DETECTION OF BACTERIOCINS FROM STARCH-UTILIZING AND LACTIC ACID-PRODUCING BACTERIA

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การตรวจหาแบคทีริโอซินจากแบคทีเรียที่สามารถใช้แป้งและผลิตกรดแลคติก

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แบคทีริโอซินเป็นสารประกอบโปรตีนที่ผลิตจากแบคทีเรียซึ่งมีฤทธิ์ยับยั้งการเจริญของจุลินทรีย์อื่นโดยเฉพาะอย่างยิ่งแบคทีเรีย และมีการนำไปใช้ประโยชน์อย่างกว้างขวางในอุตสาหกรรมอาหาร การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาและศึกษาแบคทีริโอซินที่ผลิตจากแบคทีเรียที่สามารถใช้แป้งและผลิตกรดแลคติก เพื่อเพิ่มศักยภาพของกระบวนการผลิตกรดแลคติกและการผลิตสารแบคทีริโอซิน จากการทดสอบความสามารถในการผลิตแบคทีริโอซินของแบคทีเรียที่สามารถใช้แป้งและผลิตกรดแลคติกจำนวน 119 ไอโซเลท พบหนึ่งไอโซเลทคือ A5UVU25 ซึ่งจัดอยู่ในสกุล *Lactococcus* ที่สามารถผลิตแบคทีริโอซิน แบคทีเรียไอโซเลทดังกล่าวผลิตสารได้เมื่อเจริญในอาหารเลี้ยงเชื้อที่มีน้ำตาลกลูโคสหรือแป้งมันสำปะหลังเป็นแหล่งคาร์บอน โดยผลิตแบคทีริโอซินได้สูงสุด 20.31 หน่วยสากลต่อมิลลิลิตร (เทียบกับสารมาตรฐานไนซิน) เมื่อเลี้ยงในอาหารที่ประกอบด้วย แป้งมันสำปะหลัง ทริปโตน สารสกัดจากเนื้อ สารสกัดจากยีสต์ ไดโพแทสเซียมไฮโดรเจนฟอสเฟต โพแทสเซียม-ไดไฮโดรเจนฟอสเฟต แมกนีเซียมซัลเฟต แมงกานีสซัลเฟต ไตร-แอมโมเนียมซิเตรต และทวีน 80 ปริมาณ 1.0, 1.0, 0.15, 0.4, 0.87, 0.8, 0.02, 0.005, 0.2 และ 0.1 เปอร์เซ็นต์ ตามลำดับ ในสภาวะไร้ออกซิเจนที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 18 ชั่วโมง และใช้กล้าเชื้อ (10^6 เซลล์ต่อมิลลิลิตร) 10 เปอร์เซ็นต์ แบคทีริโอซินที่ผลิตโดยไอโซเลทที่คัดเลือกนี้สามารถยับยั้งการเจริญของเชื้อทดสอบโดยเฉพาะอย่างยิ่ง *Bacillus stearothermophilus* TISTR329, *Listeria monocytogenes* DSM1327 และ *Micrococcus luteus* TISTR884 และเป็นสารโปรตีนที่มีน้ำหนักโมเลกุล 12 กิโลดาลตัน มีความเสถียรต่อไลโซไซม์และความร้อนที่ 80 องศาเซลเซียส นาน 15 นาที แต่ถูกทำลายด้วยโปรตีเอส โปรติเนสเค และความร้อนที่ 100 องศาเซลเซียส นาน 30 นาที เมื่อตรวจหายีนที่ควบคุมการผลิตแบคทีริโอซินของไอโซเลท A5UVU25 ด้วยไพรเมอร์ที่จำเพาะกับยีนที่ควบคุมการผลิตแบคทีริโอซินของแบคทีเรียแลคติกชนิดที่ใกล้เคียงกัน สามารถตรวจจับยีนได้ด้วยไพรเมอร์ที่จำเพาะกับไนซินซึ่งได้ชิ้นดีเอ็นเอขนาด 218 คู่เบส ที่มีลำดับเบสเหมือน (100 เปอร์เซ็นต์) กับยีนที่ควบคุมการสร้างไนซิน Z ของ *Lactococcus lactis* subsp. *lactis*

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา จิตราภรณ์ แสนสิทธิ์

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BACTERIOCIN/BACTERIA/CASSAVA STARCH/LACTIC ACID

Bacteriocins, proteinaceous compounds produced by bacteria that have antimicrobial activity especially against bacteria, are currently much applied widely in food industry. This study aims to detect and study bacteriocins produced by starch-utilizing and lactic acid-producing bacteria for increasing the potential production of both lactic acid and bacteriocins. A total of 119 isolates of starch-utilizing and lactic acid-producing bacteria were tested for their bacteriocin production capability. Only one isolate, A5UVU25, identified as belonging to the genus *Lactococcus*, was found to produce bacteriocins in the medium containing either glucose or cassava starch as a carbon source. The maximum production of bacteriocins, 20.31 IU/ml, was obtained when the isolate A5UVU25 was cultured in the medium containing (%): cassava starch, 1.0; tryptone, 1.0; beef extract, 0.15; yeast extract, 0.4; di-potassium hydrogen phosphate, 0.87; potassium di-hydrogen phosphate, 0.8; magnesium sulfate, 0.02; manganese sulfate, 0.005; tri-ammonium citrate, 0.2, and tween 80, 0.1, under anaerobic condition at 30°C for 18 h, and the inoculum size (10^6 cells/ml) at 10% (v/v) was used. The bacteriocin produced by the selected isolate showed its antimicrobial activity, especially against *Bacillus stearothermophilus* TISTR329, *Listeria monocytogenes* DMS1327, and *Micrococcus luteus* TISTR884. The

bacteriocin was a protein having its molecular weight of 12 kDa. It was stable to lysozyme and heat at 80°C for 15 min but it was sensitive to protease, protinase K, and heat at 100°C for 30 min. When the gene(s) encoding bacteriocin of the isolate A5UVU25 was detected using bacteriocin-specific primers of closely related lactic acid bacterial species, the amplified product (218 bp) was obtained when nisin-specific primers were applied. The amplicon had similar sequence (100% homology) to gene encoding nisin Z of *Lactococcus lactis* subsp. *lactis*.

School of Microbiology

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LIST OF ABBREVIATIONS

AU	Arbitrary unit
BLAST	Basic Local Alignment Search Tool
bp	Base pair
°C	Degree Celsius
CFU	Colony forming unit
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
dTTP	Deoxythymidine triphosphate
(k) Da	(kilo) Dalton
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
et al.	et alia (and others)
(m, μ) g	(milli, micro) Gram
h	Hour
IU	International unit
(m, μ) l	(milli, micro) Litre
(m, μ) M	(milli, micro) Molar

LIST OF ABBREVIATIONS (Continued)

(c, m) m	(centri, milli) Metre
min	Minute
(m, μ) mol	(milli, micro) Mole
%	Percent
PCR	Polymerase chain reaction
rpm	Round per minute
RU	Reading units
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	Siriraj hospital
sp.	Species
TISTR	Thailand Institute of Scientific and Technology Research
w/v	Weight by volume
v/v	Volume by volume
UV	Ultraviolet

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Bacteriocins are proteins or protein complexes produced by bacteria, and have antimicrobial activity against closely related species and various Gram-positive bacteria including food spoilage bacteria and pathogens (Gaeng *et al*., 2000; Meghrouse *et al*., 1999; Ra *et al*., 1999). Most of bacteriocins have been isolated from lactic acid bacteria involved in food fermentation. Lactic acid bacteria that found to produce bacteriocins are classified into several genera including *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus* (Hoover and Steenson, 1993). The best-known bacteriocin is nisin produced by *Lactococcus lactis*. It is the only substance which has been approved for use as food additive in many countries (Lee and Paik, 2001). There is currently much interested in the application of bacteriocins in food preservation and the inhibition of pathogenic bacteria in food industry, for example, nisin A, sakacin A, and carnobacteriocin BM1, which are bacteriocins that have antimicrobial activity against the pathogen *Listeria* sp. (Mahon and Manuselis, 2000; Naidu, 2000; Ryan *et al*., 1996).

Lactic acid bacteria produce lactic acid as the major end product of carbohydrate fermentation. They are generally associated with habitats rich in nutrients, such as milk, meat, beverages and vegetables (Kelly *et al*., 1996; Lee and

Paik, 2001), and are used extensively in food-processing products, particularly dairy, alcoholic beverage, meat, and vegetable products. Lactic acid bacteria are also used as human and animal health-promoters called probiotics (Fuller, 1997).

L(+)-Lactic acid produced by the bacterial fermentation of glucose is used as a raw material for biodegradable polymer production, and for food and cosmetic preservation (Mostra, 2002). Based on a cheap and abundant raw material, the homolactic starch-utilizing bacteria are great benefit to the lactic acid fermentation process (Rodtong, 2001; Rodtong *et al*., 2001; Rodtong and Ishizaki, 2003). The starch-utilizing bacterium strain, that could potentially produce both lactic acid and bacteriocins, will be very useful for both the acid and antibacterial compound production. This study aims to detect and investigate bacteriocins from starch-utilizing and lactic acid-producing bacteria.

1.2 Research objectives

The purposes of this research are as follows:

1) To detect bacteriocins produced by starch-utilizing and lactic acid-producing bacteria,
2) To detect gene(s) encoding bacteriocins from selected strains,
3) To basically characterize the selected bacteriocin (broad spectrum bacteriocin) produced by the selected strains.

1.3 Scope and limitations of the study

Starch-utilizing and lactic acid-producing bacteria will be collected and screened for their bacteriocin activity against test organisms which will be focused on

food spoilage bacteria and food-borne pathogens using the agar plate diffusion assay. Gene(s) encoding bacteriocins of the strains revealing broad spectrum activity of the compounds will be detected. A broad spectrum bacteriocin will be selected for the preliminary characterization.

1.4 Expected results

Bacteriocins, hopefully broad spectrum bacteriocins produced from starch-utilizing and lactic acid-producing bacteria, will be achieved. The bacteriocins could be useful for the application in food and lactate industry. Data of gene(s) encoding bacteriocins produced by starch-utilizing and lactic acid-producing bacteria will also be obtained. The results will be the prerequisite data for gene cloning as well as the detection and identification bacteriocin-producing strains. The information of bacteriocin gene(s) sequenced could be used to develop probes for the detection of bacteriocin genes in the future.

CHAPTER II

LITERATURE REVIEW

2.1 Starch-utilizing and lactic acid-producing bacteria

Starch is one of the most common storage compounds, and consists of 25% amylose and 75% amylopectin. Amylose is a long and unbranched chain of glucose in α-(1,4) linkage whereas amylopectin is a highly branched form of starch in which the backbone consists of glucose chains in α-(1,4) linkage with α-(1,6) linkages at the branch points. Amylose can be hydrolyzed by α-amylases, which cleave the α-(1,4) linkages to yield a mixture of α-glucose and α-maltose. Amylose is also hydrolyzed by β-amylases producing β-maltose. These enzymes also hydrolyze amylopectin to yield glucose, maltose, and a branched core, but it is not completely degradation. The α-(1,6) linkage in branch is hydrolyzed by α-(1,6)-glucosidase. Thus, the combined action of α-(1,6)-glucosidase and α-amylase is required to completely degrade amylopectin to glucose and maltose. Both α- and β-amylases and α-(1,6)-glucosidase are produced by several bacillus strains, for example, *Bacillus stearothermophilus*, *B. thermooleovorans, B. amyloliquefaciens, B. licheniformis, B. subtilis, B. acidopullyticus* (Kim *et al.*, 1989; Moat *et al.*, 2002; Narang and Satyanarayana, 2001). Interestingly, the amylases produced by a variety of *Bacillus* species have been studied intensively because of their industrial application. *Bacillus acidopullyticus* and *B. stearothermophilus* are thermophilic species. They produce amylases that are stable

at temperatures ranging from 58°C to 80°C. Moreover, *Bacillus licheniformis* and *B. amyloliquefaciens*, mesophilic species, also produce amylases that are active at temperature in excess of 75°C. Thus, the thermostability of their amylases has been considered interest.

Starch could be used as a raw material for their industrial production of lactic acid, and lactic acid bacteria play the most important role for lactic acid production (Guyot *et al.*, 2000). Some lactic acid bacteria are able to produce amylases, for example, *Lactobacillus plantarum*, *Lb. acidophilus*, *Lb. amylovorus*, *Lb. cellobiosus*, *Streptococcus bovis*, and *Pediococcus damnosus* (Escamilla *et al.,* 2000; Pintado *et al.,* 1999).

2.2 Lactic acid bacteria

According to Axelsson (1998), lactic acid bacteria are Gram-positive; cocci or rods; anaerobic, microaerophilic, or aero-tolerant; and catalase negative. They produce lactic acid as the major end product during fermentation of carbohydrate. They are mesophilic. Some can grow below 5°C and some as high as 45°C. With respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most can grow in the pH range of 4.0-4.5. Genera of lactic acid bacteria are *Aerococcus*, *Alloiococcus*, *Bifidobacterium*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. The classification of lactic acid bacteria into different genera is largely based on their cell morphology, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance.

Depending on species, lactic acid bacteria synthesize either L(+) or D(−) isomer of lactic acid or both. Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Embden-Meyerhof pathway (Glycolysis) results in almost exclusively lactic acid as end-product under standard conditions, and the metabolism is referred to as homolactic fermentation. The homofermenters are *Aerococcus*, *Carnobacterium*, *Enterococcus, Lactococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus,* and some species of *Lactobacillus.* In the case of heterolactic fermentation, bifidum pathway used by *Bifidobacterium*, and 6-phosphogluconate/phosphoketolase pathway used by *Leuconostoc*, *Oenococcus*, *Weissella*, and some species of *Lactobacillus,* are mainly sugar fermentation pathway that results in significant amounts of other end-products such as ethanol, acetate, and carbon dioxide in addition to lactic acid.

Lactic acid bacteria can produce a variety of antimicrobial compounds, which provide these organisms a competitive advantage over other microorganisms. The antimicrobial compounds include lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, diacetyl, as well as bacteriocins (Gould, 1995; Herbin, 1997; Mishra and Lambert, 1996; Lee and Paik, 2001). These inhibitory compounds have difference in antimicrobial property. The pH reduction by lactic acid or acetic acid has effect to cellular metabolism with retardation of the growth of several contaminated microorganisms. The antimicrobial effects of hydrogen peroxide resulted from the oxidation of sulfhydryl groups cause denaturing of enzymes that are able to destroy many pathogens. The hydrogen peroxide may also be as a precursor for the production of free radicals, which can damage DNA of other microorganisms. Carbon dioxide may exert its antimicrobial effect in several ways such as by rendering the

environment more anaerobic, inhibiting the enzymatic decarboxylation, and disrupting cell membrane with the accumulation of the gaseous phase in the lipid bilayer. Diacetyl inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization. Antimicrobial activities of bacteriocins are the insertion and pore formation, the depolarization of the target membrane, and leading to the rapid efflux of low molecular weight compounds of the target cell (Bruno and Montville, 1993; Carlsson *et al*., 1983; Gould, 1995; Mishra and Lambert, 1996; Ouwehand, 1998).

2.3 Applications of lactic acid bacteria

Lactic acid bacteria have advantages for both food and lactic acid production industries. They have been used for many years in the manufacture and ripening of fermented foods and beverages such as fermented meats, bread, fermented vegetables, and wines (Kanatani *et al*., 1995; Kelly *et al*., 1996; Lee and Paik, 2001; Martinez, 1999). The main role of lactic acid bacteria in food manufacturing is to acidify raw materials by producing large amounts of lactic acid (homofermentative bacteria), or lactic acid along with acetic acid, carbon dioxide, and ethanol (heterofermentative bacteria). In particular in dairy industry, lactic acid bacteria are responsible for milk acidification and curd formation, and applied as starter cultures for the production of dairy products (Dufour *et al*., 1991; Rattanachaikunsopon and Phumkhachorn, 2000; Vedamuthu, 1998). There are both mesophilic and thermophilic starters classified by their optimum growth temperatures, 30-35°C and 40-50°C respectively. The species of mesophilic starters including *Lactococcus lactic* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis, Leuconostoc*

lactis, and *Leuconostoc cremoris*, are used in the production of several cheese varieties, fermented milk products, and ripened cream butter. Thermophilic lactic starter cultures, usually *Lactobacillus bulgaricus*, *Lactobacillus helvaticus*, and *Streptococcus thermophilus*, are used in the manufacture of yogurt and related fermented milks, and Swiss- and Italian-type cheese (Robinson, 2002; Trepanier *et al*., 1998). Nisin, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, has been applied as food preservative in several countries. It has been used to control some food-borne pathogens, especially some species of the genera *Aeromonas, Bacillus*, *Clostridium*, *Enterococcus*, *Listeria, Micrococcus,* and *Staphylococcus* (Benkerroum, 2000; Halami, 2000; Hoover and Steenson, 1993; Lee and Paik, 2001; Meghrous *et al.*, 1999; Noonpakdee *et al.*, 2003; O'Keeffe, 1999; Rasch and Knochel, 1998; Shillinger *et al.*, 2001).

Lactic acid bacteria have also been reported to be applied as potential health-benefiting microorganisms for human and animals. This phenomenon is being exploited in the food industry in the form of probiotics. Lactic acid bacterial strains that have been reported to be used as probiotics belong to genera *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Bifidobacterium.* They have several health-promoting properties including the improvement of nutritional valve of food, control of intestinal infections, improvement of lactose digestion, and control of mineralization and serum cholesterol level. At present, probiotics have more advantages in food and pharmaceutical industries (Benno *et al.*, 1996; Fuller, 1992; Fuller, 1997; Hosoda *et al.,* 1994; Huris *et al.*, 1994; Isolauri *et al.*, 1991; Ling *et al.*, 1992; Marteau and Rambaud, 1993; Mishra and Lambert, 1996; Moore and Moore, 1995).

In lactate industry, lactic acid bacteria are used to produce lactic acid from glucose, particularly L(+)-lactic acid which is the preferred isomer as a raw material for biodegradable polymer production (Savijoki and Palva, 1997). Polylactic acid has been applied for bioplastic and biodegradable packing material production. Several cosmetic products contain lactic acid, such as soap, skin care, oral care, and hair care products. L(+)-Lactic acid is also useful in the field of biomedical or surgical applications, particularly surgical sewing thread, and the shape of screws and plates for repairing broken bones (Mostra, 2002). Several species in genera *Carnobacterium*, *Enterococcus*, *Lactococcus*, and *Vagococcus*, could potentially produce L(+)-lactic acid (Axelsson, 1998; Kagermeier-Callaway and Lauer, 1995; Teuber *et* al., 1991). Based on the cheap and abundant starchy raw material, the starch-utilizing and lactic acid-producing strains are useful for the lactic acid fermentation process (Rodtong, 2001; Rodtong *et al.,* 2001). Some of these strains have been identified as belonging to the genus *Lactococcus*.

2.4 Bacteriocins produced by lactic acid bacteria

Bacteriocins are proteins or protein complexes which have antimicrobial activity against closely related bacteria (Tagg *et al*., 1976 quote in Martinez *et al*., 1999). The concept is widened to include peptides of a fairly broad inhibitory spectrum since they have been found to inhibit several food-borne pathogens, for example, *Bacillus, Clostridium*, *Listeria*, and *Staphylococcus* (Castellano *et al.*, 2004; Gaeng *et al*., 2000; Jay, 1992; Meghrouse *et al*., 1999; Ra *et al*., 1999; Zheng *et al*., 1999).

Most bacteriocins act on sensitive cells by destabilization and permeabilization of the cytoplasmic membrane through the formation of transitory poration complexes or ionic channels, that causes the reduction or dissipation of the proton motive force (Cintas *et al.*, 1998; Herranz *et al.*, 1999; Luders *et al.*, 2003). All bacteriocin-producing strains require themselves to contain protection mechanisms in order to prevent cell death due to the action of their own bacteriocins by the expression of a specific immunity protein. The protein is generally encoded in the bacteriocin operon (Cintas *et al.*, 2001; McAuliffe *et al.*, 2000).

A large number of bacteriocins produced by lactic acid bacteria have been identified and characterized. Cintas *et al*. (2001) and Cleveland *et al*. (2001) summarized that bacteriocins can classified into three groups: Class I, lantibiotics; Class II, small heat-stable bacteriocins; and Class III, large heat-labile bacteriocins.

2.4.1 Class 1, lantibiotics

Lantibiotics are bacteriocins characterized by the presence of lanthionine-type thioester bonds between certain side chains of amino acid residues of the protein. These modifications are introduced into the peptide post-translationally. Examples of lantibiotics produced by lactic acid bacteria are shown in Table 1.

Nisin is the best well known bacteriocin in this class. It is produced by some strains of *Lactococcus lactis*, which is encoded by the 174-bp *nis*A gene (also termed *spa*N) (Gould, 1995). The pre-nisin molecule, immediately after translation, is a 57-amino acid peptide with no unusual amino acid or secondary structure. Pro-nisin is formed after the cleavage of the initial 23-amino acid leader peptide (Figure 1). The mature active molecule is formed after the dehydration steps (converting serine and threonine to dehydroalanine and dehydrobutyrine, respectively). The sulphide ring

between these modified residues and cysteine residues results in the formation of one lanthionine and four-β-methyllanthionines (Figure 2). The molecule is cationic and hydrophobic in character (Dabard *et al.*, 2001; Hindre *et al.*, 2003).

The model-type lantibiotic nisin is discussed as an example for modes of action of lantibiotics. Nisin has a broad spectrum of inhibitory activity affecting to various Gram-positive bacteria including lactic acid bacteria, bacilli, and clostridia (Olasupo *et al*., 1999). *Escherichia coli* and other Gram-negative bacteria are affected when their outer membranes are sublethally damaged. Under these conditions, other bacteriocins also show antimicrobial activity against Gram-negative bacteria. The primary target of nisin is the cell membrane. For its interaction, nisin does not need any receptor, unlike some other antimicrobial peptides. However, it does need the presence of a membrane potential (Beuchat *et al*., 1997; Bruijff *et al*., 1998).

The activity of nisin is measured in reading units (RU) or international units (IU), and is based upon the antimicrobial activity of 1 microgram (μg) of a standard batch of commercial nisin. The approximate activity of 1 μg of pure nisin is 40 RU or IU (Naidu, 2000; Yousef and Carlstrom, 2003). Nisin is the only one bacteriocin that is being commercially produced and used. It is produced by the fermentation of milk and nisin-producing lactococci, and is sold as a commercial preparation under the tradename of Nisaplin® by Aplin and Barret Ltd. (U.K.). It has been approved by the World Health Organization for use in foods since 1968, but it has been gained approval from the U.S. Food and Drug Administration since 1988. Nisin is currently approved in over 50 countries worldwide, and has found to be applied in many different food systems (Gould, 1995; Martirani, 2002).

Table 1. Lanthionine-containing bacteriocins (class I) produced by lactic acid bacteria.

Bacteriocin	Producer strain	Molecular mass (kDa)	Sensitive organism
Carnocin UI49	*Carnobacterium piscicola* UI49	4.6	*Carnobacterium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Pediococcus* sp.
Lacticin 481	*Lactococcus lactis* 481	2.9	*Clostridium tyrobutyricum*
Lactocin S	*Lactobacillus sake* 145	3.7	*Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp.
Lactococcin	*Lactococcus lactis* ADRI85L030	2.3	*Clostridium tyrobutyricum*, *Lactobacillus helveticus*, *Streptococcus thermophilus*
Nisin	*Lactococcus lactis* subsp. *lactis*	3.5	*Bacillus* sp., *Clostridium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Listeria* sp., *Micrococcus* sp., *Mycobacterium* sp., *Pediococcus* sp., *Staphylococcus* sp., *Streptococcus* sp.
Salivaricin	*Streptococcus salivarius*	2.3	*Micrococcus luteus*

Source: Nes *et al*. (1996).

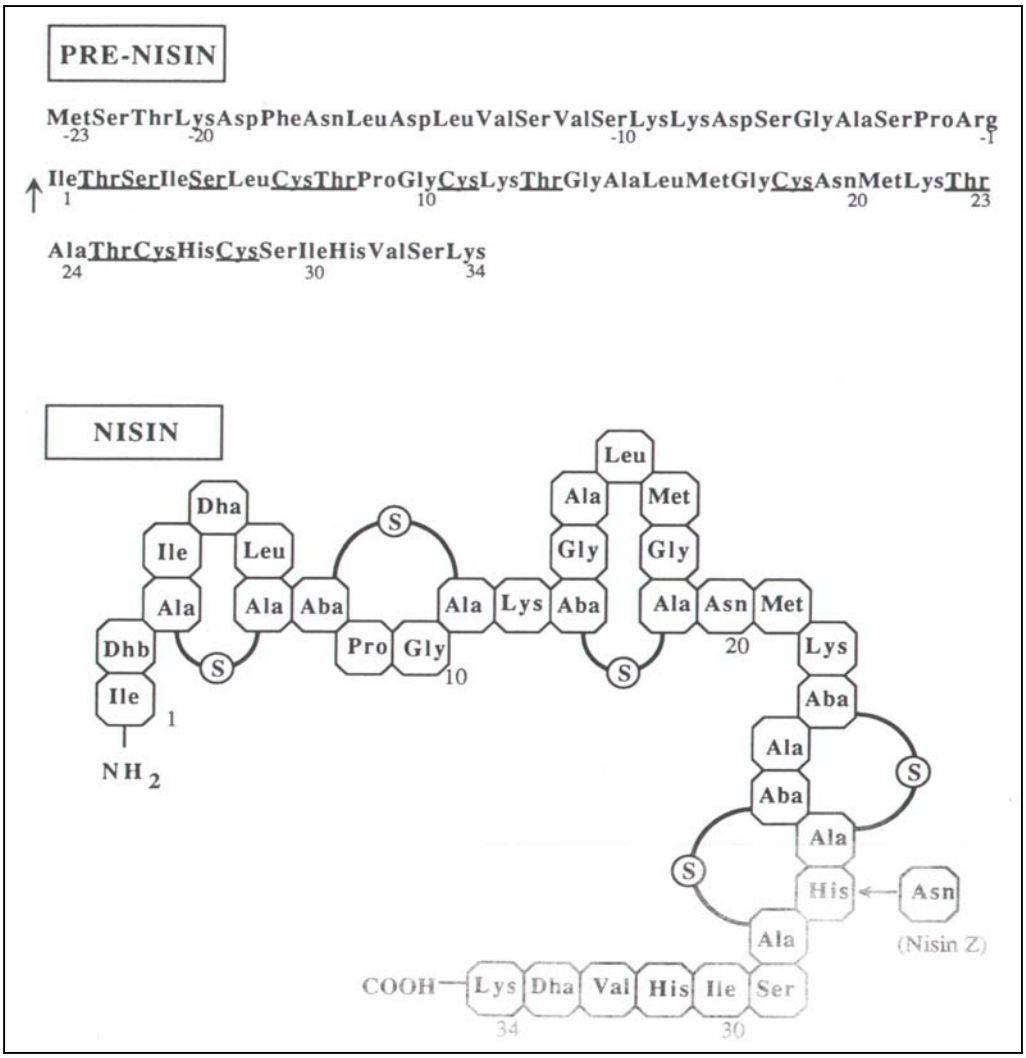


Figure 1. Structures of pre-nisin and nisin. Pre-nisin shows the amino acid sequence immediately post-translation. The cleavage site is indicated by an arrow. The residues which will be modified are underlined. Nisin shows the pentacyclic structure of the mature inhibitor. The molecule shown is nisin A, but the substitution of (Asn) for (His_{27}) in nisin Z is indicated.

Source: Gould (1995).

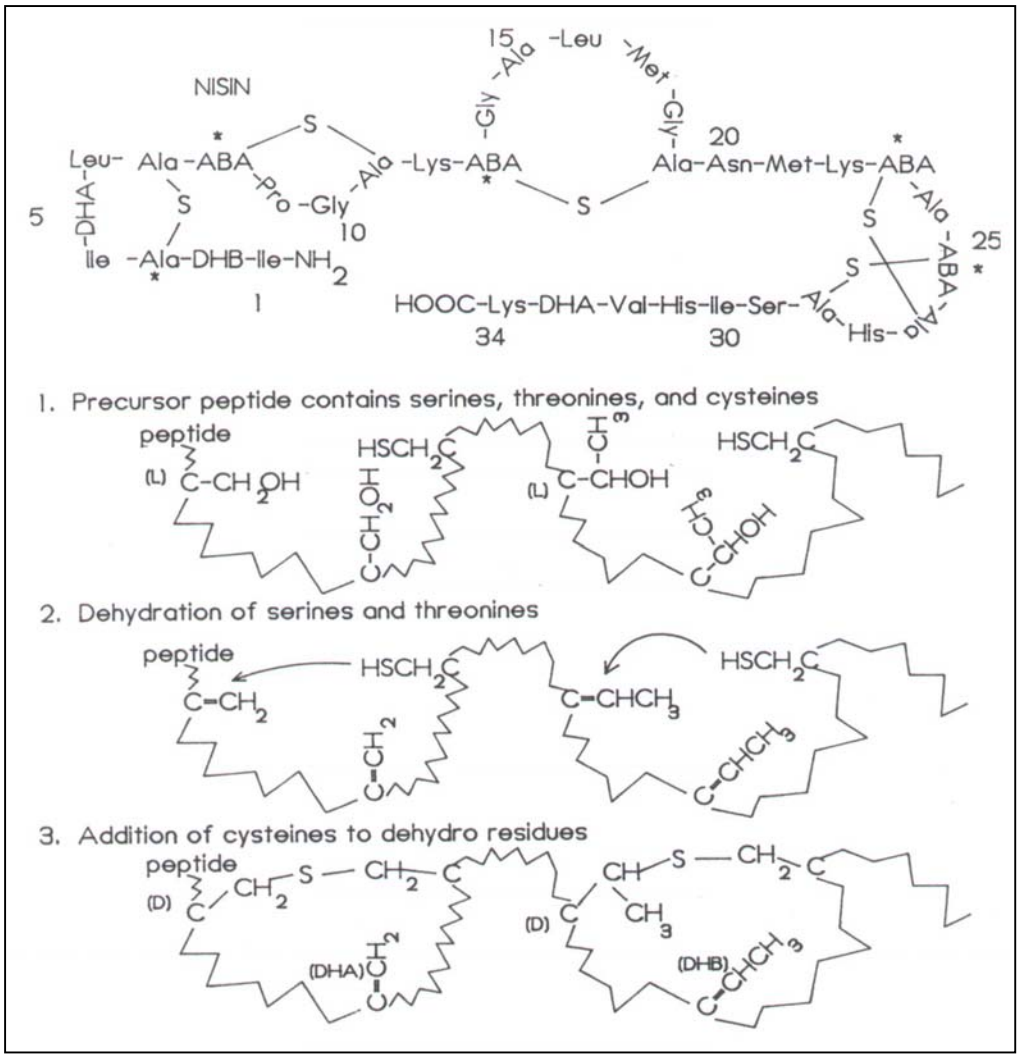


Figure 2. Structure of nisin (5, top) and scheme for formation of unusual amino acids (bottom, 1-3).

Source: Hoover and Steenson (1993).

Lacticins are bacteriocins produced by some strains of *Lactococcus lactis* (Keren *et al.*, 2004). Lacticin 481 produced by *Lactococcus lactis*, has been characterized to the sequence level. It is unrelated to nisin at the level of the primary sequence, but the 27-amino acid of the mature peptide also contains characteristic

lanthionine residues. Lactocin S is a lantibiotic produced by *Lactococcus sake*. The mature molecule is a 37-amino acid peptide with two lanthionine groups and at least four dehydrated residues. Both lacticin 481 and lactocin S are broad host range inhibitors (Gould, 1995).

2.4.2 Class II, small heat-stable bacteriocins

All of the small heat-stable bacteriocins in this class have not been shown any post-translational modification beyond the cleavage of an 18- to 21-amino acid leader regions from the pro-bacteriocin molecule (Gould, 1995). Examples of this class of bacteriocins are shown in Table 2.

A large number of bacteriocins characterizied to date belong to class II. They are synthesized as precursor peptides (pre-probacteriocins) containing an N-terminal double-glycine leader peptide, which is cleaved off concomitantly with externalization of biologically active bacteriocins by a dedicated ABC-transporter and its accessory protein (Callewaer *et al.*, 1999; Kanatani *et al.*, 1995; Matinez *et al.*, 2000). Moreover, the recently identified *sec*-dependent bacteriocins contain N-terminal signal peptide that directs bacteriocins secretion through the general secretory pathway (GSP) (Cintas *et al.*, 2001). The comparison of the N-terminal extensions of class II bacteriocins: lactacin F, leucocin A, pediocin PA-1, and lactococcins A, B, Ma, is presented in Figure 3.

Table 2. The small non-lanthionine-containing and heat-stable bacteriocins (class II) produced by lactic acid bacteria.

Bacteriocin	Producer strain	Molecular mass (kDa)	Sensitive organism
Carnobacteriocin BM1	*Carnobacterium piscicola* LV17B	4.5	*Carnobacterium* sp., *Enterococcus* sp., *Lactobacillus plantarum*, *Listeria* sp., *Pediococcus parvulus*
Lactacin B	*Lactobacillus acidophilus* N2	6.3	*Lactobacillus delbrueckii*, *Lactobacillus helveticus*
Lactacin F	*Lactobacillus johnsonii* 11088	6.3	*Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus fermentum*
Plantaricin	*Lactobacillus plantarum*	>8	*Lactobacillus* sp., *Lactococcus lactis*, *Leuconostoc* sp., *Pediococcus* sp.
Reutericin	*Lactobacillus reuteri*	2.0	*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*
Sakacin A	*Lactobacillus sake* LB 706	4.3	*Carnobacterium piscicola*, *Enterococcus* sp., *Lactobacillus sake*, *Listeria monocytogenes*

Source: Jack *et al*. (1994); Klaenhammer (1993); Ryan *et al*. (1996).

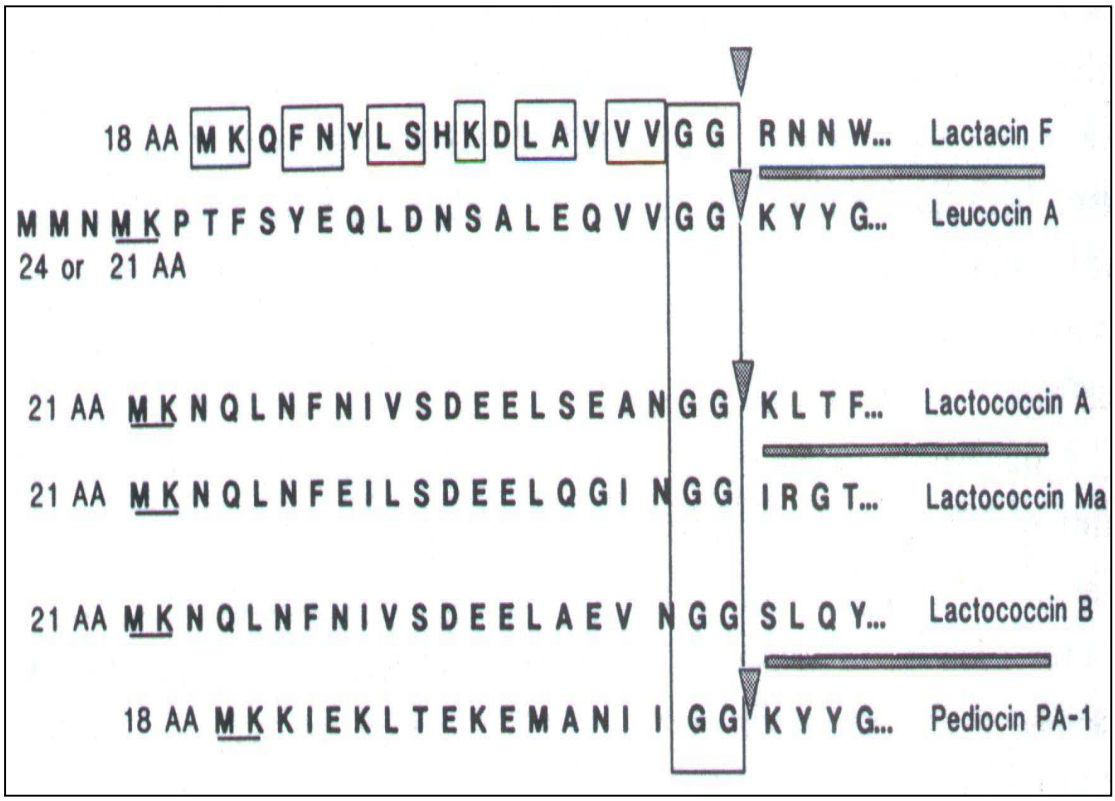


Figure 3. N-terminal amino acid sequences of six pre-peptide bacteriocins produced by different lactic acid bacteria representing genera *Lactobacillus* (lactacin F), *Leuconostoc* (leucocin A), *Lactococcus* (lactococcins A, B, and Ma), and *Pediococcus* (pediocin). Processing sites indicated by the inverted triangles, are determined by comparison of DNA sequences with the known amino acid sequences of the mature peptides. Boxed residues illustrated in lactacin F, are found in one or more of other prepeptides at the identical position within the N-terminal extension.

Source: Hoover and Steenson (1993).

A large number of class II bacteriocins has been shown to be membrane-active peptides. They destroy the integrity of membrane by the formation

of pores. In contrast to nisin, lactococcins act on the target cells regardless of their energization. Mature lactococcin A is a 54-amino acid hydrophobic peptide, which is bactericidal for other lactococcal strains. Lactococcin A acts at the cytoplasmic membrane of sensitive cells, leading to the disruption of the membrane potential and the leakage of small molecular weight compounds (Gould, 1995). Because it is only active against membrane vesicles derived from sensitive strains, the conclusion is that it needs a specific receptor protein.

However, Martinez *et al.* (2000) reported that *Lactococcus lactis* IPLA972 produced lactococcin 972 which acts on the cell wall biosynthesis of *Lactococcus lactis* Mg1614. Most of data on lactococcin 972 indicate that it is an unusual bacteriocin, and sensitive to heat and low pH. Furthermore, it is probably exported via a sec-dependent mechanism. It might hence be reasonable to consider the creation of a new subgroup of bacteriocins among these already described in class II to include bacteriocins whose target is the cell wall.

2.4.3 Class III, large heat-labile bacteriocins

Class III bacteriocins are high molecular weight and heat-labile proteins. They have been isolated from members of the genus *Lactobacillus* (Klaenhammer, 1993). This class may therefore include bacteriolytic extracellular enzymes (hemolysins and muramidases) that may mimic the physiological activities of bacteriocins. Only, Helveticin J produced by *Lactobacillus helveticus* 481, is the bacteriocin that has been investigated into great detail. It is a 37-kDa protein with narrow host range. This molecule is produced from the nucleotide sequence of the

chromosomally located *hly*J gene, and little is known about possible post-translational modifications (Gould, 1995). Examples of class III bacteriocins are shown in Table 3.

Table 3. High molecular weight and heat-labile bacteriocins (class III) produced by lactic acid bacteria.

Bacteriocin	Producer strain	Molecular mass (kDa)	Sensitive organism
Caseicin 80	*Lactobacillus casei* B80	40	*Lactobacillus casei*
Helveticin J	*Lactobacillus helveticus* 481	37	*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*
Helveticin LP27	*Lactobacillus helveticus* LP27	12.4	*Lactobacillus delbrueckii* subsp. *bulgaricus*

Source: Hoover and Steenson (1993).

2.5 Detection of bacteriocins produced by lactic acid bacteria

2.5.1 Determination of bacteriocin activity

The agar plate diffusion assay is a popular method for screening bacteriocin activity produced by lactic acid bacteria (Jack *et al*., 1995). Several techniques of the agar plate diffusion assay including agar spot test, paper disc assay, and agar well diffusion technique, are used to detect the bacteriocin activity. In these techniques, the bacteriocin activity is determined by comparing zones of inhibition around the site of bacteriocin inoculation after incubation. The zone of inhibition is the result of diffusion of the bactericidal protein through the agar medium preventing the growth of indicator organism (Figure 4). Cooper (1964) reported that the gradient or zone of inhibition was established when the charge or concentration at the source

was well defined, and a gradient was established by gradual exhaustion of the bacteriocin diffusion ($x = r - r_d$). Bacteriocin diffuse through an agar at a constant rate depending upon its molecular weight, its ionic charge, and the composition of the gel (agar). Temperature and solvent viscosity of the gel has effect to the constant of diffusion. The size of the inhibition zone is related to the diffusion rate of bacteriocins and also to the growth rate of the indicator bacteria. However, certain problems and limitations are lactic acid bacteria, the bacteriocin producers, that are able to produce other antagonistic compounds and create conditions mimicking bacteriocin activity.

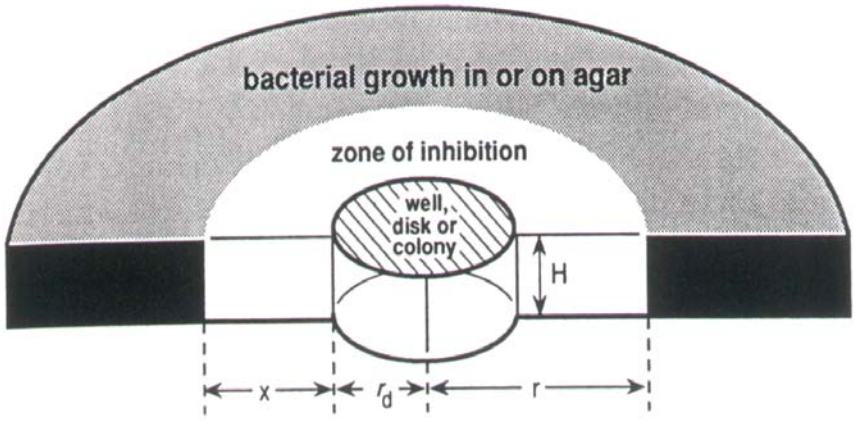


Figure 4. A zone of inhibition formed by radial diffusion of bacteriocin in a solidified medium.

Source: Hoover and Steenson (1993).

For the agar spot test, the bacteriocin-producer and indicator cultures are grown on the same solid medium under the same incubation conditions. The indicator is spread onto the surface of the agar medium, and the producing culture is spotted on top of this surface (Callewaert *et al.*, 1999; Dykes *et al.*, 2003; Karaya *et*

al., 2001). Martinez *et al*. (1999 and 2000) used the agar spot test to detect lactococcin 972 produced by *Lactococcus lactis* IPAL972, which was effective against other lactococci. Both bacteriocin-producing strain and indicator organism were grown in M17 broth at 32°C. Akcelik (1999) also used this technique for detecting bacteriocin production from *Lactococcus lactis* subsp. *lactis* LL102 grown in M17 broth. *Leuconostoc carnasum* and *Lactobacillus plantarum* grown in MRS broth and *Staphylococcus aureus, Micrococcus luteus,* and *Listeria monocytogenes* grown in nutrient broth were used as indicator strains. Effects of heat, proteolytic and lipolytic enzymes, and pH on the bacteriocin activity were determined. This strain produced bacteriocin that was heat stable, sensitive to proteolytic enzymes, but unaffected by lipolytic enzymes. The activity of bacteriocin was reduced at pH above 5. Venema *et al*. (1996) detected bacteriocin produced by *Lactococcus lactis* IL1403. This bacteriocin had effect on the growth of *Lactococcus* sp. The M17 broth supplemented with 0.5% glucose was used as a bacteriocin production medium. The agar spot test technique was also applied for the detection of lacticin NK24 produced by *Lactococcus lactis* NK24 (Lee and Paik, 2001). The lacticin NK24 was active against *Leuconostoc mesenteroides* KCCM11324, *Bacillus* sp., *Lactobacillus* sp., *Listeria* sp., *Pediococcus* sp., *Staphylococcus* sp., *Escherichia coli* KCCM32396, and *Sphingomonas paucimobilis* BNJ9964. Ohmomo *et al*. (1998) screened the bacteriocin-like activity from thermophilic lactic acid bacteria using the agar spot test. Each lactic acid bacterium was inoculated onto the MRS agar plate with a colony less than 8 mm in diameter under anaerobic condition at 43°C for 24 h. Then, the agar medium surface with colony formation was overlaid with a suspension of an indicator

The paper disc assay has also been applied for bacteriocin activity detection. Sterilize paper discs absorbed with the cell-free growth supernatant of the bacteriocin-producing strain, are placed onto freshly indicator-seeded agar medium (Ryser and Richard, 1992; Sawan and Manivannan, 2000). The paper disc assay is also applied for the detection of bacteriocin activity by Ohmomo *et al*. (1998). The thin-type of paper discs (8 mm diameter) with the adsorption capacity of 30 μl of cell-free culture filtrate were put on an agar plate containing 10^6 CFU/ml of indicator strain. The culture filtrate was adjusted to pH 6.0 with a NaOH solution before adsorption on to the paper disc. The bacteriocins showed antimicrobial actively against to *Enterococcus faecium* and *Enterococcus faecalis*.

Agar well diffusion technique is another method for bacteriocin activity detection. This technique is cutting wells into freshly indicator-seeded agar medium. The well can contain either a cell suspension of the bacteriocin-producing strain or cell-free growth supernatant (Tichaczek *et al.*, 1992). Plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*, was detected using the agar well diffusion technique (Kelly *et al.*, 1996). The bacteriocin producer was isolated from fermented corn. Test organisms *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains were grown in MRS broth at 28°C. *Listeria* and *Micrococcus* strains were grown in trypticase soy broth with 0.6% yeast extract at 37°C and 28°C, respectively. Cells of the bacteriocin producer were removed from broth at the stationary phase of growth by centrifugation at 10,000 rpm for 10 min. The supernatant fluid was adjusted to pH 6.5, treated with catalase (1 mg/ml) and filtered through a 0.45 μm pore-size cellulose acetate filter before testing the antimicrobial activity. The sensitivity of bacteriocin to proteolytic enzymes and lipolytic enzymes, detergents, pH, and temperature, were

determined. Plantaricin KW30 was found to inhibit *Lactobacillus* sp., but had no effect on *Listeria monocytogenes*, *Leuconostoc* sp., *Micrococcus* sp., and *Pediococcus* sp. It was sensitive to protiolytic enzymes but unaffected by α-amylase, lipase, and lysozyme. It was also stable to heat, pH, and treatment with surfactants. Kanatani *et al.* (1995) detected acidocin A produced from *Lactobacillus acidophilus* TK9201 using both the agar well diffusion and the agar spot test. For the agar well diffusion technique, 100 μl of each serial dilution of culture filtrate of TK9201 strain was added to each well (1 cm in diameter) cut into the plate containing sensitive indicator cells. The plate was then incubated for examining clear zones. And for the agar spot test, an overnight culture of strain TK9201 was spotted onto MRS agar and incubated at 37°C for 16 h to allow colonies to develop. Then, the mixture of indicator organism with soft-agar medium was overlaid onto the plate. After incubation for 12 h, the clear zone surrounded strain TK9201 colony was examined. *Lactobacillus* sp., *Pediococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Propionibacterium* sp., and *Listeria monocytogenes* were found to be acidocin A-sensitive strains.

2.5.2 Detection of genes encoding bacteriocins

Genes of several bacteriocins produced by lactic acid bacteria, are usually found to locate on plasmids. In some cases, a single plasmid may carry the genetic determinants for several bacteriocins. Alternatively, separate plasmids carried by different strains and subspecies may encode the same bacteriocin. For example, lactococcin A is carried on three separate plasmids ranging in size from 55 to 131 kb in two different subspecies of *Lactococcus lactis*. Moreover, two or more bacteriocins may be encoded by different plasmids in the same strain. *Carnobacterium piscicola*

LV17 harbors pCP49 encoding carnobacteriocin A, and pCP60 encoding carnobacteriocins B1 and B2. However, some of bacteriocins analysed to date have chromosomal locations. Examples are nisin, plantaracin A, sakacin P, sakacin 674, salivaricin B, lactacin B, helveticin J, and a number of pediocins (Cataloluk, 2001; Gound, 1995; Jack *et al*., 1995; Kawai, 1998 and 2004; Luesink *et al.*, 1999; Vaughan *et al.*, 2003).

Several bacteriocin genes have been cloned through a process of reverse genetics, in which a portion of the active bacteriocin has been sequenced and oligonucleotides synthesized on the basis of this information. The oligonucleotides can be used as probes to detect bacteriocin genes. Nisin is the most well known bacteriocin, produced by *Lactococcus lactis* subsp. *lactis*. The production of nisin is encoded by a cluster of genes proposed to be transcriptionally arranged as *nis*ABTCIP, *nis*RK, and *nis*FEG (Li and O'Sullivan, 2002). This cluster is located on a chromosomally located 70-kb conjugative transposon, variously termed Tn*5276* or Tn*5301*, and also linked with sucrose utilization genes.

Remiger *el al.* (1996) used both the polymerase chain reaction (PCR) and oligonucleotide-probing techniques to identify homologous genes among thirteen bacteriocin-producing *Lactobacilli.* Four well known bacteriocins; curvacin A, sakacin P, plantaricin A, and plantaricin S, were investigated, and various PCR primers were derived from their respective sequences. All amplificates were confirmed by hybridization. *Enterococcus* sp. strain S12β was used as a recipient for conjugative transfer of the nisin transposon TN*5307* from *Lactococcus lactis* ATCC 11454 to construct *Enterococcus* sp. strain N12β. The PCR technique was used to

confirm that genes extending throughout the nisin gene cluster were present in strain N12β.

Carnobacterium piscicola CP5, isolated from a French mold-ripened soft cheese, produced a bacteriocin, Carnocin CP5, which inhibited other *Carnobacterium* sp., *Enterococcus* sp., *Listeria* sp., and *Lactobacillus delbrueckii*. The bacteriocin, carnocin CP51, was partially sequenced. Then, a degenerated 24-mer oligonucleotide probe was constructed from the N-terminal sequence, and used to detect the structural gene. It was localized on a plasmid of about 40 kb (Herbin *et al*., 1997).

The lactocin S structural gene (*las*A) in seven bacteriocingenic lactobacilli isolated from fermented sausages was studied (Rodriguez *et al.*, 1995a). Two degenerate primers were synthesized to amplify a 75 bp fragment of the gene. The fragment from plasmid DNA of three lactobacilli strains were amplified, and hybridization analysis was used to confirm these results. Two strains of lactococci, *Lactococcus lactis* BB24 and *Lactococcus lactis* G18, were also screened their bacteriocin production (Rodriguez *et al.*, 1995b). The bacteriocins were broad spectrum, stable after boiling for 5 min at pH 2.0. They were resistant to the digestion of trypsin, but sensitive to α-chymotrypsin digestion. These characteristics suggested that both bacteriocins could be nisin. The suggestion was confirmed by PCR analysis using four primers designed from the single-strand DNA sequence of nisin structural gene. Their genomic DNA generated amplified bands of size revealed that they produced nisin A.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Chemicals for media preparation were beef extract, soytone, tryptone, and yeast extract (Pronadisa, HispanLab, Spain); potassium dihydrogen phosphate (Merck, Merck KGaA, Germany); di-potassium hydrogen phosphate, glucose, tween 80, and tri-ammonium citrate (Fluka, Sigma-Aldrich Chemical Company, U.S.A.); sodium chloride, magnesium sulfate, and manganese sulfate (Carlo Erba Reagenti, Montedison group, Italy).

Reagents used for PCR amplification were PCR buffer, $MgCl_2$ solution, dNTPs (dATP, dCTP, dGTP, dTTP), and *Taq* DNA polymerase (Invitrogen, Invitrogen life technologies, U.S.A.). The oligonucleotide primers were ordered from the BioService Unit (BSU) of the National Science and Technology Development Agency, Thailand. Nisin, chloramphenical and protease were purchased from Sigma-Aldrich Chemical Company (U.S.A.). Lysozyme was purchased from Merck KGaA (Germany). The molecular weight markers were purchased from Invitrogen life technologies (U.S.A.).

3.2 Instrumentation

Instruments required for the detection of bacteriocins from starch-utilizing and lactic acid-producing bacteria were located in the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima Province, Thailand. The instrument required for DNA sequencing (ABI PRISM® 377 DNA SEQUENCER, Perkin Elmer, U.S.A.) was located at the Biotechnology and Development Office, Department of Agriculture, Prathumthani, Thailand.

3.3 Collection and isolation of starch-utilizing and lactic acid-producing bacteria

Starch-utilizing and lactic acid-producing bacteria were obtained from stock cultures of the School of Microbiology, Suranaree University of Technology, and isolated from starch waste samples from cassava starch production factory in Nakhon Ratchasima Province. To isolate the lactic acid bacteria from starch waste samples, serial dilutions of samples were spread onto De Man, Rogosa and Sharpe agar (MRS) (Appendix A3). The plates were then incubated under anaerobic condition at 37°C for 48 h. Bacterial colonies grown onto MRS agar were randomly selected and isolated to obtain pure cultures. The isolates were streaked onto the Rogosa agar with modification (RAM) (Appendix A4) containing 1% cassava starch to test their starch-utilization, and also inoculated into MRS broth containing 0.0015% bromocresol purple with durham tube to test their ability to produce carbon dioxide. The homolactic starch-utilizing bacteria were selected.

The selected isolates were maintained in MRS broth and kept at -20°C with the addition of skim milk to 5% (v/v) final concentration. For cell propagation procedure, the stock cultures were taken from -20°C, thawed at room temperature. Two hundred ml of each culture were inoculated into 2 ml of MRS broth. After incubation at 37°C for 18 h, the culture was streaked onto RAM agar medium containing 1% cassava starch, and incubated under anaerobic condition at 37°C for 48 h. Then, a single colony was collected for further study.

3.4 Collection of bacteriocin test organisms

Several stains of bacteria including food spoilage and food-borne pathogens were used as bacteriocin test organisms. These test organisms were obtained from both the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand, and from stock cultures of the School of Microbiology, Suranaree University of Technology (Table 4). The media used for culturing the test organisms were tryptic soy broth (TSB) (Appendix A5) and MRS broth.

Table 4. List of test organisms used for bacteriocin activity detection.

Strain of test microorganism	Cultivation medium	Cultivation temperature (°C)
Bacillus stearothermophilus TISTR329	TSB	55
Bacillus cereus TISTR687	TSB	37
Bacillus subtilis TISTR008	TSB	37
Escherichia coli TISTR780	TSB	37
Escherichia coli TISTR887	TSB	37
Lactobacillus acidophilus TISTR1034	MRS	37
Lactobacillus brevis subsp. *brevis* TISTR855	MRS	30
Lactobacillus delbrueckii TISTR326	MRS	37
Lactobacillus delbrueckii subsp. *bulgaricus* TISTR892	MRS	37
Lactobacillus delbrueckii subsp. *lactis* TISTR785	MRS	37
Lactobacillus fermentum TISTR055	MRS	37
Lactobacillus plantarum TISTR050	MRS	37
Listeria monocytogenes DMS1327	TSB	37
Micrococcus luteus TISTR884	TSB	30
Pediococcus acidilactici TISTR051	MRS	26
Pediococcus acidilactici TISTR783	MRS	37
Pediococcus pentosaceus TISTR374	MRS	37
Pediococcus pentosaceus TISTR954	MRS	37
Pseudomonas aeruginosa TISTR781	TSB	37
Salmonella durby SH1455/95	TSB	37
Salmonella typhimurium TISTR022	TSB	37
Streptococcus thermophilus TISTR894	TSB	37
Staphylococcus aureus TISTR029	TSB	37
Staphylococcus aureus TISTR118	TSB	37
Staphylococcus aureus TISTR517	TSB	37

3.5 Screening of starch-utilizing and lactic acid-producing bacteria for bacteriocin production

To find out the suitable screening medium, some of starch-utilizing and lactic acid-producing bacteria obtained in section 3.3 selected by their potential production of lactic acid (Rodtong *et al.*, 2001) were tested for their bacteriocin production capability as the following steps. The most reliable production medium, sensitive test organism, and bacteriocin detection technique, were chosen and applied to screen other isolates.

3.5.1 Test organisms

According to Bangkok MIRCEN (1995), some antibiotic susceptibility test strains were chosen for bacteriocin test or indicator organisms in the primary screening. The test strains were *Escherichia coli* TISTR887, *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008, *Staphylococcus aureus* TISTR029, and *Staphylococcus aureus* TISTR517. The bacteria were cultivated using their appropriate media under optimal temperatures as illustrated in Table 4 for 18 h in order to use in the bacteriocin activity detection step.

3.5.2 Cultivation of starch-utilizing and lactic acid-producing bacteria for bacteriocin production

The media M17 broth (Appendix A2), MRS broth (Appendix A4), and bacteriocin screening medium (BSM) (Appendix A1) have been reported to be applied for the production of bacteriocin from some lactic acid bacteria (Coventry *et al*., 1996; Eijsink *et al*., 1996; Faye *et al*., 2000; Martinez *et al.*, 2000; Tichaczek, *et al.*, 1992). To obtain the reliable medium for the bacteriocin production of starch-

utilizing and lactic acid-producing bacteria in the screening step, these three media were applied. One loopful of 48 h culture was inoculated into 7 ml of each medium, and incubated at 30°C under anaerobic condition for 18 h. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}), then the bacterial cells were separated from culture medium by centrifugation at 12,000 rpm (Labofuge 400R, Heraeus Instruments, Heraeus Instruments GmbH, Germany) at 4°C for 10 min. The acid supernatant was collected, determined pH, and adjusted to pH 6.5 using 3 N NaOH.

3.5.3 Detection of bacteriocin activity

To obtain the reliable technique for the detection of bacteriocin activity in this study, three techniques; the agar well diffusion technique, paper disc assay, and agar spot test were applied in the preliminary screening step. Standard chloramphenical (Fluka) (0.5 mg/ml) was used as the positive control.

The agar well diffusion technique was performed as described by Ohmomo (1998) with some modifications as follows: the overnight culture of test organism was inoculated into 100 ml of TSA soft agar (0.75% agar) to the final concentration 10^6 CFU/ml. Then, 15 ml of the mixture were dispensed into a sterile Petri-dish. The plate was then allowed to solidify at room temperature for 30 min. Wells were punched by using the sterilized 6 mm cork borer. Then, 65 µl of supernatant (section 3.5.2) were added into each well in duplicate.

The paper disc assay was performed as described by Martirani *et al*. (2002) with some modifications as follows: seven ml of TSA soft agar (0.75%) with 10^6 CFU/ml of test organism were overlaid onto plate containing 10 ml of 1.5% TSA

agar. To prevent the low concentration of bacteriocin that could not be detected, two sizes of blank discs (6 mm and 9 mm diametres) (Schleicher and Schuell, Schleicher and Schuell GmbH, Germany) were used for testing. The 25 µl and the 45 µl of supernatant (section 3.5.2) were applied onto the 6 mm and 9 mm diametres of sterilized blank discs in duplicate, respectively. Then, the discs were placed onto the surface of agar medium.

The agar spot test was performed as described by Schillinger *et al*. (2001) with some modifications as follows: seven ml of TSA soft agar (0.75%) containing 10^6 CFU/ml of test organism were overlaid onto plate containing 10 ml of 1.5% TSA agar. Then, 10 µl of supernatant (section 3.5.2) were spotted in duplicate onto the surface of agar medium.

Inhibition zones were observed after 18 h incubation under optimal temperatures for each test organism. The most suitable bacteriocin production medium, sensitive test organism, and bacteriocin detection technique were chosen for further study.

3.6 Selection of bacteriocin-producing strain(s) and production of bacteriocins by the selected strain(s)

The inhibition zones in the section 3.5 were compared, the starch-utilizing and lactic acid-producing bacteria which showed a broad spectrum of bacteriocin activity were selected and used as bacteriocin-producer(s) for further investigation.

For the bacteriocin production experiment, one loopful of selected isolate grown for 48 h at 30°C on MRS agar was inoculated into 7 ml of MRS broth. The

inoculated MRS broth was incubated at 30°C for 18 h. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). Then 10% (v/v) of culture (10^6 CFU/ml) were inoculated into 15 ml test tube containing 10 ml-working volume of the selected medium. The incubation was performed at 30°C under anaerobic condition for 18 h. The bacterial cells were separated from culture medium by centrifugation at 12,000 rpm at 4°C for 10 min. The acid supernatant was collected, determined pH, and adjusted to pH 6.5 using 3 N NaOH.

3.7 Optimization of some bacteriocin production conditions

Some optimal conditions for cultivation the selected isolates were investigated to obtain the efficient bacteriocin production. The composition of the selected medium from section 3.5 was varied as the following steps. The reliable technique for bacteriocin activity detection and test organisms obtained from section 3.5 were used.

3.7.1 Type and concentration of carbon source

To obtain the carbon source based on a cheap and abundant raw material, cassava starch was used to replace the carbon source in the selected medium (section 3.5). Also various concentrations of cassava starch were applied to achieved the optimal concentration.

3.7.2 Concentration of nitrogen sources

The optimum concentration of nitrogen sources contained in the selected medium was investigated. The medium containing suitable concentration of cassava starch (section 3.7.1) was used.

3.7.3 Concentration of growth factors

Yeast extract was applied as the growth factors. The optimum concentration of yeast extract was also investigated using the medium containing the optimum concentrations of carbon and nitrogen sources.

3.7.4 Initial pH of bacteriocin production medium

The initial pH of the optimized medium for the bacteriocin production was studied. The medium was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 using 1 N HCL and 1 N NaOH, and then used for culturing the selected bacteriocin-producing isolate(s).

3.7.5 Production temperature

The suitable temperature for bacteriocin production was investigated. Various incubation temperatures; 25, 28, 30, 32, 35 and 37°C were performed for the cultivation of the selected bacteriocin-producing isolate in the suitable medium resulted from sections 3.7.1-3.7.4.

3.8 Production of bacteriocins using the optimum conditions

The selected isolate(s) was cultivated using 10 ml of the suitable medium and optimal conditions obtained from section 3.7 for 12, 14, 16, 18, 20, 24 and 48 h. Crude bacteriocins were prepared and detected as described in section 3.6. Then, the production of bacteriocins was performed with 100-ml working volume and incubating for the period that gave the maximum bacteriocin yield. In this step, the preparation of inoculum was considered. One loopful of selected isolate grown for 48 h

at 30°C on MRS agar was inoculated into 15 ml of MRS broth, and incubated at 30°C under anaerobic condition for 18 h. Then, the 18 h culture adjusted cell concentration to 10^6 CFU/ml were applied as the inoculum to inoculate into 250 ml Erlenmeyer flask containing 100 ml of the suitable medium. The inoculum size used was 10% (v/v).

3.9 Characterization of bacteriocins produced by the selected strain(s)

Crude bacteriocins prepared from section 3.8 were investigated as follows:

3.9.1 Antimicrobial spectrum

The inhibition spectrum of selected bacteriocin was investigated. All bacterial strains listed in the Table 4 were used as test organisms. These test organisms were cultured in TSB and MRS broth. The activity of bacteriocin was compared with the standard nisin (Fluka). The standard curve of nisin was prepared by testing nisin at various concentrations 10, 20, 30, 40, 50, 60, 80, 90 and 100 IU/ml against the test organism correlating with the selected bacteriocin.

3.9.2 Effects of enzymes, temperature, and pH on bacteriocin activity

Effects of enzymes, temperature, and pH on bacteriocin activity were investigated. Crude bacteriocins were dispensed into 500 μl portions in 1.5 ml microcentrifuge tubes. To test the sensitivity to enzymes, eight portions were used for 4 treatments, protease, protinase K, lysozyme treatments, and no treatment (control). The enzymes were filter-sterilized by passing through 0.45 μm cellulose acetate membrane, and treated the selected bacteriocins by adding to a final concentration

1 mg/ml. The mixtures of enzyme and bacteriocins were incubated at 37°C for 2 h. Then, the residue bacteriocin activity was determined.

To test of temperature sensitivity, twenty-eight portions were used. Each portion was treated under different conditions as following; control (no treatment), heating at 60, 70, 80, 90, 100, and 121°C, for 15 min and 30 min for each temperature, then, assayed for bacteriocin activity.

To determine the effect of pH on bacteriocin activity, twenty-eight portions were used. Each portion was adjusted, using 6 N HCL or 6 N NaOH, to pH between pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, and 14.0. The samples were incubated at 4°C for 2 h. The antimicrobial activity was determined.

3.9.3 Molecular mass and bacteriocin activity in gel

The selected bacteriocin was concentrated and partial purified using the vivaspin concentrator, molecular weight cut off 3000 kDa (Vivascience AG, Sartorius group, U.S.A.). Two ml of crude bacteriocins were centrifuged at 4°C for 2 h. The molecular weight of the selected bacteriocin was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by using Mini Protein II apparatus (BioRad). The slab gel containing 15% acrylamide as separating gel (Appendix B2.7) and 4% acrylamide as stacking gel (Appendix B2.8) were used. Samples to be analyzed were mixed with the sample buffer (Appendix B2.5) containing tracking dye at a ratio of 1:1 (v/v), heated at 65°C for 5 min. Then, 10 µl of the mixture were loaded in each well. BenchMark^{TM} Protein Ladder was used as molecular weight marker. SDS-PAGE was performed in

electrophoresis buffer (Appendix B2.6) at a constant current of 100 volts until the tracking dye reached the bottom of the gel. The SDS-PAGE gel was done in duplicate. The first piece of gel was stained with SimpleBlue (Invitrogen) according to the manufacture's protocol. Another gel was washed 3 times, 40 min for each time, with sterile deionized water. The gel was placed onto the surface of a plate filled with 4 ml of 1.5% TSA agar. Then, 7 ml of 0.75% TSA agar containing 10^6 CFU/ml of test organism was overlaid. The zone of inhibition was examined after incubation at 37°C for 18 h.

3.10 Detection and sequence analysis of genes encoding bacteriocins

Since several bacteriocin genes have been found to locate on chromosomal DNA, the genomic DNA was preferred in this study. Starch-utilizing and lactic acid-producing bacteria were selected randomly, and also were selected according to the result of section 3.5 to detect their genes encoding bacteriocin.

3.10.1 Genomic DNA extraction

Genomic DNA of the selected isolate of starch-utilizing and lactic acid-producing bacteria was extracted using the method of Vassu *et al*. (2002). One loopful of the selected isolate was inoculated into 5 ml of MRS broth, and incubated anaerobically at 37°C for 18 h. Bacterial cells were harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was discarded, and the cell pellet was washed once with 200 µl of TE buffer, then resuspended in 200 µl of lysis buffer (50 mM glucose, 25 mM Tris-HCL, and 10 mM EDTA, pH 8.0) (Appendix B1.1) containing 5 mg/ml of lysozyme. The mixture was incubated at 37°C for 30 min.

Then, 30 µl of 10% sodium dodecyl sulfate (SDS) (Appendix B1.2) and 10 µl of protinase K were added. After incubation at 37°C for 1 h, 4 µl of RNase (2 µg/µl) was added, and incubated at 37°C for 20 min. The mixture was then added with 240 µl of TE-saturated phenol and 240 µl of chlorophorm:isoamyl alcohol (25:1), mixed by inversion, and centrifuged at 4°C for 5 min. The upper layer, the DNA phase, was transferred into a new sterilize microcentrifuge tube. The DNA was precipitated with a twice volume of absolute ethanol, and collected by centrifugation at 4°C for 10 min. The supernatant was discarded. The DNA pellet was washed with 600 µl of 70% ethanol, and dried at 37°C for 1 h. Then 30 µl of the sterilize deionized water were added, and kept overnight at 4°C to allow the DNA to dissolve. The extrected DNA was detected by electrophoresis on 0.8% agarose gels performed in TSB buffer pH 8.3.

3.10.2 Amplification of bacteriocin gene(s)

Gene encoding bacteriocin of the selected isolate was detected by the polymerase chain reaction (PCR) amplification using different specific primer pairs, which were the primers for lacticin 481 (O'Sullivan *et al*., 2002), nisin A (Li and O'Sullivan, 2002) and plantaricin A (Remiger *et al*., 1996) and genomic DNA of the bacterium. The PCR amplification was performed as described in Table 5 using the thermocycle (i-cycle, BioRad, U.S.A.).

The PCR amplified products were analyzed by electrophoresis on 1% agarose gel (1× TSB buffer pH 8.3) using 100 bp DNA ladder (Invitrogen) as a molecular weight standard. The gel was run at 80 V for 90 min.

Table 5. Sequences of specific primers and the PCR amplification of genes encoding bacteriocins**.**

Primer	Sequence of primer (5'-3')	PCR reaction mixture	Amplification condition
481 /F	GGAGCATACCCTGTTCC	1 µm of each primer	93°C for 1 min
481 /R	TATTTAGTGCTTTTTCCC TCTTTA	0.4 mM of each dNTP 3 mM of $MgCl_2$ 1X buffer 0.05 U of *Taq* DNA-polymerase 100 ng of genomic DNA Tatal 50 µl	Repeat the following for 30 cycles 93°C for 1 min 62°C for 1 min 72°C for 1 min
Source: O'Sullivan *et al*. (2002)			
NisA /F	GGATAGTATCCATGTCTG	0.6 µm of each primer	92°C for 2 min
NisA /R	CAATGATTTCGTTCGAAG	0.2 mM of each dNTP 1.5 mM of $MgCl_2$ 1X buffer 0.05 U of *Taq* DNA-polymerase 100 ng of genomic DNA Tatal 50 µl	Repeat the following for 30 cycles 92°C for 30 s 55°C for 45 s 72°C for 1 min 72°C for 3 min
Source: Li and O'Sullivan. (2002)			
plnA5p S7	GTACAGTACTAATGGGAG CTTACGCCATCTATACG	0.6 µm of each primer 0.2 mM of each dNTP 1.5 mM of $MgCl_2$ 1X buffer 0.05 U of *Taq* DNA-polymerase 200 ng of genomic DNA Tatal 100 µl	94°C for 3 min Repeat the following for 30 cycles 94°C for 30 s 53°C for 1 min 72°C for 1 min
Source: Remiger *et al*. (1996)			

3.10.3 Analysis of bacteriocin gene sequence

The PCR amplicon was characterized by sequencing and comparing the nucleotide sequence to GenBank database (http://www.ncbi.nlm.nih.gov). The sequencing work was done at the Biotechnology and Development Office, Department of Agriculture, Bangkok, Thailand.

PCR fragment to be sequenced was eluted from 1% agarose gel with QIA-quick and purified by using a QIA-quick PCR purification kit (Invitrogen) according to the manufacture's protocol. Then, the PCR for sequencing was performed in a total volume of 10 μl containing 1 μl of terminator ready reaction mix, 1 μl of template (approximately 100 ng), 1 μl of 5 μM of primer, and 4 μl of the sterilize deionized water. The PCR amplification was performed in 25 cycles, each cycle consisting of a denaturation step at 96°C for 10 s, a primer annealing step at 50°C for 5 s, and a primer extension step at 60°C for 4 min. Then, the extension product was transferred into a 1.5 microcentrifuge tube, and added with 16 μl of deionized water and 64 μl of 95% ethanol. The tube was vortex briefly and incubated at 4°C for 15 min. After centrifugation at 14,000 rpm for 20 min at 4°C, the supernatant was discarded. The tube was added with 70% ethanol, and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was discarded.

The precipitated DNA was dried, and dissolved in deionized water. Next, sequencing was performed using the ABI PRISM® 377 DNA SEQUENCER (Perkin Elmer, CA) according to the manufacture's protocol.

The nucleotide sequence data obtained from DNA sequencing software of ABI PRISM® 377 DNA SEQUENCER was interpreted and converted to single

letter code in text file format by the Chromas 1.56 program (Technelysium Pty. Ltd). The sequence was also corrected by manual inspection of the chromatogram. The sequence was compared to local alignment search of the GenBank database using the BLAST version 2.2.9 program of the National Center for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Collection and isolation of starch-utilizing and lactic acid-producing bacteria

One hundred and ten isolates of starch-utilizing and lactic acid-producing bacteria were obtained from stock cultures of the School of Microbiology, Suranaree University of Technology. Their capability to utilize cassava starch and produce lactic acid has been reported (Rodtong, 2001; Rodtong *et al.*, 2001; Rodtong and Ishizaki, 2003).

For the isolation of starch-utilizing and lactic acid-producing bacteria from starch waste samples, 57 isolates of lactic acid bacteria randomly selected from colonies grown on MRS agar, were tested for their starch-utilization using RAM agar containing 1% cassava starch. Thirty seven out of 57 isolates were able to utilize cassava starch. Homolactic starch-utilizing isolates were screened using MRS broth containing 0.0015% bromocresol purple with durham tube. Only 9 isolates were found to produce acid without gas. These isolates were collected for further investigation.

A total of 119 isolates of starch-utilizing and lactic acid-producing bacteria obtained from stock cultures and isolated from starch waste sample, were then tested for their bacteriocin production.

4.2 Screening of starch-utilizing and lactic acid-producing bacteria for bacteriocin production

To obtain the suitable medium for screening of starch-utilizing and lactic acid-producing bacteria, three media, M17 broth, MRS broth, and BSM broth, were tested for cultivating 48 isolates from a total of 119 (section 4.1). The 48 isolates, were selected randomly based on their lactic acid production capability (Rodtong and Ishizaki, 2003). To find out the most suitable bacteriocin activity detection, three methods; the agar well diffusion technique, paper disc assay, and agar spot test, were used for the preliminary screening step. Test organisms selected were *Escherichia coli* TISTR887, *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008, *Staphylococcus aureus* TISTR029 and *Staphylococcus aureus* TISTR517. Then, the most suitable medium, method for bacteriocin activity detection, and the sensitive test organism, were chosen and applied for screening bacteriocin production from the other 71 isolates.

One loopful of each bacterial isolate was incubated into each medium, then incubated under anaerobic condition at 30°C for 18 h. The bacterial growth was determined spectrophotometically at 600 nm (A_{600}). Bacteriocins were harvested by centrifugation. The acid supernatant containing bacteriocins was neutralized to pH 6.5 for the bacteriocin activity assay. For the bacterial growth, it was found that the starch-utilizing and lactic acid-producing bacteria could give their good growth both in MRS broth and BSM broth (A_{600} more than 0.5), which was better than in M17 broth (A_{600} lower than 0.5). The majority of MRS (initial pH 7.0) and M17 (initial pH 7.5) broth cultures showed more acidity (pH 4.5-5.5) than BSM broth cultures, which initial pH of 7.0 changed to 5.8-6.3. This might be because the BSM medium

contained lower concentration of glucose (0.2%) than MRS (1% glucose) and M17 (0.5% glucose) media resulting in low concentration of lactic acid produced. The neutralized supernatant of the first selected 48 isolates was tested for bacteriocin activity. Only one isolate, A5UVU25, was detected to produce bacteriocin when cultured in the BSM broth. None of isolates tested was found to produce bacteriocins in M17 and MRS media. Some isolates might produce bacteriocins in low concentration when cultured in M17 and MRS media. But they also produced high acid. When the pH of the cultured medium was adjusted using 3 N NaOH to obtain the neutral pH, the bacteriocin concentration was diluted until their bacteriocin activity could not be detected.

For the selection of test organism to be used in this screening step, *Staphylococcus aureus* TISTR029 was found to be the most sensitive test organism. While *Escherichia coli* TISTR887, *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008 and *Staphylococcus aureus* TISTR517 were not sensitive to bacteriocins produced from the preliminary selected strain. And among three bacteriocin detection techniques, only the agar well diffusion assay was successfully for the detection of bacteriocin activity.

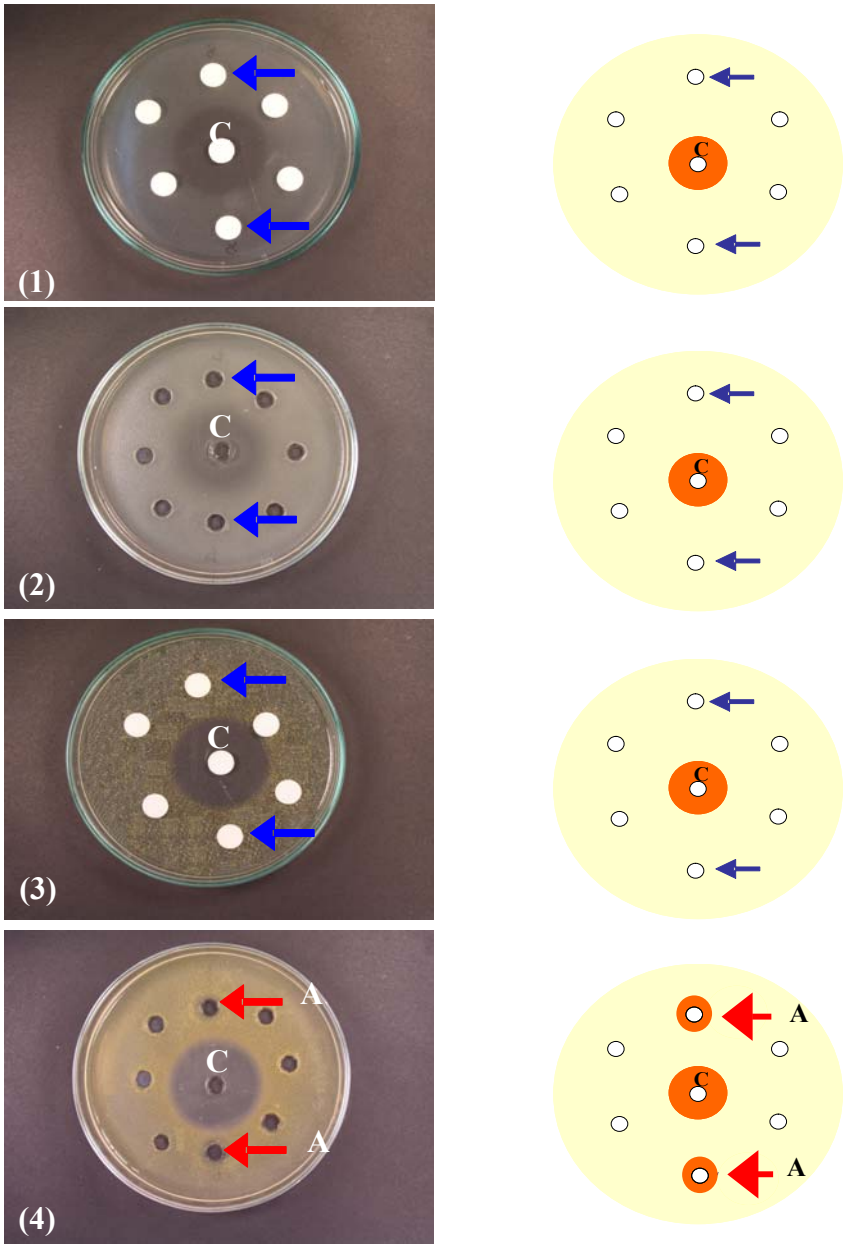


Figure 5. Antimicrobial activity of bacteriocin produced by isolate A5UVU25 when tested against *Escherichia coli* TISTR887 using the paper disc assay (1) and the agar well diffusion technique (2), and *Staphylococcus aureus* TISTR029 using the paper disc assay (3) and the agar well diffusion technique (4). Arrows indicate positions of crude bacteriocin from isolate A5UVU25 (A) (7mm inhibition zone diametre). Standard chloramphenical was used as the positive control (C) (20 mm inhibition zone diametre).

No inhibition zone was obtained from both the paper disc method and agar spot test. Thus, the agar well diffusion technique using 65 µl of crude bacteriocins and the test organism *Staphylococcus aureus* TISTR029 were chosen for testing the bacteriocin activity of starch-utilizing and lactic acid-producing bacterial isolates. Results from 48 isolates, the BSM medium, the agar well diffusion technique and the sensitive test organism, *Staphylococcus aureus* TISTR029, were selected and applied to other 71 strains. When the remainder isolates were tested for their bacteriocin production, none of them showed their capability to produce the antibacterial substances. The isolate A5UVU25 was therefore selected for further investigation.

4.3 Selection of bacteriocin-producing strain and production of bacteriocin by the selected strain

From the screening step, only one isolate, A5UVU25, out of 119 isolates of starch-utilizing and lactic acid-producing bacteria could produce bacteriocin when cultured in BSM broth and detected by testing against *Staphylococcus aureus* TISTR029 using the agar well diffusion technique. The isolate A5UVU25 was selected. It was obtained from stock cultures of the School of Microbiology, Suranaree University of Technology, and previously identified as belonging to the genus *Lactococcus* (Rodtong and Ishizaki, 2003). When the isolate A5UVU25 was cultivated in 10-ml-working volume of BSM medium under anaerobic condition at 30°C for 18 h, it could produced 12.30 IU/ml of bacteriocins compared to the standard nisin and tested against *Staphylococcus aureus* TISTR029.

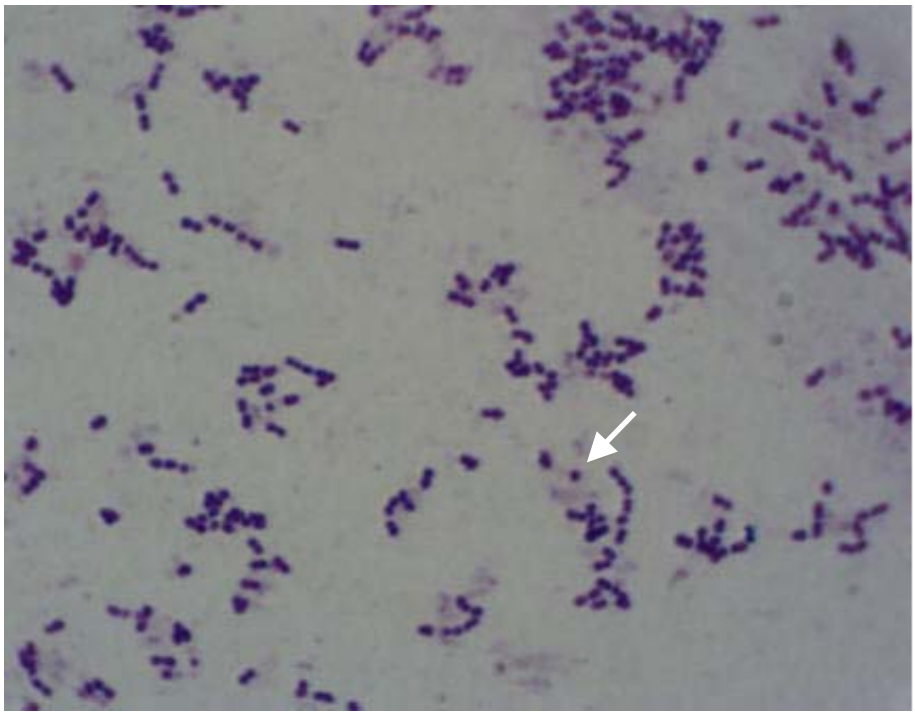


Figure 6. Gram stain of isolate A5UVU25, bright field microscopy (×1000).
An arrow indicates cell of the isolate A5UVU25.

4.4 Optimization of some bacteriocin production conditions

To obtain the maximum production of bacteriocins and low production cost, some components of BSM medium and production conditions including carbon and nitrogen sources, growth factors, initial pH of medium, and incubation temperature, for culturing the isolate A5UVU25 were investigated. The BSM medium composed of (g/l); glucose, 2; tryptone, 10; beef extract, 2; yeast extract, 4; K_2HPO_4, 8.7; KH_2PO_4, 8; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; tri-ammonium citrate, 2; and tween 80, 1 ml. The bacteriocin activity was detected by using agar well diffusion technique and test organism *Staphylococcus aureus* TISTR029.

4.4.1 Type and concentration of carbon source

For the suitable type of carbon source in the BSM medium, glucose was replaced with cassava starch. The various concentrations: 0, 5, 10, 15 and 20 g/l, were investigated to obtain the optimal concentration. After cultivating the isolate A5UVU25 for 18 h, the bacteriocin activity was detected. Results showed that glucose could be replaced with cassava starch. The highest bacteriocin activity was obtained when 10 g/l of cassava starch was applied to the BSM broth (Figure 7A). Bacteriocins could be produced when using the BSM medium containing either glucose or cassava starch (for all concentrations tested). The increase in concentration of cassava starch to 15 and 20 g/l could not increase the bacteriocin concentration. Thus, cassava starch (10 g/l) was chosen to be used as the carbon source in BSM medium for bacteriocin production by the isolate A5UVU25.

4.4.2 Concentration of nitrogen sources

Nitrogen source is another crucial substrate for bacteriocin production. Since two components, tryptone and beef extract, of BSM medium could be served as nitrogen source, varied concentrations of tryptone (g/l): 0, 5, 10, 15, and 20, were added to the BSM medium containing cassava starch to obtain the optimal concentration. It was found that both with and without the addition of tryptone into the BSM medium, the isolate A5UVU25 could produce bacteriocin (Figure 7B). But the maximum production was achieved when using both 10 and 15 g/l of tryptone. So, the low concentration of 10 g/l tryptone was used for further optimization.

Various concentrations of beef extract (g/l): 0, 0.5, 1.0, 1.5, 2.0 and 2.5, were added to the BSM medium containing cassava starch and optimum concentration

of tryptone, to obtain the optimal concentration. Results showed that the highest bacteriocin activity was obtained when 1.5, 2.0 and 2.5 g/l of beef extract were applied (Figure 7C). Thus, the low concentration of 1.5 g/l of beef extract was chosen to be used in BSM medium.

4.4.3 Concentration of growth factors

Since the original BSM medium contained 4 g/l of yeast extract, various concentration of the component (g/l): 1, 2, 3, 4 and 5, were applied in BSM medium containing (g/l) 10, 10, and 1.5 of cassava starch, tryptone, and beef extract, respectively. It was found that the isolate A5UVU25 could produce maximum bacteriocin when using both 4 and 5 g/l of yeast extract (Figure 7D). So, the low concentration of 4 g/l of yeast extract was used for further optimization.

Up to this step, the suitable medium for cultivating isolate A5UVU25 was BSM medium containing 10 g/l of cassava starch, 10 g/l of tryptone, 1.5 g/l of beef extract, and 4 g/l of yeast extract. The medium was used to determine the suitable initial pH as well as the optimal temperature to obtain the maximum bacteriocin yield.

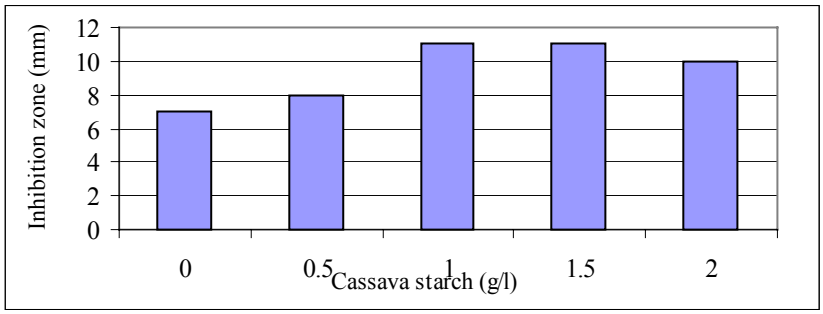
4.4.4 Initial pH of bacteriocin production medium

To obtain the maximum bacteriocin production, the initial pH of the suitable medium from section 4.4.3 was varied at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Results showed that the isolate A5UVU25 could not produce bacteriocin when cultivated in medium with both initial pH 5.0 and 5.5. The highest bacteriocin activity was obtained when the medium was adjusted to both initial pH 6.5 and 7 (Figure 8A). But the bacteriocin activity decreased when the initial pH of medium was adjusted to

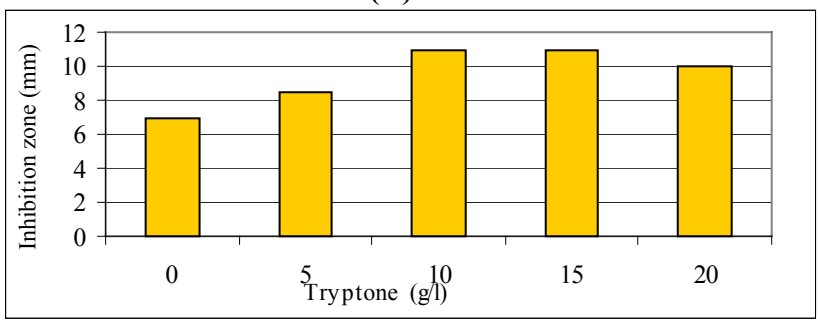
both pH 7.5 and 8.0. This indicated that the initial pH of medium at 6.5 or 7 could support for the bacteriocin production. Therefore, the initial pH of medium at 7.0 was chosen for further optimization.

4.4.5 Production temperature

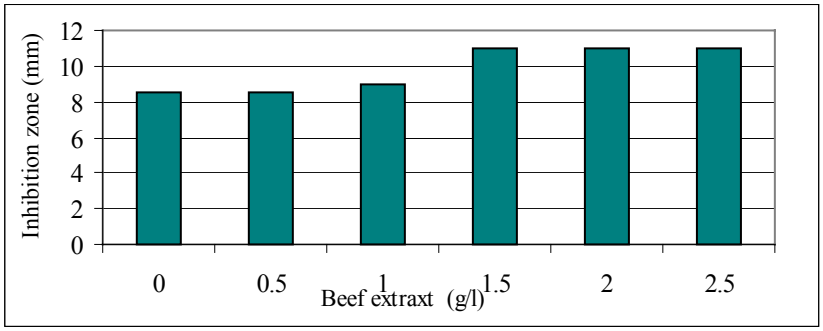
The optimal temperature for bacteriocin production was determined by cultivating the isolate A5UVU25 in the optimized medium for 18 h. The incubation temperatures were varied at 25, 28, 30, 32, 35 and 37°C based on the range of its growth temperatures. Results showed that the isolate A5UVU25 could not produce bacteriocin when it incubated at both 25 and 37°C. But it could produce bacteriocin when cultivated at 28, 30 and 35°C (Figure 8B). And the maximum bacteriocin production was obtained when the isolate was incubated at 30°C.



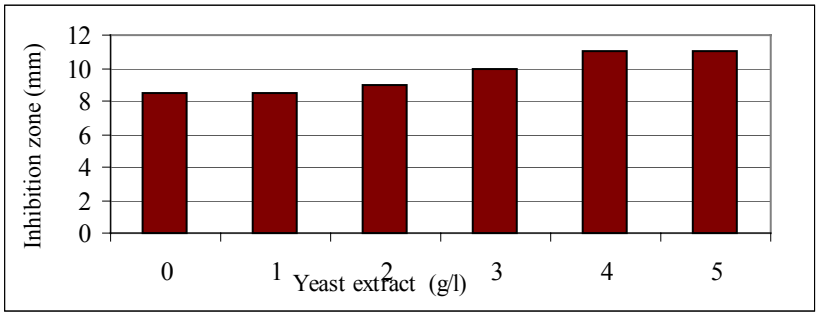
(A)



(B)

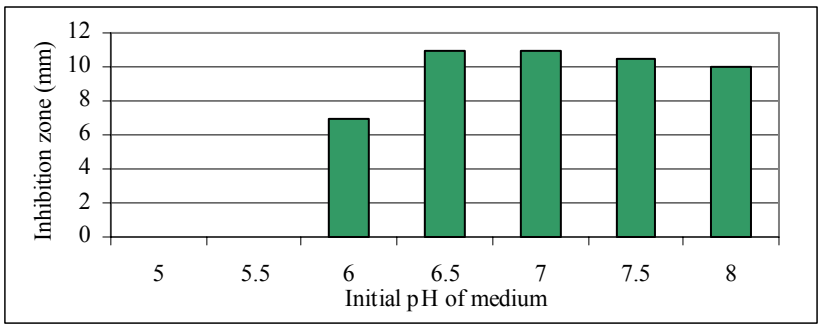


(C)

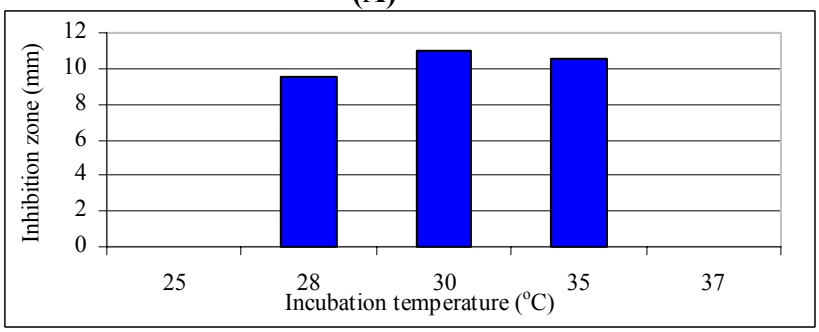


(D)

Figure 7. Inhibition zones of bacteriocin activity of isolate A5UVU25 against *Staphylococcus aureus* TISTR029 when the isolate A5UVU25 was cultured in BSM basal medium with various concentrations of cassava starch (A), tryptone (B), beef extract (C), and yeast extract (D).



(A)



(B)

Figure 8. Inhibition zones of bacteriocin activity of isolate A5UVU25 against *Staphylococcus aureus* TISTR029 when the isolate A5UVU25 was cultured in the optimized medium with various initial pHs of medium (A), and in the most suitable production medium at various incubation temperatures (B).

Table 6. Comparison of components of BSM medium and the optimized medium.

Component	BSM medium (g/l)	Optimized medium (g/l)
Glucose	2	-
Cassava starch	-	10
Tryptone	10	10
Beef extract	2	1.5
Yeast extract	4	4
K_2HPO_4	8.7	8.7
KH_2PO_4	8	8
$MgSO_4.7H_2O$	0.2	0.2
$MnSO_4.4H_2O$	0.05	0.05
tri-Ammonium citrate	2	2
Tween 80	1 ml	1 ml

4.5 Production of bacteriocins using the optimum conditions

Time course of bacteriocin production from isolate A5UVU25 was investigated to obtain the most suitable incubation time. The isolate was cultured in the optimized medium and incubated under anaerobic condition at 30°C. The activity of bacteriocin was detected when the isolate was cultured for 12, 14, 16, 18, 20, 24, and 48 h. It was found that the bacteriocin production commenced at 16 h of incubation because 8-mm diametre of inhibition zone was observed. And the highest bacteriocin activity was found at 18-, 20-, and 24-h incubation times (Figure 9). The bacteriocin activity decreased when the isolate was cultivated longer than 24 h. Thus, the incubation period chosen for the bacteriocin production was 18 h. The bacteriocin

was then produced by the isolate using the optimum production conditions and incubation period for its characterization.

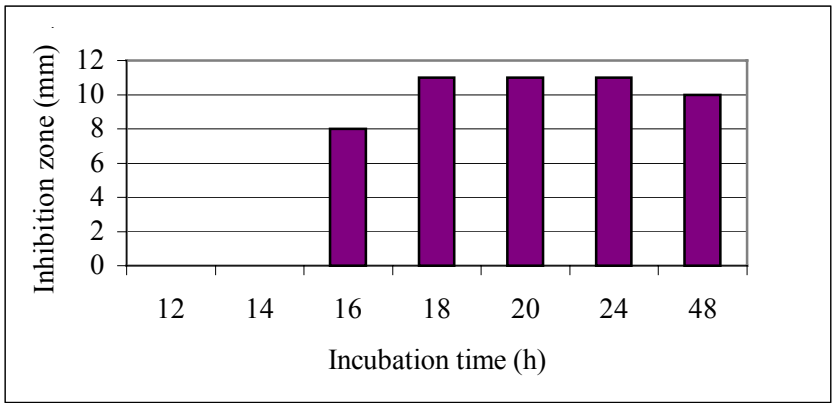


Figure 9. Inhibition zones of bacteriocin activity of isolate A5UVU25 against *Staphylococcus aureus* TISTR029 using the optimum production conditions.

4.6 Characterization of bacteriocins produced by the selected strain

4.6.1 Antimicrobial spectrum

When the neutralized crude bacteriocin of isolate A5UVU25 was tested against 25 strains of test organisms. The bacteriocin could inhibit the 16 out of 25 indicator strains (Table 8). It showed inhibitory spectrum towards various Gram-positive bacterial strains of genera *Bacillus*, *Lactobacillus*, *Listeria*, *Micrococcus*, *Pediococcus*, and *Staphylococcus*, but had no effect to Gram-positive bacteria *Streptococcus thermophilus* and *Staphylococcus aureus* TISTR517, and Gram-negative bacterial strains of genera *Escherichia*, *Pseudomonas*, and *Salmonella*. The largest inhibition zone (15 mm diametre) was obtained when *Bacillus stearothermophilus* TISTR329 was used as a test organism, and the bacteriocin concentration compared to the standard nisin was 20.31 IU/ml.

Table 7. Antimicrobial activity of bacteriocins produced by isolate A5UVU25.

Strain of test microorganism	Inhibition zone (mm)
Bacillus stearothermophilus TISTR329	15
Bacillus cereus TISTR687	0
Bacillus subtilis TISTR008	0
Escherichia coli TISTR780	0
Escherichia coli TISTR887	0
Lactobacillus acidophilus TISTR1034	12
Lactobacillus brevis subsp. *brevis* TISTR855	12
Lactobacillus delbrueckii TISTR326	11
Lactobacillus delbrueckii subsp. *bulgaricus* TISTR892	12
Lactobacillus delbrueckii subsp. *lactis* TISTR785	12
Lactobacillus fermentum TISTR055	12
Lactobacillus plantarum TISTR050	12
Listeria monocytogenes DMS1327	11
Micrococcus luteus TISTR884	13
Pediococcus acidilactici TISTR051	12
Pediococcus acidilactici TISTR783	12
Pediococcus pentosaceus TISTR374	12
Pediococcus pentosaceus TISTR954	12
Pseudomonas aeruginosa TISTR781	0
Salmonella durby SH1455/95	0
Salmonella typhimurium TISTR022	0
Streptococcus thermophilus TISTR894	0
Staphylococcus aureus TISTR029	11
Staphylococcus aureus TISTR118	11
Staphylococcus aureus TISTR517	0

4.6.2 Effects of enzymes, temperature, and pH on bacteriocin activity

Protease sensitivity is a key criterion for the characterization of an inhibitory substance as bacteriocins (Holo *et al.*, 1991). When the crude bacteriocin produced by isolate A5UVU25 was treated with protease, protinase K, and lysozyme at the concentration of 1 mg/ml with incubating at 37°C for 2 h, protease and protinase K had great effects but lysozyme had no effect to bacteriocin activity (Figure 10). These indicated that the substance which produced by A5UVU25 was protein, which was sensitive to the proteolytic enzymes.

For temperature sensitivity test, the bacteriocin was treated under different temperatures for 15 or 30 min. Then, the bacteriocin activity was detected using *Bacillus stearothermophilus* TISTR329 as the test organism. It was found that the bacteriocin produced by A5UVU25 was stable at 60 and 70°C for 30 min, and 80°C for 15 min. But its activity was completely lost when heated at 100°C for 30 min and 121°C for 15 min (Figure 11A).

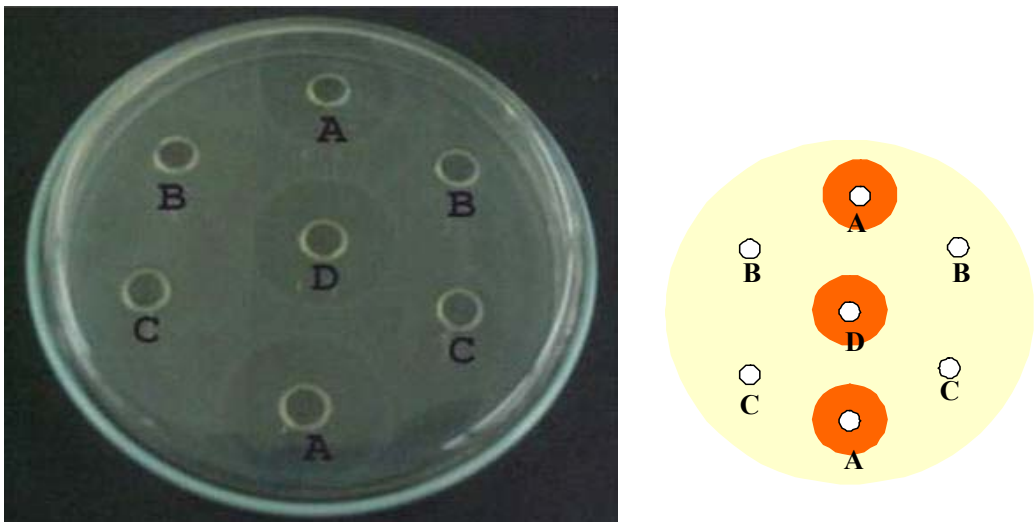


Figure 10. Stability of bacteriocin activity of isolate A5UVU25 to enzymes: lysozyme (A), protease (B), protinase K (C), and control (D). *Bacillus stearothermophilus* TISTR329 was used as the test organism.

When the pH stability of bacteriocin was tested, it was found that the bacteriocin activity was stable at pH ranged from 6.0-8.0 but slightly decreased at pH 5.0 and 9.0 (Figure 11B). But when the crude bacteriocin was adjusted to pH between 1.0-4.0 and 10.0-14.0 it was difficult to neutralized to pH 6.5 for bacteriocin activity assay. The crude bacteriocin became so diluted, which resulted in no activity detected.

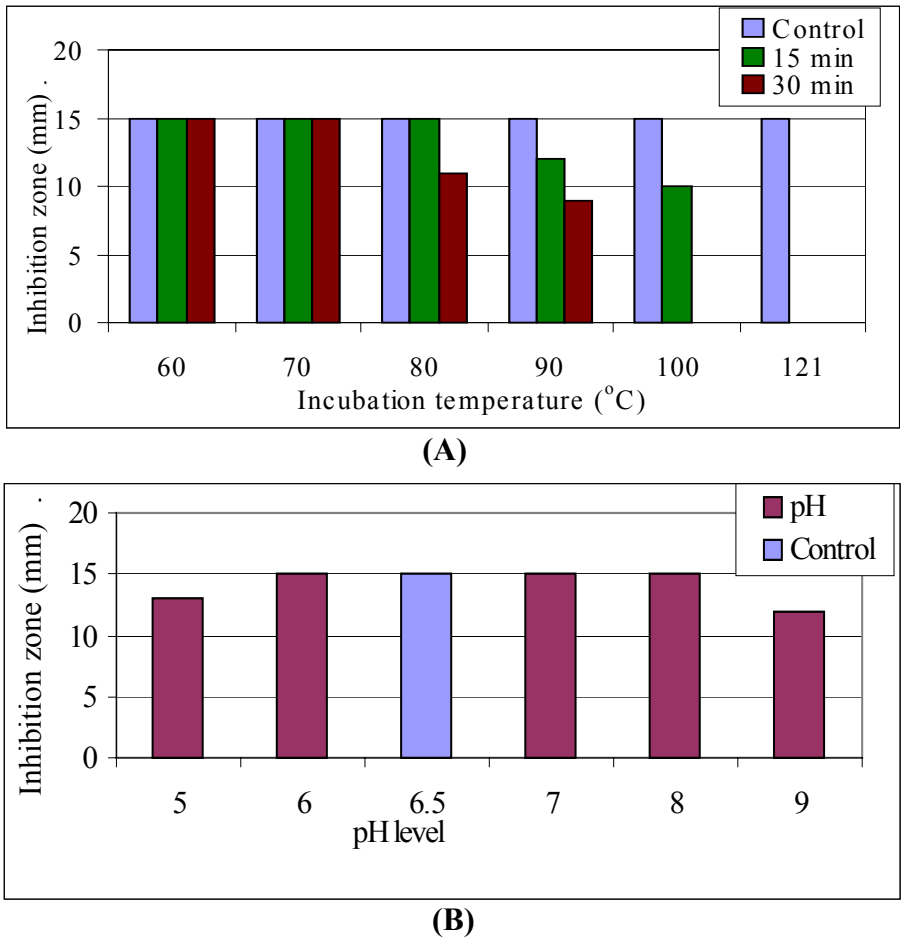


Figure 11. Stability of bacteriocin activity of isolate A5UVU25 to heat (A) and pH (B). *Bacillus stearothermophilus* TISTR329 was used as the test organism.

4.6.3 Molecular mass and bacteriocin activity in gel

For the beginning of this step, the molecular mass or molecular weight of the partial purified bacteriocin produced by isolate A5UVU25, was examined using sodium dedocyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two gels of SDS-PAGE were performed. The first gel was stained with SimpleBlue (invitrogen). Result showed that, at least six bands of protein were observed (Figure 12).

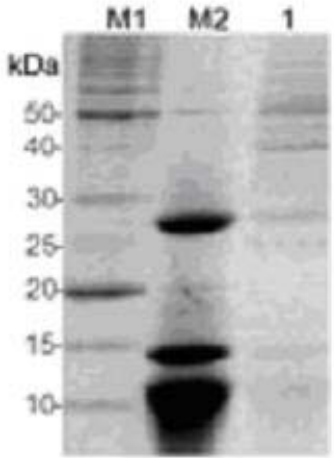


Figure 12. SDS-PAGE of partial purified bacteriocin produced by isolate A5UVU25. Lanes: M1, BenchMarkTM Protein Ladder (Invitrogen); M2, Ultra Low Range (Sigma); and 1, partial purified bacteriocin.

The unstained gel was washed 3 times with sterile deionized water to get rid of undesired chemicals and use for the determination of bacteriocin activity in gel. The gel was overlaid with 7 ml of 0.75% TSA agar containing 10^6 CFU/ml of Staphylococcus aureus TISTR029. After incubation at 37°C for 18 h, the location of

active bacteriocin band was observed. The zone corresponded to the protein band of 12 kDa was found presented (Figure 13).

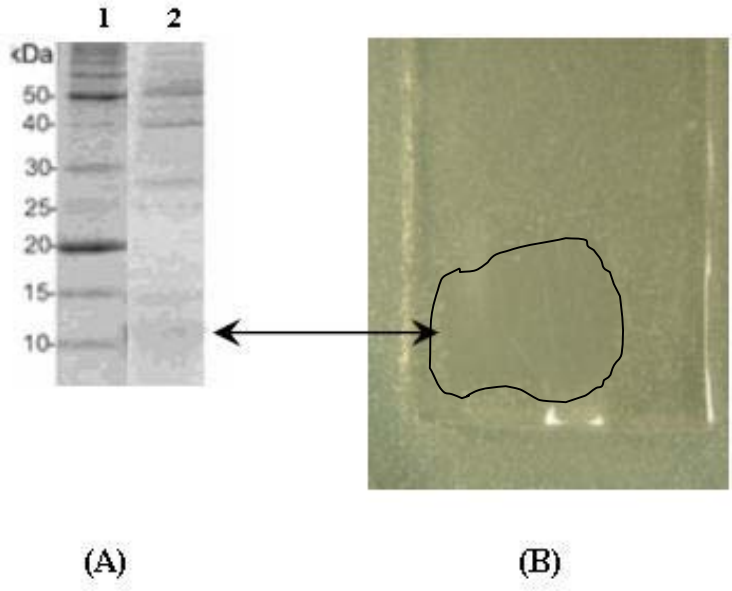


Figure 13. SDS-PAGE gels of partial purified bacteriocin: (A) Stained gel; lanes: 1, BenchMarkTM Protein Ladder (Invitrogen); and 2, partial purified bacteriocin. (B) Unstained gel overlaid with soft agar containing *Staphylococcus aureus* TISTR029. Arrow indicates the location of inhibition zone on unstained gel corresponding to the protein band on stained gel.

4.7 Detection and sequence analysis of gene(s) encoding bacteriocins

Since only one isolate, A5UVU25, was found to produce bacteriocin which could be detected by agar well diffusion technique, 70 isolates of starch-utilizing and lactic acid-producing bacteria were also randomly selected according to their identification results and bacteriocin-producing species reported in literatures.

Genomic DNAs of 18-h cells (Figure 14, for example) from 70 isolates, including genomic DNA of isolate A5UVU25, were extracted and used as a template for gene encoding bacteriocin detection.

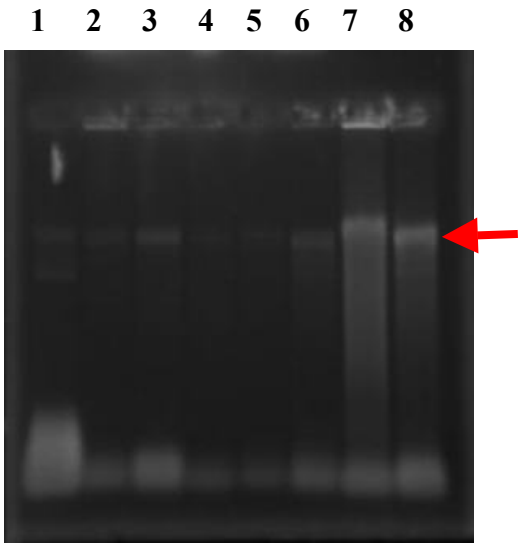


Figure 14. Agarose gel electrophoresis of genomic DNA (arrow) extracted from 8 isolates of selected starch-utilizing and lactic acid-producing bacteria (lanes 1-8).

The specific primers of lacticin 481, nisin A and plantaricin A genes were then used for the amplification of genes encoding bacteriocins using the polymerase chain reaction. Only isolate A5UVU25 was successfully amplified with primers generated from nisin A gene. The PCR amplification product was approximately 300 bp (Figure 15).

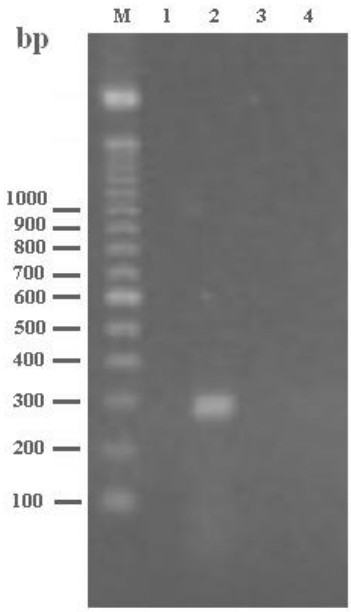


Figure 15. Agarose gel electrophoresis of PCR fragment obtained from the amplification of genomic DNA of isolate A5UVU25 using 3 pairs of specific primers of bacteriocin gene(s). Lanes: M, 100bp DNA ladder (Invitrogen) as a molecular weight marker; 1, negative control; lanes 2-4, PCR primers of: 2, nisin A; 3, lacticin 481; and 4, plantaricin A genes.

The nucleotide sequence of the PCR amplicon (300 bp) was analyzed (Figure 16). Then, the nucleotide sequence was compared to bacteriocin-encoding gene sequences that have been reported using BLAST version 2.2.9 program of the GenBank database of the National Center for Biotechnological Information (NCBI). The 218-bp nucleotide sequence was found to have 100% homology with nisin Z of *Lactococcus lactis* subsp. *lactis* accession number AF420259 (Lee *et al*., 2004) and D10768D01142 (Araya, 1992), and have 99% homology with nisin Z of *Lactococcus*

lactis subsp. *lactis* accession number M27277 (Dott *et al*., 1992); AF465351 (Johansen *et al*., 2002); and *Streptococcus lactis* accession number M24527 (Kaletta and Entian 1989) (Figure 17).

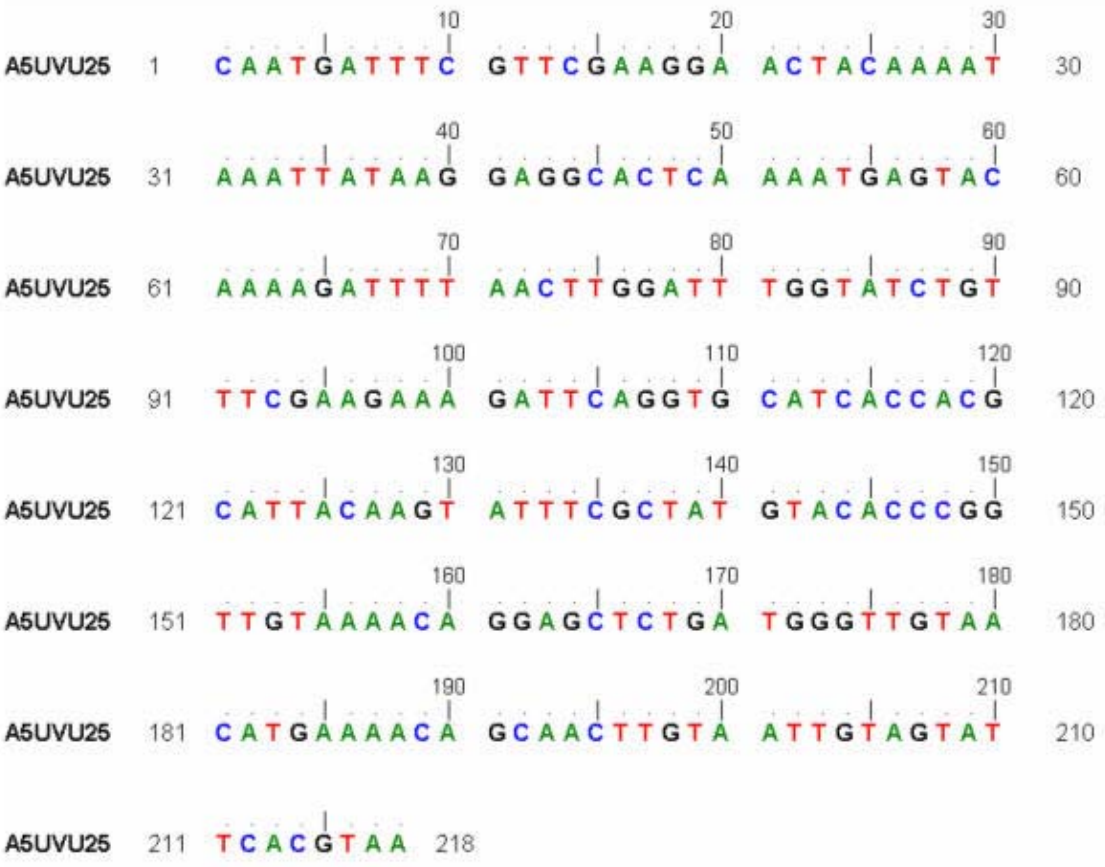


Figure 16. Nucleotide sequence (218 bp) of PCR product amplified with primers generated from nis A gene.

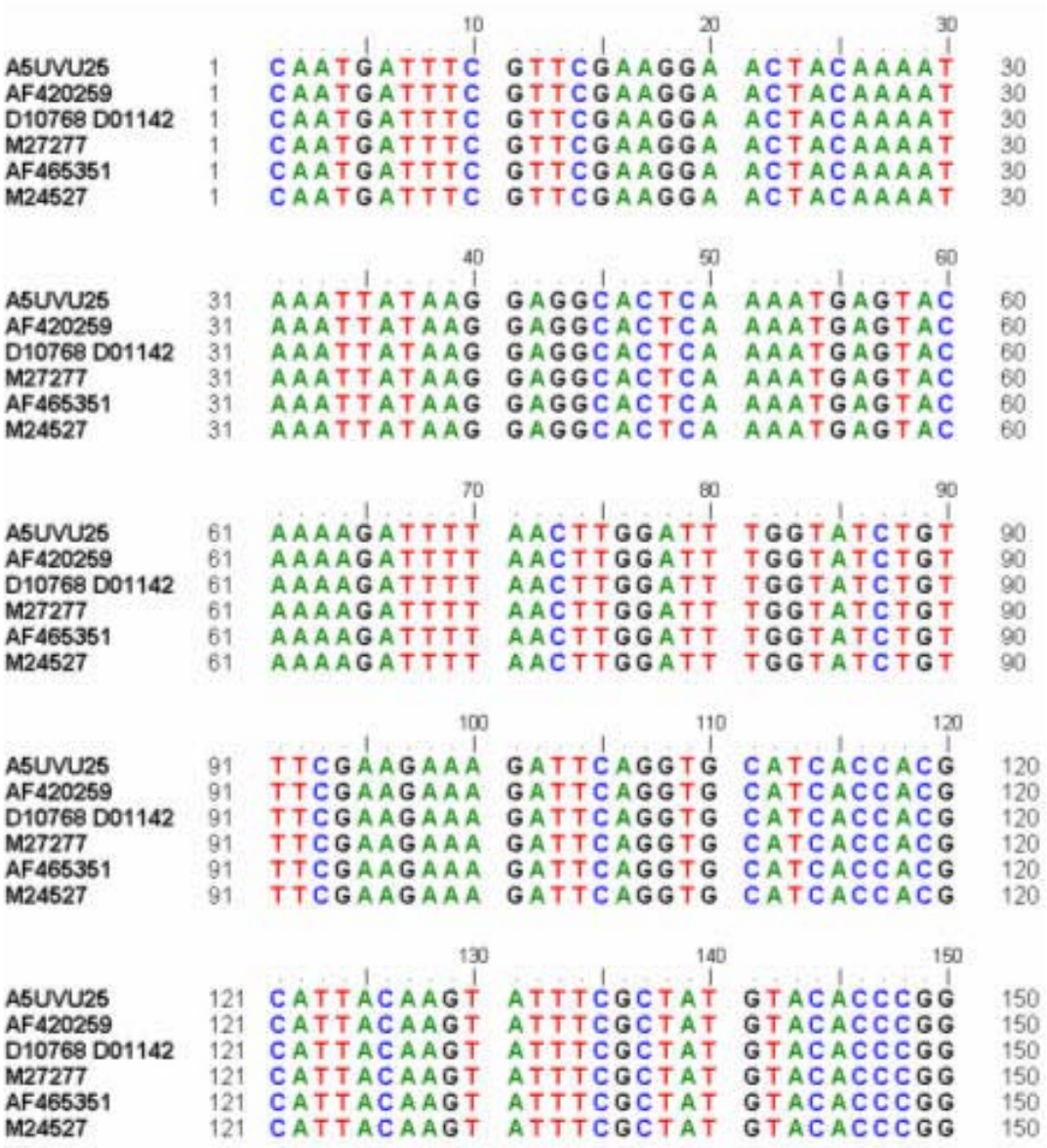


Figure 17. Nucleotide sequence (from the 5′terminus) aligment of PCR fragment from the amplification of genomic DNA of isolate A5UVU25 using specific primers of nisin A gene (top) and nisin Z gene sequences from GenBank (*Lactococcus lactis*, AF420259; *Lactococcus lactis*, D10768D01142; *Lactococcus lactis*, M27277; *Lactococcus lactis*, AF465351; and *Streptococcus lactis*, M24527) (bottom).

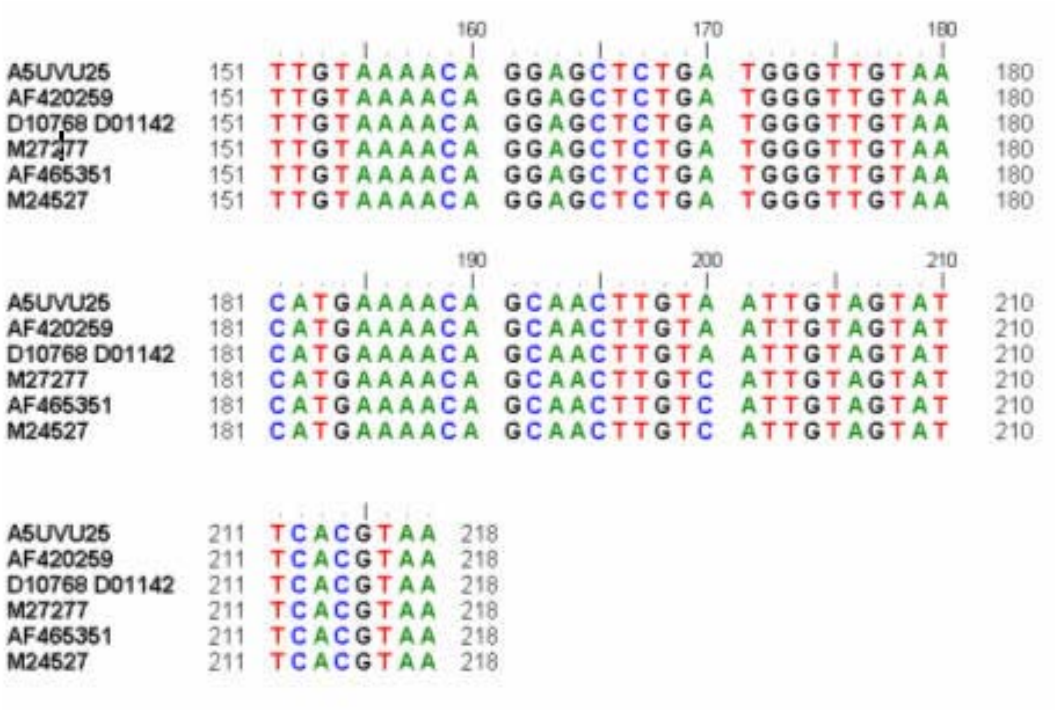


Figure 17. (Continued)

CHAPTER V

CONCLUSIONS

One hundred and nineteen isolates of starch-utilizing and lactic acid-producing bacteria were screened for their bacteriocin production. For the primarily screening, only one isolate, A5UVU25, obtained from stock cultures of the School of Microbiology, Suranaree University of Technology, was able to produce bacteriocin using the BSM broth. None of the tested isolates produced bacteriocins in both MRS and M17 media. The bacteriocin produced by the isolate could be detected by using only the agar well diffusion technique. There was no positive result when using both agar spot test and the paper disc assay. The bacteriocin of isolate A5UVU25 showed antimicrobial activity against the Gram-positive coccus, *Staphylococcus aureus* TISTR029, but did not inhibit growth of *Escherichia coli* TISTR887, *Bacillus cereus* TISTR687, and *Bacillus subtilis* TISTR008. The isolate A5UVU25 was previously identified as belonging to the genus *Lactococcus.*

The optimization of some bacteriocin production conditions was performed to obtain the highest yield of bacteriocin and low production cost. The isolate A5UVU25 could produce bacteriocin in the BSM medium containing cassava starch. The optimized medium composed of (g/l); cassava starch, 10; tryptone, 10; beef extract, 1.5; yeast extract, 4; K_2HPO_4, 8.7; KH_2PO_4, 8; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; tri-ammonium citrate, 2; and tween 80, 1 ml, at the initial pH of

7.0. The maximum bacteriocin production was achieved when cultured the isolate A5UVU25 in the optimized medium and incubated under anaerobic condition at 30°C for 18 h, which the inoculum size (10^6 cells/ml) at 10% (v/v) was used.

At the optimal production conditions, 12.30 IU/ml of bacteriocin activity compared to the standard nisin could be obtain from the isolate A5UVU25 using *Staphylococcus aureus* TISTR029 as test organism. The bacteriocin produced could inhibit the growth of several Gram-positive test organisms including *Bacillus stearothermophilus* TISTR329, *Lactobacillus acidophilus* TISTR1034, *Lactobacillus brevis* subsp. *brevis* TISTR855, *Lactobacillus delbruechii* TISTR326, *Lactobacillus delbruechii* subsp. bulgaricus TISTR892, *Lactobacillus delbruechii* subsp. lactis TISTR785, *Lactobacillus fermentum* TISTR055, *Lactobacillus plantarum* TISTR050, *Listeria monocytogenes* DMS1327, *Micrococcus luteus* TISTR884, *Pediococcus acidilactici* TISTR051, *Pediococcus acidilactici* TISTR783, *Pediococcus pentosaceus* TISTR374, *Pediococcus pentosaceus* TISTR954, *Staphylococcus aureus* TISTR029, and *Staphylococcus aureus* TISTR118. But it could not inhibit the growth of some other Gram-positive bacteria which were *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008, *Streptococcus thermophilus* TISTR894, and *Staphylococcus aureus* TISTR517 and Gram-negative bacteria *Escherichia coli* TISTR780, *Escherichia. coli* TISTR887, *Pseudomonas aeruginosa* TISTR781, *Salmonella durby* SH1455/95, and *Salmonella typhimurium* TISTR022. The bacteriocin concentration tested against *Bacillus stearothermophilus* TISTR329 was 20.31 IU/ml when compared to the standard nisin. The bacteriocin was sensitive to protease and protinase K enzymes and heat at 100°C for 30 min. The activity also decreased at pH of 5.0 and 9.0 for 2 h. But it stable to lysozyme, heat at 80°C for 15 min, and at pH range of 6.0-8.0 for 2 h. This

indicated that the obtained antimicrobial compound was the heat-stable protein. The molecular weight of the bacteriocin was 12 kDa.

The gene encoding bacteriocin of the isolate A5UVU25 could be amplified by using nisin gene-specific primers. The 218 bp of amplified product was sequenced. The obtainable nucleotide sequence had 100% homology to the lantibiotic bacteriocin nizin Z gene sequence of *Lactococcus lactis* subsp. *lactis*.

From this study, the starch-utilizing and lactic acid-producing bacterium isolate A5UVU25 could produce both lactic acid and bacteriocin using cassava starch, cheap and abundant raw material, that would be very useful for both lactic acid and bacteriocin production. Moreover, this heat-stable bacteriocin would be applied for both food and lactate industry to prevent the growth of some contaminants, food spoilage bacteria, and food-borne pathogens. Data of gene encoding bacteriocin from this isolate would be the prerequisite data for gene cloning as well as bacteriocin-producing strain detection or identification. However, further investigation has to be performed.

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APPENDICES

APPENDIX A

MICROBIOLOGICAL MEDIA

1. Bacteriocin screening medium (BSM)

tri-Ammonium citrate	2.00	g
Beef extract	2.00	g
Tryptone	10.00	g
Tween 80	1.00	g
Yeast extract	4.00	g
K_2HPO_4	8.70	g
KH_2PO_4	8.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Glucose	2.00	g

Final pH 7.0

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 13 psi pressure at 121°C for 15 min.

2. M17 medium

di-Sodium β-glucophosphate	19.00	g
Beef extract	5.00	g
Lactose	5.00	g
Glucose	5.00	g
Soy peptone	5.00	g
Tryptone	2.50	g
Yeast extract	2.50	g
Acorbic acid	0.50	g
$MgSO_4.7H_2O$	0.25	g

Final pH 7.5

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 13 psi pressure at 121°C for 15 min.

3. De Man Rogosa and Sharpe medium (MRS)

Peptone	10.00	g
Meat extract	10.00	g
Yeast extract	5.00	g
Glucose	10.00	g
Tween 80	1.00	g
K_2HPO_4	2.00	g
Sodium acetate	2.00	g
di-Ammonium citrate	0.20	g

$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g

Final pH 7.0

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 115°C for 10 min.

4. Rogosa agar with modification medium (RAM) (Rodtong and Ishizaki, 2003)

Tryptone	5.00	g
K_2HPO_4	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.076	g
$FeSO_4.7H_2O$	0.050	g
Cassava starch	10.00	g

Final pH 7.0

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 13 psi pressure at 121°C for 15 min.

5. Tryptic soy broth (TSB)

Tryptone	17.0	g
Soytone	3.0	g
Dextrose	2.5	g
Sodium chloride	5.0	g
Dipotassium phosphate	2.5	g

Final pH 7.3

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 13 psi pressure at 121°C for 15 min.

APPENDIX B

REAGENTS

1. Reagents for genomic DNA extraction

1.1 Lysis buffer; (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA)

Tris Base	3.950	g
EDTA	3.722	g
Glucose	9.000	g

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved, adjusted pH to 8.0, and brought volume up to 1 l.

1.2 SDS (10% w/v)

Sodium dodecylsulfate	100	g

Preparation of reagent: the component was added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l.

2. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.1 Acrylamide stock solution

Acrylamide	300	g
Bisacrylamide	8	g

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l. Store at 4°C away from light.

2.2 Tris (1.5 M)

Tris Base	181.5	g

Preparation of reagent: the component was added to distilled water, mixed thoroughly until dissolved, adjusted pH to 8.8, and brought volume up to 1 l.

2.3 Tris (0.5 M)

Tris Base	60	g

Preparation of reagent: the component was added to distilled water, mixed thoroughly until dissolved, adjusted pH to 6.8, and brought volume up to 1 l.

2.4 Ammonium persulfate (APS) (10% w/v)

Ammonium persulfate	100	g

Preparation of reagent: the component was added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l.

2.5 SDS-gel loading buffer (2×)

0.5 M Tris, pH 6.8	12.5	ml
10% SDS	20.0	ml
Glycerol	10.0	ml
2-mercaptoethanol	1.0	ml

Bromophenol blue	1.0	mg

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l. Divide in to 1 ml aliquot and store at -20°C.

2.6 Electrophoresis buffer

Tris Base	6.0	g
Glycine	28.8	g
SDS	2.0	g

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l.

2.7 Separating gel SDS-PAGE (15% w/v)

Sterilize deionized water	236	ml
Acrylamide stock	500	ml
1.5 M Tris, pH 8.8	250	ml
10% SDS	10	ml
10% APS	15	ml
TEMED	1	ml

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l.

2.8 Stacking gel SDS-PAGE (4% w/v)

Sterilize deionized water	600	ml
Acrylamide stock	134	ml
0.5 M Tris, pH 6.8	250	ml
10% SDS	10	ml
10% APS	5	ml
TEMED	1	ml

Preparation of reagent: all components were added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l.

APPENDIX C

STANDARD CURVE OF NISIN

Various concentrations of nisin (10, 20, 30, 40, 50, 60, 80, 90 and 100 IU/ml) were tested against *Bacillus stearothermophilus* TISTR329 which correlated to the selected bacteriocin. Inhibition zones were measured. Nisin concentration and average inhibition zone diametre valves were used for standard curve preparation by calculating using linear regression equations (Crowe and Crowe, 1969) as follows:

$$Y = ab^x$$

or $$\text{Log } Y = \log a + x \log b$$

$$\sum_{i=1}^{n} \log Yi = n(\log a) + (\log b) \sum_{i=1}^{n} Xi \qquad (1)$$

$$\sum_{i=1}^{n} Xi \log Yi = (\log a) \sum_{i=1}^{n} Xi + (\log b) \sum_{i=1}^{n} Xi^2 \qquad (2)$$

Xi = inhibition zone (mm)

Yi = nisin concentration (IU/65 μl)

Table 1 C. Statistic data for nisin standard curve.

Number	Yi	log Yi	Xi	X^2i	Xi log Yi
1	0.65	-0.19	11	121	-2.09
2	1.30	0.11	13	169	1.43
3	1.95	0.29	18	324	5.22
4	2.60	0.41	20	400	8.2
5	3.25	0.51	23	529	11.73
6	3.90	0.59	24	576	14.16
7	4.55	0.66	26	676	17.16
8	5.20	0.72	27	729	19.44
9	5.85	0.77	28	784	21.56
10	6.50	0.81	28.5	812.25	23.085
		4.68	218.5	5120.25	119.895

Xi = inhibition zone against *Bacillus stearothermophilus* TISTR329 (mm)

Yi = nisin concentration (IU/65 μl)

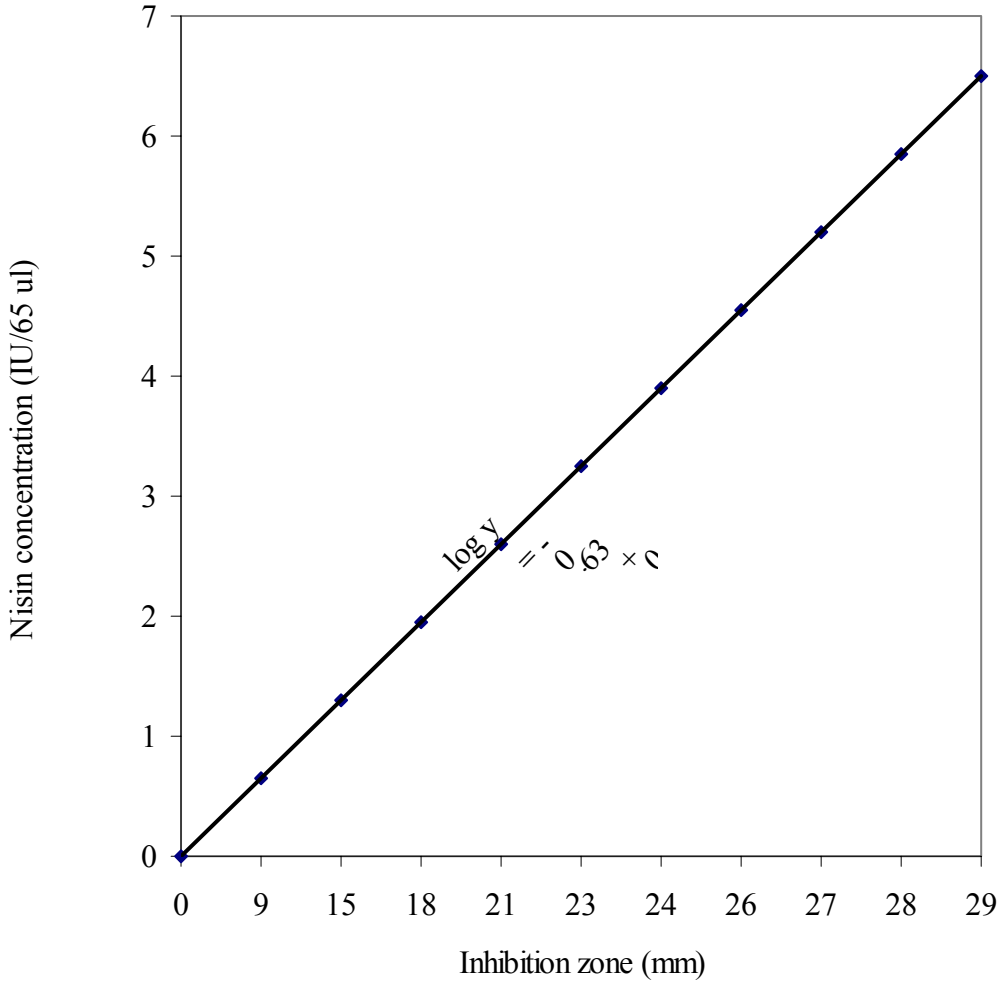

Figure 1C. Standard curve of nisin.

APPENDIX D

NUCLEOTIDE SEQUENCE DATA

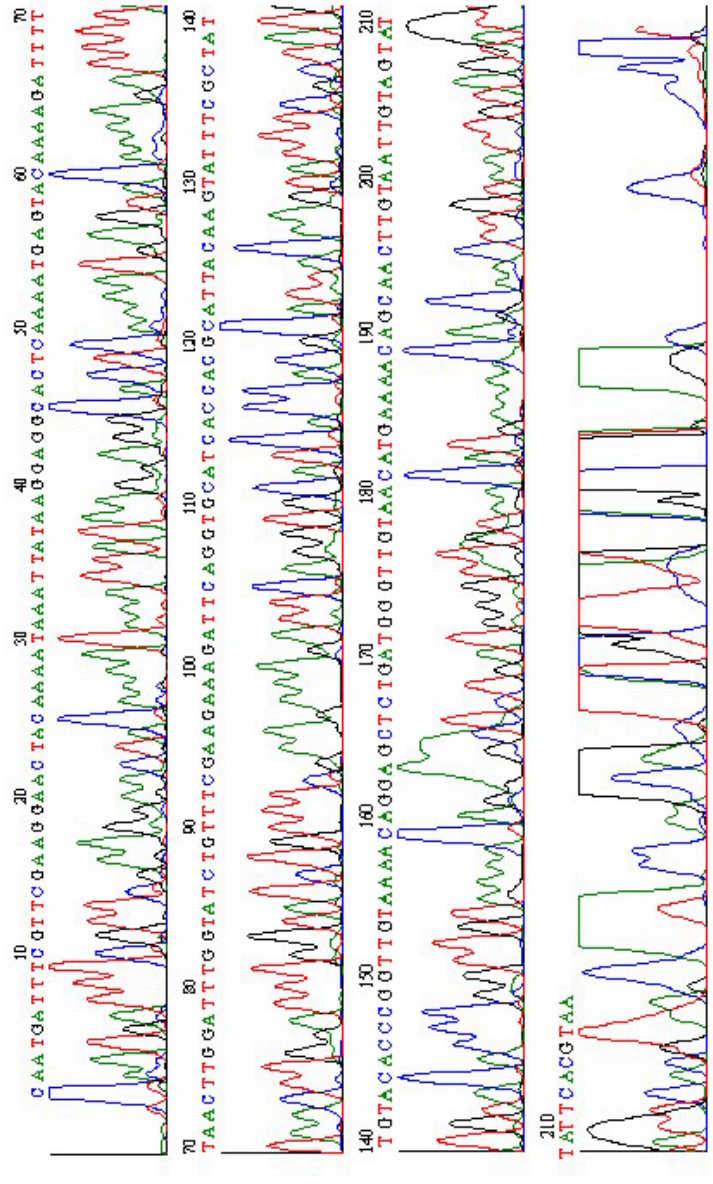

Figure 1D. The sequence electrophenogram of gene coding bacteriocin of isolate A5UVU25 amplified using nisin gene-specific primer.

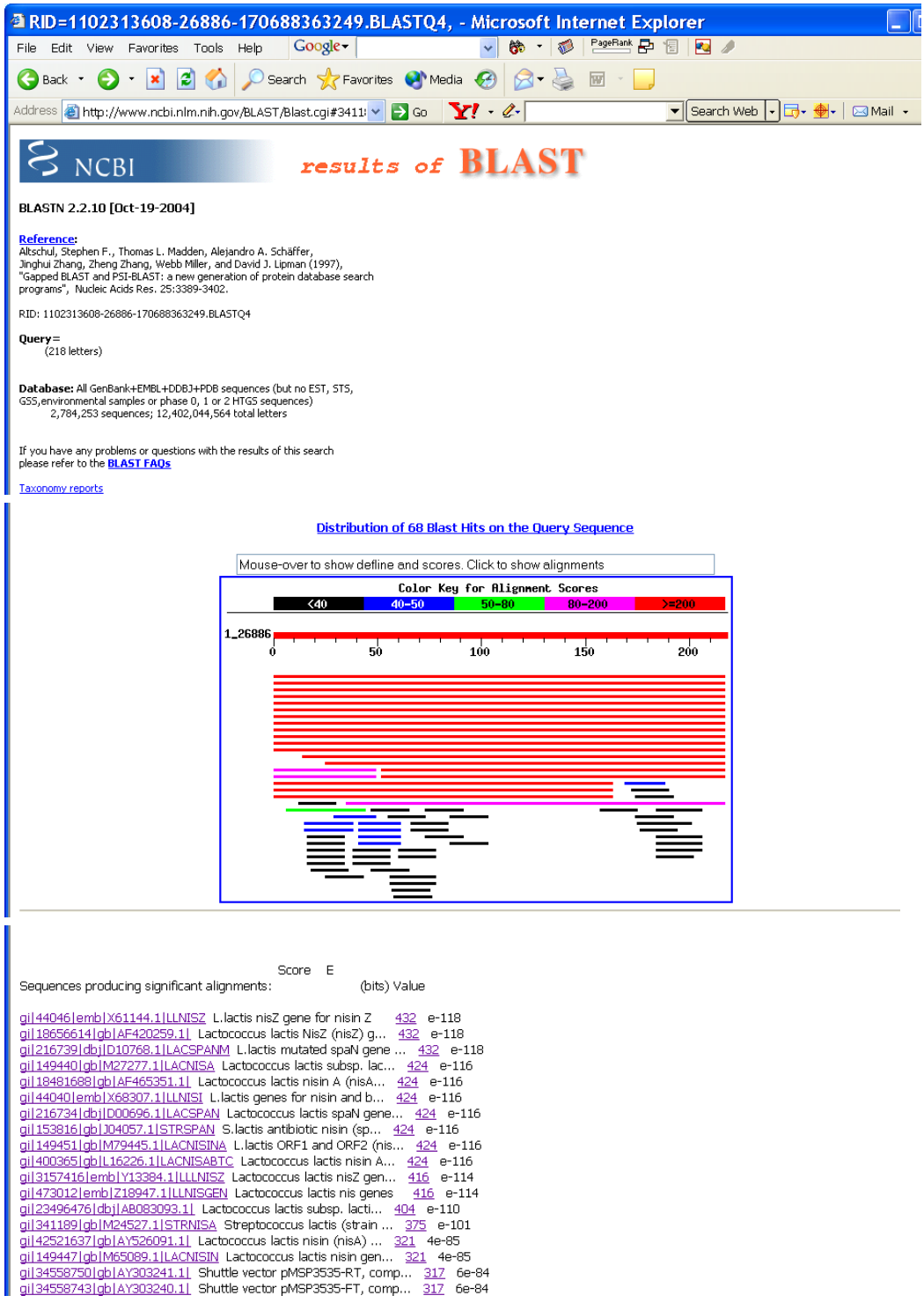

Figure 2D. The compared result of PCR product obtained from nucleotide database provided by the National Center for Biotechnology Information (NCBI) using the BLAST.

APPENDIX E

LIST OF PRESENTATIONS

Rodtong, S., Sansit, J., Chimsoongnern, P., Vechklang, K., and Ishizaki, A. (2001). **Strain improvement of starch-utilizing bacteria by mutagenesis to enhance L-lactic acid production.** BioThailand 2001, November 7-10, 2001, Queen Sirikit National Convention Center, Bangkok, Thailand. (Poster Presentation).

Sansit, J. and Rodtong, S. (2004). **Bacteriocin production by homolactic starch-utilizing bacteria.** The 15th Annual Meeting of the Thai Society for Biotechnology and JSPS-NRCT Symposium, February 3-6, 2004, Lotus hotel Pang Suan Kaew , Chiang Mai, Thailand. (Poster Presentation).

Sansit, J. and Rodtong, S. (2004). **Bacteriocins from starch-utilizing and lactic acid-producing bacteria.** The 4th National symposium on Graduate Research, August 10-11 2004, Pang Suan Kaew Hotel, Chiang Mai, Thailand. (Poster Presentation).

P-MICRO-GENETIC-12

Strain improvement of starch-utilizing bacteria by mutagenesis to enhance L-Lactic acid production

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Lactic acid is one of typical organic acids produced by microbial fermentation of glucose. The increase in demand of L-lactic acid as a raw material of a biodegradable polymer is going on. Therefore, investigations aiming to obtain efficient lactate production and high productivity have been being attempted by several investigators. This study aims to obtain potential mutant strains for homolactic production from cassava starch, a cheap and abundant raw material which could be of great benefit to the fermentative production of lactic acid. *Lactococcus lactis* IO-1, a homolactic fermenting and non-starch-utilizing strain, was used as the reference strain for comparing L-lactic acid production capability. Two strains of starch-utilizing and homofermentative bacteria (isolates SUT-1 and SUT-5) isolated from cassava starch waste samples in Nakhon Ratchasima Province, Thailand, were selected for strain improvement using two mutagens: Ultraviolet (UV) light and N-Methyl-N′-Nitro-N-Nitrosoguanidine (MNNG) to enhance their lactic acid production from cassava starch. The selected strains were identified as belonging to different strains of the genus *Lactococcus*, and could produce the maximum amounts of lactic acid of about 10 and 9 g/l respectively in the suitable medium containing 2% cassava starch. While *Lactococcus lactis* IO-1 could produce lactic acid at the concentration of around 11 g/l in the same basal medium containing 2% glucose. The selected bacteria were exposed to either UV light or MNNG for three rounds. Mutants were selected to compare their lactic acid production capabilities. Two mutants produced approximate 10% of lactic acid higher than their original strains were finally selected and maintained for using as the potential strains for the direct production of lactic acid from cassava starch. After subculturing the cultures for sixth times, the mutants could still produce the similar amounts of lactic acid as before the first subculturing.

th
The 15 Annual Meeting of the Thai Society for Biotechnology on
"Sustainable Development of SMEs Through Biotechnology"

JSPS - NRCT Symposium
"The Forefront of Bioinformatics Application"

Bacteriocin Production by Homolactic Starch-Utilizing Bacteria

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Bacteriocins, proteinaceous bactericidal compounds produced by bacteria, are currently much interest in the application in food industry. Lactic acid, especially L(+)-lactic acid produced by the bacterial fermentation of glucose, is used as a raw material for biodegradable polymer production, and for food and cosmetic preservation. Based on a cheap and abundant raw material, the starch-utilizing bacteria that could potentially produce both lactic acid and bacteriocins will be very useful for both the acid and antimicrobial compound production. This study aims to investigate bacteriocin production by homolactic starch-utilizing bacteria. A total of 119 isolates of starch-utilizing and lactic acid-producing bacteria were screened for their bacteriocin production capability. A homofermentative isolate identified as belonging to the genus *Lactococcus* showed its bacteriocin activity against the Gram-positive coccus, *Staphylococcus aureus* TISTR 029, when detected by the agar well diffusion technique. The crude bacteriocin did not inhibit growth of *Escherichia coli* TISTR 887, *Bacillus cereus* TISTR 687, and *Bacillus subtilis* TISTR 008. The selected isolate could not produce bacteriocin in MRS medium. But the medium containing cassava starch could support its production under anaerobic condition at 30°C for 18 hours when 10% (v/v) inocula of late-exponential phase (10^6 CFU/ml) were applied. The suitable production medium composed of (g/l); cassava starch, 10; tryptone, 10; beef extract, 2; yeast extract, 4; K_2HPO_4, 8.7; KH_2PO_4, 8; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; Tween 80, 1; and citric acid diammonium salt, 2. The gene coding for bacteriocin was also detected by amplifying using nisin gene-specific primers. The characterization of bacteriocin and gene encoding bacteriocin of *Lactococcus* isolate has been being performed.

Key words : bacteriocin, lactic acid, starch, fermentation

Bacteriocins from Starch-Utilizing and Lactic Acid-Producing Bacteria

จิตรากรณ์ แสนสิทธิ์ และ สุรีลักษณ์ รอดทอง
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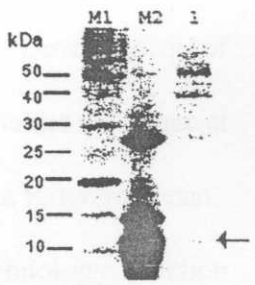
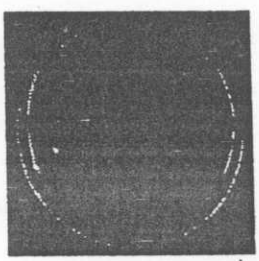
บทคัดย่อ: แบคทีริโอซินเป็นสารประกอบโปรตีนที่ผลิตจากแบคทีเรียซึ่งมีสมบัติยับยั้งการเจริญของจุลินทรีย์อื่นโดยเฉพาะอย่างยิ่งแบคทีเรีย และมีการนำไปใช้ประโยชน์อย่างกว้างขวางในอุตสาหกรรมอาหารและการหมัก การศึกษาครั้งนี้มุ่งเน้นถึงการผลิตและศึกษาสมบัติของแบคทีริโอซินจากแบคทีเรียที่สามารถใช้แป้งและผลิตกรดแล็กติก เพื่อเพิ่มศักยภาพของกระบวนการผลิตกรดแล็กติกและการผลิตสารแบคทีริโอซิน จากการศึกษาพบแบคทีเรียไอโซเลท A_5UVU_{25} สามารถผลิตแบคทีริโอซินที่มีผลยับยั้งการเจริญของแบคทีเรียแกรมบวกหลายสายพันธุ์รวมถึง *Listeria monocytogenes* DMS 1347 ได้ สารที่ผลิตมีขนาดโมเลกุล 10-15 กิโลดาลตัน มีความเสถียรต่อความร้อนถึงอุณหภูมิ 80°C เป็นเวลา 15 นาที และยีนที่เกี่ยวข้องกับการผลิต (ซิ้นดีเอ็นเอขนาด 273 คู่เบส) มีลำดับเบสเหมือนกับ Nisin Z (99% Homology)

ระเบียบวิธีวิจัย: เพาะเลี้ยงแบคทีเรียไอโซเลท A_5UVU_{25} ซึ่งคัดเลือกได้จากเชื้อทั้งสิ้น 119 ไอโซเลท ในอาหารเลี้ยงเชื้อที่มีแป้งมันสำปะหลังเป็นแหล่งคาร์บอนเพื่อผลิตแบคทีริโอซิน พร้อมทั้งศึกษาสภาวะที่เหมาะสมในการผลิต และศึกษาสมบัติของแบคทีริโอซินตาม Lee and Paik (1) รวมถึงลำดับของยีนที่เกี่ยวข้องกับการผลิตแบคทีริโอซินตาม Rodriguez *et al.* (2)

ผลการวิจัย อภิปราย และสรุปผลการวิจัย: แบคทีเรียที่นำมาศึกษาจัดอยู่ในสกุล *Lactococcus* ตามลักษณะทางสัณฐานและชีวเคมี (3) สามารถผลิตแบคทีริโอซินในอาหารเลี้ยงเชื้อที่มีแป้งมันสำปะหลัง การผลิตแบคทีริโอซินสูงสุดเมื่อเลี้ยงในอาหารที่ประกอบด้วย (%) $MnSO_4.4H_2O$, 0.005; $MgSO_4.7H_2O$, 0.02; Tween 80, 0.1; Beef extract, 0.2; Citric acid diammonium salt, 0.2; Yeast extract, 0.4; KH_2PO_4, 0.8; K_2HPO_4, 0.87; Tryptone, 1 และแป้งมันสำปะหลัง, 1 ในสภาวะไร้ออกซิเจนในที่ 30°C เป็นเวลา 18 ชั่วโมง แบคทีริโอซินที่ผลิตนี้สามารถยับยั้งการเจริญของเชื้อทดสอบแกรมบวกคือ *Bacillus stearothermophilus* TISTR 329, *Lactobacillus acidophilus* TISTR 1034, *L. delbrueckii* TISTR 326, *L. fermentum* TISTR 055, *Listeria monocytogenes* DMS 1347, *Micrococcus luteus* TISTR 884, *Pediococcus acidilactici* TISTR 051, *P. acidilactici* TISTR 783, *P. pentosaceus* TISTR 374, *Staphylococcus aureus* TISTR 029 และ *S. aureus* TISTR 118 จากการศึกษาสมบัติของแบคทีริโอซินพบว่าเป็นสารที่มีน้ำหนักโมเลกุลประมาณ 10-15 กิโลดาลตัน (รูปที่ 1) มีความเสถียรต่อเอนไซม์ Lysozyme และความร้อนที่อุณหภูมิ 80°C (นาน 15 นาที) ในขณะที่เอมไซม์ Protease และ Protinase K (รูปที่ 2) และความร้อนที่อุณหภูมิ 100°C (นาน 15 นาที) สามารถทำลายแบคทีริโอซินได้ แบคทีริโอซินที่ผลิตได้มีความเข้มข้นเท่ากับ 76 µg/ml เมื่อเทียบกับ Chloramphenical และมีค่าเท่ากับ 25 IU เมื่อเทียบกับสาร Nisin มาตรฐาน และยีนที่ตรวจพบ (ซิ้นดีเอ็นเอขนาด 273 คู่เบส) มีลำดับเบสเหมือนกับแบคทีริโอซิน ชนิด Nisin Z (99% Homology)



รูปที่ 1 SDS-PAGE ของแบคทีริโอซินจากไอโซเลท A_5UVU_{25} ช่องที่: M1, MW Marker (Invitrogen, USA); M2, MW Marker (Sigma, USA); และ 1, ลูกศรแสดงตำแหน่งของสารที่ออกฤทธิ์



รูปที่ 2 ความเสถียรของแบคทีริโอซินเมื่อทดสอบกับเอนไซม์ Lysozyme (A) Protease (B) Protinase K (C) และสารก่อนการทดสอบ (D) และใช้ *Bacillus stearothermophilus* TISTR 329 เป็นเชื้อทดสอบ

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คำสำคัญ: แบคทีริโอซิน แบคทีเรียแล็กติก แป้งมันสำปะหลัง

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5.1 Rodtong, S., Sansit, J., Chimsoongnern, P., Vechklang, K., and Ishizaki, A. (2001). **Strain improvement of starch-utilizing bacteria by mutagenesis to enhance L-lactic acid production.** BioThailand 2001, November 7-10, 2001.

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