1) after 7 to 11 days of ripening, this microorganism is unable to grow or survive in the presence of the bacteriocin-producing strain in any of the samples examined until the end of ripening (21 days). The pathogen often reaches counts higher than 10^4 cfu ml^{-1} in control samples. *Lb.*

plantarum WHE 92 does not interfere with the ripening process. The *L. lactis* LL108 strain producing pediocin PA-1 lacks several properties required for application in milk and dairy products. Therefore, in order to fully exploit the commercial potential of a pediocin-producing dairy starter culture, *L. lactis* MM210, a strain that had been used previously in Cheddar cheese manufacturing, was selected as an alternative host for the *ped* operon-encoding plasmid. The presence of the plasmid encoding heterologous pediocin production in the *Lactococcal* starter culture does not affect its cheese-making quality (42).

Other Foodstuffs

With respect to other foodstuffs, the addition of pediocin PA-1 to liquid whole egg containing *L. monocytogenes* showed an immediate reduction in listerial counts and led to a greater reduction during heating when compared with the levels found after heating untreated liquid whole egg. The addition of pediocin PA-1 during fermentation of a vegetable (kimchi) also notably decreases *L. monocytogenes* counts and inhibits its growth during a minimum of 16 days (42).

CHAPTER II
OBJECTIVES

Fermentation of various foods by lactic acid bacteria is one of the oldest of biopreservation. The process involving the use of lactic acid bacteria presented in the material to make biochemical reactions to produces a variety of substances to preserve food, adding texture, aroma, and taste to the products. Studying lactic acid bacteria in these fermented foods therefore help us to understand roles of these lactic acid bacteria and patterns of fermentation, so that they can be improved and may be leading to a new variety of fermented food products.

Many kinds of fermented food products including fish, meat, pork, vegetable and fruits are produced worldwide including Thailand (43). Nham, Plaa–ra, pickled pak-sain and pickled mango are examples of popular Thai fermented food products. They are most famous not only consumed domestically, but they are also exported to other countries (44).

Thai fermented food are generally of a household scale where the fermentation is usually carried out by the natural microflora or natural contaminants of the raw materials and this resulting in sometimes the failure of the fermentation process or sometimes the products are contaminated with some pathogenic bacteria (45). Meanwhile, chemical synthetic food preservatives have been added to various foods to prevent contamination, but it is not preferable by people who care about their health. They need a way to control quality of fermentation processing and contamination without the use of chemical preservatives.

Lactic acid bacteria play an important role in food fermentation and preservation and give the unique characteristics of fermented products, they are also produce many antimicrobial substances that are active against other bacteria such as food contaminated bacteria and pathogenic bacteria. The most interesting antimicrobial

substance is bacteriocin. Bacteriocins are antimicrobial peptides that can be used to control food poisoning and food spoilage microflora contaminated during fermentation process (7). These bacteriocin-producing lactic acid bacteria or their bacteriocins may be useful in extending shelf life of fermented food (3).

In Thailand, various species of lactic acid bacteria in fermented food products were identified, *Lactobacillus* sp. and *Pediococcus* sp.were commonly found in nham , a traditional fermented meat product (43). *Lactobacillus farciminis, Lactobacillus pentosus, Lactoba plantarum, lactobacillus* sp. and *Leuconostoc* sp. identified in fermented fish products such as Plaa-ra, Plaa-som and Som –fak (43, 46). Some of these strains produced bacteriocin such as plantaricin W from *Lactobacillus plantarum* PMU 33 strain isolated Som-fak (46) and Pongtep Wilaipun *et al* (47) also found two–synergistic peptide bacteriocin produced by *Enterococcus faecium* isolated from Thai fermented fish (Pla-ra) (47).

Studying of bacteriocin–producing lactic acid bacteria originally isolates from Thai traditional fermented food products may be useful to use them as starter cultures or co-protective culture for improving the microbial safety of many fermented food products. The bacteriocin may also be useful as biopreservative in food in a similar way as nisin. Since very few studies have been on the bacteriocin producing strains from Thai fermented food products, so the purpose of this study is to isolate bacteriocin producing strains from Thai fermented food products and further purify to its homogeneity.

The specific purposes of this thesis are

1. To isolate and detecte LAB that produce bacteriocins from Thai fermented food products.
2. To charactering and purify one of the interesting bacteriocin peptide including its amino acid composition and the gene encode.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains and culture conditions.

All bacteria used in this study and their culture condition are listed in Table 7. The bacteria were maintained as frozen stocks at -80°C in the appropriate broth containing 30% (v/v) sterile glycerol. Throughout the experiments, strains were sub-cultured every 1 week on agar media and kept at 4ºC. Before use in experiments cultures were propagated twice in broth overnight. Agar media were prepared by adding 1.5% (w/v) agar to liquid media and soft agar media were prepared by adding 0.75% (w/v) agar to liquid media (48).

3.1.2 Food sources

Bacteriocins producing-lactic acid bacteria were isolated by direct plating methods, from 30 fermented samples, including 1 samples of fermented meat, 3 samples of fermented pork, 4 samples of fermented seafood,10 samples of fermented fish, 3 samples of fermented fruits and 9 samples of fermented vegetables (Table 8).

Table 7 The bacterial strains and culture conditions.

Bacterial strains	Culture conditions (Media, Temperature)
Gram positive bacteria	
Lactococcus lactis subsp. *lactis* ATCC 19435	MRS, 30°C
Lactococcus lactis subsp. *Cremoris* TUA 1344L	MRS, 30°C
Lactococcus lactis WNC20	MRS, 30°C
Lactobacillus plantarum ATCC 14917	MRS, 30°C
Lactobacillus sakei subsp. *sakei* JCM 1157	MRS, 30°C
Pediococcus pentosaceus JCM 5885	MRS, 30°C
Streptococcus thermophilus ATCC 19258	MRS, 30°C
Enterococcus faecalis JCM 5803	MRS, 30°C
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 1642	MRS, 30°C
Micrococcus lutues IFO 12708	TSBYE, 37°C*
Staphylococcus aureus TISTR 118	NB, 37°C*
Bacillus circulans JCM 2504	TSBYE, 30°C*
Bacillus subtilis JCM 1465	TSBYE, 37°C*
Bacillus coagulans JCM 2257	MRS, 30ºC*
Listeria monocytogenes IFRPD 2068	TSBYE, 37°C*
Listeria innocua ATCC 33090	TSBYE, 37°C*
Gram negative bacteria	
Salmonella sp. TISTR 101	NB, 37ºC*
Salmonella anatum spp.	NB, 37°C*
Escherichia coli ATCC 11775	LB,37°C *

Abbreviations: ATCC, American Type Culture Collection; IFO, Institute for Fermentation, Osaka; IFRPD, Institute of Food Research and Product Development; JCM, Japan Collection of Microorganisms; TISTR, Thailand Institute of Science and Technology Research; TUA, Tokyo University of Agriculture. The asterisk refers to with shaking condition.

Table 8 The list of fermented food samples.

Type of fermented food	Samples name	Quantity	Source
Fermented meats	Nham nua*	1	Bangkok
Fermented pork	Rice sausage*	1	Bangkok
	Nham *	2	Bangkok
Fermented seafood	Hoi-dong*	1	Samutsongkhram
	Koong jom*	3	Bangkok
Fermented fish	Plaa-som fak*	1	Bangkok
	Plaa- nham*	2	Bangkok
	Plaa-som*	3	Bangkok
	Kai – plaa som*	2	Bangkok
	Plaa –jao*	2	Bangkok
Fermented fruits	Pickled olive*	2	Ratchaburi
	Pickled mango*	1	Ratchaburi

Table 8 The list of fermented food samples (continued).

Type of fermented food	Samples name	Quantity	Source
Fermented vegetables	Pickled Chiniese Mustard*	2	Ratchaburi
	Pickled bamboo shoots*	1	Ratchaburi
	Pickled mushroom*	1	Bangkok
	Pickled eggplant*	1	Bangkok
	Pickled pak sain *	3	Bangkok
	Pickled pak nam*	1	Samutsongkhram

* Definition of the samples name.

* Nham nua: Thai fermented beef with salt, minced garlic and boiled rice.

Rice sausage: Thai north - east style sausage composed of fermented pork, rice and garlic.

Nham: Thai fermented pork with salt, minced garlic and boiled rice.

Hoi-dong: Thai fermented clams.

Koong jom: Thai fermented small shrimps.

Plaa-som fak: Thai product composed of minced fish fillet, salt, ground boiled rice.

Plaa- nham: Thai fermented mince fish, garlic and boiled rice.

Plaa-som: Thai light salt fermented whole fish (fresh water fish).

Kai–plaa som: Thai light salt fermented egg from fresh water fish.

Plaa–jao: Thai fermented small fish.

Pickled olive: Fermented olive preparing by soaking or storing it in water with salt and /or sugar.

Pickled mango: Fermented green mango preparing by soaking or storing it in water with salt.

Pickled Chinese mustard: Chinese fermented cabbage in salt and sugar water.

Pickled bamboo shoots: Fermented bamboo shoots in salt and sugar water.

Pickled mushroom: Fermented bamboo shoots in salt and sugar water

Pickled eggplant: Fermented eggplant in water with salt and vinegar.

Pickled pak sain: Fermented pak sain in salt water.

Pickled pak nam: Fermented pak nam in salt water.

3.1.3 Chemicals

Chemicals	Companies
Acetic acid	Merck
Acetonitrile (HPLC grade)	Lab-Scan
Acrylamide	Sigma
Agarose	Gibco
Amberlite XAD-16	Sigma
Anaerobicult® A	Merck
Anaerobic jar	Merck
Broad range, protein marker	BioLabs
Catalase	Sigma
Chloroform	Merck
Disodium hydrogen phosphate	Merck
Dipotassium hydrogen phosphate	Baker
dNTP	Amersham
EDTA	Sigma
Ethanol	Merck
Ethidium bromide	BDH
Formaldehyde	BDH
Glucose	Sigma
Glycerol	Carlo elba
Hydrochloric acid	Merck
Isopropanol (2-propanol)	Merck
λ *Hin*dIII	Amersham
Lysozyme	Sigma

3.1.3 Chemicals (continued)

Chemicals	Companies
Manganese sulfate	Sigma
2-Mercaptonethanol	Sigma
N,N'-Methylene-bis-Acrylamide	Sigma
Meat extract	Difco
Methanol	Merck
MRS	Merck
Nova-Pak C_{18}	Water
Peptone	Gibco
Phenol (saturated with Tris)	USB
Proteinase K	Sigma
Silver nitrate	Sigma
Sodium dodecyl sulfate	Sigma
Sodium acetate	Merck
Sodium chloride	Merck
Sodium di hydrogen phosphate	Merck
Sodium hydroxide	Merck
Sodium thiosulfate	Fluka
SP-sepharose	Amersham
Sucrose	Merck
Taq DNA polymerase	Qiagen
TEMED	Promega
Tricine	Sigma
Trifluoroacetic acid (sequencing grade)	Perkin Elmer
Trisma base	Sigma
Tryptic soy broth	Difco
Tween80	Merck
Yeast extract	Scharlau

3.2 Methods

3.2.1 Detection and isolation of bacteriocins producing - lactic acid bacteria from 30 fermented food samples by direct plating method.

The direct plating method (49) was used as screening of bacteriocin producers of lactic acid bacteria isolated from food samples. *Lactobacillus sakei* subsp *sakei* JCM 1157 was used as indicator strain. A 10% (w/v) food sample in diluent (0.85% NaCl) was mixed and 10 fold serially diluted. Pour plates of serial dilutions (1ml aliquot) in MRS media were incubated under anaerobic conditions (Anaerobic generating Kit, Merck) for 24 - 48 hours at 30°C. The plates that have 100-200 colonies were overlaid with indicator strain (*Lactobacillus sakei* subsp. *sakei* JCM 1157) with addition of catalase at a final concentration of 50 U/ml to eliminate the potential inhibitory effect of H_2O_2 produced by colonies than incubated at 30ºC overnight. The colonies that produced clear inhibition zone were picked up and cultured. Heat-inactivated cell-free supernatants were used for check the ability to secrete antimicrobial substance in broth. Heat-inactivated cell-supernatant was obtained by centrifugation at 10,000 rpm for 10 min, followed by heated at 100°C 10 min. Inhibition was tested by spotting 10 µl of the supernatant onto soft agar lawn (0.75%) seeds with 10 ul of overnight grown indicator strain and incubated overnight at 30°C. Only cultures producing an inhibitor in broth were kept for further studies at -80°C. To proof that the suspected colonies can produce proteinaceous antimicrobial substance by treated with proteinase K enzyme. Heat-inactivated cell-free supernatant was treated with proteinase K enzyme at final concentration of 1 mg/ml. The Proteinase K enzyme dissolved in 50 mM Sodium phosphate buffer pH 7.0 as stock solution at 10 mg/ml. The reaction mixtures (45 ul of heat-inactivated cell-free supernatant incubated with 5 ul of 10 mg/ml stock enzyme solution) were incubated at 37°C for 4 hr. After incubation enzyme activity was terminate by heating 100°C for 5 min. Untreated supernatant was used as control. The residual bacteriocin activity was assayed against indicator strain *L. sakei* subsp. *sakei* JCM 1157(48, 49).

3.2.2 Characterization and identification of the bacteriocin- producing isolate.

3.2.2.1 General morphology and phenotypic characterization.

Bacteriocin-producing strain was examined microscopically for cellular morphology by wet mount preparation and Gram-stain phenotype. The catalase reaction was tested, a drop of overnight broth culture was dropped onto slide glass and a drop of 3% hydrogen peroxide was mixed and observed for the gas bubbles.

3.2.2.2 Bacteriocin activity assay.

Bacteriocin activity was assayed based on the critical dilution of antagonistic activity caused by a bacteriocin-producing culture. Generally a two-fold series of dilutions of the bacteriocin sample was prepared and a standardized plating method was used for evaluating the antagonistic effect of aliqouts from each dilution. After incubation, arbitrary endpoint was selected which is often the last dilution exhibiting inhibition (10). Firstly, to prepare the heat-inactivated cell-free supernatant, a single colony was activated in 1 ml MRS broth at 30°C without shaking for overnight and 10 µl of the culture was inoculated to 1 ml of MRS broth and grown at 30°C without shaking for 16-18 hours. Then the bacterial culture was centrifuged at 10,000 rpm for 10 min. Cell-free supernatant was heated at 100°C for 5 min and twofold serial diluted. Secondly spot-on-lawn method was used for evaluating the antagonistic effect of aliquots from each dilution from heat-inactivated cell-free supernatant, the indicator lawn of *Lactobacillus sakei* subsp. *sakei* JCM1157 was prepared by overlaid solidified MRS agar (1.5% agar) with 5 ml of MRS soft agar (0.75% agar) inoculated with 10 µl of *Lactobacillus sakei* subsp. *sakei* JCM 1157 overnight culture with addition of catalase at a final concentration of 50 U/ml to eliminate the inhibitory effect of the H_2O_2. After solidification of the soft agar, the plate was dried for 10 min (oven 60°C). The 10 µl portions of serial twofold dilutions of heat-inactivated cell-free supernatant were spotted on to the indicator lawn,

incubated at 30°C for overnight. The bacteriocin activity was expressed in arbitrary units (AU). One unit was defined as the reciprocal of the highest dilution showing the inhibition of the indicator lawn and was expressed in activity units (AU) per milliliter (6)

$$\text{Bacteriocin activity (AU/ml)} = \frac{\text{The highest serial two-fold dilution} \times 1{,}000}{\text{Volume } (\mu l)}$$

3.2.2.3 Spectrum of activity determination of bacteriocin-producing isolate.

Spectrum of activity determination using heat-inactivated cell-free supernatant was determined by spot-on-lawn method. Briefly, the tested bacterial lawn was prepared by overlaying solidified MRS agar (1.5% agar) with 5 ml of appropriate soft agar (0.75% agar) seeded with 10 μl of tested strains with addition of 50 U/ml of catalase to eliminate the inhibitory effect from H_2O_2. After solidification, 10 μl of heat-inactivated cell–free supernatant was spotted on the surfaces of the solidified soft agar seeded with the various tested strains (Table 7). After incubated overnight at appropriate conditions as illustrated in Table 7, the antagonistic effect was determined and recorded.

3.2.3 Purification of the bacteriocin produced by bacteriocin- producing isolate.

All steps of the purification were performed at room temperature. Pick 1 single colony of the isolate to 3 ml MRS broth, was incubated at 30ºC without shaking overnight. One ml of cell culture was inoculated in 10 ml of MRS broth and grown at 30°C without shaking for overnight then inoculated in 1 litre of MRS broth and grown at 30°C without shaking for 17 hours to reach early stationary phase. The cells were removed by centrifugation at 10,000 rpm for 10 min at 4ºC (Sorvall). Supernatant was heated at 100°C for 15 min to inactivated proteases activity before further purification. The heat-inactivated cell-free supernatant was used as the starting material for bacteriocin purification. The protein concentration was estimated

by using the absorbance 280 nm: A_{280}=1, estimated protein concentration is 1 mg/ml (50).

3.2.3.1 Amberlite XAD-16 adsorption.

Twenty gram of Amberlite XAD-16 was soaked in 50% isopropanol and stored at 4ºC. Before use, 50% isopropanol was removed from Amberlited XAD-16 and washed by deionized distilled water several times (until no smell). Twenty grams of Amberlite XAD-16 was added to 1 liter of heat-inactivated cell-free supernatant. The mixture was left for 4 hours with shaking at room temperature. The mixture was transferred to a chromatographic column, the matrix (Ambarlite XAD-16) was washed with 100 ml deionized distilled water and 100 ml of 40% (v/v) ethanol in distilled water and the bacteriocin was eluted with 100 ml of 70%(v/v) isopropanol in distilled water, followed by washed with 100 ml of 100% 2-propanol. The eluted was evaporated to get rid of isopropanol reducing the volume to 30 ml and pH was adjusted to 5.7 with 5N NaOH. This fraction is called Amberlite fraction. The Amberlite fraction was determined for bacteriocin activity by spot on lawn method. The protein concentration was estimated by using absorbance at 280 nm (50).

3.2.3.2 Cation exchange chromatography.

Thirty milliliter of Amberlite fraction was applied to a column containing 10 ml SP-sepharose equilibrated with 20 mM sodium phosphate buffer pH 5.7 with a flow rate of 0.5 -1 ml/min. The column was first washed with 100 ml of the starting buffer to remove the unbound components. The column was eluted with stepwise salt concentration between 0.2-1 M in 20 mM sodium phosphate buffer. The bacteriocin activity was determined by spot on lawn method. The concentration of protein was estimated by using the absorbance at 280 nm. The 0.5 M salted eluted containing antimicrobial activities were subjected to reverse phase chromatography.

3.2.3.3 Reverse-phase chromatography

Final purification step was performed by reverse phase chromatography on a Nova-Pak C_{18} cartridge (3.9 mm X 300 mm) integrated in a high-performance liquid chromatography system (Waters, Millford, MA, USA). The gradient proportion was generated by 0.1% TFA and acetonitrile containing 0.1% TFA. The 5 ml of SP-sepharose fraction containing bacteriocin activity was applied to a column by manual injection to Waters 600 controller pump (Waters, Millford, MA, USA) using 5 ml sample loop. The column was washed with 20 % acetonitrile for 10 min and washed with 20% to 30% acetronitrile for 20 min then eluted with 30%-70% acetonitrile for 20 min. Polypeptides were detected spectrophotometrically by measuring the optical density at 220 and 280 nm using Waters 2996 Dual λ Absorbance Detector (Waters, Milford, MA, USA). The protein concentration was estimated by using the absorbance at 280 nm (50).

3.2.3.4 Tricine-SDS-PAGE

The protein profile of each purification step was determined by tricine SDS-PAGE analysis. Tricine buffer system was used instead of glycine buffer system to improve the seperation power of proteins. Preparation of tricine SDS-PAGE was performed according to the method of Schägger H (51) with slightly modification by using Mini ProteinII apparatus (BioRad). The slab gel (6x8x0.75 mm) containing 16.0% acrylamide as separating gel and 4% acrylamide as stacking gel was used. The 16.0% acrylamide was prepared for separating gel which consisted of 2.5 ml of 49.5% acrylamide mix (49.5% acrylamide, 3% bis-acrylamide), 2.5 ml of 3M Tris-HCl pH 8.45 with 0.3% SDS, 2 ml 50% (v/v) glycerol, 25 µl of 10% ammonium persulfate and 3.25 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED). The 4% stacking gel was prepared from 0.5 ml of 49.5% acrylamide mix, 1.5 ml of 3M Tris-HCl pH 8.45 with 0.3% SDS, 2 ml of deionized distilled water, 20 µl of 10% ammonium persulfate and 2 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED). The anode buffer (200 mM Tris pH 8.9) was loaded on the bottom in gel apparatus tray and the cathode buffer (100 mM Tris, 100 mM Tricine, 0.1%SDS) was

loaded on the top into wells. Twenty µl samples of each purification step composed of the crude extraction, Amberlite fraction, SP-sepharose fraction and HPLC fraction which concentrated by speed - vacuum to be analyzed were treated with 5 µl of 5Xsample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% Bromophenol blue, 5% β-mercaptoethanol). The mixtures were vigorously mixed and heated at 100ºC for 2 min, quick spin down. Fifteen µl of each sample mixture was subjected on each slot previous prepared SDS-polyacrylamide gel (51). Broad-range protein marker 2-212 kDa [protein (dalton); insulin A, B chain (2,340-3,400); aprotinin (6,517); lysozyme (14,313); trypsin inhibitor (20,040-20,167); triosephosphate isomerase (26,625); lactate dehydrogenase (36,487); MBP2 (42,710); glutamic dehydrogenase (55,561); serum albumin (66,409); phosphorylase b (97,184); β-galactodidase (116,351); MBP-β-galactosidase (158,194); Myosin (212,000)] was used as molecular size marker. The electrophoresis was run at constant current 30 mA until the tracking dye reach about 2 cm above the edge of the gel. The protein profiles were visualized by silver stain. The electrophoresis gel was soaked with the fixing solution (10 ml methanol, 11 ml 3A DDW, 3.5 ml formaldehyde) for 30 min followed by wash the gel with the 3A DDW for 2 times of 30 min. Then the gel was immersed in sensitizing solution (0.02% (w/v) sodium thiosulfate) for 30 min followed by washed the gel for 5 min. Soak the gel in staining solution (0.1% silver nitrate) for 30 min and wash the gel with 3A DDW for seconds. The protein patterns was developed by immerse the gel into the developer solution (3 g of di-sodium carbonate, 200 µl formaldehyde, 250 µl 0.02% Sodium thiosulfate) until notice the appearance of yellowish brown band then reaction was terminated with 5% acetic acid followed by washed the gel with 3A DDW. The treated gel was mounted by the cellophane over the glass plate and kept at room temperature until the gel was completely dried. .

3.2.4. Identified of purified bacteriocin by mass spectroscopy analysis.

The Mass spectrum was acquired on a MALDI –TOF mass spectrometer equipped with delayed extraction technology (Bruker Daltonics, Bremen, Germany). The spectrum was acquired in reflector mode with nitrogen laser for

desorption/ionization of the sample. Angiotensin_I_[$(M+H)^+$ = 1296.68480] ,Substance_P_[$(M+H)^+$ = 1347.73540] ,Bombesin [$(M+H)^+$ = 1619.82230], ACTH_clip (1-17)[M+H]+ = 2093.08620 ,ACTH_clip[$(M+H)^+$ = 2465.19830, Somatostatin [$(M+H)^+$ = 3147.47100]. were used as the calibrants for external mass calibration. Sample was prepared as followed method: first prepared matrix solution by saturated alpha-cyano-4-hydroxycinnamic acid saturated with 0.1% TFA-acetonitrile (2:1), and then mixed 5 uL of matrix solution with 1 ul of sample (10 X HPLC fractions). The mixture was spotted a stainless steel Microchannel plate (MCP). The spotted-plate was allowed to dried at room temperature before analysis by mass spectrometer (12, 50).

3.2.5 PCR analysis and DNA sequencing.

The isolates produced bacteriocin in broth were collected and identified whether they were nisin or plantaricin W producing strains by colony PCR technique with nisin and plantaricin W specific primer. One isolate was selected for further studied including purification and gene encoding bacteriocin.

3.2.5.1 DNA extraction for PCR analysis

Chromosomal DNA and large plasmid of the lactic acid bacteria were isolated according to the method of Anderson and McKay (52) as followed. One single colony was activated in 5 ml MRS broth at 30°C without shaking for 14-24 hours. 300 µl of the culture was inoculated to 5 ml of MRS broth and grown at 30°C without shaking for 4 hours. The cells were harvested by centrifugation at 10,000 rpm for 3 min. The pellet was suspended in 379 µl STE buffer (6.7% sucrose, 50 mM Tris, 1 mM EDTA pH 8). The 96.5 µl of lysozyme solution (10 mg/ml in 25 mM EDTA pH 8.0) was added, followed by incubation at 37°C for 60 min. Then 48.2 µl of solution A (0.25 M EDTA, 50 mM Tris pH 8.0) and 27.6 µl solution B (20% SDS, 50 mM Tris, 20 mM EDTA) were added, mixed immediately and incubated at 37°C for 10 min to complete lysis, then vortex for 30 sec. 49.6 µl of 2M Tris pH 7.0 was added with gentle mixed for 3 min, followed by 71.7 µl of 5M NaCl.

The proteins were removed from DNA by extraction with 700 µl phenol (saturated with Tris buffer) and 700 µl chloroform-isoamyl (24:1). The aqueous phase was transferred to a new tube and 1 volume of isopropanol was added, incubated at -80°C more than 30 min. The DNA pellet was collected by centrifugation at 10,000 rpm for 5 min. The pellet was washed with 70% ethanol, before dissolving in 100 µl of sterile deionized distilled water and kept at 4°C for further use. The plasmid pattern was determined on 0.8% agarose gel electrophoresis.

3.2.5.2 Primer designation

3.2.5.2.1 Nisin Primer

These nisin primers were previously used by Lacroiw, *et al* (53). For amplification of the nisin operon and are located at positions –100 to –81 and 221 to 201 of the *nis*Z structural gene, generating a 320-bp amplicon. Nucleotide sequence contained *nis*Z structural gene which was reported in GenBank database (X61144) and the primer location were shown in Figure 3. The Forward and reverse primers were synthesized from BioService Unit, BIOTEC, Thailand. Estimated Tm (melting temperature) can be calculated as Tm a 4(G+C) + 2(A+T).

Forward primer:

Nisin1; 5’-CGCGAGCATAATAAACGGCT-3'

Tm = 60°C, (20 mers)

Reverse primer:

Nisin2; 5’-GGATAGTATCCATGTCTGAAC-3'

Tm = 60°C, (21 mers)

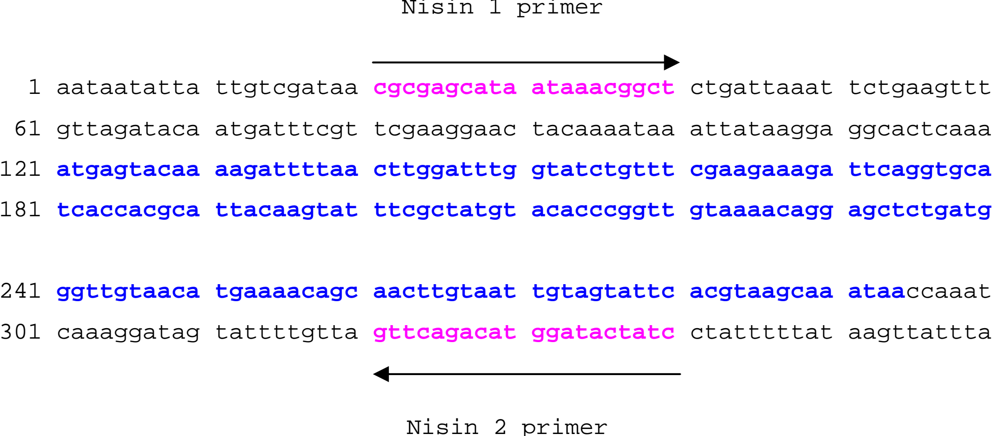

Figure 3 Nucleotide sequence of the precursor nisin structural gene (X61144). The nisin structural gene (*nis*Z) are indicated in blue. Primers are pink which are indicated by the arrows.

3.2.5.2.2 Plantaricin W Primer

Specific primers Plw1 and Plw2 were designed from DNA sequence region containing the two-component plantaricin W structural gene (*plw*) containing *plw*α and *plw*β gene of *Lactobacillus plantarum* LMG 2379 which was reported in GenBank database (AY007251). Primers were designed by Primer design MS-dos program to select the most appropriate primer pair without any primer dimers or hairpins forming. These primers was used in PCR reaction to amplify a 591 bp DNA fragment and estimated Tm (melting temperature) can be calculated as Tm a 4(G+C)+ 2(A+T). The 5' end of the coding strand primer (Plw1) is located 39 nucleotides upstream of the start codon of *plw*β gene, and the 3' end of the complementary strand primer (Plw2) is located 121 nucleotides downstream of *Plw*α gene as shown in Figure 4 (48).

Forward primer:

Plw1; 5'-GCGCTTGCCAATGAACAAAT-3'

Tm = 60°C, (20 mers)

Reverse primer:

Plw2; 5'-TATCTTCTCCCCAAACTCAC-3'

Tm = 60°C, (20 mers)

```
  1 aaatgttttt aataaaatat ataaaattat gttgatttta aaaatgactg tattataata
                                     Plw1 primer
 61 aaagttgttc aggtaagcgc ttgccaatga acaaatatga aaaggggtct tatatatgac
121 taaaactagt cgtcgtaaga atgctattgc taattattta gaaccagtcg acgaaaaaag
181 tattaatgaa tcttttgggg ctggggatcc ggaagcaaga tccggaattc catgtacaat
241 cggcgcagct gtcgcagcat caattgcagt ttgtccaact actaagtgta gtaaacgttg
301 tggcaagcgt aagaaataaa tgagtatcaa cttttaggag ggtttttatg aaaatttcta
361 agattgaagc tcaggctcgt aaagattttt ttaaaaaaat cgatactaac tcgaacttat
421 taaatgtaaa tggtgccaaa tgcaagtggt ggaatatttc gtgtgattta ggaaataatg
481 gccatgtttg taccttgtca catgaatgcc aagtatcttg ta actaattt ggtataaaac
541 taaaaataat gggacaaaga tcaatttagt taataaatta gttgcataag ctgatatatc
601 tgctaagcat ttggtttttg tcctattctt tttagctata tacatatgtg agtttgggga
                                                          Plw 2 pri-
661 gaagataact attgcaaaaa ataaaatata ttggtcaagc acaggaaacg gaatgtggac
      mer
```

Figure 4 Nucleotide sequence of the precursor plantaricinW structural gene (AY007251).

The plantaricin structural gene (*plw*) containing *plw*α and *plw*β gene of *Lactobacillus plantarum* LMG 2379 are indicated in green and blue respectively. Primers are pink which are indicated by the arrows.

3.2.5.2.3 Pediocin Primer

Amplification of pediocin gene, specific primers pediocin1 and pediocin2 were designed from DNA sequence region contained the pediocin structural gene (*ped A*) of *Pedioccocus acidilactici* PAC1.0 (54) which was reported in GenBank database (M 83924). Primers were designed by Primer 3 design program (online free program htpp:// Frodo.wi.mit.eau/cgi-bin/primer 3/primer3 _www.cgj) to select the most appropriate primer pair. These primers were used in PCR reaction to amplify a 369 bp DNA fragment and estimated Tm (melting temperature) can be calculated as Tm a 4(G+C)+ 2(A+T). The 5' end of the coding strand primer was located 151 nucleotides upstream of the start codon of *ped A* gene, and the 3' end of the complementary strand primer was located 29 nucleotides downstream of *ped A* gene as shown in Figure 5

Forward primer:

Pediocin 1; 5'-ATACTG CGTTGATAGC CAGG-3'

Tm = 60°C, (20 mers)

Reverse primer:

Pediocin 2; 5'- CCCTTTATTGATGCCAGCTC-3'

Tm = 60°C, (20 mers)

```
                                                      Pediocin primer 1
                                                   ------------------------->
 901ataaactgtg tcataactta aaagatactg cgttgatagc caggtttcaa aaattgacca
 961agatcgttaa ccagttttgg tgcgaaaata tctaactaat acttgacatt taaattgagt
1021gggaactaga ataagcgcgt attaaggata atttaagaag aaggagattt ttgtgatgaa
1081aaaaattgaa aaattaactg aaaaagaaat ggccaatatc attggtggta aatactacgg
1141taatggggtt acttgtggca aacattcctg ctctgttgac tggggtaagg ctaccacttg
1201cataatcaat aatggagcta tggcatgggc tactggtgga catcaaggta atcataaatg
1261ctagcattat gctgagctgg catcaataaa gggtgattt tatgaataag actaagtcgg
                      <------------------------
                           Pediocin primer 2
```

Figure 5 Nucleotide sequence of the precursor ped A gene (M83924).

The pediocin structural gene (*pedA*) of *Pediococcus acidilactici* PAC1.0 is indicated in green respectively. Primers are red which are indicated by the arrows.

3.2.5.3 PCR analysis for nisin and plantarcin W producing strain

The PCR reaction was performed by using colony of isolates as a template for both specific primers for nisin and plantaricin W. The 50 μl of PCR reaction contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 10x buffer, 0.2 mM of each primer and 1 U of Taq polymerase (QIAGENT). Before the PCR cycle begun, hot start was used to completely separate double stranded DNA of colony at 95°C for 5 minutes. Each cycle of PCR included denaturation for 30 sec at 95°C, 30 sec of primer annealing at 55°C and extension for 60 sec at 72°C, except for 5 minutes in the last cycle. For aliquot of 15 ul μl were analyzed by 1.5% agarose gel electrophoresis for nisin product and 1.0% agarose for plantaricin W product.

3.2.5.3.1 PCR analysis for pediocin gene

The PCR amplification was performed in a 50 ul of the PCR reaction mixture in a DNA thermo cycler (Perkin Elmer Cetus model TCI) as follows: 2 μl of plasmid extraction of WNK19 isolate or about 100 ng of purified DNA plasmid was added to 50 μl of the PCR reaction mixture The 50 μl of PCR reaction contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 10x buffer ,0.2 mM of each primer and 1 U of Taq polymerase (QIAGENT). Before the PCR cycle begun, hot start was used to completely separate double stranded DNA at 95°C for 5 minutes. Each cycle of PCR included denaturation for 30 sec at 95°C, 30 sec of primer annealing at 55°C and extension for 60 sec at 72°C, except for 5 minutes in the last cycle. Aliquots of 4 μl were analyzed by 1.5% agarose gel electrophoresis.

3.2.5.4 DNA sequencing and analysis

The 369 bp amplification fragment obtained from PCR using the extracted DNA plasmid of WNK19 isolate was used for submitted to DNA sequencing. The DNA sequencing was performed on both strands using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit on a ABI Prism 377 DNA sequencer (PE Applied Biosystem, Foster City, Calif.) by BioService Unit, BIOTEC, Thailand. A database search was performed by using the Blast program of

the National Center for Biotechnology Information, Bethesda, Md (http://www.ncbi.nlm.nih.gov).

3.2.6 Agarose gel-electrophoresis

Agarose ge-electrophoresis was performed in Tris borate buffer (89 mM Tris-HCl, 89 mM boric acid, and 25 mM EDTA pH 8.0). 0.8%, 1.0% and 1.2% agarose gel were prepared in Tris borate buffer. The agarose solution was melt in microwave oven and cool down before pouring in electrophoresis set with comb chamber. The 25-200 ng of DNA samples were mixed with 1/3 volume of loading dye (0.1% bromophenol blue, 40% ficoll 400 and 5 mM EDTA) were loaded into the slots of agarose gel. The 150 ng of λ DNA *Hin*d III or 100 bp DNA ladder was used as size standard marker. The electrical current, 100 Volts was applied to the gel for 1-2 hours. Pattern of DNA bands was visualized by staining in 2 μg/ml ethidium bromide solutions for 1 min and destaining in distilled water for 20 min. The gel was photographed under UV translumination using Gel Doc (BioRad).

CHAPTER IV
RESULTS

4.1 Detection and isolation of bacteriocin-producing lactic acid bacteria from fermented food samples.

A total of 35,717 bacterial colonies from 30 different Thai fermented food and vegetables samples (Table 8) were screened for bacteriocin producing isolates by direct plating method (section 3.2.1) using *Lactobacillus sakei* subsp. *sakei* JCM1157 as the indicator strain (Figure 6). The results show that 739 colonies recorded positive inhibition zones (Table 9). The detection rate was 2.07%. One hundred and sixteen isolates produced antimicrobial substances in broth but only 24 isolates stably produced antimicrobial substances in broth after sub-culturing five times as listed in Table 10.

The proteinaceous nature of antimicrobial substances was tested by treating heat-inactivated cell free supernatant (100°C, 10 min) with proteinase K enzyme (1 mg/ml) at 37 C°for 1 hr. Results shown in Table 10 indicated antimicrobial substance produced by the 24 isolates were all inactivated, which meant that they are bacteriocin. Isolates were further test for heat stability by treating heat-inactivated cell-free supernatants at 121°C for 15 min. Results shown also in Table 10 indicated the bacteriocin produced by WNK 3-4 (pickled pak sain), WNK 18-19 (nham) and WNK 20-24 (kai-plaa som) were stable at this autoclaving condition. These bacteriocins are potentially useful in food industry. The bacteriocins produced by WNK 8, 9, 10, 12 and 13 were partially stable and the rest were not stable at this condition (121°C, 15 min).

Figure 6 The inhibition zones around bacteriocin producing strains detected by direct plating method.

Table 9 An antimicrobial-producing colonies isolated from various food samples.

Samples name	Total colonies tested	Number colonies producing clear zone	Percentage of detection rate*
Nham nua	251	9	3.59
Nham	5,737	4	0.07
Rice sausage	382	8	2.09
Hoi-dong	3	0	0.00
Koong jom	1,210	53	4.38
Plaa-som fak	1,169	88	7.53
Plaa- nham	4,508	158	3.50
Plaa-som	2,514	156	6.21
Kai – plaa som	1,607	112	6.97
Plaa –jao	0	0	0.00
Pickled olive	7,075	0	0.00
Pickled mango	3,000	0	0.00
Pickled Chinese mustard	2,333	0	0.00
Pickled bamboo shoot	2,000	0	0.00
Pickled mushroom	200	0	0.00
Pickled eggplant	185	0	0.00
Pickled paksain	2,499	111	4.44
Pickled paknam	1,044	40	3.83
Total	35,717	739	2.07

* Percentage of detection rate was determined by dividing number of colonies producing clear zone by total colonies tested for each sample.

Table 10 Effect of heat and enzyme treatment on the bacteriocins produced from 24 isolates.

Bacteriocin producing isolates	Food source	Diameter of inhibition zone (cm)	Diameter of inhibition zone after treated with proteinase K enzyme (cm)	Diameter of inhibition zone after incubating at 121°C, 15 min.(cm)
1.WNK1	Pickled paksain	1.0	0.0	0.0
2.WNK2		1.0	0.0	0.0
3.WNK3		1.8	0.0	1.8
4.WNK4		2.0	0.0	2.0
5.WNK5	Pickled paknam	1.0	0.0	0.0
6.WNK6		1.0	0.0	0.0
7.WNK7		1.8	0.0	0.5
8.WNK8	Plaa-nham	1.3	0.0	1.0*
9.WNK9		1.5	0.0	1.3*
10.WNK10	Plaa-som fak	1.7	0.0	1.0*
11.WNK11		1.1	0.0	0.0
12.WNK12		1.7	0.0	1.0*
13.WNK13		2.0	0.0	1.0*
14.WNK14		1.3	0.0	0.0
15.WNK15		1.3	0.0	0.0
16.WNK16		1.2	0.0	0.0
17.WNK17	Nham	1.5	0.0	0.0
18.WNK18		1.8	0.0	1.3
19.WNK19		1.8	0.0	1.3
20.WNK20	Kai – plaa som	2.0	0.0	1.7
21.WNK21		2.0	0.0	2.0
22.WNK22		2.0	0.0	2.0
23.WNK23		2.0	0.0	1.8
24.WNK24		2.0	0.0	2.0

* refer to partial inhibition,where the inhibition zone was not totally clear.

4.2 Antimicrobial spectrum of bacteriocins produced by selected isolates.

Bacteriocin producing isolates were selected for antimicrobial spectrum activity from all food sample according to similar size of clear zones they produced (WNK2, 4 from pickled paksain; WNK5, 6 and 7 from pickled paknam; WNK8, 9 from plaa nham; WNK10, 12, 13 and 14 from plaa-som fak; WNK20, 21 and 24 from kai-plaa som). The spectrum of activity of those bacteriocin producing–isolates were determined against a range of indicator strains comprising lactic acid bacteria and food borne pathogenic strains. The results are shown in Table 11.

The antimicrobial activities of bacteriocins from 17 isolates could be divided into three groups: a broad, somewhat broad and narrow spectrum of antimicrobial activity. Bacteriocins from the first group (WNK4, 8, 9, 10, 13, 21 and 24) were able to inhibit most of the tested indicator strains of closely related lactic acid bacteria *Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc, Pediococcus* and some of pathogenic bacteria, such as those in the genus of *Staphylococcus*, *Streptococcus*, *Listeria* and *Bacillus*. These isolates might be class I lantibiotic bacteriocins similar to nisin because of their broad spectrum of antimicrobial activity. Interestingly, isolates WNK 8, 9, and 13 could inhibit *Lactococcus lactis* WNC 20, a nisin producing strain, while the rest could not.

The second group, WNK12, 14, and 19, showed growth inhibition of *Enterococcus faecalis, Lactobacillus sakei* subsp. *sakei, Lactobacillus plantarunm, Leuconostoc mesenteriodes* and *Pediococcus pentosaceus.* These bacteriocins have antilisterial activity and are similar to class II bacteriocins.

The third narrow spectrum group, WNK17 and 18, inhibited growth of four indictor strains of *Lactobacillus sakei* subsp. *sakei*, *Lactobacillus plantarunm*, *Leuconostoc mesenteriodes* and *Pediococcus pentosaceus.* The rest of isolates (WNK 2, 5, 6, and 7) showed a very narrow antimicrobial spectrum, inhibiting only one or two strains of *Lactobacillus sakei* subsp. *sakei* and *Pediococcus pentosaceus*.

From the results of the spectrum of antimicrobial activity we suggest that some isolates might be producing bacteriocins of broad spectrum class I lantibiotic or antilisterial class II bacteriocins. However, our laboratory has isolated nisin-producing *Lactococcus lactis* WNC 20 strain from nham (55) and plantaricin W-producing

Table 11. Antimicrobail spectrum of bacteriocins secreted by selected bacteriocin.

Indicator strain	Antimicrobial spectrum																
	WNK2	WNK4	WNK5	WNK6	WNK7	WNK8	WNK9	WNK10	WNK12	WNK13	WNK14	WNK17	WNK18	WNK19	WNK20	WNK21	WNK24
M. lutues IFO 12708		+				+	+	+		+					+	+	+
B subtilis JCM 1465		+				+	+	+		+						+	+
*B. circulans*IFRPD 2037		+				+	+	+		+						+	+
B. coagulans JCM 2257		+				+	+	+		+						+	+
L. innocua ATCC 33090		+				+	+	+	+	+	+			+		+	+
L. monocytogenes IFRPD 2068		+				+	+	+	+	+	+			+		+	+
L. sakei subsp. *Sakei* JCM 1157	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L. plantarum ATCC 14917		+				+	+	+	+	+	+	+	+	+	+	+	+
E. faecalis JCM 5803		+				+	+	+	+	+	+			+	+	+	+
L. mesenteroides subsp.*mesenteroides* JCM 1642		+		+		+	+	+	+	+	+	+	+	+	+	+	+
P. pentosaceus JCM 5885		+				+	+	+	+	+	+	+	+	+	+	+	+
L. lactis subsp. *Cremoris* TUA1344L		+				+	+	+		+					+	+	+
L. lactis subsp. *lactis* ATCC 19435	+	+				+	+	+		+					+	+	+
L. lactis WNC20						+	+			+							
S. thermophilus ATCC 19258		+				+	+	+		+						+	+
S. aureus TISTR 118		+				+	+	+		+						+	+
Salmonella sp. TISTR 101																	
Salmonella anatum spp																	
Escherichia coli ATCC 11775																	

Blank mean no inhibitory zone.
+ mean show clear inhibitory zone.

Lactobacillus plantarunm PMU33 strain from fermented fish (46). Both of these are class I lantibiotics. It should be possible to use PCR technique with specific primers for nisin and plantaricin gene to check the identity of bacteriocin from these 24 isolates.

4.3 Detection of nisin and plantaricin W from 24 isolates.

As the colony PCR technique that we used (method 3.2.5.3) was easy and convenient to perform, we decided to test all 24 isolates.

4.3.1 Detection of nisin-producing isolates.

The PCR reaction was performed by using colonies as a templates for amplifying nisin gene. The primers used are located at positions–100 to–81 and 221 to 201 of the *nis*Z structural gene as described in method 3.2.5.2 generating a 320 bp amplicon. Hot start was used to completely separate double stranded DNA at 95°C for 5 minutes. The 30 cycles amplification consisted of denaturation for 30 sec at 95°C for 30 sec of primer annealing at 55°C and extension for 60 sec at 72°C, with 5 minutes of 72°C in the last cycle. The results are shown in Figure 7. The 320 bp amplicon was obtained from genomic DNA of isolate WNK4, 21, 23, 24 (shown in lane4, 21, 23, 24), which were identical to amplicon from genomic DNA of nisin-producing strain *Lactococcus lactis* WNC20 (lane a). From these results, WNK4, 21, 23, 24 were nisin-producing strains. No DNA fragments were amplified from genomic DNA of a plantaricin W-producing strain *Lactobacillus plantarum* PMU33 (lane b) and non bacteriocin-producing strain *Lactobacillus sakei* subsp *sakei* JCM 1157 (lane c).

4.3.2 Detection of plantaricin W-producing isolates.

Colony PCR technique was used to look for the presence of plantaricin W gene among the 24 isolates. Specific primers and amplification reactions are described in method 3.2.5.3. The results are shown in Figure 8. The 591 bp DNA product was amplified from genomic DNA of WNK8 (lane8), WNK9 (lane9) and WNK13 (lane13), which were identical to that amplified from genomic DNA of

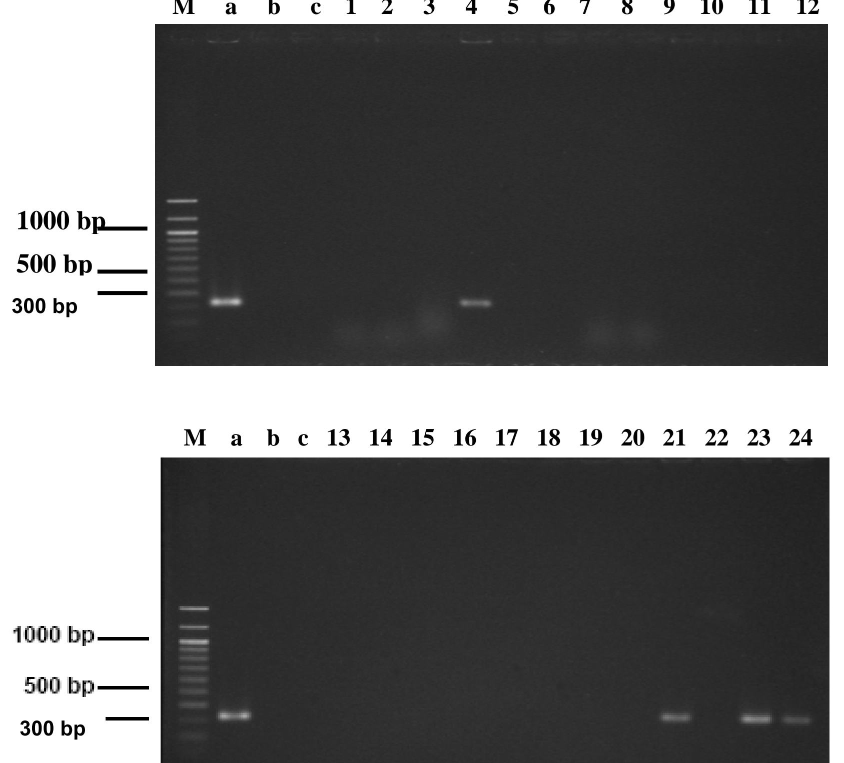

Figure 7 Ethidium bromide staining of PCR products with nisin specific primer in 1.5 % agarose gel-electrophoresis.

Agarose gel-electrophoresis of PCR products with nisin gene-specific primer from 24 isolates was stained with ethidium bromide and visualized under UV light. The band of 320 bp of PCR product shows positive nisin-producing strain.

lane M, 100 bp ladder DNA markers

lane a, *Lactococcus lactis* WNC20 (positive control for nisin-producing strain)

lane b, *Lactobacillus plantarum* PMU33 (negative control)

lane c, *Lactobacillus sakei* subsp. *sakei* JCM 1175 (negative control)

lane 1, WNK1; lane2, WNK2; lane 3, WNK3; lane 4, WNK4; lane 5,WNK5 ;

lane 6, WNK6; lane7, WNK7; lane 8, WNK8; lane 9, WNK9; lane 10, WNK10;

lane 11, WNK11; lane12, WNK12; lane 13, WNK13; lane 14, WNK14;

lane 15,WNK15; lane16, WNK16; lane 17, WNK17; lane 18, WNK18;

lane 19, WNK19; lane 20, WNK20; lane 21, WNK21; lane 22, WNK22 ;

lane 23, WNK23; lane 24, WNK24.

plantaricin W-producing strain *Lactobacillus plantarum* PMU33 (lane b). From these results, WNK 8, 9 and 13 were planricin W (plw) bacteriocin producing strains. No PCR product was found from genomic DNA of nisin-producing strain *Latococcus lactis* WNC20 (lane a) and non-bacteriocin producing strain *Lactobacillus sakei* subsp. *sakei* JCM1175 (lane c).

The results from PCR experiments showed that group I broad spectrum, bacteriocins from 7 isolates were identified as nisin and plantaricin W. Bacteriocin from the other 17 isolates were neither nisin nor plantaricin W. Of these isolates, WNK19 was chosen for further purification because the bacteriocin was in the somewhat broad group, had antilisterial activity and also could inhibit pathogenic strain of *Enterococcus faecalis* and the other lactic acid bacteria of *Lactobacillus sakei, Lactobacillus plantarunm, Leuconostoc mesenteriodes* and *Pediococcus pentosaceus* stain. Bacteriocin from WNK 19 was also heat stable at autoclaving condition (121°C, 15 min) which is useful to food industry.

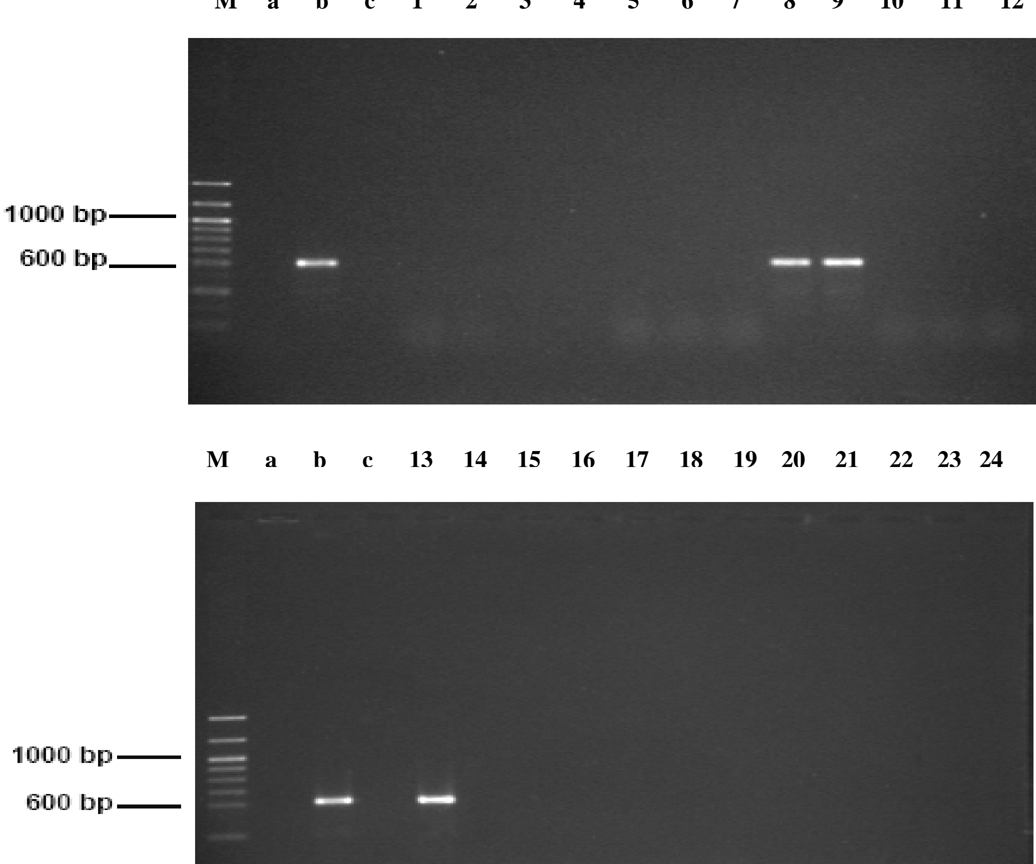

Figure 8 Ethidium bromide staining of PCR products with plantaricin W-specific primer in 1.5% agarose gel-electrophoresis.

Agarose gel-electrophoresis of PCR products with plantaricin W gene-specific primers from 24 isolates were stained with ethidium bromide and visualized under UV light. The band of 591 bp of PCR product shows positive strain.

lane M, 100 bp ladder DNA markers

lane a, *Lactococcus lactis*WNC 20 (negative control)

lane b, *Lactobacillus plantarum* PMU33 (positive control for plantaricin W-producing strain)

lane c, *Lactobacillus sakei* subsp. *sakei* JCM 1175 (negative control)

lane 1, WNK1; lane 2, WNK2; lane 3, WNK3; lane 4, WNK4; lane 5 ,WNK5;

lane 6, WNK 6; lane 7, WNK7; lane 8, WNK8; lane 9, WNK9; lane 10, WNK10;

lane 11, WNK11; lane 12, WNK12; lane 13, WNK13; lane 14, WNK14;

lane 15, WNK15; lane 16, WNK16; lane 17, WNK17; lane 18, WNK18;

lane 19, WNK19; lane 20, WNK20 lane 21, WNK21; lane 22, WNK22;

lane 23, WNK23; lane 24, WNK24.

4.4 Characterization and identification of the bacteriocin produced from WNK 19 isolate.

Morphologically, the cells of strain WNK19 were cocci in shape. The strain was Gram-positive bacterium. They usually occured in pairs or tetrads as shown in Figure 9. WNK19 strain was catalase negative and was considered as a lactic acid bacterium. Bacteriocin activity of WNK19 strain was determined by spot-on-lawn method (section 3.2.2.2) Two-fold serial dilutions of heat-inactivated cell-free supernatant were tested by spotting 10 µl of each dilution onto the surface of solidified indicator lawn. After incubating at 30°C overnight, the inhibition zone of undiluted heat-inactivated cell-free culture supernatant was observed with a diameter about 1.8 cm as shown in Figure 10. The inhibition zone was still seen after 128-fold of dilution. Therefore the bacteriocin activity of WNK19 strain was calculated as 12,800 AU/ml as shown below.

$$\text{Bacteriocin activity (AU/ml)} = \frac{\text{The highest dilution poviding inhibition zone X 1,000}}{\text{Volume } (\mu l)}$$

$$= \frac{128 \text{ X } 1{,}000}{10}$$

$$= 12{,}800$$

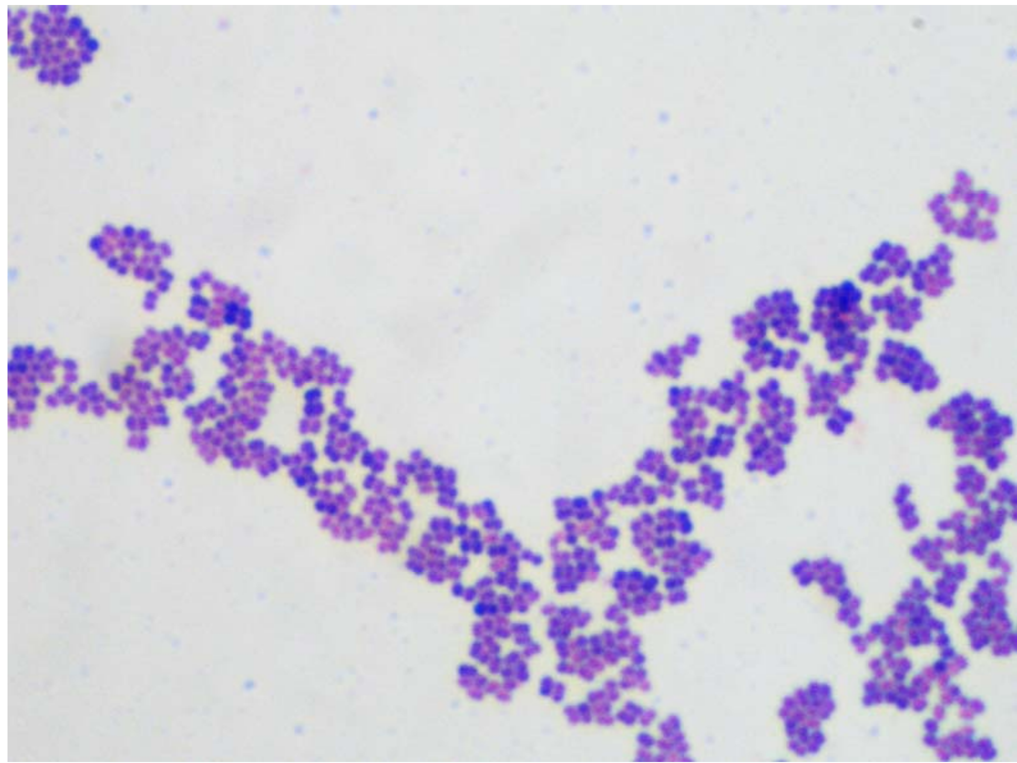

Figure 9 Morphology of WNK19 isolate stained with Gram stain under light microscope. WNK19 shows Gram-positive cocci characteristics.

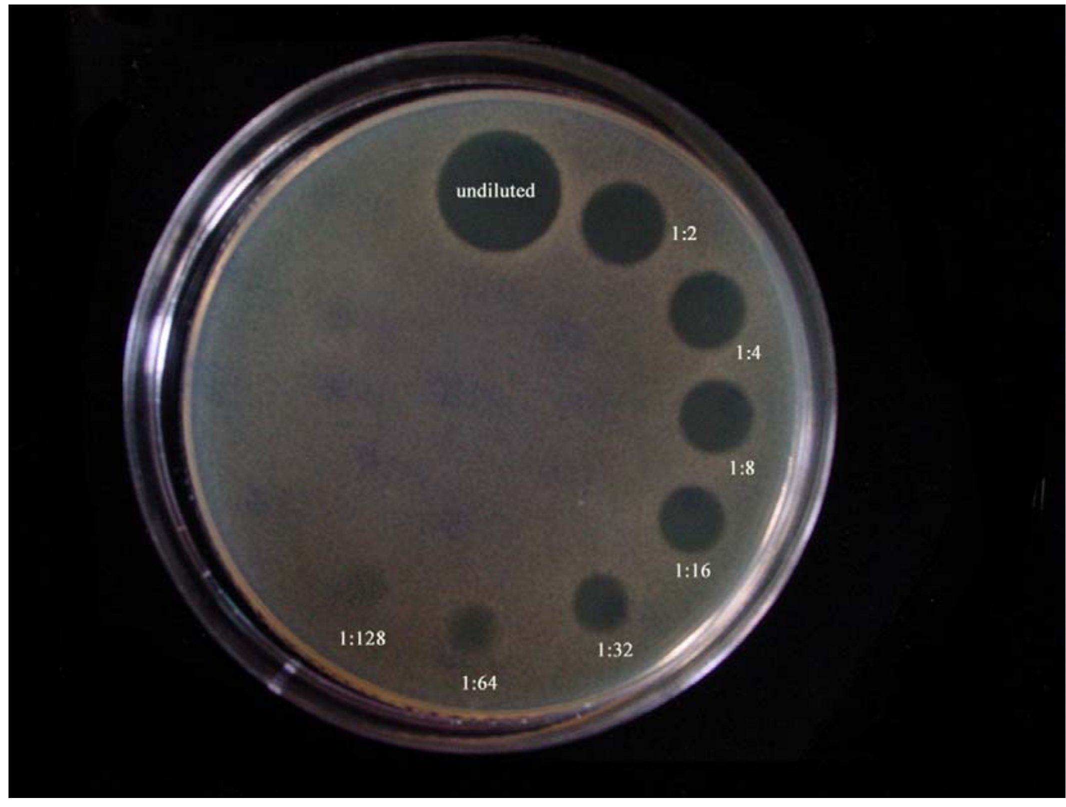

Figure 10 Bacteriocin activity assay.

Crude extract was 2-fold serially diluted and 10 µl aliquot of each dilution was spotted onto the indicator lawn of *Lactobacillus sakei* subsp. *sakei* JCM1157. The plate was incubated at 30°C overnight.

4.5 Purification of the bacteriocin produced by bacteriocin-producing WNK19 isolate.

Preliminary results showed that bacteriocin of WNK19 might be a member of class II bacteriocin, which is cationic and relatively hydrophobic. We decided to purify bacteriocin of this stain (WNK19) by 3 steps of purification, hydrophobic interaction chromatography with Amberlite XAD-16, cation-exchange chromatography using SP-sepharose and reverse-phase high performance liquid chromatography with C_{18} column (described in 3.2.3). These steps of purification have been used to purify entercin P and enterocin L50-class II bacteriocins (10, 56).

A summary of WNK19-bacteriocin purification is shown in Table 12. In the first step, bacteriocin was purified with hydrophobic-interaction chromatography by soaking Amberlite XAD-16 resin with heat-inactivated cell-free supernatant (100°C, 15 min), to allow the bacteriocin to adsorb to hydrophobic resin. The bacteriocin was eluted from Amberlite XAD-16 with 70% isopropanol (100 ml). The results produced bacteriocin with a specific activity about 1.14 x 10^5 AU/mg with 1.2 fold increase in specific activity from the initial specific activity (≈1.0 x 10^5 AU/mg) and 39.60% yield of initial total bacteriocin activity (1.28 x 10^7 AU). Protein was concentrated from 126 mg of 1000 ml supernatant to 44.65 mg in 100 ml of 70% isopropanol and this fraction was concentrated in a rotary evaporator to remove isopropanol (30 ml) before loading onto SP-sepharose column. After washing the column, the bacteriocin was eluted with stepwise NaCl gradient from 0.2 to 1.0 M NaCl (shown in Figure 11). The bacteriocin was eluted out in fraction 7 with 0.6 M NaCl. Then 5 ml of 0.6 M NaCl fraction was subjected to reverse-Phase HPLC. At this step bacteriocin purification was increased about 2-fold with specific activity of 2.3 x 10 ^{5} AU/mg, but only 8.16% yield of 4.45 mg protein remained. In the last step bacteriocin was purified to homogeneity by reverse-phase HPLC using C_{18} column, eluting with a linear gradient of 20-80% acetronitrile as described in 3.2.3.3. Elution profile is shown in Figure 12. The chromatogram did not show a single peak. However, bacteriocin activity was eluted with 50-60% acetronitrile. Therefore, the HPLC condition was further modified with an other preparation by eluting the column with 30-70% acetronitrile for 20 min, after which the HPLC column was eluted with

20% acetronitrile for 20 min. The results are shown in Figure 13. A peak of protein with activity was found in fraction number 44 at 56% acetronitrile (Figure 13). The specific activity of the bacteriocin (Table 12) was increased 5-fold (about 5 x 10^5 AU/mg). The purified bacteriocin contained about 0.4 mg protein, 1.6% yield from the crude extract.

This purified bacteriocin was used for analysis with MALDI-TOF MS to obtain the molecular mass. Each step of purification was also investigated by tricine SDS - PAGE according to method 3.2.3.4. The results are shown in Figure 14. The crude supernatant fraction in lane 1 showed many protein bands. Lane 2 was loaded with Amberlite XAD-16 fraction and showed only a thick protein band at molecular weight about 20 kDa, which might be due to aggregation of proteins. Lane 3, SP- sepharose fraction, was also showed many protein bands less than those in lane 1, with one band of more higher intensity than the other bands at molecular weight of approximately 5 kDa. Lane4, from reverse-phase HPLC fraction, shows a single band of approximately 5 kDa. The appearance of protein band at approximate 5 kDa in reverse-phase HPLC fraction was similar to the protein band in SP-sepharose fraction, indicating that it might be the band of our bacteriocin.

Table 12 Purification table of bacteriocin of WNK 19.

Purification stages	Total volume (ml)	Bacteriocin activity (AU/ml)	*Total activity (AU)	**Total Protein (mg)	#Specific activity (AU/mg)	!% yield	$Fold of purification
Crude supernatant	1,000	12,800	12,800,000	126	101,587	100.00	1
Adsorbed on Amberlite XAD-16	99	51,200	5,120,000	44.65	113,523	39.60	1.2
Binding to SP-sepharose	10.2	102,400	1,024,000	4.45	234,715	8.16	2
Reverse phase HPLC	2	102,400	204,800	0.411	498,297	1.60	5

*Total activity is determined by the multiplication of volume by activity.

**Total protein is estimation by A_{280} multiplied by volume; A_{280}=1, protein = 1 mg/ml.

#Specific activity is total activity divided by total protein.

!% yield is the remaining total activity as a percentage of the initial total activity.

$Purification fold is the increase in the specific activity.

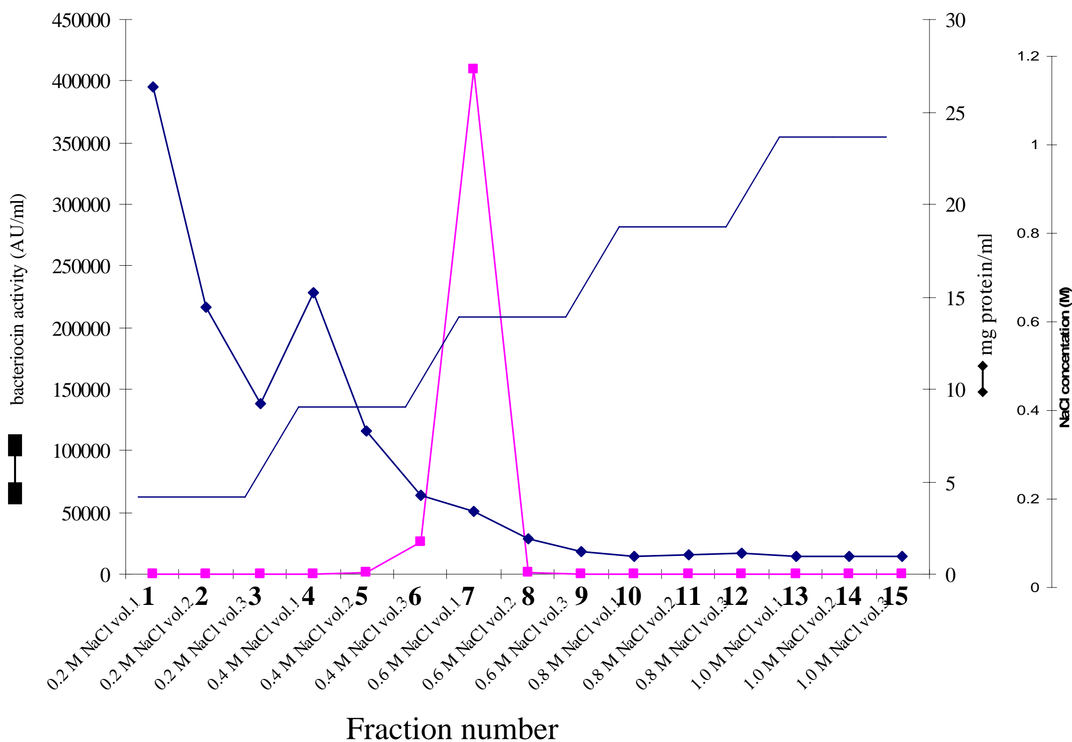

Figure 11 Elution profile of bacteriocin on SP- sepharose cation exchange chromatography.

Thirty ml of Amberlite fraction was applied to SP-sepharose equilibrated with 20 mM sodium phosphate buffer pH 5.7. Column was eluted with a stepwise NaCl gradient ranging from 0.2-1.0 M NaCl. A_{280} (♦), bacteriocin activity (▪), NaCl concentration (-).

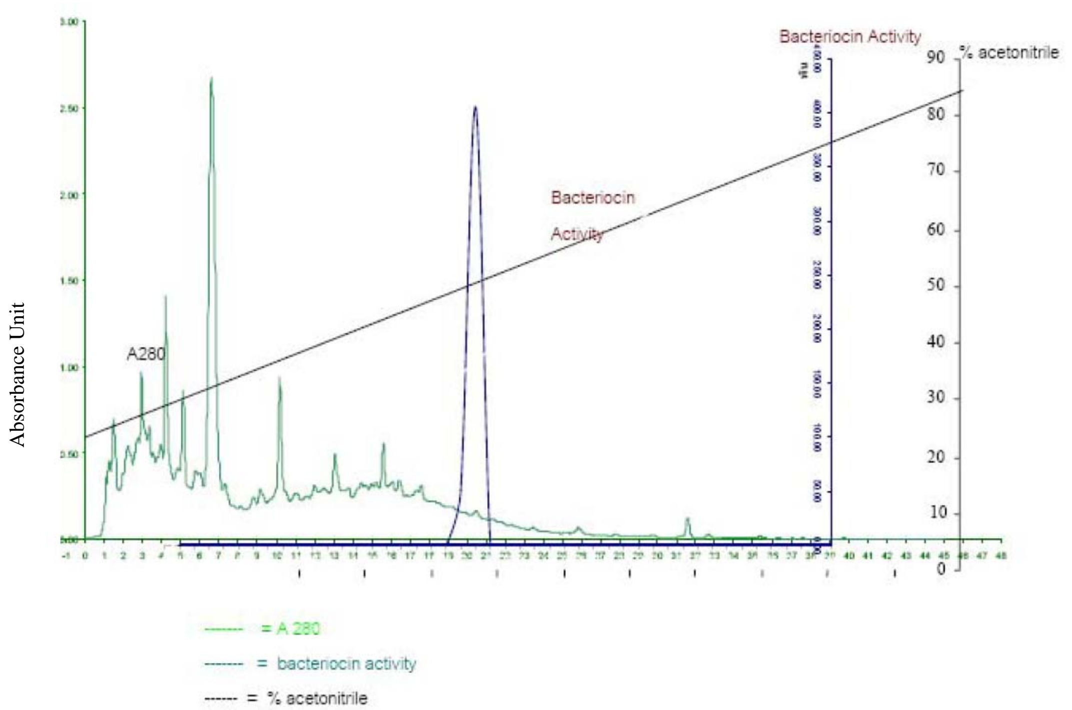

Figure 12 Reverse phase HPLC chromatogram of purified bacteriocin from WNK19 isolate.

Bacteriocin from 0.6 M salt fraction was applied to NovaPak C18 column and eluted using a linear gradient of acetonitrile (20-80%) containing 0.1%(v/v) TFA at a flow rate of 1 ml/min for 45 min. Elution profile was detected at 280 nm (—). Bacteriocin activity (—) and gradient profile (—) are shown.

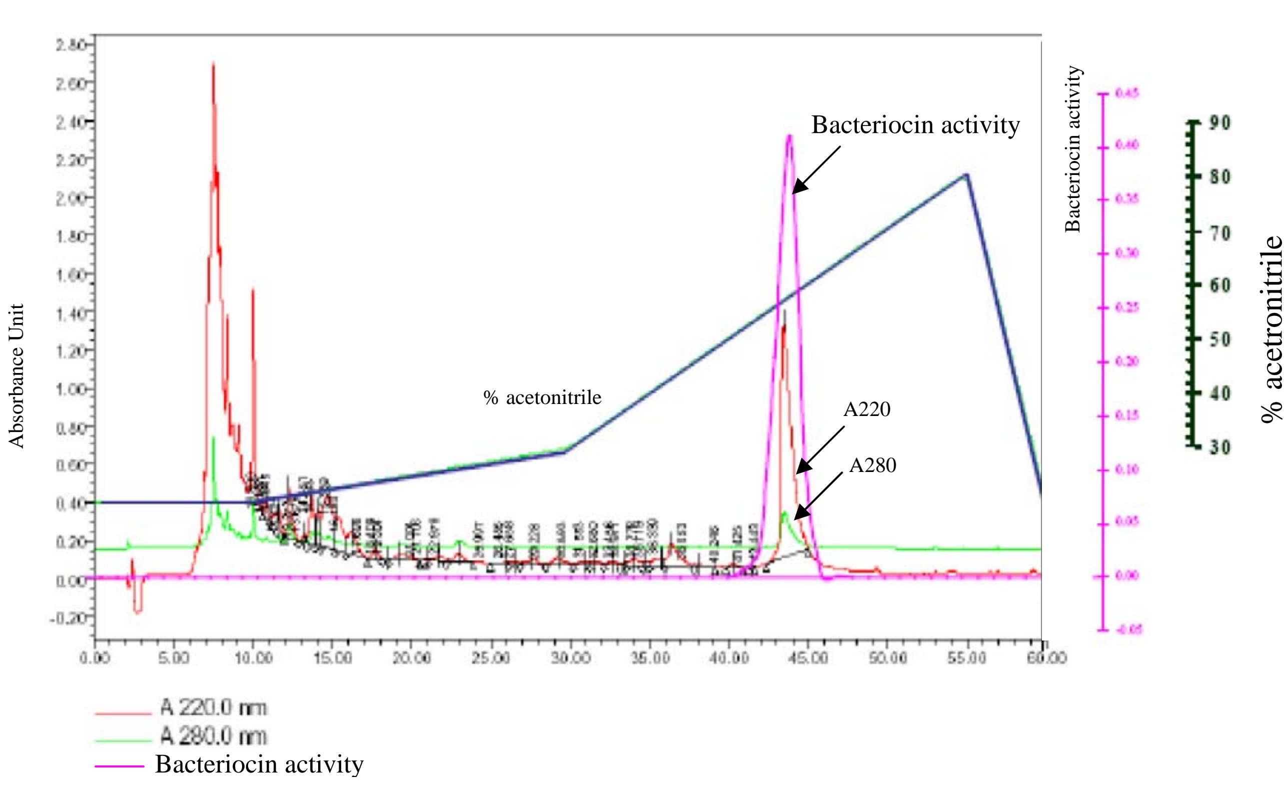

Figure 13 Modified reverse-phase HPLC chromatogram of purified bacteriocin from WNK19 isolate.

Bacteriocin from 0.6 M salt fraction was applied to NovaPak C18 column are eluted by a linear gradient of acetonitrile (30-70%) containing 0.1% (v/v) TFA at a flow rate of 1 ml/min for 60 min. Elution profile was detected at 280 nm (—)and 220 nm (—). Bacteriocin activity (—) and gradient profile (—) are shown.

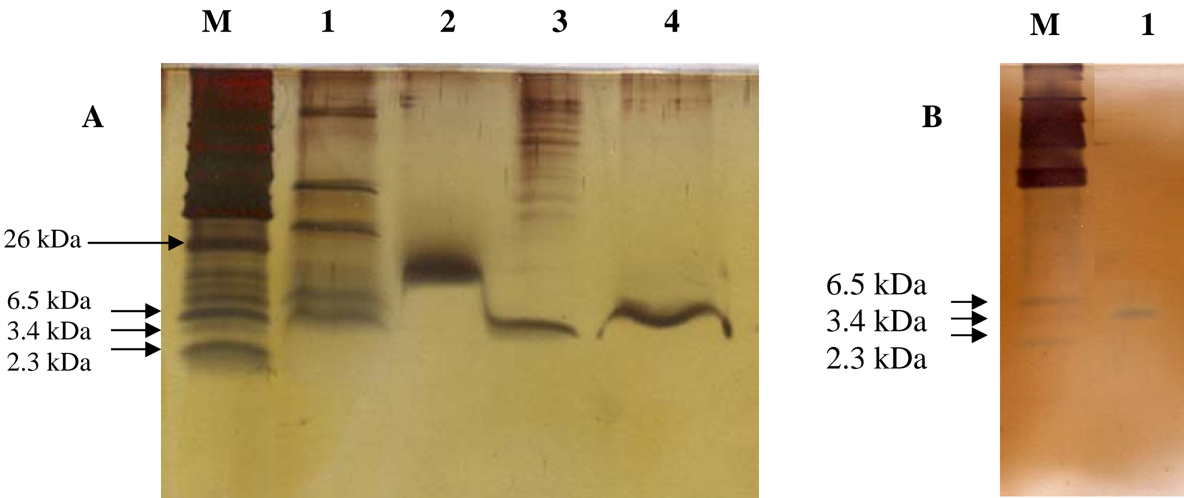

Figure 14 A. Tricine SDS-PAGE analysis of proteins from each purification step of WNK19 bacteriocin purification.

Twenty µl aliquot of each purification step was subjected to 16.0% tricine-SDS-PAGE at constant 30 mA current at room temperature. Protein pattern was visualized by silver staining.

Lane M, Protein markers, 2-212 kDa

Lane 1, Crude bacteriocin of heat-inactivated cell-free supernatant.

Lane 2, Amberlite fraction; 70 % isopropanol eluant from Amberlite XAD 16.

Lane 3, SP-sepharose fraction; 0.6 M NaCl fraction.

Lane 4, Reverse-phase HPLC fraction; bacteriocin purified by reverse-phase HPLC.

B. Tricine-SDS-PAGE analysis of reverse phase HPLC fraction.

One ug of purified bacteriocin was loaded on 16.5% tricine-SDS-PAGE at constant 30 mA current at room temperature. Protein pattern was visualized by silver staining.

Lane M, Protein marker, 2-212 kDa.

Lane 1, Bacteriocin from reverse-phase HPLC, showing a single band.

4.6 Mass spectrometry analysis of purified bacteriocin by MALDI–TOF.

To determine the molecular mass of the purified bacteriocin of WNK19, fraction 44 containing homogenous purified peptide with bacteriocin activity from reverse phase HPLC was concentrated about 10–fold by speed-vacuum and subjected to MALDI–TOF MS analysis according to the protocol described in section 3.2.4. The molecular mass of bacteriocin of WNK19 was 4625.485 Da (Figure 15). The molecular mass of this bacteriocin was close to that estimated by Tricine SDS-PAGE, which was approximately 5 kDa. The molecular mass of this WNK 19 bacteriocin was used to search in the database of molecular weights of bacteriocin. The search result showed that the molecular weight of WNK 19 bacteriocin of 4625.485 was close to that of pediocin PA-1 produced by *Pediococcus acidilactici* reported (55). If bacteriocin from WNK 19 is pediocin PA-1, than strain WNK19 is a pediocin-PA-1 producing strain.

4.7 Confirmation that pediocin PA-1 is produced by WNK19 strain.

To confirm that the WNK19 bacteriocin was pediocin PA-1 bacteriocin, PCR analysis using the published sequences of the *ped A* structural gene was performed (57). Primers complementary to sequence occurring at the 5' end 151 nucleotides upstream of the start codon, and 3' end located 29 nucleotides downstream of *ped A* structural gene were used to amplify *ped A* gene from the purified plasmids (Figure 16) of WNK19 strain (method 3.2.5.3.1). The result is shown in Figure 17. A 369-bp fragment was amplified from purified plasmids of WNK19 strain (lane 1), which was the expected size of the Pediocin PA-1 structural gene. No DNA fragment was amplified using purified plasmid DNA extracted from *Lactobacillus sakei* subsp. *sakei* JCM 1175, a non pediocin-producing strain (lane 2), or when no DNA template was present, a negative control (lane 3).

The amplified PCR product of WNK19 strain was subsequently sequenced as shown in Figure 18 (Bioservice Unit, BIOTEC, Thailand). Figure 18 shows the nucleotide sequence and the deduced amino acid sequence of the 369 bp amplicon of WNK 19 strain. The sequence contains 369 bp open reading frame (OFR), which is 100% identical to that of *ped A* of *Pediococcus acidilactici* PAC1.0, (57).

A ribosome-binding site was located at 9 bp upstream of *ped A*. The ORF of *ped A* encodes a 62-amino-acid precursor of pediocin PA-1, from which an 18-amino-acid N-terminal peptide is removed to produce the mature Pediocin PA-1. Henderson, J. T. *et al.,* 1992 (59) describe Pediocin PA-1 as a small heat-stable protein with molecular weight about 4.6 kDa containing 44 amino acids, which are consistent with the molecular mass of WNK19 bacteriocin (4625.485 Da) obtained by MALDI–TOF MS analysis. The molecular mass and properties such as heat-stability, antilisterial activity and tetrad cocci LAB indicate that the WNK19 strain is *Pediococcus* spp. producing pediocin PA-1 bacteriocin.

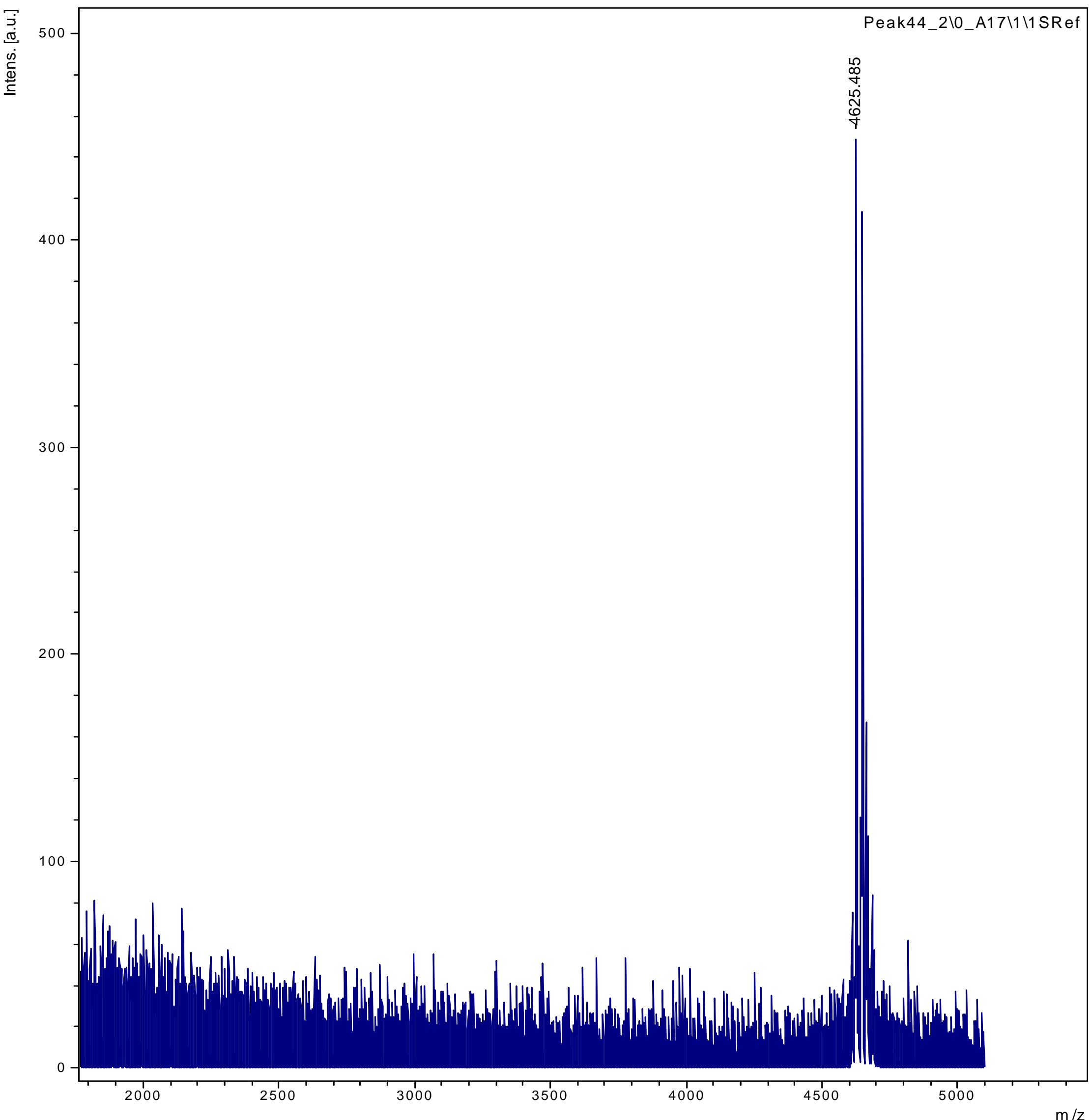

Figure 15 MALDI-TOF mass spectrum of the purified bacteriocin from reverse-phase chromatography.

Concentrated bacteriocin from HPLC fraction was mixed with saturated alpha–cyano-4 hydroxycinamic acid analyed in MALDI-TOF mass spectrometer. The molecular weight of bacteriocin was 4625.485 Da.

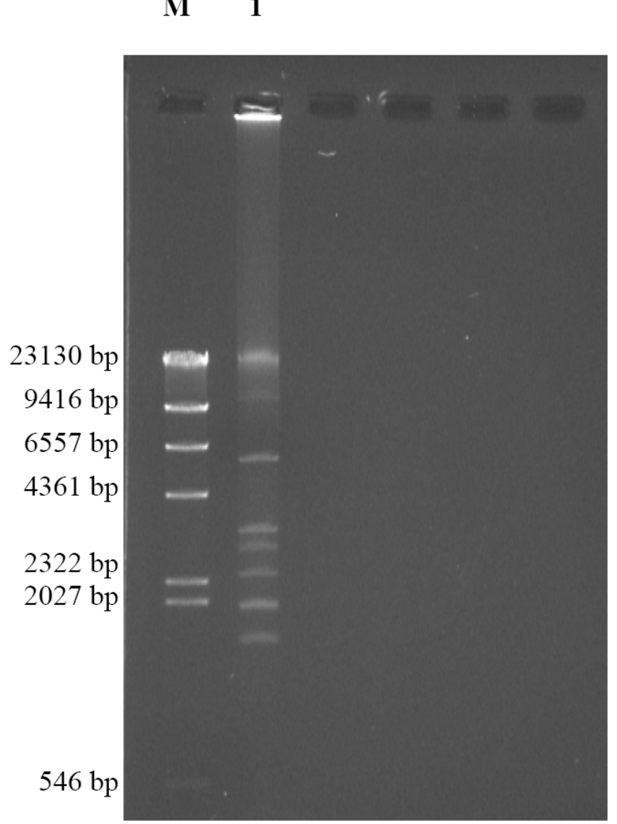

Figure 16 Ethidium bromide staining of plasmid DNA isolated from WNK19 isolate. The plasmid pattern of WNK19 isolate is show in 0.8% agarose gel-electrophoresis. Lane M, lamda *Hin*dIII DNA marker. Lane 1 plasmid DNA of WNK19 isolate.

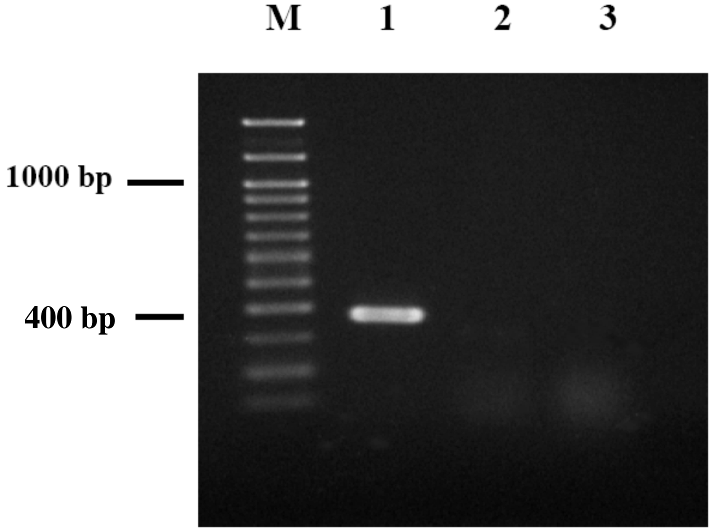

Figure 17 Ethidium bromide staining of PCR products with pediocin PA-1-specific primer in 1.5% agarose gel-electrophoresis.

Agarose gel-electrophoresis of PCR product with pediocinPA-1 gene-specific primers from WNK19 was stained with ethidium bromide and visualized under UV light. The band of 369 bp of PCR product shows *ped A* containing strain.

Lane M, 100 bp ladder DNA markers;

Lane 1, WNK19 strain;

Lane 2 *Lactobacillus sakei* subsp. *sakei* JCM 1175 (nonpediocin-producing strain);

Lane 3, water as negative control.

4.7.1.3 DNA sequencing and analysis

```
ATACTGCGTTGATAGCCAGGTTTCAAAATTGACCAAGATCGTTAACCAGTTTTGGTGCGAAATATCTAACTAATACTTGA
                                                                                 +1
CATTTAAATTGAGTGGGAACTAGAATAAGCGCGTATTAAGGATAATTTAAGAAGAAGGAGATTTTTGTG ATG AAA AAA
                                                         RBS            M   K   K

ATT GAA AAA TTA ACT GAA AAA GAA ATG GCC AAT ATC ATT GGT GGT AAA TAC TAC GGT AAT
 I   E   K   L   T   E   K   E   M   A   N   I   I   G   G   K   Y   Y   G   N

GGG GTT ACT TGT GGC AAA CAT TCC TGC TCT GTT GAC TGG GGT AAG GCT ACC ACT TGC ATA
 G   V   T   C   G   K   H   S   C   S   V   D   W   G   K   A   T   T   C   I

ATC AAT AAT GGA GCT ATG GCA TGG GCT ACT GGT GGA CAT CAA GGT AAT CAT AAA TGC TAG
 I   N   N   G   A   M   A   W   A   T   G   G   H   Q   G   N   H   K   C   *

CATTATGCTGAGCATCAATTCAATAAAGGG
```

Figure 18 Nucleotide sequence and deduced amino acid sequence of the *ped A* gene isolated from WNK19 isolate.

The amino acid sequence is shown below the nucleotide sequence. Primers are underlined. Stop codon is shown by asterisk. The *PedA* gene encodes a 62 amino acid precursor of pediocin PA-1, from which an 18 amino acid N –terminal peptide is removed. The amino acid sequence of mature peptid of pediocin PA-1 from WNK 19 strain is shown in bold. The ribosomal binding site (RBS) is underlined.

CHAPTER V
DISCUSSION

The aim of this study was to isolate and detect bacteriocin-producing lactic acid bacteria from Thai fermented food samples. A total of 739 isolates from 35,717 colonies screened produced clear inhibition zones. The rate of detection was 2.07 % which was about 10 fold higher than either of Coventry *et al*.(49) who screened about 600,000 colonies from dairy and meat products (0.2% detection). The higher incidence of percent detection rate in this our study compared with the study of Coventry *et al* (49) could be due to the differences of indicator strains used and food sources. They used a set of 4 indicator strains, which might not be as sensitive as our indicator strain, *Lactobacillus sakei* subsp. *sakei*. They used not only fermented food but also fresh meat, packaged meat, unflavored and flavored milk and cream. However, other studies have reported 0.2 -32 % detection rate (49, 58).

From 739 isolates only 116 secreted antimicrobial substances in broth and of only, 24 isolates (WNK1-24) stably secreted antimicrobial substances. All antimicrobial activity of heat-inactivated cell-free supernatants (100°C, 10 min) of the 24 isolates were inactivated by proteinase K treatment suggesting that the antimicrobial substances were bacteriocins. Some of them were also resistant at autoclave condition (121°C, 15 min). The reason why we heated supernatant at 100°C for 10 min before testing the bacteriocin activity because we would like to destroy all remaining bacterial cells and protease enzymes in supernatant and most bacteriocins are small peptides that can tolerate heat at 100°C for 10 min.

Heat-inactivated cell-free supernatant of 17 isolates from 24 selected isolates were chosen to test against a set of lactic acid bacteria (*Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc*) and a number of pathogenic strains of *Listeria*, *Staphylococcus*, *Streptococcus, Enterococcus and Bacillus.* The results showed that the antimicrobial activity spectrum of these isolates could be divided into three

groups: broad, somewhat broad and narrow antimicrobial spectrum. The first group (WNK4, 8, 9, 10, 13, 21 and 24), the broad spectrum group, could inhibit lactic acid bacteria in genera of *Lactobacillus, Lactococcus*, *Leuconostoc, Pediococcus* and pathogenic bacteria, such as in the genus of *Staphylococcus*, *Streptococcus*, *Listeria*, *Enterococcus* and *Bacillus*. As member of the first group are heat resistant (121°C, 15 min) and have broad spectrum of antimicrobial activity, they can be classified as class I bacteriocins. Nisin, a most studied class I for example can inhibit closely related bacteria and some pathogenic strains of *Staphylococcus sp., Streptococcus sp., Listeria sp.*, *Enterococcus sp.* and *Bacillus sp*, similar to our first group. Interestingly, bacteriocin of WNK8, WNK9 and WNK13 isolates could inhibit *Lactococcus lactis* WNC20, which are a nisin-producing strain, suggesting that these 3 isolates (WNK8, 9 and 13) may not be nisin-producing strains. The reason is in general, bacteriocin producing strains possess a specific mechanism (immunity) that affords self-protection against the toxicity of their own bacteriocins (15, 56, and 59).

Class II bacteriocins have more narrow activity spectra compared to class I bacteriocins. In general class II bacteriocins inhibit bacteria in the genus *Listeria* and also inhibit Gram-positive bacteria in genera of *Enterococcus, Lactobacillus and Pediococcus.* Most of them were not active against *Streptococcus sp., Staphylococcus sp.* and *Bacillus sp.* However acidocin A and pediocin PA-1 of class II bacteriocins (pediocin-like peptides), are exception and can inhibit growth of *Streptococcus,* with the extent of sensitivity varing from strain to strain. The second group of our tested isolates, WNK12, 14, and 19 (somewhat broad group), inhibited the growth of *Listeria monocytogenes, Listeria innocua, Enterococcus faecalis, Lactobacillus sakei, Lactobacillus plantarum, Leuconostoc mesenteriodes* and *Pediococcus pentosaceus*. Bacteriocin of WNK12, 14, and 19 isolates were also heat resistant at autoclave condition (121°C, 15 min) suggesting that they might be classified as class II bacteriocins.

From PCR amplification using specific primers genes of class I bacteriocin for nisin and plantaricin W. We found that WNK4, 21, 23, and 24, were identified as nisin-producing strains. These isolates produced bacteriocin that could not inhibit *Lactococcus lactis* WNC20, a nisin producing strain strongly suggests that they were nisin producing strains too. WNK 8, 9, 13 were identified to be plantaricin W-

producing strains. The first group isolate belonged to class I lantibiotic, bacteriocin producing bacteria.

The second group bacteriocin (WNK12, 14, and 19) which was expected to be class II bacteriocins were identified as either nisin or plantaricin W according to PCR amplification experiments. These three strains were of interest for further studies. We decided to choose WNK19 for further purification.

WNK19 was purified by 3-step purification scheme according to the characteristics of class II bacteriocins small peptides (larger than 3000 Da), cationic, amphiphilic, relatively hydrophobic (15, 23, 60, 61). The three steps of purification were: (i) hydrophobic interaction chromatography with Amberlite XAD-16, (ii) cation-exchange chromatography using SP-sepharose and (iii) reverse-phase HPLC with C_{18} column. Results showed that in the first step, protein was concentrated from 0.126 mg/ml of supernatant to 0.447 mg/ml of 70% isopropanol, with 1.2 fold increase in specific activity and 39.60% yield. In the second step the purification, there was 2-fold increase in specific activity and only 8.16% yield of 4.45 mg protein. In the 3rd step of purification, bacteriocin of WNK19 strain was purified to homogeneity by reverse-phase HPLC (as shown by a single band on tricine SDS-PAGE with molecular weight of approximately 5 kDa in Figure 14 b). About 0.4 mg protein yield and specific activity of 5 x 10^5 AU/mg (5-fold increase) with only 1.6 % recovery of bacteriocin activity was achieved in our purification.

Fractions from each step of purification were analyzed by tricine SDS-PAGE (Figure 14). Seventy percent isopropanol elute from Amberlite XAD-16 fraction was loaded onto Lane 2 and showed only a thick protein band at molecular weight about 20 kDa. The protein pattern was totally different than lane 1 from supernatant. This might be due to the effect of solvent on the protein load in the gel. This problem may be corrected by precipitating protein using TCA and dissolving in buffer before loading onto the gel. The 0.6 M NaCl, salt eluate from cation exchange column shown on the tricine SDS-PAGE in lane 2 revealed many bands. A single protein band at approximately 5 kDa was seen with the HPLC eluate fraction in lane 4. This showed that our bacteriocin was purified to homogeneity. The purity might be about 100 fold increased as compare to lane 1 from the supernatant. However, the

purification step in table 12 showed only a 5-fold increase of purification which should not be corrected. This lower number of fold of purification in the table 12 may come from the method that we used to determine the bacteriocin activity by dilution assay, which is not very accurate as compared to microtiter plate assay, which measures OD of cells. The protein concentration was measured by using absorbance at 280 nm, which might not be as good as Bradford assay. However, Ennahar *et al* reported similar results with very low yield of purification of pediocin ACH/PA-1 (class II bacteriocin) of about 1.6% with specific activity at 6.4 x10^5 AU/mg protein (60).

Our purified pediocin PA-1 was active in the nanogram range (0.5AU/nanogram); which is about the same as previously reported, 0.6 AU/ng (55), but higher than that of purified plantaricin W (0.02AU/nanogram) from *Lactobacillus planrarum* PMU33 (46).

The results from MALDI-TOF MS analysis showed major mass of WNK 19 bacteriocin of 4625.485 Da, which is close to the molecular weight of 4624.3 Pediocin PA-1 in the previous report (62). The second peak from MALDI-TOF mass spectrum was a little bigger than the molecular mass of the first peak. It might be possible that the higher mass may come from oxidation of amino acid in the bacteriocin molecule from the first peak. Bacteriocin mass spectrum was further studied by ESI-MS, which revealed two molecular masses of bacteriocin of 4625.2574 Da and 4641.6578 Da (Appendix C). The difference of the two molecular mass was 16.4004 which was close to mass of oxygen atom. It was previously reported that pediocin PA-1 might be oxidized at methionine residue to produce methionine sulfoxide (62)

To confirm that WNK 19 bacteriocin was Pediocin PA-1, PCR technique using specific primers of pediocin PA-1 structural gene (*PedA*) was used. *Ped A* encodes a 62-amino acid pediocin PA-1 precursor called prepediocin PA-1. The 18 N-terminal residues of prepediocin are removed by cellular protease at double glycine position at N-terminal concomitantly with secretion, resulting in mature Pediocin PA-1. The 369 bp PCR product obtained from amplification of the WNK19 genomic DNA was sequenced. The deduce amino acid sequence is shown in Figure 18. From these 44 amino acid residues was calculated for a molecular mass of 4625.07 Da (see appendix

A) which it very close to the mass 4625.485 of WNK19 bacteriocin obtained by MALDI-TOF MS analysis. It can be concluded that pediocin PA-1 had molecular weight of 4625 Da.

The sequence alignment showned 100% homology to that of *Ped A* gene of *Pediococcus acidilactici* PAC1.0 isolate from fermented fruit (Figure 20, Appendix B). This is somewhat surprising because sequence polymorphisms usually happens every 100-200 bp. This bacteriocin might be very indispensable as its sequence is highly conserved.

Many (but not all) of the bacteriocins of Gram-positive bacteria are encoded by plasmid-borne genes (3), such as the two-peptide lantibiotic lacticin 3147 that is located on a 60-kb conjugative plasmid. For Pediocin PA-1, it is reported that the Pediocin PA-1 gene is located on a 9.4 kbp plasmid (63). Our plasmid pattern of WNK19 strain shows the presence of a plasmid of about 9.4 kbp, which migth contain Pediocin PA-1 operon. This may be confirmed by plasmid curing technique or PCR analysis of the extracted 9.4 kbp plasmid.

Pediocin PA-1 was found to be produced mostly by *Pediococcus sp* (42). However, one report (60) showed that pediocin PA-1 was produced from *Lactobacillus plantarum* WHE92 isolated from cheese.

Pediococcus sp, is usually associated with vegetable and meat products. Several pediocin PA-1-producing strains were identified as having different names, such as pediocin AcH, pediocin JD, pediocin 347 and mesentericin 5. But they have been shown to be the same as pediocin PA-1 by molecular technique.

Pediocin PA-1 producing LAB have been empirically used as starter cultures for many fermented meat product such as Wisconsin bacon (6) and fermented cucumbers (6, 22). At the present enormous literatures on this bacteriocin with a potential use as antimicrobial substance have been reported.(15, 22, 64, 65) The use of pediocin PA-1 for food bio-preservative has also been commercially exploited in the form of ALTA 2431, which is a food biopreservative ingredient of pediocin PA-1 produced by *Pediococcus acidilactici* (3). It use is covered by several US and European patents. Finally, we are confident that pediocin PA-1 or other bacteriocins will be included in the next generation of food preservatives.

CHAPTER VI
CONCLUSION

The total of 35,717 isolates from 30 fermented food samples were screened for bacteriocin by direct plating method using *Lactobacillus sakei* subsp. *sakei* JCM1157 as the indicator strain. The detection rate was 2.07 %. One hundred and sixteen isolates from 739 isolates produced antimicrobial substances in broth. Only twenty-four isolated stably produced in broth bacteriocins which were proteinaceous in nature and were inactivated by proteinase treatment.

Seventeen isolates from 24 isolates were chosen for antimicrobial spectrum activity testing. According to similar size of clear zone, antimicrobial spectra of bacteriocins from the 17 isolates could be divided into three groups; a broad (WNK4, 8, 9, 10, 13, 21 and 24), somewhat broad (WNK12, 14, and 19) and narrow spectrum of antimicrobial activity (WNK2, 5, 6, 7, 17 and 18). The isolates from the broad spectrum group inhibited the growth of bacteria in genera of *Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc, Pediococcus* and a number of pathogenic bacteria in the genus of *Staphylococcus*, *Streptococcus*, *Listeria* and *Bacillus.* This broad spectrum of activity group might be class I lantibiotic bacteriocin because of their broad spectrum of antimicrobial activity and heat stability. Bacteriocin of the second group (somewhat broad antimicrobial activity spectrum group), WNK12, 14, and 19, inhibited growth of *Listeria, Enterococcus, Lactobacillus, Leuconostoc* and *Pediococcus,* and might be class II bacteriocin, which is called antilisterial bacteriocin group. The group with narrow spectrum of antimicrobial activity (WNK 2, 5, 6,7,17 and 18) inhibited only lactic acid bacteria such as *Lactobacillus sakei* and/or *Pediococcus pentosaceus*.

Colony PCR technique was used to detected nisin- and plantaricin W-producing strains of the 24 isolates. WNK4, 21, 23, 24 were nisin-producing strain and WNK8, 9, and 13 were plantaricin W-producing strains and the remaining isolates were neither nisin- nor plantaricin W-producing strains.

Of these isolates WNK19, which had somewhat broad antimicrobial activity spectrum was chosen for further studies. WNK19 isolate produced heat stable, antilisterial bacteriocin. On these evidences, bacteriocin of WNK19 might be a member of class II bacteriocins. Purification of WNK 19 bacteriocin was achieved by 3 steps employing (i) hydrophobic interaction chromatography with Amberlite XAD-16 and eluting with 70% isopropanol, (ii) cation-exchange chromatography using SP-sepharose eluting with 0.6 M NaCl, and (iii) reverse-phase HPLC with C_{18} column eluting with 56% acetonitrile. Bacteriocin was purified to homogeneity at this step as shown a single peak of protein eluted with bacteriocin activity. This purified bacteriocin fraction had a molecular weight of 4625.458 by MALDI –TOF MS analysis. This molecular weight of bacteriocin of WNK 19 was nearly identical to that of Pediocin PA-1(4624.3 Da) produced by *Pediococcus acidilactici* PAC1.0.

Plasmids contained in WNK19 showed a pattern of 8 bands. The 9.4 kbp plasmid of this isolate might contain the gene for Pediocin PA-1 production. To confirm that bacteriocin of WNK19 strain contained Pediocin PA-1 gene, PCR amplification was performed with primers specific for Pediocin PA-1 structural gene. A 369 bp product was amplified and its sequence showed 100 % identical to that of *Ped A* gene of *Pediococcus pentosacues.*

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APPENDIX

APPENDIX A

A. The calculation of molecular weight of Pediocin PA-1

Table 13 Atomic masses and average natural abundances of some stable isotopes.

Isotope	Mass	Abundance,%
^{1}H	1.007825	99.985
^{2}H	2.014102	0.015
^{12}C	12.000000	98.90
^{13}C	13.003354	1.10
^{14}N	14.003074	99.634
^{15}N	15.000108	0.366
^{16}O	15.994915	99.762
^{17}O	16.999133	0.038
^{18}O	17.999160	0.200
^{19}F	18.998405	100.00
^{28}Si	27.976927	92.23
^{29}Si	28.976491	4.67
^{30}Si	29.973761	3.10
^{31}P	30.973763	100
^{32}S	31.972074	95.02
^{33}S	32.971461	0.75
^{34}S	33.967865	4.21
^{36}S	35.967091	0.02
^{35}Cl	34.968855	75.770
^{37}Cl	36.965896	24.231
^{79}Br	78.918348	50.69
^{81}Br	80.916344	49.31
^{127}I	126.904352	100.00

```
        10         20         30         40
KYYGNGVTCG KHSCSVDWGK ATTCIINNGA MAWATGGHQG NHKC
```

Figure 19 The amino acid composition of Pediocin PA-1

44 deduced amino acid residues from DNA sequence of 396 bp amplified fragment of WNK 19 strain which was identical to Pediocin PA-1 bacteriocin.

Molecular weight Calculation

Composition	Number	Mass	Total mass
Carbon(C)	196 x	12.000000	= 2352.000
Hydrogen (H)	297x	1.007825	= 299.3240
Nitrogen (N)	61x	14.003074	= 854.1875
Oxygen (O)	60x	15.994915	= 959.6949
Sulfur (S)	5x	31.972074	= 159.8604
			= 4625.067

Calculated Molecular weight of Pediocin PA-1 = 4625.07 Da

APPENDIX B

B.DNA sequencing aliment of *Ped A*

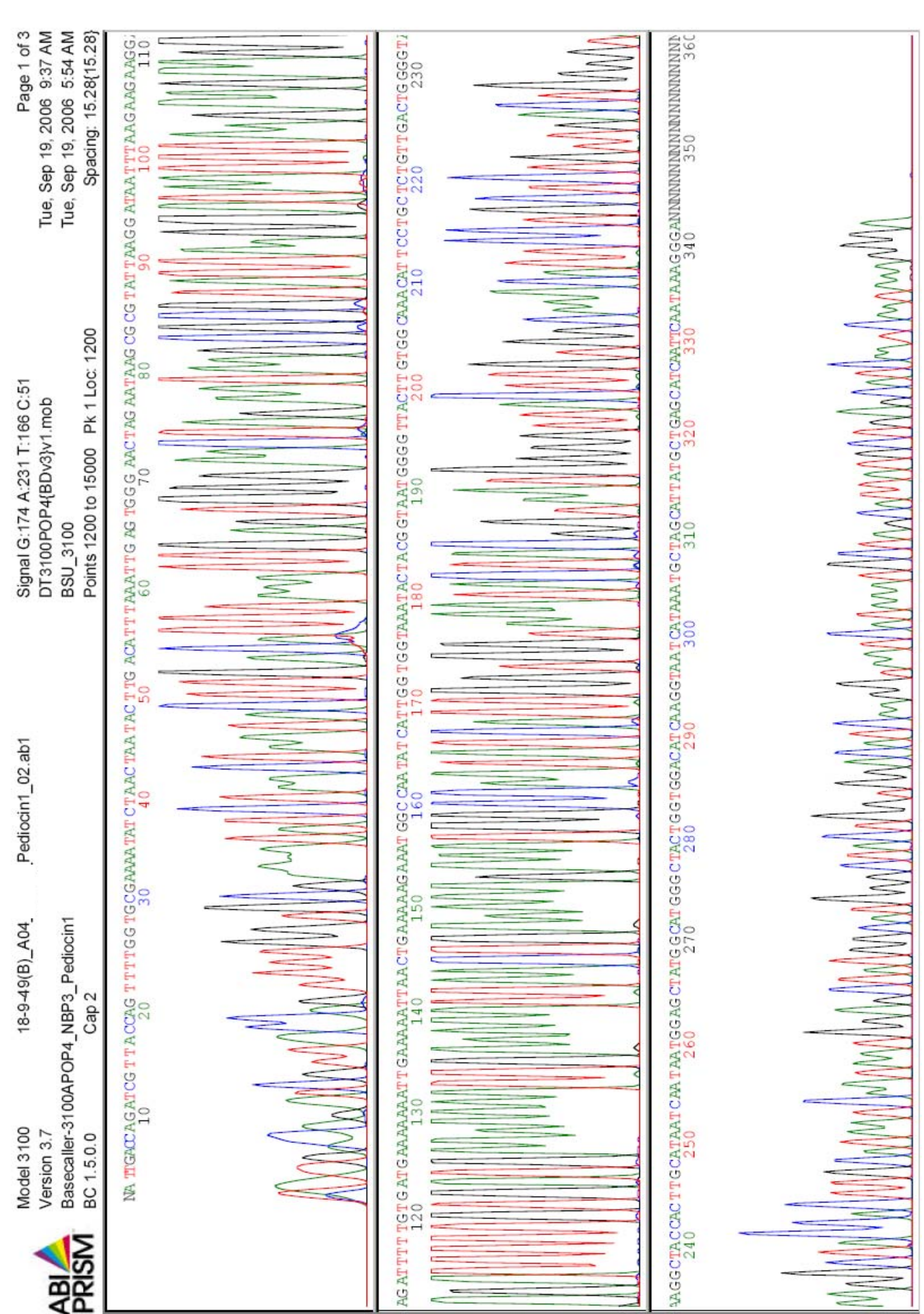
```
WNK19           -------------------------------NATTGACCA-GATCGTTA-CCAGTTTTGG
Ped A 921       AAAGATACTGCGTTGATAGCCAGGTTTCAAAAATTGACCAAGATCGTTAACCAGTTTTGG
                                                ******** ******** **********


WN    K19
TGCGAAAATATCTAACTAATACTTGACATTTAAATTGAGTGGGAACTAGAATAAGCGCGT
Ped A 981       TGCGAAAATATCTAACTAATACTTGACATTTAAATTGAGTGGGAACTAGAATAAGCGCGT
                ************************************************************
                                      RBS
WNK19           ATTAAGGATAATTTAAGAAGAAGGAGATTTTTGTGATGAAAAAAATTGAAAAATTAACTG
Ped A 1041      ATTAAGGATAATTTAAGAAGAAGGAGATTTTTGTGATGAAAAAAATTGAAAAATTAACTG
                ************************************************************


WNK19           AAAAAGAAATGGCCAATATCATTGGTGGTAAATACTACGGTAATGGGGTTACTTGTGGCA
Ped A 1101      AAAAAGAAATGGCCAATATCATTGGTGGTAAATACTACGGTAATGGGGTTACTTGTGGCA
                ************************************************************


WNK19           AACATTCCTGCTCTGTTGACTGGGGTAAGGCTACCACTTGCATAATCAATAATGGAGCTA
Ped A 1161      AACATTCCTGCTCTGTTGACTGGGGTAAGGCTACCACTTGCATAATCAATAATGGAGCTA
                ************************************************************


WNK19           TGGCATGGGCTACTGGTGGACATCAAGGTAATCATAAATGCTAGCATTATGCTGAGCATC
Ped A 1221      TGGCATGGGCTACTGGTGGACATCAAGGTAATCATAAATGCTAGCATTATGCTGAGC-TG
                ********************************************************  *


WNK19           AATTCAATAAAGGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Ped A 1281      GCATCAATAAAGGGGTGATTT---------------------------------------
                   ***********
```

Figure 20 DNA sequence aliment of amplified DNA of WNK 19 and DNA sequence of *Ped* A

396 bp amplified fragment of WNK19 was aligned with the previously report DNA sequence of Pediocin precursor gene (M83924). *Ped A* gene is indicated in green. Ribosome binding site are underline and bold.

```
Query: Pediococcus pentosaceus 16S rRNA gene, strain DSM 20336 (T)
       (gi|12734032) Length=1569

Score = 1612 bits (813), Expect = 0.0
Identities = 813/813 (100%), Gaps = 0/813 (0%)
Strand=Plus/Minus

Query  31    AAGGTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTAC  90
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1478  AAGGTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTAC  1419

Query  91    AAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTT  150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1418  AAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTT  1359

Query  151   CGTGTAGGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTTTTAAGAGATTAGCTTAA  210
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1358  CGTGTAGGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTTTTAAGAGATTAGCTTAA  1299

Query  211   CCTCGCGGTCTCGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATA  270
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1298  CCTCGCGGTCTCGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATA  1239

Query  271   AGGGGCATGATGATTTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCAC  330
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1238  AGGGGCATGATGATTTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCAC  1179

Query  331   TAGAGTGCCCAACTTAATGCTGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTA  390
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1178  TAGAGTGCCCAACTTAATGCTGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTA  1119

Query  391   ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATTCTGTCCCCG  450
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1118  ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATTCTGTCCCCG  1059

Query  451   AAGGGAACCTCTAATCTCTTAGACTGTCAGAAGATGTCAAGACCTGGTAAGGTTCTTCGC  510
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1058  AAGGGAACCTCTAATCTCTTAGACTGTCAGAAGATGTCAAGACCTGGTAAGGTTCTTCGC  999

Query  511   GTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCTTTGA  570
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  998   GTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCTTTGA  939

Query  571   GTTTCAACCTTGCGGTCGTACTCCCCAGGCGGATTACTTAATGCGTTAGCTGCAGCACTG  630
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  938   GTTTCAACCTTGCGGTCGTACTCCCCAGGCGGATTACTTAATGCGTTAGCTGCAGCACTG  879

Query  631   AAGGGCGGAAACCCTCCAACACTTAGTAATCATCGTTTACGGCATGGACTACCAGGGTAT  690
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  878   AAGGGCGGAAACCCTCCAACACTTAGTAATCATCGTTTACGGCATGGACTACCAGGGTAT  819

Query  691   CTAATCCTGTTCGCTACCCATGCTTTCGAGCCTCAGCGTCAGTTGCAGACCAGACAGCCG  750
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  818   CTAATCCTGTTCGCTACCCATGCTTTCGAGCCTCAGCGTCAGTTGCAGACCAGACAGCCG  759

Query  751   CCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCA  810
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758   CCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCA  699

Query  811   CTGTCCTCTTCTGCACTCAAGTCTCCCAGTTTC  843
             |||||||||||||||||||||||||||||||||
Sbjct  698   CTGTCCTCTTCTGCACTCAAGTCTCCCAGTTTC  666
```

Figure 21 Aliment of *Pediococcus pentosaceus* 16S ribosomal RNA and 16S rDNA amplified fragment of WNK 19 strain.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Pediocin1_02.ab1
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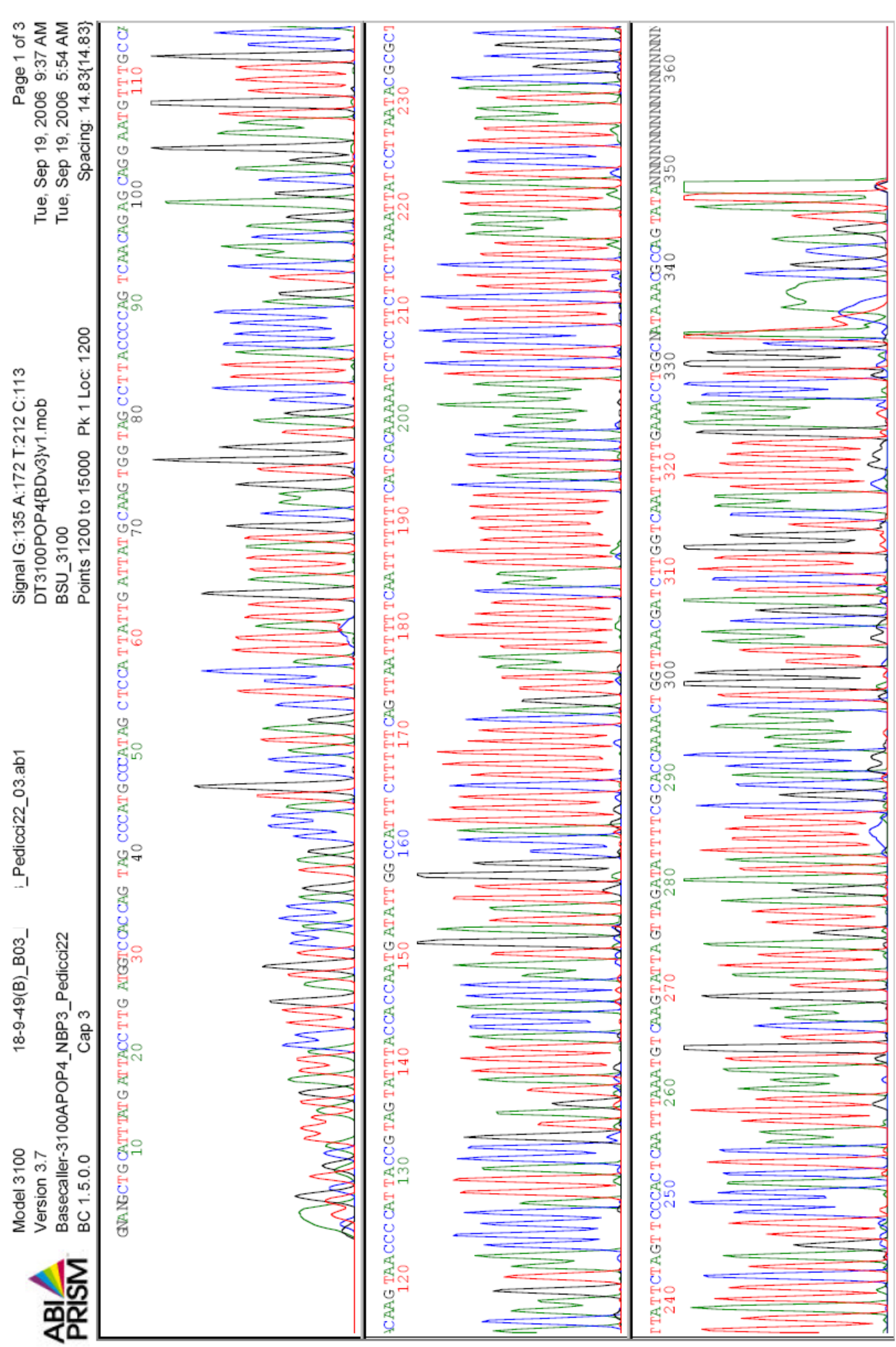
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Version 3.7
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BC 1.5.0.0
18-9-49(B)_B03_
Cap 3
_Pedicci22_03.ab1
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Points 1200 to 15000 Pk 1 Loc: 1200
Page 1 of 3
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Tue, Sep 19, 2006 5:54 AM
Spacing: 14.83{14.83}

APPENDIX C

Mass Spectrum Deconvolution Report

Analysis Info

Analysis Name	D:\Data\SC\Peptide-46.d	Acquisition Date	7/7/2006 1:43:32 PM
Method	HighMass_gradient_pos.m	Operator	Administrator
Sample Name	Peptide-46	Instrument	micrOTOF 72
Comment	II-ASR-11-F2		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	210.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	800.0 V	Set Pulsar Push	383 V
Scan End	20000 m/z	Skimmer 1	80.0 V	Set Reflector	1300 V
		Hexapole 1	35.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V

Peptide-46.d: +MS, 0.4-0.5min #(11-12

550.630

925.6230 5+ (A)

Component	Molecular Mass	Molecule		Absolute Abundance	Relative Abundance
A	4622.0684	4625.2574	[M + H]+	4361	100.00
B	4638.0753	4641.6578	[M + H]+	2331	53.45

Component A Detail

Actual Peak	Charge	Isotopic Mass([M + H]+)	Predicted Peak
771.1860	6+	4622.0770	771.1846
771.3576	6+	4623.1064	771.3518
771.5036	6+	4623.9823	771.5190
771.6842	6+	4625.0662	771.6863
771.8532	6+	4626.0802	771.8535
772.0208	6+	4627.0858	772.0207
772.1921	6+	4628.1137	772.1879
772.3725	6+	4629.1961	772.3551
772.4965	6+	4629.9396	772.5224
925.2238	5+	4622.0878	925.2199
925.4258	5+	4623.0978	925.4206
925.6230	5+	4624.0837	925.6213
925.8239	5+	4625.0880	925.8219
926.0239	5+	4626.0884	926.0226
926.2231	5+	4627.0840	926.2233
926.4258	5+	4628.0976	926.4239
926.6215	5+	4629.0761	926.6246
926.8306	5+	4630.1215	926.8253
1156.2740	4+	4622.0726	1156.2730
1156.5310	4+	4623.1006	1156.5238
1156.7771	4+	4624.0849	1156.7746
1157.0282	4+	4625.0892	1157.0255
1157.2791	4+	4626.0931	1157.2763
1157.5351	4+	4627.1168	1157.5271
1157.7778	4+	4628.0878	1157.7780
1158.0222	4+	4629.0654	1158.0288

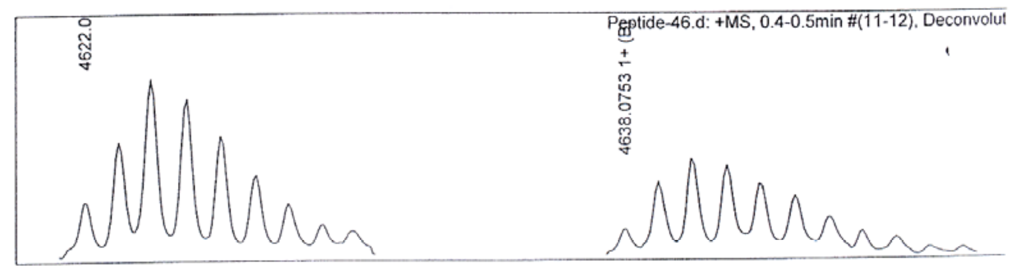
Molecular Mass ([M + H]+): 4622.0684 Std. Deviation: 0.044266
Average Mass ([M + H]+): 4625.2574

Mass Spectrum Deconvolution Report

Component B Detail

Actual Peak	Charge	Isotopic Mass([M + H]+)	Predicted Peak
774.0258	6+	4639.1156	774.0196
774.1868	6+	4640.0819	774.1868
774.3589	6+	4641.1140	774.3541
774.5052	6+	4641.9921	774.5213
774.6872	6+	4643.0838	774.6885
774.8618	6+	4644.1316	774.8557
928.4254	5+	4638.0956	928.4213
928.6230	5+	4639.0839	928.6220
928.8217	5+	4640.0772	928.8226
929.0244	5+	4641.0909	929.0233
929.2241	5+	4642.0890	929.2240
929.4270	5+	4643.1038	929.4247
929.6215	5+	4644.0761	929.6253
929.8170	5+	4645.0539	929.8260
930.0204	5+	4646.0709	930.0267
1160.2812	4+	4638.1012	1160.2747
1160.5341	4+	4639.1130	1160.5255
1160.7753	4+	4640.0777	1160.7764
1161.0270	4+	4641.0845	1161.0272
1161.2824	4+	4642.1059	1161.2780
1161.5299	4+	4643.0962	1161.5289
1161.7821	4+	4644.1050	1161.7797
1162.0290	4+	4645.0927	1162.0305
1162.2728	4+	4646.0678	1162.2814
1162.5313	4+	4647.1016	1162.5322
1162.7682	4+	4648.0493	1162.7830

Molecular Mass ([M + H]+): 4638.0753 Std. Deviation: 0.0303269
Average Mass ([M + H]+): 4641.6578



Component	Molecular Mass	Molecule		Absolute Abundance	Relative Abundance
A	4622.0684	4625.2574	[M + H]+	4361	100.00
B	4638.0753	4641.6578	[M + H]+	2331	53.45

Component A Detail

Actual Peak	Charge	Isotopic Mass([M + H]+)	Predicted Peak
771.1860	6+	4622.0770	771.1846
771.3576	6+	4623.1064	771.3518
771.5036	6+	4623.9823	771.5190
771.6842	6+	4625.0662	771.6863
771.8532	6+	4626.0802	771.8535
772.0208	6+	4627.0858	772.0207
772.1921	6+	4628.1137	772.1879
772.3725	6+	4629.1961	772.3551
772.4965	6+	4629.9396	772.5224

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925.2238	5+	4622.0878	925.2199
925.4258	5+	4623.0978	925.4206
925.6230	5+	4624.0837	925.6213
925.8239	5+	4625.0880	925.8219
926.0239	5+	4626.0884	926.0226
926.2231	5+	4627.0840	926.2233
926.4258	5+	4628.0976	926.4239
926.6215	5+	4629.0761	926.6246
926.8306	5+	4630.1215	926.8253
1156.2740	4+	4622.0726	1156.2730
1156.5310	4+	4623.1006	1156.5238
1156.7771	4+	4624.0849	1156.7746
1157.0282	4+	4625.0892	1157.0255
1157.2791	4+	4626.0931	1157.2763
1157.5351	4+	4627.1168	1157.5271
1157.7778	4+	4628.0878	1157.7780
1158.0222	4+	4629.0654	1158.0288

Molecular Mass ([M + H]+): 4622.0684 Std. Deviation: 0.044266
Average Mass ([M + H]+): 4625.2574

Component B Detail

Actual Peak	Charge	Isotopic Mass([M + H]+)	Predicted Peak
774.0258	6+	4639.1156	774.0196
774.1868	6+	4640.0819	774.1868
774.3589	6+	4641.1140	774.3541
774.5052	6+	4641.9921	774.5213
774.6872	6+	4643.0838	774.6885
774.8618	6+	4644.1316	774.8557
928.4254	5+	4638.0956	928.4213
928.6230	5+	4639.0839	928.6220
928.8217	5+	4640.0772	928.8226
929.0244	5+	4641.0909	929.0233
929.2241	5+	4642.0890	929.2240
929.4270	5+	4643.1038	929.4247
929.6215	5+	4644.0761	929.6253
929.8170	5+	4645.0539	929.8260
930.0204	5+	4646.0709	930.0267
1160.2812	4+	4638.1012	1160.2747
1160.5341	4+	4639.1130	1160.5255
1160.7753	4+	4640.0777	1160.7764
1161.0270	4+	4641.0845	1161.0272
1161.2824	4+	4642.1059	1161.2780
1161.5299	4+	4643.0962	1161.5289
1161.7821	4+	4644.1050	1161.7797
1162.0290	4+	4645.0927	1162.0305
1162.2728	4+	4646.0678	1162.2814
1162.5313	4+	4647.1016	1162.5322
1162.7682	4+	4648.0493	1162.7830

Molecular Mass ([M + H]+): 4638.0753 Std. Deviation: 0.0303269
Average Mass ([M + H]+): 4641.6578

BIOGRAPHY

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