PEDIOCIN SCREENING KIT FOR BIOTECHNOLOGICAL APPLICATION AND EDUCATION

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ABSTRACT

Several lactic acid bacteria (LAB) were isolated from Nham samples. They were tested for bacteriocin activity and the presence of the corresponding gene by inhibiting growth of closely related bacteria and polymerase chain reaction (PCR), respectively. LAB-producing clear halo zones were identified by 16S rRNA gene sequencing. *Pediococcus pentosaceus* P7, which showed the highest inhibitory effect against the indicator strains (*Vibrio harveyi* and *Lactobacillus plantarum*), was selected and the presence of its pediocin A gene (*pedA*) was confirmed by PCR. The nucleotide sequence of the *pedA* gene is 100% homologous to the corresponding pediocin structural genes of other LABs.

Ped. pentosaceus P7, and other two bacteriocin-producing strains isolated from Nham samples, *Enterococcus faecium* F103 and *Lactococcus lactis* F141 harboring pediocin, enterocin and nisin genes, respectively, were selected and used for a practical laboratory designed for the third-year undergraduate students of Biotechnology, Mahidol University, as part of a Microbial Physiology and Genetics course. This five-session laboratory module aimed to screen foods for LAB and to test isolated LAB for the presence of bacteriocins. Traditional Thai fermented foods were first screened for bacteriocin-producing LAB using microbiological methods. This was followed by a simple and rapid DNA extraction and by a multiplex polymerase chain reaction (PCR) using three pairs of specific primers to test for the presence or absence of various bacteriocin genes in the isolated LAB. PCR amplicons of 332, 412, and 608 bp indicated the presence of pediocin, enterocin, and nisin genes, respectively, whereas no amplicon band indicates the absence of these bacteriocins. The laboratory provided the students with experience in the use of microbiological and multiplex PCR methods and showed how the molecular biology techniques can be related to their daily lives. The module could easily be adapted to the study of LABs in fermented foods from other countries.

Besides the laboratory designed for the students, in this study, the development of multiplex-overlapping PCR (mPCR) method for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus* was also conducted. This mPCR proved to be a simple, rapid method of determination and reliable method for differentiation of both pediococcal species as well as the detection of pediocin gene in a single PCR reaction.

KEY WORDS: PEDIOCIN / BACTERIOCIN / *PEDIOCOCCUS PENTOSACEUS* / *PED. ACIDILACTICI* / LAB / UNDERGRADUATE LABORATORY PRACTICE / BIOTECHNOLOGY / MPCR

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ชุดตรวจสอบ pediocin สำหรับการประยุกต์ใช้ทางเทคโนโลยีชีวภาพและการศึกษา
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บทคัดย่อ

ได้คัดเลือกเชื้อ lactic acid bacteria (LAB) จากตัวอย่างแหนมและทดสอบความสามารถในการสร้าง “bacteriocin” และการมียีนนี้โดยดูผลการมีฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียที่มีพันธุกรรมใกล้เคียงกันและตรวจหายีนด้วยเทคนิค PCR ตามลำดับ ได้จำแนกชนิดของเชื้อ LAB ที่ยับยั้งการเจริญเติบโตของเชื้อตัวทดสอบได้แก่ *Vibrio harveyi* และ *Lactobacillus plantarum* โดยการหาลำดับนิวคลีโอไทด์ของบริเวณ 16S rRNA ยีน ได้เลือก *Pediococcus pentosaceus* P7 ซึ่งเป็นเชื้อที่ยับยั้งการเจริญเติบโตของเชื้อตัวทดสอบได้แก่ *V. harveyi* และ *Lb. plantarum* ได้ดีที่สุดเพื่อตรวจสอบการปรากฏของยีน pediocin (*pedA*) ด้วยวิธี PCR จากการหาลำดับนิวคลีโอไทด์ของชิ้นส่วนดีเอ็นเอ (*pedA*) พบว่ามีความคล้ายคลึง 100% กับลำดับนิวคลีโอไทด์ของ *pedA* ยีน ที่พบในแบคทีเรีย LAB ชนิดอื่นๆ

ได้นำ *Ped. pentosaceus* P7 และเชื้ออีกสองสายพันธุ์ bacteriocin คือ *Enterococcus faecium* F103 and *Lactococcus lactis* F141 ซึ่งมียีนในการสร้าง pediocin, enterocin, และ nisin ตามลำดับมาใช้ในการออกแบบปฏิบัติการสำหรับนักศึกษาชั้นปีที่สาม ภาควิชาเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล เพื่อเป็นส่วนหนึ่งของรายวิชา “Microbial Physiology and Genetics” ภาคปฏิบัติการซึ่งประกอบด้วยแบบปฏิบัติการห้าคาบเพื่อแยกและคัดเลือก LAB ที่สร้าง bacteriocin จากอาหารหมักของไทยโดยวิธีการทางจุลชีววิทยาเป็นลำดับแรก และตามด้วยการสกัด DNA อย่างง่ายและรวดเร็ว ได้ใช้วิธี multiplex PCR และใช้ไพรเมอร์ที่ทำการออกแบบอย่างจำเพาะสามคู่เพื่อตรวจสอบการปรากฏของ ยีน bacteriocin ในเชื้อ LAB ที่แยกมา หากได้ผลการเพิ่มขยายยีนด้วยวิธี PCR ขนาด 332, 412, และ 608 คู่เบส จะบ่งชี้การปรากฏของยีน pediocin, enterocin, และ nisin ตามลำดับ ซึ่งการไม่ปรากฏของท่อนยีนขนาดดังกล่าวแสดงว่าไม่มี ยีน bacteriocin การทดลองนี้ให้ประสบการณ์แก่นักศึกษาในการใช้วิธีการทางจุลชีววิทยาและวิธี multiplex PCR และแสดงให้นักศึกษาเห็นว่าอณูชีววิทยาสามารถเกี่ยวข้องกับชีวิตกระจำวันของนักศึกษา นอกจากนี้แบบปฏิบัติการนี้สามารถนำไปปรับใช้เพื่อการศึกษาเชื้อ LABs ในอาหารหมักจากประเทศอื่นๆได้ง่ายด้วย

นอกจากการออกแบบการทดลองสำหรับนักศึกษาแล้ว การศึกษาในครั้งนี้ได้มีการพัฒนาและประยุกต์ใช้วิธี multiplex-overlapping PCR (mPCR) เพื่อการตรวจหา pediocin ยีน และการจำแนก species ระหว่าง *Ped. acidilactici* and *Ped. pentosaceus* ในครั้งเดียวกัน วิธีการ mPCR นี้เป็นวิธีที่ง่าย รวดเร็ว และเป็นวิธีที่เชื่อถือได้สำหรับการจำแนก species ของเชื้อ *Pediococcus* ทั้งสองชนิด และสามารถตรวจหายีน pediocin ในปฏิกริยา PCR เดียว

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LIST OF ABBREVIATIONS

bp	base pair
˚C	degree Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
et al.	Et.alli (Latin), and other
etc.	et cetera (Latin), and other things, and so on
g	gram
h	hour (s)
i.e.	Ed est (Latin), that is
kb	kilobase (1,000 bp)
kDa	kilodalton
L, l	litre
LAB	lactic acid bacteria
LB	Luria-Bertani
mg	miligram
mg/ml	milligram per milliliter
min	minute
ml	milliliter
mM	milimolar
ORF	open reading frame
MRS	de Man, Rogosa, Sharpe
PCR	polymerase chain reaction
ped	pediocin structural gene
PED	PEDIOCIN
RNA	ribonucleic acid
rpm	round per minute
SDS	Sodium Dodecyl Sulfate

LIST OF ABBREVIATIONS
(Continued)

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second(s)
TBE	Tris-Borate EDTA
TE	Tris-EDTA buffer
TSA	Tryptic Soy Agar
U	Unit
V	voltage
v	volume
µg	microgram
µl	microlitre
%	percent

CHAPTER I
INTRODUCTION

Biotechnology and its applications are now having a significant impact on everyday life (Edmondston, 2000). In Thailand, the Thai government sees biotechnology as a key factor for developing the country, regarding biotechnology development in Thailand as possessing high potential. As biotechnology will play a vital role in the country's development in line with government policy and the national agenda, the National Biotechnology Policy Framework (2004-2009) was endorsed by the National Biotechnology Policy Committee which proposed six major goals, including key strategies, for biotechnology development in Thailand. This encompasses sustainable competitiveness, healthcare for all, equitable income distribution and a self-sufficient economy. The emphasis will be placed on applying core technologies, e.g. genomics, bioinformatics, plant and animal breeding by means of molecular markers, to accelerate development in the following areas: agriculture/food, medical care and environment protection, and new knowledge creation for the development of higher value-added products, as well as to knowledge-based policy development and strategic planning. The core technologies will also help to promote biotechnology business, including high-end products with high value and new types of services where modern technology is required.

Because of rapid developments in the field of DNA technology, current molecular biology methods such as polymerase chain reaction (PCR) need to be taught in undergraduate laboratory courses. Many researchers (Baker et al., 2002; Bowlus & Grether, 1996; Brinegar & Levee, 2004) have described the use of PCR methodology in exploratory student laboratory exercises, arguing that it allows the students to generate their own meaningful data for the analysis and also provides them with essential laboratory skills in the modern laboratory methods. The positive laboratory experience will clearly show how such methods can be applied to their daily lives, and it may also stimulate some students to continue their education in molecular biology.

The PCR is a molecular technique that has been widely used in research to amplify a specific region of DNA template to millions of copies in a few hours (Bowlus & Grether, 1996). The technique is very useful in targeting specific regions of a chromosome for study, and it allows the investigation of DNA that might otherwise be undetectable by usual techniques. Since it was introduced in 1985, PCR has revolutionized the field of DNA analysis. In teaching, the principle of PCR has facilitated student understanding of DNA structure and replication. PCR has previously been shown to be useful in student laboratory practice, particularly to detect genetically modified organisms (GMOs) (Brandner, 2002; Holst-Jensen et al., 2003; Thion et al. 2002) and to identify and characterize pathogenic microorganisms (Claros & Quesada, 2000; Wang et al., 1997).

Lactic acid bacteria (LAB) are widely used in food fermentation of dairy, meat, vegetable, and bakery products (Marrug, 1991). LAB are known to produce a variety of antibacterial substances including bacteriocins that can inhibit the growth of undesirable bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus* spp., and *Clostridium* spp. (Jack et al., 1995; Klaenhammer, 1993).

Bacteriocins are produced by bacteria and comprise peptides or proteins that have antibacterial activity against other bacteria genetically related to the producer strains (Jack et al., 1995; Klaenhammer, 1993). Bacteriocins, and especially those from LAB (Jack et al., 1995), have been considered for use as biological control agents in foods. They could provide healthier foods by allowing a reduction in the level of chemical additives or the physical treatments currently employed during food processing.

The undergraduate program for the Bachelor of Science degree in Biotechnology at Mahidol University in Thailand aims at producing graduates who are well equipped with skills and basic knowledge in biotechnology, capable of integrating their knowledge and applying it fruitfully for the benefit of society and the country, with professional ethics in mind and responsibilities in their related career practices, and who are able to conduct research and development work (Biotechnology, 2006), a research in training students into biotechnology community of practice should be done. The biotechnology students usually have an opportunity to learn abstract concepts, for example, prior to this study, a class was given an

opportunity to learn PCR in a lecture setting, but they had not been given a chance to practice the PCR procedure. To address this problem, as a part of this study, a laboratory module that includes training in a microbiological method to screen Thai traditional fermented foods for the presence of LAB and a multiplex PCR to test the LAB for the presence of bacteriocins were designed and implemented with a class of third-year undergraduate students of Biotechnology as a part of Microbial Physiology and Genetics (SCBT302) course. The laboratory module was designed after consideration of limitations such as time, funding and level of student experience.

This study was to investigate students learning through a sociocultural perspective of learning in biotechnology laboratory setting. The following science education research questions were posted in this study:

1. Does the experiment on the screening of LAB producing bacteriocins improve students' understanding of PCR and its applications in biotechnology?
2. Does the experiment on the screening of LAB producing bacteriocins promote students feel they have learned practical biotechnology skills?
3. From a sociocultural perspective of learning, how does the screening of LAB with bacteriocins help students to learn and compare between microbiological method and molecular biology technique?

To explore these questions, this study was conducted in the biotechnology laboratory and with a class of 49 students who were studying SCBT302: Microbial Physiology and Genetics. In addressing the first two questions, this study developed two methods, microbiological and PCR, for screening of LAB for bacteriocins in Thai foods the students usually consume. Because by using the microbiological method the students could be able to observe bacteriocin activity, and by using the PCR methods, using three pairs of specific primers, they can test for the presence or absence of various bacteriocin genes in the isolated LAB.

Thus, to give students more understanding about screening of LAB for bacteriocins by microbiological and PCR methods knowledge (principles), skills, inquiry, and enjoyment, a laboratory unit intervention was designed. In this study,

materials belong to Thailand and were developed as products to serve the government's goals for biotechnology development together with undergraduate program for the Bachelor of Science degree in Biotechnology. The microbiological and PCR methods that have been widely used in scientific research were used for the unit of teaching and learning in a biotechnology laboratory.

Besides the laboratory designed for the students, the developing and applying the use of multiplex-overlapping PCR (mPCR) method for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus* was also conducted.

CHAPTER II
OBJECTIVES

1. Science

1.1 To screen and characterize bacteriocin-producing LAB in Thai fermented foods by microbiological and PCR methods.

1.2 To design specific primers for detecting gene encoding bacteriocin by PCR.

1.3 To develop and apply the use of multiplex-overlapping PCR (mPCR) for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus*.

2. Education

2.1 To design and apply the use of simple microbiological and PCR methods for screening of LAB and bacteriocin for education in biotechnology.

2.2 To improve students' understanding of PCR and its applications in biotechnology.

2.3 To promote students feel they have learned practical biotechnology skills.

CHAPTER III
LITERATURE REVIEW

1. Scientific review

Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, non-spore-forming, non-respiring, cocci, coccobacilli, or rods bacteria. They are chemoorganotrophic and grow only in complex media. They need a fermentable carbohydrate, as energy sources, for growth. They produce lactic acid as the major end product during the fermentation of carbohydrates. They generally lack catalase, although in rare cases pseudocatalase can be detected in cultures grown at a low sugar concentration (Wood & Holzapfel, 1995).

Many LAB have important roles in the production of fermented foods, and some of these bacteria have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms (Stiles & Hastings, 1991). In food, they contribute to the taste, aroma and texture of fermented products and inhibit the growth of food spoiling bacteria by the production of growth-inhibiting substances including bacteriocins and the production of large amounts of lactic acid. LABs, e.g. *Pediococcus*, play an essential role in fermentation of food and feed products (meat, vegetables, fruits, beverages, dairy products, and silage) (Jack et al., 1995). Many LABs are known to have a positive impact on human and animal health. However, not all of the LABs are useful, some of them are involved in food spoilage, e.g., meat and canned foods by *Brochothrix*, beer and wine by *Lactobacillus* or may even be pathogens, e.g., the *Streptococcus* (Stiles & Holzapfel, 1997). Therefore, it is necessary to establish a detailed classification system for LAB and to develop reliable and rapid identification methods (Wood & Holzapfel, 1995) in order to recognize them.

Pediococci are Gram-positive, non-motile, non-sporing, cells spherical, never elongated, 1.0-2.0 μm in diameter. Division occurs alternatively in two planes at right

angles to form tetrads under favorable condition, although sometimes only pairs of cells are seen. Single cells are rare, and chains are not formed. Facultative anaerobes, some strains are inhibited on incubation in air. Chemoorganotrophic, the cells require nutritionally rich media and fermentable carbohydrate (mainly mono- and disaccharides). Glucose is fermented with the production of acid but no gas; the major product is DL or L(+)-lactate. Catalase negative, cytochromes are absent. Nitrate is not reduced. The optimum growth temperature is 25-40°C. Pediococci occur in vegetable material and food products; nonpathogenic to plants or animals (Holt et al., 1994). The main characters for distinguishing among the species are the range of sugares fermented, hydrolysis of arginine, growth at different pH levels (7.0 and 4.5), and the configuration of lactic acid produced.

With the recent transfer of *Pediococcus urinaeequi* to *Aerococcus* (*A. urinaeequi*) and *Ped. halophilus* to *Tetragenococcus* (*T. halophilus*) (Anon, 2007). Then, the genus *Pediococcus* consists of eleven species (Anon, 2007): *Ped. acidilactici*, *Ped. pentosaceus, Ped. claussenii, Ped. cellicola, Ped. stilesii, Ped. inopinatus, Ped. damnosus, Ped. parvulus, Ped. dextrinicus, Ped. siamensis, Ped. ethanolidurans.* Thus, the genus *Pediococcus* can be described as "the only acidophilic, homofermentative LAB that divide alternatively in two perpendicular directions to form tetrad".

Pediococcus species are major lactic acid microflora in fermenting foods and dairy products (Nigatu et al., 1998). However, some pediococci are also among the most prevalent contaminants in breweries. For example, *Ped. damnosus* is regarded as the most significant spoilage species (Satokari et al., 2000).

There are two major important species, *Ped. acidilactici* and *Ped. pentosaceus* that have been isolated and widely used in the fermentation of vegetables (Knorr, 1998), meats (Luchansky et al., 1992), dough (Nigatu et al., 1998), fruit juices (Knorr, 1998), dairy products (Bhowmik & Marth, 1990), and silage (Hudson et al., 2000). Both species show the same characteristics such as growth at 35ºC and 40ºC, pH 4.2 – 7.5 (some strains can grow at pH 8.5), growth with 4% and 6.5% NaCl, hydrolysis of arginine, and not forming acid from dextrose and starch (Holt et al., 1994). With these similar characteristics, it is difficult to differentiate these two species from each other.

1.1.1 Classification of LAB

The classification of LAB into different genera and species is largely based on morphology, mode of glucose fermentation, configuration of the lactic acid produced, growth at different temperatures, ability to grow at high salt concentrations, and acid or alkaline tolerance (Table 1) (Axelsson, 1998).

1.1.1.1 Conventional taxonomy

Based on morphology, LABs can be divided into rods (*Carnobacterium and Lactobacillus*) and cocci (all other genera). One exception is the newly described genus *Weissella*, which is the first genus in the LAB group that, by definition, can include both cocci and rods. Furthermore, cell division in two perpendicular directions in a single plane, leading to tetrad-forming genera are *Aerococcus*, *Pediococcus*, and *Tetragenococcus* (Wood & Holzapfel, 1995).

Under standard conditions, i.e., non-limiting concentrations of glucose and growth factors (amino acids, vitamins, and nucleic acid precursors) and limited oxygen availability, the mode of glucose fermentation has been used to divide all LAB into two groups (Wood & Holzapfel, 1995); the homofermentative, converting glucose almost quantitatively to lactic acid; and the heterofermentative, fermenting glucose to lactic acid, ethanol/acetic acid, carbon dioxide, formate or succinate.

The formation of different isomeric forms of lactic acid during fermentation of glucose can also be used to distinguish between leuconostocs and most heterofermentative lactobacilli, as the former produce only D-lactic acid and later a racemate (DL-lactic acid). A summary of the differentiation of the LAB genera with classical phenotypic tests is shown in Table 1. Some of the characters listed in Table 1 have been useful in the classification at the species level, e.g. salt and pH tolerance, growth at certain temperatures, and configuration of the lactic acid produced.

However, the taxonomic revisions and the description of genera rather than physiological studies suggest that the LAB comprise the following: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Axelsson, 1998).

Table 1. Differential characteristics of lactic acid bacteria[a]

Character	Rods		Cocci							
	Carnobacterium	*Lactobacillus*	*Aerococcus*	*Enterococcus*	*Lactococcus* / *Vagococcus*	*Leuconostoc* / *Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*[b]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[c]	-[e]	±	-	-	-	+	-	-	-	+
Growth at10ºC	+	±	+	+	+	+	±	-	+	+
Growth at45ºC	-	±	-	+	-	-	±	±	-	-
Growth in 6.5% NaCl	ND[f]	±	+	+	-	±	±	-	+	±
Growth in 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	±	-	+	±	±	+	-	-	±
Growth at pH 9.4	-	-	+	+	-	-	-	-	+	-
Lactic acid[d]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

[a] +, positive; -. negative; ±, response varies between species; ND, not determined.

[b] *Weissella* strains may also be rod-shaped.

[c] Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[d] Configuration of lactic acid produced from glucose.

[e] Small amounts of CO_2 can be produced, depending on media.

[f] No growth in 8% of NaCl has been reported.

[g] Production of D-, L- or DL-lactic acid varies among species.

From; Axelsson, (1998)

The conventional approach to LABs taxonomy was based on morphological and physiological features and on biochemical tests, as mentioned above. This was expanded to include e.g., cell wall composition, whole-cell protein analysis, and other characteristics of the cells, such as fermentation reactions, lactate dehydrogenase.

Determination of the cell wall composition has traditionally been important in Gram-positive bacteria. The peptidoglycan type of Gram-negative bacteria is rather uniform and provides little information. Cell walls of Gram-positive bacteria, in contrast, contain various peptidoglycan types, which may be genus or species specific (Schleifer & Kandler, 1972). Murein types in lactobacilli were comprehensively summarized by Hammes and Vogel (1995). *Lb. delbrueckii* group have the Lys–D-Asp type. *Leuconostoc* group have the Lys (monoamino, monocarbonyl) amino acid type (Lys-L-Ser-L-Ala_2 or Lys–L-Ala_2. Representative species of the *Lb. casei-Pediococcus* group have either the Lys–D-Asp type or the diaminopimelicdirect type. The procedure is time-consuming, although a rapid screening method has been proposed (Schleifer & Kandler, 1972).

The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be reliable for comparing and grouping large numbers of closely related LAB strains (Descheemaeker et al., 1994). It was possible to solve specific identification problems for, e.g., lactococci strains (Descheemaeker et al., 1994). The technique has proven to be useful for grouping of large numbers of strains (Descheemaeker et al., 1994).

Using fermentation reactions as a tool for identification and classification for example, examination of the presence of fructose-1,6-bisphosphate aldolase of lactobacilli in which resulted in the subdivision of these bacteria into three physiological groups: the obligately homofermentative lactobacilli, the facultatively heterofermentative species, and the obligately heterofermentative species which lack fructose-1,6-bisphosphate aldolase. Therefore, the three phylogenetic subgroups, the *Lb. delbrueckii* group, the *Lb. casei-Pediococcus* group, and the *Leuconostoc* group, respectively, had been established (Hammes & Vogel, 1995).

Lactate dehydrogenase (LDH) has been an important characteristic in the classification of LAB. The electrophoretic mobility of LDH in starch gels or in

polyacrylamide gels was used mostly for the discrimination of phenotypically very similar species (e.g., *Lb. acidophilus*, *Lb. crispatus*, *Lb. gallinarum*, *Lb. gasseri*, and *Lb. johnsonii* (Fujisawa et al., 1992). NAD-dependent D-LDH profiles have also been used for the differentiation of *Leuconostoc* species (Dicks & van Vuuren, 1990). Conventional taxonomy usually involves the use of a large variety of methods to conclude the results. In addition, there are certain species of LAB which cannot be readily distinguished by phenotypic characters as conventional taxonomy because phenotypic responses can be also affected by environmental conditions. Thus, for the design of more stable identification methods, it is necessary to apply genotypic tests (Schleifer et al., 1995).

1.1.1.2 DNA-based method for bacterial identification

Classification of LAB is becoming dependent on more sophisticated relying on molecular biology methods such as ribotyping (Barney et al., 2001; Satokari et al., 2000), 16S rRNA gene sequencing (Barney et al., 2001; Omar et al., 2000), randomly amplified polymorphic DNA (RAPD) (Mora et al., 2000; Nigatu et al., 1998) and pulsed-field gel electrophoresis (PFGE) have been used for LAB species differentiation (Barros et al., 2001).

Application of molecular genetic techniques to determine LAB has resulted in significant changes in their taxonomic classification (Stiles & Holzapfel, 1997). Molecular characteristics that have become important taxonomic tools are plasmid profiling, the mol% G + C content of the DNA, restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP), pulse-field gel electrophoresis (PFGE), PCR, randomly amplify polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and 16S rRNA gene sequencing. Thus, various molecular genetic methods have been used to discriminate among LAB species or subpopulations within a species.

1.1.1.2.1 Plasmid profiling

Plasmid profiling was formerly considered suitable for the typing of individual strains within a species. Although in some LAB, plasmids seem to occur very frequently in high numbers (e.g. *Lb. plantarum*), other species appear to be rather

poor in plasmid content. Due to the lack of plasmids in various LABs and as a result of the instability of extrachromosomal DNA, methods that use chromosomal DNA are superior to this technique in the respective species and have become more popular (Duffner & O'Connell, 1995).

1.1.1.2.2 Mol % G + C content

Determination of the moles percent guanosine plus cytosine (mol% G + C) is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. Generally, the range observed is not more than 3% within a well-defined species and not more than 10% within a well-defined genus. The DNA base composition of LAB is less than 50 mol% G + C (Vandamme et al., 1996). For examples, *Ped. acidilactici* JCM 8797^T, *Ped. dextrinicus* JCM 5887^T, *Ped. parvulus* JCM 5889^T, *Ped. pentosaceus* JCM 5890^T and *Lactococcus lactis* subsp. *lactis* IL1403, EMR4, CECT 4433 had mol% G + C content of 40.8, 38.7, 39.6, 37.6, and 35.33, respectively (Cai et al., 1999; Gonzalez & Saiz-Jimenez, 2002).

1.1.1.2.3 Restriction endonuclease analysis (REA)

The use of restriction endonuclease analysis (REA) is another approach for classifying LAB (Johansson et al., 1995). REA involves the digestion of chromosomal DNA with a set of restriction endonucleases. The fragments obtained are usually separated in an agarose gel with the use of conventional electrophoresis. The selection of an appropriate restriction enzyme or a set of enzymes is important for obtaining revealing patterns. The banding patterns obtained from different strains are then subject to multivariate data analysis. This method also requires a highly standardized system but has a very high resolution capacity and can be used as a classification tool at subspecies, species, and genus level (Johansson et al., 1995). REA was applied successfully to differentiate between strains of *Lb. casei*, *Lb. rhamnosus*, and *Lb. reuteri* (Ahrne´ & Molin, 1997; Stahl & Molin, 1994).

1.1.1.2.4 Restriction fragment length polymorphism (RFLP)

A restriction fragment length polymorphism (RFLP) molecular typing, when the chromosomal DNA of a strain is digested with a restriction enzyme, separated in

agarose gels blotted onto a filter, and hybridized with a gene probe targeted are performed, a banding pattern is obtained. For ribotyping, an rRNA-specific probe was used. Depending on the complexity of the patterns, they can be analysed manually or by computer technique. Similar to the actual rRNA sequences, which are more conserved than many other genes, the organization of the rRNA genes is also conserved to a certain degree in related species and this is reflected in the banding patterns or "fingerprints". It appears that the method is useful for species and subspecies recognition e.g., lactococci (Rodrigues et al., 1991) and *Lb. plantarum* (Johansson et al., 1995).

1.1.1.2.5 Pulse-field gel electrophoresis (PFGE)

A highly discriminatory and reproducible method of pulse-field gel electrophoresis (PFGE) has been used to differentiate strains. PFGE differs from conventional agarose electrophoresis in that the orientation of the electric field across the gel is periodically changed in contrast to being unidirectional and constant in standard electrophoresis. This type of electrophoresis enables the separation of high-molecular-weight fragments generated by restriction of the genome to a low number of fragments. Zapparroli et al. (1998) stated a high discriminatory power of PFGE indicating the existence of a genetical polymorphism in 11 strains of *Lb. sanfranciscensis*.

1.1.1.2.6 PCR

The PCR technique is becoming more and more useful for classification purposes. With this technique, it is possible to amplify a gene or a part of a gene from a very limit number of cells. One of the targets for amplification is obviously rRNA genes. PCR can also be used in combination with probing techniques (Klijn et al., 1991) with the obvious advantage of being much more sensitive than direct hybridization. Applications of PCR are randomly amplify polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).

1.1.1.2.7 Randomly amplify polymorphic DNA (RAPD)

Another method for bacterial identification is to obtain a banding pattern or fingerprint from a particular strain by randomly amplify polymorphic DNA (RAPD) method. Short oligonucleotide primers (8-10 bases) with a more or less random sequence are allowed to bind to the total DNA from a strain. Under amplified conditions, a number of fragments of different sizes are amplified and then separated in gels. The pattern can subsequently be analyzed with computerized methods, similar to ribotyping. RAPD has been shown to be applicable and suitable for distinguishing strains of *Lb. plantarum* than ribotyping (Johansson et al., 1995). The typing efficiency is markedly increased when several single primers are applied. The combination of RAPD profiles generated with different primers was also shown to enable differentiation of sourdough *Lactobacillus* and *Weissella* strains (Corsetti et al., 2003). In addition, an analysis of *rpoB* gene was used to differentiate in both rods and cocci of wine LAB species (Claisse et al., 2007). The technique efficiently identified several species of LAB but advanced software was required to analyze the lengths of gel bands since the sizes of restriction fragments of the amplicons in several LAB species were very similar. The simplicity of this method makes it very attractive, but reproducible results require highly standardized conditions. The method of breaking the cells to obtain a crude DNA preparation is one of the critical steps in this regard (Johansson et al., 1995).

1.1.1.2.8 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) consists of three steps: (1) digestion of total cellular DNA with restriction enzymes and the ligation of halfsite-specific adapters to the restriction sites of all fragments, (2) the selective amplification of these fragments with two PCR primers that have corresponding adaptor- and restriction site sequences, (3) the separation of amplified products by polyacrylamide gel electrophoresis (PAGE). Introduction of deliberate mismatches at the 3' ends of primers influences the number of amplified fragments and the discriminatory power of the patterns. AFLPs were shown by Gancheva et al. (1999) to be applicable to LAB differentiating of strains belonging to the *Lb. delbrueckii* group.

1.1.1.2.9 16S rRNA gene sequencing

Within bacteria, sequence information from the gene coding for the small subunit of ribosomal RNA, 16S rDNA, is widely regarded as one of the most valid criteria for determining relationships between closely related groups, such as species or genera (Weisburg et al. 1991). Therefore, the 16S rRNA gene is the most widely accepted gene used for bacterial classification and identification (Weisburg et al., 1991). Sequence-based analysis of the 16S rRNA gene represents a highly accurate and versatile method for bacterial classification and identification. The features of this molecular target, the universal distribution among bacteria, and the presence of species-specific variable regions make it a useful tool not only for bacterial phylogenetic analysis but also for the detection and identification (Weisburg et al., 1991). The 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new "gold standard" for identification of bacteria to the species level (Woo et al., 2003). The conservative nature of the gene, its universal distribution and the availability of information in public databases (GenBank, EMBL, DDBJ and RDP) make the 16S rRNA gene very useful for phylogenetic studies and taxonomy.

The most powerful new approach for bacterial identification is based on using a PCR-based strategy combined with sequencing of 16S ribosomal RNA encoding genes (Hugenholtz et al., 1998). It allows the identification of closely related microorganisms by, first, amplifying the 16S rRNA gene directly from isolated colonies using universal primers directed at conserved regions at both ends of the gene, and then sequencing the PCR product (O'Sullivan, 2000).

The size of 16S rRNAs is about 1550 bp (Martinez-Murcia et al., 1999). The species-specific sequences are contained in the first half of the 16S rRNA gene (V1-V3 region). Both the V1 and V2 region provided strong evidence for identification of LAB. An approximately 500 bp region of the 16S rRNA gene, which contained the V1 and V2 variable regions, was amplified from the isolates by the PCR. The sequence of this region of the 16S rRNA gene was sufficiently variable to allow clear differentiation among each of the strains such as *Lb. amylovorus, Lb. crispatus, Lb. gallinarum, Lb. gasseri and Lb. johnsonii* from the type strain of the *Lb. acidophilus* (Kullen et al., 2000). The sequence containing both the V1 and V2 region provided strong evidence for the identification of several tested LABs such as *Carnobacterium*

maltaromaticum, *Lb. curvatus*, *Lb. sakei*, *Lb. plantarum*, *Lc. lactis* subsp. *cremoris*, *Lc*. *lactis* subsp. *lactis*, and *Leuc*. *mesenteroides* (Balca´zar et al., 2007). The method described was found to be a very simple, rapid, specific, and low-cost tool for the identification of unknown strains of LAB. Sequencing of the 500 bp from the 5' region of the 16S rRNA gene would be a useful substitution for conventional full-sequence 16S rRNA gene sequencing (Woo et al., 2003).

With automated sequencing systems and convenient direct PCR sequencing methods, it has become a quite easy task to determine at least a part of the 16S rRNA sequence from any bacterium in a short time. Comparative 16S rRNA sequencing for identification to genus level may be defined at sequence similarities of 95% (Ludwig et al., 1998) while above 97% is considered to be the cutoff value indicating species identity (Stackebrandt & Goebel, 1994). The percent homologies of 16S rRNA gene within the two major lactobacillus groups vary from 90.8 to 99.3% (*Lb. delbrueckii* group) and 90.3 to 99.0% (*Lb. casei-Pediococcus* group) (Collins et al., 1992).

1.2 Bacteriocin

Bacteriocins, as defined by Tagg et al. (1976), are proteinaceous compounds that kill closely related bacteria. The narrow specificity of their action and their protein nature distinguish them from antibiotics. Several bacterial species produce bacteriocins as substances used in defense systems to compete for growth and survival of themselves against other genetically related species. According to Klaenhammer (1993), 99% of LAB may make at least one bacteriocin. Since the late 1920s, when the first reports on the antimicrobial activity of lactococcal bacteriocin, nisin, were made, a larger number of LAB bacteriocins have been identified, particularly in the last few years.

The majority of bacteriocins produced by LAB have been characterized by the initial definition of a proteinaceous inhibitor, estimation of molecular mass (via retention in dialysis membranes, ultrafiltration, molecular sizing, or mass spectrometry), and determination of susceptible strains. Recent developments in the biochemical and genetic characterization of many of these compounds have elucidated their probable structures and mechanisms of action (Hennessy, 1993; Klaenhammer, 1993).

1.2.1 Classification of bacteriocins from LAB

Bacteriocin of LAB have been classified, depending on the basis of their primary structure, into three main classes (Hennessy, 1993; Klaenhammer, 1993), class I: the lantibiotics; class II: the small heat-stable non-lantibiotics; and class III: large heat-labile bacteriocins.

The lantibiotic bacteriocins in class I are small (<5 kDa), heat-stable, cationic, amphiphilic peptides that contain lanthionine formed by post-translational side-chain modifications of a precursor peptide before they are exported from the cell (Hechard & Sahl, 2002). The lantibiotic bacteriocins have post-translational side-chain modifications by the dehydration of serine and threonine molecules to form didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively. Then the SH group of the cysteine molecule is added at C=C of Dha or Dhb to form a thioether amino acid, called lanthionine. The lanthionine resulting from this addition reaction are, in contrast to disulfide bridges, acid tolerant and can be identified in peptide hydrolysates as lanthionine (originating from Dha and Cys) and 3-methyllanthionine (from Dhb and Cys). Examples of lantibiotic are nisin and lactocin produced by *Lc. lactis* subsp. *lactis* (Steen et al., 1991), and *Lb. sake* 145 (Mortverdt & Nes, 1990), respectively. Lantibiotic bacteriocins have broad spectrum activity (Hechard & Sahl, 2002).

Class II bacteriocin contains small (<10 kDa), heat-stable, cationic and hydrophobic peptides of 20–60 amino acids in length that are formed directly, without posttranslational modification. The inhibition spectrum of class II bacteriocin is rather narrow, limited to species or strains related to the producers. Class II bacteriocins have been further classified into three subclasses, IIa, IIb, and IIc, on the basis of their primary structure.

Subclass IIa bacteriocins possess a highly conserved N-terminus which has the YGNGV consensus amino acid sequence and includes, disulfide bridges and several conserved residues. They show very strong antilisterial activity. They are found in a wide variety of LAB including *Pediococcus* (Marugg et al., 1992) and *Lactobacillus* (Reenen et al., 2006). Examples are pediocin AcH/PA-1 from *Ped. pentosaceus* and *Ped. acidilactici* (Miller et al., 2005), enterocin P and plantaricin 423 from *E. faecium* (Cintas et al., 1997) and *Lb. plantarum* 423 (Reenen et al., 2006), respectively.

Divercin V41, Mesentericin Y105, leucocin A, produced by *Carnobacterium divergens* V41 (Richard et al., 2004), *Leuconostoc mesenteroides* Y105 (Biet et al., 1998) and *Leuc. gelidum* (van Belkum & Stiles, 1995), respectively, are also classified as part of this subclass.

Subclass IIb contains bacteriocins whose activity depends on the complementary action of two peptides. The primary structure of the two peptides is clearly different and no significant similarity has ever been described between all these bacteriocins (Hechard & Sahl, 2002). Examples of these bacteriocins are lactacin F, plantaricin EF and JK, and lactococcin G which are produced by *Lb. johnsonii* VPI11088 (Fremaux et al., 1993), *Lb. plantarum* C11 (Moll et al., 1999) and *Lc. lactis* LMG 2081 (Hechard & Sahl, 2002), respectively.

Subclass IIc bacteriocins possess a sec-independent leader peptide in which there is an ATP-binding cassette at the C terminal. They are thiol-activated peptides requiring reduced cysteine residues for activity (Klaenhammer, 1993). This sec-independent leader peptide functions in transportation of bacteriocins out of the cell. Examples of bacteriocin in this subclass are lactococcin A produced by *Lc. lactis,* and plantaricin A produced by *Lb. plantarum* C11 (Hechard & Sahl, 2002).

Class III contains large bacteriocins (>30 kDa) that are heat-labile proteins. The structural gene that has been cloned for only one of the class is helveticin J, produced by *Lc. helveticus* 481 (Joerger & Klaenhammer, 1986).

Bacteriocin genes can be present on the bacterial chromosome or on plasmids, for example, nisin and pediocin, respectively. These bacteriocin genes are occurring in operon, composed of several open reading frames (ORFs), e.g., nisin and pediocin which are shown in Figure 1. The genes *nisA* and *pedA* encode the bacteriocin, which are nisin and pediocin, respectively. Other genes in the operon encode for accessory proteins for immunity and transporter functions.

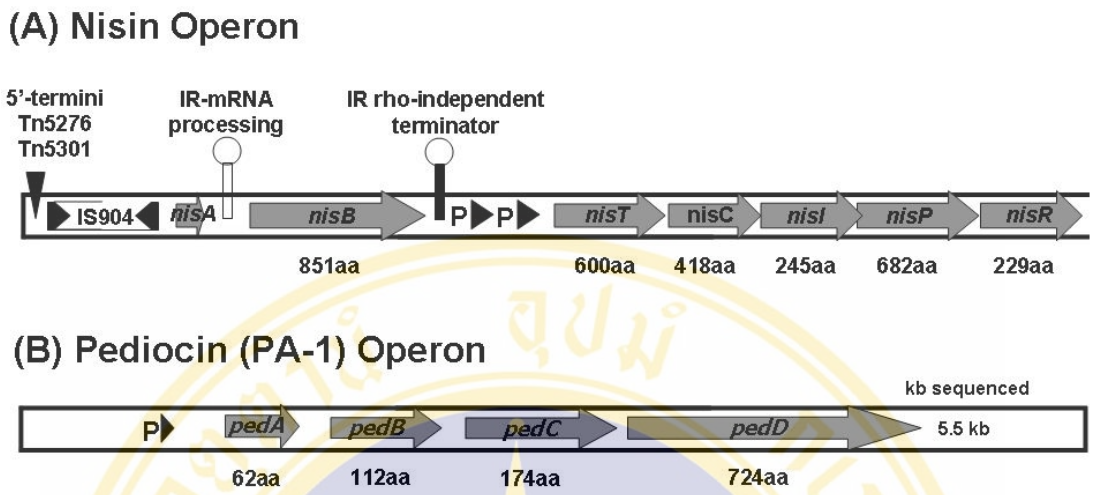

Figure 1. Organization of nisin A (A), and pediocin PA-1 (B) operons. ORFs are indicated by shaded arrows and the numbers of amino acid residues (aa) are denoted under each ORF. Putative or incomplete ORFs are not shaded. Inverted repeats (IR) suspected in mRNA processing ( ) and rho independent terminators ( ) are illustrated. The left ends of nisin-sucrose-conjugative transposons Tn5276 and Tn5301 are denoted (▼). Promoters (P,▶) are illustrated. Adapted from Klaenhammer (1993).

1.2.2 Mode of action

Mode of action of bacteriocins from Gram-positive bacteria, whether they are modified (class I bacteriocins, lantibiotics) or unmodified (class II), is based on interaction with bacterial membrane (Hechard & Sahl, 2002).

Nisin and related lantibiotics kill sensitive bacterial cells by a combination of two mechanisms: (1) inhibition of the peptidoglycan biosynthesis through interaction with lipid II. They possess ability to use the lipid-bound cell wall precursor lipid II, a precursor in cell-wall synthesis, as a docking molecule. Thus cell wall biosynthesis is inhibited through arresting the precursor in the membrane. (2) subsequent formation of highly effective and targeted of lethal pores in the cytoplasmic membrane (Wiedemann et al., 2001). Binding to anionic phospholipids for initial membrane interaction by the positive charges in the carboxyl-terminal region of nisin (Breukink et al., 1997) have been described. Insertion of lantibiotics into lipid monolayers such as the ability of nisin to interact with anionic phosphatidylglycerol correlates with its antimicrobial activity (Demel et al., 1996). The formation of pores leads to dissipation of the membrane potential and promotes a rapid efflux of small

metabolites such as amino acids or ATP, which consequential immediately stops all cellular biosynthetic process such as DNA, RNA, proteins and polysaccharides (Hechard & Sahl, 2002).

Class II bacteriocins's activity mainly induces membrane permeabilization and leakage of molecules from sensitive bacteria (Chikindas et al., 1993; Herranz et al., 2001).

Subclass IIa bacteriocins tend to dissipate proton motive force (PMF) via dissipation of transmembrane potential ($\Delta\psi$) and/or transmembrane pH gradient (ΔpH). Pediocin PA-1 dissipates the $\Delta\psi$ and causes release of amino acids accumulated and other low molecular weight compounds. Binding to anionic phospholipids for initial membrane interaction by the positive charges in the carboxyl-terminal region of pediocin PA-1 (Chen et al., 1998) have been described. Anionic cell surface polymers like teichoic acid and lipoteichoic acid have been suggested to play a role in the initial interaction with cationic bacteriocins (Jack et al., 1995). According to 'barrel-stave' model (Ennahar et al., 2000), the initial step of class IIa bacteriocin interaction with the membrane surface is generally believed to be an electrostatic binding mediated by a putative membrane-bound receptor-type molecule (Figure 2). Recently, a mannose PTS permease (EII_t^{Man}) has been proposed to be a target molecule for mesentericin Y105 (Dalet et al., 2001) and leucocin A (Ramnath et al., 2000).

Several class IIb and IIc bacteriocins also need proton motive force for activity, e.g. lactococcin G induces a release of amino acids, such as alanine or leucine, accumulated through PMF dependent mechanism (Moll et al., 1996), lactacin F dissipates $\Delta\psi$, induces a leakage of K^+ and decreases the intracellular ATP concentration (Abee et al., 1994).

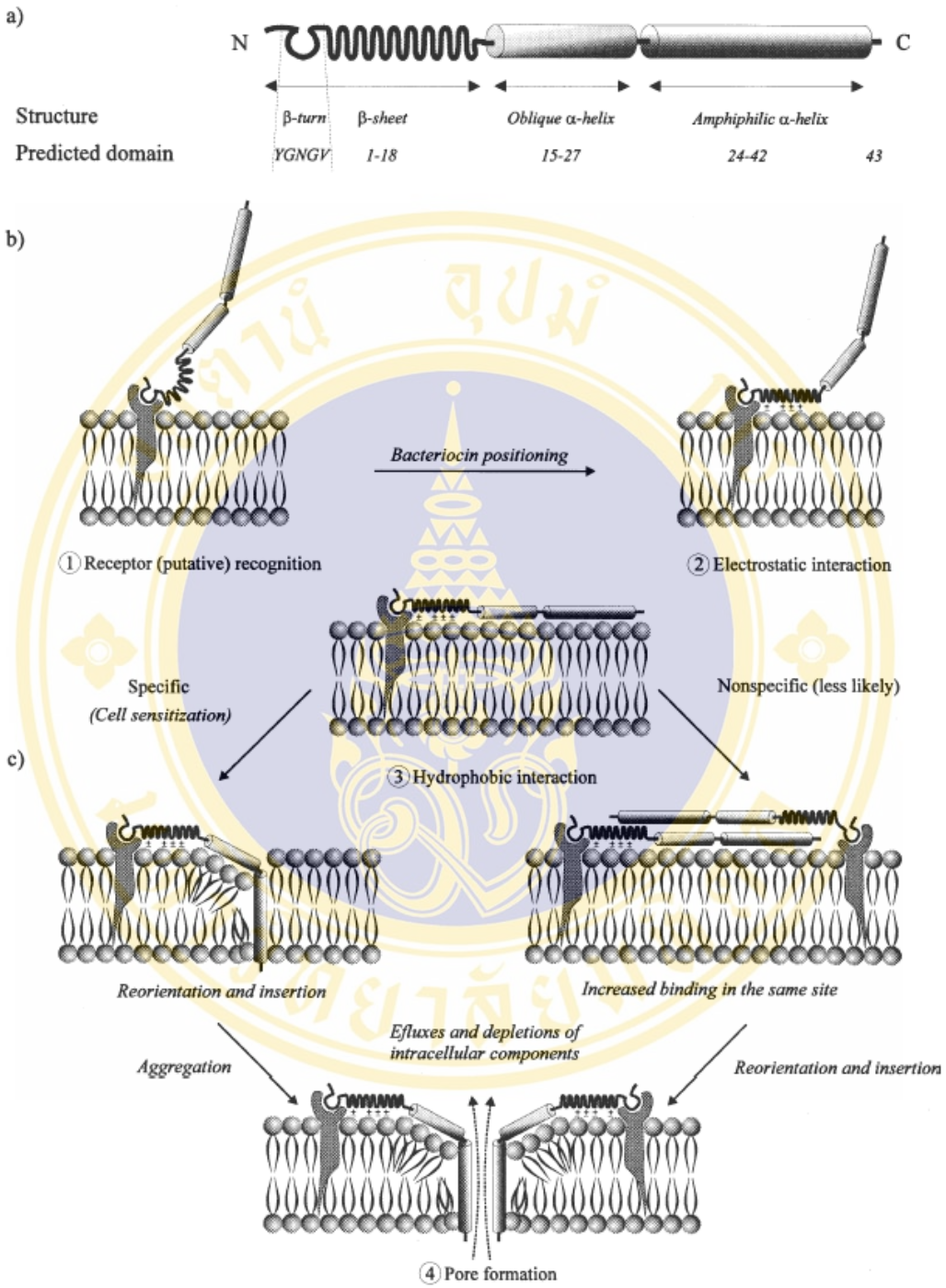

Figure 2. Schematic representation of structure of a model class IIa bacteriocin and the predicted location its domains with respect to target cell membrane: a) bacteriocin predicted structural domain; b) possible interactions of each domain with the membrane surface; c) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide in a) is shaded dark and hydrophilic face is shaded light. From: Ennahar et al. (2000)

1.2.3 Role of bacteriocin and its application

As the majority of bacteriocin-producing LABs are natural food isolates, bacteriocins are ideally suited to food applications. Bacteriocins of LAB are attractive in the food industry because they may be used as natural biopreservatives to control undesirable bacteria in a food-grade and natural manner (Jack et al., 1995). Also, the use of these bacteriocins may allow a significant reduction in the level of chemical additives and/or in the intensity of the physical treatments currently employed during food processing. Therefore, they could also help to provide healthier foods. The classic example is nisin produced by some *Lc. lactis* isolates and structurally characterized in 1971, thus, nisin and nisin-producing strains have had a long history of application in food preservation, especially of dairy products (Jack et al., 1995). Recognition that *Lc. lactis* strains are naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested has led the U.S. Food and Drug Administration (FDA) to accord GRAS (generally recognized as safe) status to nisin. Today, nisin is accepted as a safe food preservative by over 45 countries, and it is the most widely used commercial bacteriocin. Over the past decade the recurrence of listeriosis outbreaks, combined with the natural resistance of the causative agent, *Listeria monocytogenes*, to traditional food preservation methods such as its ability to grow at near-freezing temperatures has focused the attention of bacteriocin researchers that has resulted in the isolation of a large number of class IIa bacteriocins, all of which are highly active against *L. monocytogenes*.

In addition, the rapid rise and spread of multi-resistant bacterial pathogens have forced the consideration of alternative methods of combating infection. One of the limitations of using broad-spectrum antibiotics is that they kill almost any bacterial species not specifically resistant to the drug. Bacteriocins provide an alternative solution. With their relatively narrow spectrum of killing activity, they can be considered “designer drugs” which target specific bacterial pathogens. Given the diversity of bacteriocins produced in nature, it is a relatively simple task to find bacteriocins active against specific human pathogens. Studies of mersacidin and epidermin, ribosomally synthesized peptides of the lantibiotic class, have suggested that they may be at least as effective as some currently used therapeutic agents for the

treatment of staphylococcal infections in mice (Limbert et al., 1991) and acne in humans (Ungermann et al., 1991), respectively.

2. Educational review

2.1 Perspectives of teaching and learning in science

2.1.1 Behaviorism

One of the first scientific approaches to understand learning was to observe an actual behaviour. Behaviourism began by trying to explain simple behaviours – observable and predictable responses following specific conditions called stimuli (Lefrançois, 1999). Learning was viewed as potential changes in behaviour as a function of experience. There were three works that can be taken jointly as the foundation of behaviourism: Thorndike's early animal research; Pavlov's investigations of the conditioned reflex; and Watson's article, *Psychology as the behaviourist views it* (Mackenzie, 1977). Most of these principles are highly teacher-centred. That is, they emphasize the role of the teacher in organizing and transmitting information and in controlling important aspects of the learning situation (Lefrançois, 1999). This early research saw largely as a passive process. Changes in observable behaviour were viewed as results of learning, with no mention of processes which are occurred in the learners' minds. Prior to the late 1950s, published research in the USA had a predominantly behaviourist tone (Duit & Treagust, 1998). After this time, researchers began to see the limitations of behaviourism and a new theory of learning emerged.

2.1.2 Constructivism

Constructivism is a theory of learning originating from the work of a developmental psychologist, Jean Piaget (1954). The term constructivism includes a wide range of theoretical positions (Geelan, 1997) and has been widely used to refer to views about learning, teaching, and curriculum development.

In constructivism, learning is viewed as conceptual development. Piaget (1954) proposed that children's learning is a process of personal, individual, intellectual construction arising from their activities in the world. Knowledge is viewed as an

individual construction. Learning is not viewed as transfer of knowledge but as the learners actively constructing their knowledge on the basis of the knowledge they already hold from their previous experience.

The heart of the constructivist view is the idea that the conceptions held by each individual guide their understanding. The learners have their own prior knowledge. They have to make sense of the world through an existing conceptual structure, so whatever knowledge is constructed by the learners, this will be an interpretation of experience in terms of extant knowledge (Tobin & Tippins, 1993).

In his work, Piaget (1954) explained how he thought the categories of sensorimotor intelligence were organized the learner's world view. He introduced three terms, assimilation, accommodation, and equilibration to describe the process of learning. Assimilation is the process of the individual's adaptation to new concepts with the novel inputs basically fitting into an already-existing cognitive structure. Accommodation indicates an adaptation process where restructuring of the already-existing structure is necessary when the inputs do not fit the existing cognitive structure. If the inputs do not fit the existing cognitive structure, there is a cognitive conflict. Then, an interplay of assimilation and accommodation can restore the balance, and the process called "equilibration" occurs.

Posner et al. (1982) suggested that there are several important conditions which must be fulfilled before an accommodation is likely to occur.

"1) There must be dissatisfaction with the existing conception.

2) A new conception must be intelligible. The individual must be able to grasp how experience can be structured by a new concept sufficiently to explore its inherent possibilities.

3) A new conception must appear initially plausible. Any new concept adopted must, at least, appear to have the capacity to solve the problems generated by its predecessors.

4) A new concept should suggest the possibility of a fruitful research program. It should have the potential to be extended, to open up new areas of inquiry" (p. 214).

By the late 1960s, Piaget's ideas of individual constructivism came to influence learning in science. Extensive research into children's alternative conceptions in science stimulated considerable interest in constructivist's views of learning and has spawned a range of constructivist's approaches to the teaching of science (Hodson, 1990). Since the mid-seventies, most studies on teaching and learning science appear to follow a constructivist's orientation (Tobin & Tippins, 1993).

From this perspective, learning in the classroom setting is seen to require well-designed practical activities that challenge learners' prior conceptions, encouraging learners to reorganize their knowledge. Knowledge cannot be transmitted directly from one knower to another, but must be actively constructed by the mental activity of learners (Driver et al., 1994) and thus learning is perceived as more learner-centred.

Student's constructions are not always the same as the scientific view (Driver et al., 1994; Treagust et al., 1996). Constructivism, thus, saw learning as involving a process of conceptual change. Teaching approaches in science based on this perspective focus on providing learners with experiences that induce cognitive conflict and encourage learners to develop new knowledge schemes that are better adapted to experience.

Hodson (1990) stated that constructivist views of learning do provide some clear pointers towards teaching strategies that might assist students in the task of conceptual reconstruction by: identifying student ideas and views; creating opportunities for students to explore their ideas and test their robustness in explaining phenomena; accounting for events and making predictions; providing stimuli for students to develop, modify and, where necessary, change their ideas and views; and supporting their attempts to re-think and reconstruct their ideas and views.

The constructivist's approach had a powerful impact on how educators view good teaching practice. That is, individual constructivism illustrates how the traditional and teacher-centered approach of transmitting knowledge is inadequate for stimulating learning in the classroom (Rodriguez, 1998). However, even though science educators have learned a great deal from individual constructivist research in the last two decades, the approach of this epistemology appears to have mainly benefited science education as a research field, but not science instruction in the school and university classroom (Rodriguez, 1998). In practice, many teachers have

not changed the way they teach. Therefore, more activities to inform teachers about individual constructivism research findings and how to make them work in practice must be undertaken (Duit, 1995).

There are criticisms of constructivism. In constructivism, new knowledge does not come from experiences alone, sense impressions have to pass through the filter of prior conceptions. The claim that experiences are the key source of learning is not sufficient (Treagust et al., 1996). It is suggested that conceptual change approaches in science education overemphasize the individual learning and neglect the social issues in knowledge construction processes, and view knowledge primarily as something stored in the individual mental system, as a mental model of the world outside (Duit & Treagust, 1998). Knowledge construction requires an active process of interpretation within a social and cultural setting by a learner. Social constructivist's ideas have attracted growing attention in science education over the past years, with educators seeking ways to employ these ideas to address the limitations of "purely" constructivist's approaches (Hennessy, 1993).

2.1.3 Social constructivism

Over the past few decades, perspectives on learning science have been changed (Solomon, 1994) from personal constructivism to social constructivism, which includes learning that is influenced by the social setting (Hennessy, 1993). The view that learners do not learn in isolation but by being part of the surrounding community was proposed by John Dewey (Oxford, 1997). He suggested a triangular relationship modeling on the social construction of ideas between the individual, the community and the world. He believed that ideas are meaningful only if they are part of an acceptable theory, instrumentally useful for creating positive action, constructed by participants in society, and related to the reference points provided by society. Therefore, the social constructivism perspective reflects person-world relationships (Duit & Treagust, 1998).

Like Dewey in the 1950s, Lev Vygotsky (1978) contributed significantly to social constructivist's epistemology. He recognized that ideas have social origins; they are constructed through communication with others. The individual's cognitive system is a result of communication in social groups and cannot be separated from

social life. Vygotsky and Dewey focused on the individual powerfully rooted in the group context (Shayer, 2003).

Vygotsky (1978) introduced the concept of the zone of proximal development (ZPD); that is, the distance between children's actual development, determined with the help of independently solved tasks, and the level of potential development, shown in tasks solved by the children under the guidance of adults and in cooperation with their more competent peers. Lantolf (1993) emphasized that the ZPD is negotiated between the teacher and the student or between the students and peers or others.

For Vygotsky (1978), teachers act as facilitators or guides and providers of assistance. The teachers support students by providing any form of assistance that might help students develop their language and cultural skills. Vygotsky's idea of assistance might include a hint or clue, a word of praise, a suggestion, a learning strategy, an intensive review, or anything that the particular student needs at a given time. When the learner needs the greatest assistance, the teacher gives "scaffolding" to ensure that the learner's constructs will continue to become stronger and more complex. As the learner requires less help, the teacher slowly removes the scaffolding that previously supported the learner, and the learner becomes increasingly self-directed.

In social constructivist's approaches that have been employed in science education, the idea of situated cognition usually plays a key role (Hennessy, 1993). Brown et al. (1989) described the basic ideas of situated cognition:

> "The activity in which knowledge is developed and deployed, it is now argued, is not separate from or ancillary to learning and cognition. Nor it is neutral. Rather, it is an integral part of what is learned. Situations might be said to co-produce knowledge through activity. Learning and cognition, it is now possible to argue, are fundamentally situated." (p. 32).

From the perspective of situated cognition, learning means change from one everyday context to a new science context or, changes from the practices of one culture to another. This view recognizes the critical role of the social and physical circumstances when interpreting those actions, and of including thinking as a part of

culturally organized activity which is carried out within a community. Hennessy (1993) described this view of learning as a process of enculturation or individual participation in socially organized practices, in which specialized local knowledge, rituals, practices, and vocabulary are developed. This perspective of learning sees knowledge as being shared through engagement in socially shared activity and discourse.

The situated cognition framework has important implications for classroom learning. As learning can be facilitated through a series of processes such as scaffolding, coaching, and encouraging, cognitive apprenticeship is often seen as a method for introducing the learner into the new culture as the expert guides the apprentice (the novice). By increasingly participating in activities within the community in question step-by-step, the apprentice becomes a member of that community.

Salomon and Perkins (1998) proposed six perspectives of the notion of social learning and individual learning, which are now considered to be used in this study:

1. Active social mediation of individual learning. In the same way that a person in society or a team helps an individual to learn, in the laboratory, teachers, instructors or peers can be considered and expected to help and enhance each individual's learning of a particular scientific context. This could be seen as an instructor training a student to use a PCR machine.
2. Social mediation as participatory knowledge construction. As social constructivists view learning "less as the socially facilitated acquisition of knowledge and skill and more as a matter of participation in a social process of knowledge construction" (Salomon & Perkins, 1998, p. 4), students learning as a group could help to foster learning outcomes in the laboratory, as students come to understand the meaning of tasks through their participation in the group.
3. Social mediation by cultural scaffolding. As tools and information sources can help the learners learn, well-designed laboratory experiences and instructions could act as a scaffold for student learning.
4. The social entity as a learning system. "The group constitutes a collective learning system, a system that will function better or worse as a learner

depending on how well its structures address critical conditions of learning" (Salomon & Perkins, 1998, p. 5) If students are asked to work in laboratory groups, each individual is considered to be a part of their group, and all of the group members could share and make decisions on the completion of their task.

5. Learning to be a social learner. Each individual student in the group could learn to learn through participation with their peers by asking questions or helping each other.
6. Learning social content. In the group, students could learn about how to get along with others or how to collaborate in reaching decisions and taking collective actions.

A social constructivist's view of learning appears to be suited to guiding thinking about science education towards the aim of making science teaching and learning more effective (Duit & Treagust, 1998). In summary, a social constructivism view of teaching and learning is seen as a useful theoretical background to this study. This has certain implications for teaching and learning in the screening of LAB for bacteriocins by microbiological and PCR methods laboratory intervention. A unit design involving some training, participation, scaffolding, and group learning would see students working together, discussing methods and results, and negotiating tasks. This could promote more meaningful engagement correlated with the objective of the task provided.

2.1.4 Sociocultural perspectives of learning

Recent ideas have suggested that not only social but also cultural views of learning play an important role in learning. Teaching science can be viewed as cultural transmission and learning science can be viewed as culture acquisition, where culture means the beliefs, way of life, art, and customs that are shared and accepted by people in a particular society. Human mental processes such as learning are situated within their historical, cultural and institutional setting (Wertsch, 1991). Social interaction such as communication, expectations, etc. takes place within the learners culture. Cultures which students live in or are exposed to, including the family, and

peers at school, are viewed as influencing the learning of science. By providing cultures of science which agree with a student's everyday culture and science instruction which supports the student's view of the world, enculturation occurs by participation in shared activities. Enculturation, which is the acquisition of meaningful knowledge, situated in some activity and made sense of within specific contexts and cultures, facilitates the entry of an individual into a specific culture. Cobern and Aikenhead (1998) suggested that when enculturation into science occurs, scientific thinking enhances a person's everyday thinking. Science knowledge is developed in the science community by the influence of the science is social and cultural context. In this view, teaching and learning in a science laboratory can be viewed as enculturation into science.

A second influence of culture in science teaching and learning is the influence of a student's culture on their learning. For example, undergraduate laboratory for Thai students usually arranged in groups of five. Students simply follow instructions to complete their tasks. They usually separate into small teams or individuals to do each subtask, then they gather data as a group. The students seldom ask questions to teachers or teaching assistants, but respect and obey them. But if teachers or teaching assistants raise an interesting question, students have the ability and willingness to find the answer. For this reason, if laboratory tasks are designed in a way that allow students to work closely with their peers, teachers and teaching assistants, this may make students to think more critically about their tasks. Students may communicate, participate and learn together with peers, instructors, and teaching assistants. Using this suggestion, if the screening of LAB for bacteriocins by microbiological and PCR methods was designed in a way that, laboratory learning situation is developed which considers students' culture and they can share knowledge, all intended learning outcomes could occur.

Learning occurs across cultural borders from the worlds of peers, community, and family into the world of science. Rogoff (1995) described a sociocultural approach as involving three planes corresponding to personal, interpersonal, and community processes. The developmental processes corresponding to these three planes are participatory appropriation, guided participation and apprenticeship. Participatory appropriation "refers to how individuals change through their

involvement in one or another activity, in the process becoming prepared for subsequent involvement in related activities" (Rogoff, 1995, p. 142), e.g. by participating in the screening of LAB for bacteriocins by microbiological and PCR methods, students learn how to do overlaying and PCR and understand the processes involved in the laboratory practice. "[G]*uided participation* refers to the processes and systems of involvement between people as they communicate and coordinate efforts while participating in culturally valued activity" (Rogoff, 1995, p. 142), e.g. students learning in the PCR laboratory by communication with their peers as they guide each other to finish the task. Apprenticeship "involv[es] active individuals participating with others in culturally organized activity that has as part of its purpose the development of mature participation in the activity by the less experienced people" (Rogoff, 1995, p. 142), e.g. students learn how to screen LAB for bacteriocins by microbiological and PCR methods by participation as apprentices with their instructors.

Another perspective of the sociocultural view of learning is mediated action, which is a process involving the potential of cultural tools that are used to shape action in social interaction. Wertsch et al. (1995) discussed Vygotsky's views of language as a cultural tool that emerged in the service of the forms of mental functioning they mediate. Language is a tool of learning as it is used for communication in the learning context. Even instruments such as the PCR machine have their own language. In the culture of biotechnology, after teachers have introduced the principles of PCR, students can realize that the PCR machine facilitates DNA denaturation, annealing, and extension. Thus, the PCR machine also carries the principle of PCR. In the PCR laboratory, students can learn from their interaction with several kinds of cultural tools, such as the laboratory setting itself, books, internet sources, etc., in which language plays an important role.

Learning is seen as an exercise in the use of existing cognitive resources (Olson, 1995). In the Thai learning culture, students' learning could be influenced by cultural tools, which are reinforced by interaction and discussion among peers. The teaching methods developed also allow the incorporation of the content or aspects of science cultures into a student's everyday culture and enable students to enjoy and construct

meaning in science. This teaching method could enhance teaching and learning outcomes in Thai culture.

The biotechnology students bring with them the understanding of potential importance of PCR on DNA amplification from their lectures into the laboratory. Providing a laboratory context relevant to their daily life e.g. applications of PCR to the food they eat, could provide motivation to construct meaningful personal knowledge. Students can use and understand the PCR principle for the detection of bacteriocin genes in LAB which are present in the foods they eat. The potential of biotechnology relevant to students' daily lives was also emphasized.

In summary, views of teaching and leaning in science have changed, from behaviorism that emphasized the transmission of knowledge from the teachers to the learners, through constructivism that regarded learners' prior knowledge as allowing each individual to construct their own concepts, to social constructivism and the sociocultural view of learning that sees mental processes as related to a social, cultural, and historical setting (Wertsch et al., 1995). This sociocultural view can be used to develop a laboratory exercise that promotes learning outcomes related to PCR, where the process of laboratory learning is situated in a particular social context. It involves part of the culture of the learning community, where the proper assistance is available. In a community of laboratory learners, other people such as teachers and peers could help the learners negotiate their own zone of proximal development (ZPD). These people provide scaffolding, consisting of multiple forms of assistance, that can be removed as the learner becomes more proficient in the culture. Thus, the sociocultural view is seen as the foundation of learning in the laboratory classroom for the screening of bacteriocins in LAB by microbiological and PCR methods.

2.2 Perspectives of teaching and learning in science laboratory

Since the aim of science education is to help students develop an understanding of the natural world, the activities in which students can observe or interact with the real objects and materials are designed for the laboratory situation. Hofstein and Lunetta (2004) defined science laboratory activities as learning experiences in which students interact with materials and/or with models so as to observe and understand the natural world. The fundamental purpose of any laboratory task is to help students

to make links between domains of real objects and observable things, and the abstract domain of ideas (Millar et al., 2002).

There are a number of science educators (e.g. Hodson, 1993; Lazarowitz & Tamir, 1994; Leach, 2002) who have proposed the goals of practical work as developing inquiry, knowledge, skills, attitudes, experiences, and understanding of nature of science. These goals are closely related to the four major categories of intended laboratory learning outcomes described by Hegarty-Hazel (1990). These learning outcomes are: acquiring technical skills, learning about scientific inquiry, learning scientific knowledge, and developing scientific attitudes.

Technical skills are concerned with the acquisition of practical skills and techniques. Hodson (1993) framed the arguments for practical work as a means of developing laboratory skills into two categories; the acquisition of content-free, generalizable skills, and development of the basic professional and research skills needed for future scientists and technicians. All laboratory skills involve technically skilful or careful movement and the manipulation of equipment. Hegarty-Hazel (1990) proposed that the laboratory where students manipulate apparatus and perform techniques individually or in small groups could provide for the learning of technical skills more effectively than teacher demonstrations. Laboratory technical skills involved the senses, the brain, and the muscles. These internal conditions of learning require an overview of the skills routine while external conditions require practice and feedback for improvement of accuracy and speed and the quality of skills. Because of this, teachers should provide students with a satisfying reason for the laboratory tasks, such as future learning or use in society, a specification of the technique and an overview of the steps in performance, followed by detailed feedback on the performance.

Scientific inquiry requires the development of intellectual skills. It is concerned with the natural world and is guided by certain beliefs and assumptions. In science education, scientific inquiry is divided into three main components: students' science process skills, general enquiry processes, and the nature of scientific inquiry (Klopfer, 1990).

1) Students' science process skills: observing and measuring, seeing, seeking solutions to problems, interpreting data, generalizing and building testing, and revising theoretical models.
2) General enquiry processes: problem-solving, uses of evidence, logical and analogical reasoning, clarification of values, decision-making.
3) The nature of scientific inquiry: epistemological reflection on connections with the philosophy of science.

Scientific knowledge is shown in the facilitation of science content. There are many types of scientific knowledge, symbols, rules or facts which have obvious value to a student to use for further learning, science concepts, empirical laws, principles, and their applications. A personal involvement in practical work helps students acquire further scientific knowledge as well as developing technical skills. A student carrying out a practical experiment, or seeing it demonstrated, would be more likely to store memories relating to the experiment, recall the steps involved and its theoretical underpinning (Atkinson, 1990). Teachers can enhance the learning of scientific knowledge in the laboratory by stressing the purpose of the practical work and its relationship with theory.

Scientific attitude is attached to activities that aim to enhance motivation, enjoyment, and interest. In science education, attitudes are divided into attitudes to science (students' favourable or unfavourable reactions to some objects, including interest, enjoyment and satisfaction) and scientific attitudes (personality traits related to habitual styles of thinking which includes open-mindedness, honesty, objectivity, willingness to suspend judgement and disinterestedness) (Gardner & Gauld, 1990; Gauld & Hukins, 1980). Science educators have seen that student attitudes to laboratory work are influenced by a variety of factors. Laboratory work provides opportunities for enhancing students' scientific attitudes and their enjoyment of science in relation to teaching methods. When students enjoy the activities provided they will develop positive attitudes to science (Henry, 1975).

2.3 Educational research in teaching and learning in science laboratory

2.3.1 General concept

A number of works have been published concerning students' understanding and conceptual difficulties regarding scientific concepts and phenomena (Gabel, 1994) and how students use or do not use conceptual knowledge when performing experimental work. These studies have set up special teaching situations, focusing on students' scientific discussion and actions during practical work (Psillos & Niedderer, 2002a). For example, Kariotoglou (2002) developed experiments concerning empirical laws in order to engage students in data-processing activities for teaching the concepts of fluids. The researcher aimed at the students' familiarization with the phenomena, concepts and experiments. Students had to predict and carry out experiments, and then, in groups, discuss the results compared with their predictions. After the analysis of video, audio tape, and interviews, the results showed that students seem to unify the concepts of liquids and gases as fluids at the conceptual level. The researcher suggested that the familiarization phase is very important because it contributes to the creation of a broad conceptual and methodological framework of thinking and intervening for students who are not familiar with such scientific procedures. In addition, peer-to-peer levels of discussion, which can make students feel comfortable to express their views, can be implemented at some stages of the laboratory teaching.

Some research studies described frameworks for constructing and analyzing practical work. They presented a framework of tools, methods and basic research results related to research and development of practical work environments. The aim was to capture and classify the varieties of laboratory work, to reflect design problems of determining its effectiveness, and to summarize some basic finding on students' epistemological beliefs in relation to practical work. Some of them are considered to be useful for designing laboratory instruction. For example, Millar et al. (2002) and Tiberghien et al. (2001) presented a model of the process of developing a practical work task and evaluating its effectiveness. Two senses of "effectiveness" were identified: the match between what students are intended to do in the task and what they actually do (effectiveness 1); and the match between what students are intended to learn from the task and what they actually learn (effectiveness 2). They also described a scheme which can be used to produce a profile of any practical work task. This provides a useful tool for systematically exploring the effectiveness of practical

work tasks. Leach (2002) considered how students' understanding of the nature of science influences their actions and learning during practical work via survey questions. He presented some basic hypotheses and findings about students' epistemological conceptions of images of data and measurement, nature of investigation, nature of theory, nature of explanation, and of the nature of public scientific knowledge related to practical work in the form of hypotheses. The results showed that some students understood the nature of science but some of them held alternative conceptions. These kinds of hypotheses are important to consider in laboratory design in order to emphasize the nature of science during laboratory work.

Some studies investigated what happens during the action phase of practical work, examining both students and teachers in an attempt to make the relationship between doing, thinking and learning easier to understand. For example, Bécu-Robinault (2002) and Beney and Séré (2002) studied the links between doing-thinking-learning during standard practical work by using audio and video analyses. The results showed that the traditional lab sheet information is not sufficient to lead students to make links between the theories and models and the experimental field. They also described the difference between what was expected by teachers and what students actually did. The results showed that students lacked knowledge of procedures and suggested that practical work was irreplaceable in learning procedural knowledge. The researchers suggested that if the objectives for procedural knowledge are not taken seriously, standard practical work will be limited to objectives like familiarity with apparatus, experimental skills and motivation. This study has compared between the screening of LAB for bacteriocins by microbiological and PCR methods. It shows the necessity to emphasize PCR principles to students, and to examine students' understanding of the principles of PCR while they are carrying out the experiment and interpreting data.

Some research studies focus on teacher and student perceptions of the nature and purpose of practical work. For example, Pekmez, et al. (2005), and Wilkinson and Ward (1997) examined science students' and teachers' thinking on the nature and purpose of practical work. Data were collected through individual interviews with science teachers about their classroom practice. The results showed that both students and teachers believed that the most important aim of laboratory work was to make

science more interesting and enjoyable through actual experience, and laboratory work was related to theory work. But students found that it was difficult to see the relevance of laboratory works to everyday life, and that this should be made clearer. This may be enhanced by developing laboratory which is involved their daily lives, such as the laboratory class on the screening of LAB and bacteriocins in food samples by microbiological and PCR methods, that are seen to be more relevant or useful in students' daily lives.

2.3.2 Educational research for undergraduate level

There are a large number of studies focused on open-ended undergraduate laboratory work in which students are required to make some decisions for themselves as to how to act in various types of projects. These studies are focused mainly on aspects of the understanding and use of scientific procedures on the part of the students, as well as on the improvement of their epistemological knowledge when engaged in investigative work, for example, Evangelinos et al. (2002); Guillon and Séré (2002); Leach (2002); Lewis (2002); Ryder (2002). These researchers investigated open-ended laboratory work to introduce epistemological knowledge, and to allow students to go through investigation strategies in open-ended projects. These studies familiarized students with different procedures. The results showed that the projects were successful in developing the students' understandings of the nature and processes of scientific research, and students did begin to develop awareness through their work. They thought for themselves in deciding research questions, solving problems, interpreting results, deciding the next steps. Students developed organizational skills such as planning, and time management. They also realized that in the real laboratory, it is often difficult to obtain the necessary data. This is important in the laboratory on the screening of LAB for bacteriocins by microbiological and PCR methods where the students have to bring their own food samples to detect the presence of the bacteriocin genes. Students may not get the anticipated PCR results, but they still need to provide the empirical reasons for that lack. Thus, these open-ended discussions should be included in the PCR laboratory.

There are several other reports focused on inquiry-based teaching and learning in the laboratory (e.g., Luckie et al. (2004); Kolkhorst et al. (2001); DiPasquale et al.

(2003); Petrie (2005); Collins and Bell (2004); Chaplin (2003); Myers and Burgess (2003); Wilterding and Luckie (2002)). In these inquiry-based laboratories, students worked in small groups, posed scientific questions, formulated hypotheses, proposed experimental designs, performed investigations and then presented their findings. Research results showed that these activities stimulate undergraduate students' cognitive development of scientific concepts, develop laboratory skills to become independent problem solvers and critical thinkers, and develop an understanding of the scientific process. Observations of student behaviours reflected a high level of enthusiasm and engagement in laboratory activities. Russell and French (2002) studied participation, achievement, and attitude in both traditional cook-book and inquiry-based introductory biology laboratories through observations and interviews. They found that there were no differences in participation between men and women, and there was higher achievement, and more positive attitude development, when using inquiry-based laboratories. These results suggest the design of laboratory class should allow every student in the group to contribute. Laboratory work can support equal participation and increase attitudes, in addition to making students feel empowered by having ownership of their projects.

Other research studies focused on undergraduate students' conceptions in science. Using interviews, Gopal et al. (2004) investigated conceptions held by evaporation, condensation and vapour pressure of second-year chemical engineering students. The results showed some misconceptions. Many students changed their initial incorrect answers when presented with further physical evidence as the interview progressed. The study points to the importance not only of practical work, but of associated conceptual discussions that allow students to reflect on and refine their conceptions. This research suggests the interviews or discussions about e.g. PCR principles should be implemented at some stages of the screening of LAB for bacteriocins by microbiological and PCR methods laboratory. Ross et al. (2005) suggest that using analogies, modelling, and role play strategies following a traditional lecture, together with online resources and textbooks, can make students increasingly use metacognitive skills to aid their further understanding of abstract concepts of photosynthesis, and the submicroscopic world of atoms and molecules. Similar methods can be included in some stages of the screening of LAB for

bacteriocins by students' laboratory on microbiological and PCR methods, with guidance from lecturers and teaching assistants. These suggestions could be used to enhance positive attitudes towards further study and raise students' self-esteem about learning strategies.

CHAPTER IV
MATERIALS AND METHODS

1. Science

1.1 Chemicals and reagents

Bacterial culture media were obtained from Oxoid (Hampshire, England). Agarose was obtained from F.M.C. (USA). All oligonucleotide primers were synthesized by Proligo Primers & Probes (Proligo Singapore Pty Ltd, Singapore). *Taq* DNA polymerase and ThermoPol Reaction Buffer for PCR were purchased from NEW ENGLAND BioLabs Inc. TBE (0.089 M Tris, 0.089 M Borate and 0.002 M EDTA for 1X) was purchased from AMRESCO, Ohio, USA. A 100 base pair (bp) DNA ladder (GeneRuler^{TM} 100 bp DNA Ladder Plus, Fermentas, USA) was used as standard size markers. All chemicals were either analytical or molecular biology grades.

1.2 Bacterial strains

The bacterial strains used in this study for Experiments 1, 2, and 3, are listed in Table 2. They were either purchased from German Collection of Microorganisms and Cell Cultures (DSMZ) (seven isolates), Thailand Institute of Scientific and Technological Research (TISTR) (19 isolates) and K535 stock culture or newly isolated from Nham samples. Some of TISTR strains were originated from American Type Culture Collection (ATCC), NODAI Research Institute Culture Collection (NRIC), Noda Institute for Scientific Research (NISL), Japan Collection of Microorganism (JCM), Tokyo University of Agriculture (TUA), and Agricultural Research Service Culture Collection (NRRL). Those strains, therefore, will have two code numbers (as shown in Table 2).

Table 2. Bacterial strains used in this study

Isolates or strains	Sources*
Experiment 1	
B. subtilis	K535 stock culture
S. aureus	K535 stock culture
Lb. plantarum ATCC 8014	TISTR 050
Ped. pentosaceus JCM 2027	TISTR 419
E. coli	K535 stock culture
V. harveyi 639	K535 stock culture
V. harveyi 1114	K535 stock culture
V. cambelli	K535 stock culture
V. parahaemolyticus	K535 stock culture
Aeromonas sp. 30-3c	K535 stock culture
Aeromonas sp. 30-3p	K535 stock culture
Experiment 2	
Ped. pentosaceus P7	Isolated from Nham
Experiment 3	
Ped. pentosaceus P7	Isolated from Nham
En. faecium F103	Isolated from Nham
Lc. lactis F141	Isolated from Nham
Experiment 4	
Ped. acidilactici ATCC 8042	TISTR 051
Ped. acidilactici NRIC 0124	TISTR 952
Ped. acidilactici DSM 20284	TISTR 953
Ped. acidilactici NRIC 1096	TISTR 1117
Ped. acidilactici NISL 7113	TISTR 397
Ped. pentosaceus JCM 5885	TISTR 783
Ped. pentosaceus JCM 2027	TISTR 419
Ped. claussenii DSM 14800	DSMZ
Ped. cellicola DSM 17757	DSMZ
Ped. stilesii DSM 18001	DSMZ
Ped. inopinatus DSM 20285	DSMZ
Ped. damnosus DSM 20331	DSMZ
Ped. parvulus DSM 20332	DSMZ
Ped. dextrinicus DSM 20335	DSMZ
Ped. halophilus (*Tetragenococcus halophilus*)	TISTR 429
Ped. urinaeequi (*Aerococcus urinaeequi*) ATCC 29722	TISTR 394
Lb. plantarum ATCC 8014	TISTR 050
Lb. casei ATCC 7469	TISTR 047
Lb. brevis NRIC 0134	TISTR 860
Lb. acidophilus ATCC 4355	TISTR 1034
Lb. bulgaricus TUA 093L	TISTR 451
Lb. delbrueckii NRRL B-763	TISTR 326
En. faecalis ATCC 19433	TISTR 379
Leu. dextranicum ATCC 8086	TISTR 377
Leu. mesenteroides ATCC 10830	TISTR 053
Lc. lactis JCM 7638	TISTR 1401

* Note: Some isolates used in Experiment 3 also have other TISTR assigned numbers.

1.3 Bacterial culture condition

All LAB were grown in MRS medium at 30˚C with agitation. Agar was added to make 1.5% for solid medium. For long-term storage, 600 µl of overnight cultures were transferred to sterilized vial containing 300 µl 45% glycerol to obtain a final concentration of 15% and kept at -80ºC.

1.4 Growth curve and pH change

The growth curve and pH change of the seven selected strains (F1 from fish Nham and P1, P3, P4, P5, P7 and P8 from pork Nham) were studied. One colony of the selected strains was inoculated into 5 ml MRS broth and incubated at 30˚C with shaking for overnight. The overnight culture was 1% subcultured into 100 ml fresh MRS broth and incubated at 30˚C with shaking. Five milliters of samples were taken at 0, 6, 12, 18, 24, 36 and 48 h. The samples, 0.5 ml, were ten-fold serially diluted from 10^{-1} to 10^{-6} with 0.85% NaCl. The one hundred microliters of three appropriate dilutions were spread onto MRS plates (each dilution on three plates), and the plates were then incubated at 30˚C for 24 h. The viable count method was performed from the plates containing 30-300 colonies for CFU/ml determination. Another portion of the sample, 4.5 ml, was centrifuged at 10,000 rpm and the pH of the culture supernatant was measured by pH meter.

1.5 Experiment 1: Screening of LAB for bacteriocins

1.5.1 Isolation of LAB

LAB producing bacteriocins were isolated from Nham (fermented pork and fish sausages) samples. One gram of Nham samples was mixed in a tube containing 5 ml of de Man Rogosa Sharpe (MRS). After incubating at 30°C for 1 hour with continuously shaking; the incubated samples was ten-fold serially diluted from 10^{-1} to 10^{-6} with 0.85% NaCl. The one hundred microliters of the dilutions 10^{-4}, 10^{-5}, and 10^{-6} was spread onto MRS plates (each dilution on two plates), and the plates were then incubated at 30˚C for 24 h.

1.5.2 Bacteriocin activity test

The plates containing 30–300 colonies were selected for primary screening by overlaying with a tester strain, *Vibrio harveyi* 639. It was later known that *V. harveyi*

is sensitive to lactic acid (Rodrussamee, 2007). The selected plated were overlaid with 6 ml soft agar containing 1 ml of bacterial culture (precultured for overnight and then subcultured into TSB plus 3% NaCl and further incubated for 4 hours before mixing with soft agar) and 5 ml of TSA plus 3% NaCl and 0.8% agar. Before mixing with bacterial culture, molten agar was kept in the shaking water bath at 60˚C. The molten agar was cooled to about 45˚C before adding the cell cultures to prevent cell death. The mixture was vigorously mixed using a vortex mixer, and then the contents were gently overlaid onto each plate and allowed to completely cover the agar surface. After the overlaid agar was solidified, the plates were turned upside down and incubated at 30˚C for 24 h. Colonies with a clear halo zone were picked by a toothpick and subcultured on new MRS plates.

The positive strains producing clear halo zones from the primary screening were selected and performed secondary bacteriocin activity test by determining the ability of the LAB strains to inhibit growth of *V. harveyi* 639, *V. harveyi* 1114, and *V. cambelli*, *V. parahaemolyticus*, *Aeromonas* sp. 30-3c, *Aeromonas* sp. 30-3p, *E. coli*, *B. subtilis*, *S. aureus*, *Lb. plantarum* TISTR 050, *and Ped. pentosaceus* TISTR 419 (all, except *B. subtilis*, *S. aureus*, *Lb. plantarum* TISTR 050 and *Ped. pentosaceus* TISTR 419 are Gram-negative bacteria. *Vibrio* spp. and *Aeromonas* spp. are pathogens of shrimp and fish, respectively), the overlay method was used. LAB colonies isolated from Nham samples on MRS agar plates (after incubation at 30˚C for 24 h) were overlaid with all of the tester strains mentioned above (precultured for overnight and then subcultured into TSB plus 3% NaCl or LB and further incubated for 4 h, 1 ml was inoculated in 5 ml TSA containing 3% NaCl and 0.8% agar or LA containing 0.8% agar). For *V. harveyi* 639, *V. harveyi* 1114, and *V. cambelli*, *V. parahaemolyticus*, *Aeromonas* sp. 30-3c, and *Aeromonas* sp. 30-3p, TSB plus 3% NaCl and TSA containing 3% NaCl and 0.8% agar were used. For *E. coli*, *B. subtilis*, and *S. aureus*, LB and LA containing 0.8% agar were used. For *Lb. plantarum* TISTR 050 *and Ped. pentosaceus* TISTR 419, MRS containing 0.8% agar were used. After the overlaid agar was solidified, the plates were turned upside down and incubated at 30˚C for 24 h. Inhibition zone around colony of LAB was then observed.

The bacteriocin activity of LAB in supernatant was assayed and compared by using agar well diffusion method described by Tagg and McGiven (1971). The

bacteriocin activity was determined using culture broth (CB), culture broth boiled for 10 min (CBb10), filtered culture broth (F) obtained by centrifugation of the cultures at 10,000 rpm for 5 min and filter sterilization through a 0.22 µm pore membrane (Millipore, Bedford, Mass., U.S.A.), and filtered culture broth boiled for 10 min (Fb10). MRS agar plates were spreaded with 100 µl of overnight cultured of the tester strains. Wells (5 mm in diameter) were made in the agar layer using cork borer and 50 µl of each fraction, CB, CBb10, F, and Fb10, were placed in each well. Plates were incubated at 30˚C for 24 hr. After incubation inhibition zone around the wells was examined. The tester strains are shown in Table 2.

1.5.3 Bacterial identification by 16S rDNA

Identification of LAB strains were performed by determining 16S rDNA using universal primers for 16S rDNA of bacteria, UFUL and URUL (Nilsson et al., 2003) (Table 3). 16S rRNA gene was amplified using a final concentration of primers and other components as follows: 0.4 µM for UFUL and URUL primers, 1X ThermoPol Reaction Buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% TritonX-100, pH 8.8 @ 25°C, NEW ENGLAND BioLabs Inc.), and 0.4 mM dNTPs. DNA template was prepared by pipetting 1.5 ml of overnight culture broth of each strain into Eppendorf tubes, centrifuged at 8,000 rpm for 5 min, discarded the supernatant, added 200 µl of TE buffer, vortexed well, boiled for 10 min, and then put on ice immediately for 1 min, recentrifuged at 10,000 rpm for 10 min. Five microliters of DNA template (approx. 100 ng) prepared from boiled cells and 5 U of *Taq* DNA polymerase (NEW ENGLAND BioLabs Inc.) were added to obtain a final volume of 20 µl. The PCR amplification (GeneAmp System 2400, PERKIN ELMER, Massachusetts, USA) was started by heating at 95ºC for 5 min, followed by 25 cycles of three steps consisting of 1 min at 95°C, 30 s at 60°C, and 1 min at 72°C. The reaction was terminated by 5 min at 72°C for final extension.

1.6 Experiment 2: Amplification and sequencing of pediocin gene from *Ped. pentosaceus* P7

1.6.1 Primer design

The primers, Fppba and Rppba (Table 3), for detection the presence of pediocin gene were designed from sequence of a 9.4 kb plasmid pSRQ11 harboring pediocin PA-1 operon (Marugg et al., 1992). A study on pediocin operon from *Ped. pentosaceus* P7 was also parallelly conducted by Rodrussmee (2007).

1.6.2 Amplification of pediocin gene

Pediocin gene was amplified using a final concentration of primers (Fppba and Rppba) and other components as follows: 0.4 μM for Fppba and Rppba, 1X ThermoPol Reaction Buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% TritonX-100, pH 8.8 @ 25°C, NEW ENGLAND BioLabs Inc.), and 0.4 mM dNTPs. Five microliters of DNA template (approx. 100 ng) prepared from boiled cells as described above and 5 units of *Taq* DNA polymerase (NEW ENGLAND BioLabs Inc.) were added to obtain a final volume of 20 μl. The PCR amplification (GeneAmp System 2400, PERKIN ELMER, Massachusetts, USA) was started by heating at 95ºC for 5 min, followed by 25 cycles of three steps consisting of 1 min at 95°C, 30 s at 56°C, and 1 min at 72°C. The reaction was terminated by 5 min at 72°C for final extension.

1.6.3 Agarose gel electrophoresis

Amplicons were resolved by 1% agarose gel electrophoresis in 1X TBE (0.089 M Tris, 0.089 M Borate and 0.002 M EDTA for 1X, AMRESCO, Ohio, USA) buffer and electrophoresed at 100 V. DNA was stained with ethidium bromide and visualized under UV transilluminator. A 100 base pair (bp) DNA ladder (GeneRuler^{TM} 100bp DNA Ladder Plus, Fermentas, USA) was used as standard size markers.

1.6.4 Nucleotide sequencing

The nucleotide sequence of the PCR products was determined using Fppba and Rppba (Table 3) by automate DNA sequencing (Perkin Elmer Applied Biosystems, USA).

1.6.5 Sequence analysis

The open reading frame of investigated sequence was analyzed by Vector NTI package. The investigated sequence was compared with other genes by BLASTN analysis (www.ncbi.nlm.nih.gov). The homology and identity with other pediocin genes were analyzed.

1.7 Experiment 3: Screening of pediocin, enterocin, and nisin genes from lactic acid bacteria

1.7.1 Preparation of DNA template

The large plasmid from *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* were isolated according to the 'Miniprep' plasmid DNA alkaline lysis method, the large plasmids of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* were isolated by a similar alkaline lysis method but NaOH concentration were adjusted to 0.15N instead of 0.2N.

1.7.2 Primer design

For screening of pediocin, enterocin and nisin genes from LAB, three pairs of these three bacteriocin genes specific primers pedF, pedR, entAF, entAR, nisRF and nisRR (Table 3) were designed from conserved regions identified by the alignment of the genes for pediocin (GenBank accession no. AY083244.3, AY705375.1, M83924.1, AY316525.1, U02482.2), enterocin (AF099088.1, AF240561.1, X94181.1), and nisin (AY303239.1, AY303240.1, AY303241.1, X76884.1, Z22813.1). To simplify the interpretation, the specific annealing sites of primers were selected to generate only a single amplicon for each gene to be visualized as a single band on agarose gels following PCR (see Table 3 for size of amplified sequences).

1.7.3 Amplification of pediocin, enterocin and nisin genes

The pediocin, enterocin and nisin genes were amplified using a final concentration of primers and other components as follows: 0.4 μM for pedF, pedR, entAF, entAR, nisRF and nisRR, 1X PCR buffer (10 mM Tris HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. Five microliters of DNA template mixture of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* (approx. 100 ng each) prepared by Miniprep method as described above, and 2.5 units of *Taq* DNA polymerase were

added to obtain a final volume of 20 µl. The PCR amplification (GeneAmp System 2400, PERKIN ELMER, Massachusetts, USA) was performed as the following PCR conditions, initial denaturation at 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 30 s, extension at 72˚C for 45 s, and a final extension at 72˚C for 5 min.

1.7.4 Agarose gel electrophoresis

Amplicons were resolved by 1% agarose gel electrophoresis in 1X TBE (0.089 M Tris, 0.089 M Borate and 0.002 M EDTA for 1X, AMRESCO, Ohio, USA) buffer and electrophoresed at 100 V for 45 min. DNA was stained with ethidium bromide and visualized under UV transilluminator. A 100 base pair (bp) DNA ladder (GeneRuler^{TM} 100bp DNA Ladder Plus, Fermentas, USA) was used as standard size markers.

1.8 Experiment 4: Application of multiplex-overlapping PCR method for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus*

1.8.1 Preparation of DNA template

DNA templates for PCR reactions were prepared from bacterial colonies grown on MRS plates for two days at 30ºC. One colony was resuspended in 50 µl of sterilized TE (10 mM Tris-HCl, 1 mM EDTA pH 7.5) buffer and boiled for 10 min. Supernatant containing released DNA was collected after centrifugation at 10,000 rpm for 10 min and was directly used as template in PCR amplification.

1.8.2 PCR primers

The target sequences used in this study were localized within the sequences encoding 16S rRNA gene specific to *Ped. acidilactici* and *Ped. pentosaceus*. BLASTN database searches were performed, and primers were designed from conserved regions to distinguish *Ped. acidilactici* and *Ped. pentosaceus* from other bacteria and to identify both species (Figure 3) based on sequences information available in the GenBank database. Primers PpF and PaR for distinguishing *Ped.*

pentosaceus and *Ped. acidilactici*, respectively, were in opposite orientation and overlapped (Figure 3B). For simultaneous detection of pediocin gene, primers for amplification of pediocin gene were also included in the PCR reaction. Specific details of primers are listed in Table 3.

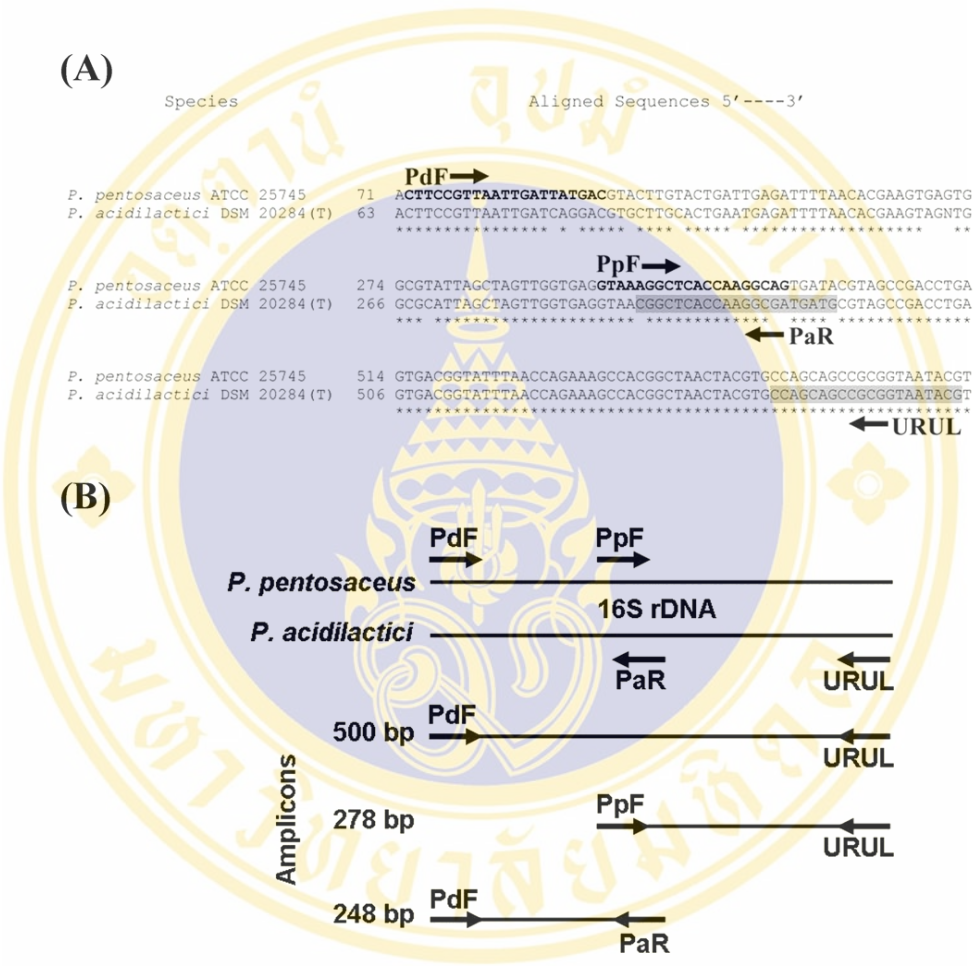

Figure 3. Alignment of a partial 16S rRNA gene sequence of *Ped. acidilactici* DSM 20284^T (GenBank accession no. AJ305320) and *Ped. pentosaceus* ATCC 25745 (GenBank accession no. CP000422) with primers annealing sites (A) and schematic map of multiplex-overlapping PCR (B). Forward and reverse primers are indicated in bold letters and shaded areas, respectively. Arrows indicate the direction of DNA synthesis.

1.8.3 Multiplex-overlapping PCR amplification

Target genes were amplified using a final concentration of primers and other components as follows: 0.4 μM for PdF and URUL, 0.2 μM for PpF, PaR, PedF, and

PedR. 1X ThermoPol Reaction Buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% TritonX-100, pH 8.8 at 25°C, NEW ENGLAND BioLabs Inc.), and 0.4 mM dNTPs. Five microliters of DNA template (approx. 100 ng) prepared from boiled cells and 5 U of *Taq* DNA polymerase (NEW ENGLAND BioLabs Inc.) were added to obtain a final volume of 20 µl. The PCR amplification (GeneAmp System 2400, PERKIN ELMER, Massachusetts, USA) was started by heating at 94ºC for 5 min, followed by 30 cycles of three steps consisting of 30 s at 94°C, 30 s at 52°C, and 35 s at 72°C. The reaction was terminated by extension step for 5 min at 72°C for final extension.

1.8.4 Agarose gel electrophoresis

Amplicons were resolved by 2% agarose gel electrophoresis in 1X TBE (0.089 M Tris, 0.089 M Borate and 0.002 M EDTA for 1X, AMRESCO, Ohio, USA) buffer and electrophoresed at 50 V. DNA was stained, visualized as described above using a 100 base pair (bp) DNA ladder as standard marker.

Table 3. Primers used for LAB identification, amplification of pediocin gene, species differentiation between *Ped. acidilactici* and *Ped. pentosaceus*, and bacteriocin genes amplification.

Primer name[a]	Target gene	Primer size (bp)	Amplicon size (bp)	T_m (°C)	Primer sequence (5'-3')	Annealing with template DNA of	References
UFUL	16S rDNA	20		60.0	GCCTAACACATGCAAGTCGA	All bacterial strains	Nilsson et al., 2003
URUL	16S rDNA	20	500	66.0	CGTATTACCGCGGCTGCTGG	All bacterial strains	Nilsson et al., 2003
Fppba	*ped*	21		62.0	GCATCACCACTCGTATCGATA	Pediocin gene	This study
Rppba	*ped*	21	812	60.0	CTTATCTACTAACGCTTGGCT	Pediocin gene	This study
PdF	16S rDNA	21		54.8	CTTCCGTTAATTGATTATGAC	*Ped. acidilactici* and *Ped. pentosaceus*	This study
URUL	16S rDNA	20	500	66.0	CGTATTACCGCGGCTGCTGG	All bacterial strains	Nilsson et al., 2003
PpF	16S rDNA	20		62.4	GTAAAGGCTCACCAAGGCAG	Only *Ped. pentosaceus*	This study
URUL	16S rDNA	20	278	66.0	CGTATTACCGCGGCTGCTGG	All bacterial strains	Nilsson et al., 2003
PdF	16S rDNA	21		54.8	CTTCCGTTAATTGATTATGAC	*Ped. acidilactici* and *Ped. pentosaceus*	This study
PaR	16S rDNA	21	248	62.0	CATCATCGCCTTGGTGAGCCG	Only *Ped. acidilactici*	This study
pedF	*ped*	20		60.0	GGTAAGGCTACCACTTGCAT	Pediocin gene	This study
pedR	*ped*	20	332	62.0	CTACTAACGCTTGGCTGGCA	Pediocin gene	This study
entAF	*entA*	20		60.0	GGGTACCACTCATAGTGGAA	Enterocin gene	This study
entAR	*entA*	21	412	60.0	CCAGCAGTTCTTCCAATTTCA	Enterocin gene	This study
nisRF	*nisR*	21		62.0	CTATGAAGTTGCGACGCATCA	Nisin gene	This study
nisRR	*nisR*	20	608	60.0	CATGCCACTGATACCCAAGT	Nisin gene	This study

[a] F = forward primer; R = reverse primer

2. Education

2.1 Research design

The research design was based on science education research questions and on the following principles of intervention.

1. Students studying Biotechnology need to learn the principle of PCR and how to use PCR machine (PI1).
   1.1. Students understand the principle of PCR.
   1.2. PCR is an important tool for research in Biotechnology.
   1.3. PCR can help students to understand DNA structure and replication.
2. Students need to know bacteriocin and its application (PI2).
   2.1. Students understand the nature and structure of bacteriocin.
   2.2. Bacteriocins are used as biological control
   2.3. Bacteriocins are used in food preservations
   2.4. Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method.
3. Teaching and learning can be described by sociocultural views of learning (PI3).
   3.1. Learning in Biotechnology is enhanced through social interaction such as group work.
   3.2. Teachers and teaching assistants, and well-designed laboratory protocols with clear instructions, provide scaffolding for students.
   3.3. Using PCR to detect bacteriocin genes in foods in the laboratory can lead students into the biotechnological research culture.
   3.4. An application of bacteriocins is indirectly used in many kinds of fermented food in Thailand. Students need to know about these applications because they can be used instead of food preservatives.
4. Four learning outcomes can be enhanced in this Biotechnology laboratory class (PI4).
   4.1. Knowledge: PCR principles and the applications of bacteriocin can be emphasized during the lab sessions.

4.2. Skills: PCR skills for bacteriocin screening should be used as a model for student's development of practical skills (training students to use PCR and biological screening methods).

4.3. Critical inquiry: Encourage students to interpret their laboratory results such as giving reason to explain their empirical data when they can not obtain the expected PCR results, etc. In addition, bioinformatics information can be learned trough internet access.

4.4 Scientific attitude: Students can see PCR as closely relevant to their daily lives. This should bring about students motivation and enjoyment.

2.1.1 Laboratory design for students

Microbiological and PCR methods were developed for students. The overlay method was selected to allow student be able to screen for LAB producing bacteriocins by microbiological method. In screening of LAB for bacteriocins by PCR method, three pairs of three different bacteriocin genes specific primers, pediocin, enterocin, and nisin were designed (Table 3). These three bacteriocin gene specific primers were designed from sequences available in GenBank database. Since *Ped. pentosaceus* P7, *En. faecium* F103, and *Lc. lactis* F141, screened from Nham samples in science Experiment 1, harbor pediocin, enterocin, and nisin genes, respectively, they were used as positive control in both microbiological and PCR methods for the students' experiment.

A class of 49 students in the second semester of their third year of study was divided into 10 groups. Each group was asked to bring two fermented food samples purchased from the local market to the class. They were instructed to select fermented meat (Nham), fermented fish mixed with rice (som-fak), or fermented vegetables. Prior to performing in the laboratory, the following topics were covered in lectures: LAB in fermented foods and dairy products, methodology for DNA isolation and analysis, PCR method, and agarose gel electrophoresis for DNA. Also prior to working in the laboratory, the students were instructed to read the laboratory manual for background information on LAB and bacteriocins, detailed protocols of LAB isolation, and methods for bacteriocin screening by both microbiological and

PCR. At the beginning of each laboratory session, the instructor reviewed the laboratory protocols and answered the questions raised by the students.

Five 3-hr laboratory sessions were divided as follows: period 1 — isolation of LAB from foods using the specific medium, de Man Rogosa Sharpe (MRS) (Man et al., 1960); period 2 — screening for bacteriocin-producing bacteria using the overlay method; period 3 — selection of colonies with clear halo zones from the overlay method, replica plating on new MRS agar plates, and practice in bioinformatics; period 4 — analysis of the bacteriocin-producing bacteria by multiplex PCR; and period 5 — agarose gel electrophoresis and interpretation of PCR results.

2.1.1.1 Screening of LAB for bacteriocin by microbiological method

Day 1. *Screening Bacteriocin-Producing LAB by Microbiological Methods* – One gram of food sample (Nham samples spiked with three LAB and two food samples brought to the laboratory by the students) was added to the tubes with MRS broth (5 ml; one sample per tube) using sterilized forceps or spoon, mixed well, and shaken at 30˚C for 1 h. These samples are then used for DNA template isolation and LAB cultivation. For DNA template isolation, the samples are processed as follows: 1) particulate matter was allowed to settle by gravity for 1 min; 2) the upper solution of each sample (1.5 ml) was pipetted into a microcentrifuge tube; 3) the sample was centrifuged at 8,000 rpm (4,300 X g) (MiniSpin plus, Eppendorf, Germany) for 5 min and the supernatant was discarded; 4) TE buffer (200 µl) was added and mixed well; 5) this was centrifuged again at 8,000 rpm (4,300 X g) for 5 min and the supernatant discarded; 6) another 200 µl of TE buffer was added and mixed well; and 7) the solution was stored at -20˚C for the experiment on Day 4. For LAB cultivation, the samples are processed as follows: food samples are mixed in MRS broth vigorously; tenfold 0.5 ml of incubated samples was serially diluted from 10^{-1} to 10^{-6} with 0.85% NaCl; a sterilized glass spreader was used to spread 0.1 ml of the dilutions 10^{-4}, 10^{-5}, and 10^{-6} onto MRS plates (each dilution on two plates), and the plates were then incubated at 30˚C for 24 h.

Day 2 – Students select the plates containing 30–300 colonies for overlaying with tester strain (*Lb. plantarum*). *Lb. plantarum* was used instead of *V. harveyi* because *Lb. plantarum* is a Gram positive LAB, and it is not sensitive to lactic acid.

They overlay 1 ml of *Lb. plantarum*, previously grown for 4 h in MRS broth, by pipetting into tubes containing 5 ml of MRS broth + 0.8% agar kept in the shaking water bath at 60˚C. The molten agar is cooled to about 45˚C before adding the cell cultures to prevent cell death. This is vigorously mixed using a vortex mixer or by rotating the tube between two hands, and then the contents are gently overlaid onto each plate and allowed to completely cover the cultured agar plate surface. After the overlaid agar solidifies, the plates are turned upside down and incubated at 30˚C for 24 h.

Day 3 – Students observe clear zones on plates from Day 2. They use a toothpick to pick colonies that are surrounded by a clear zone (note that these bacterial colonies will be under the overlaid agar) to subculture on two MRS plates (nine colonies per plate). Students turn the plates upside down and these are incubated at 30˚C for 24 h. During this session, students learn how to search for nucleotide sequences of pediocin, enterocin, and nisin genes from the GenBank database (www.ncbi.nlm.nih.gov) and how to perform alignment and primer design using the clustalW program (www.ebi.ac.uk/clustalw/). Students also checked the primers for similarity by searching and for primer–dimer formation using BLAST program (www.ncbi.nlm.nih.gov/BLAST/).

2.1.1.2 Screening of LAB for bacteriocin by PCR method

Day 4. *Detection of Bacteriocin Genes by PCR-DNA Template Preparation by Boiling Method* – 1) DNA template from LAB strains: students pipetted 1.5 ml of instructor-provided culture broth containing *Ped. pentosaceus, En. faecium,* and *Lc. lactis* into Eppendorf tubes, one tube per sample; this was centrifuged at 8,000 rpm (4,300 X g) for 5 min and the supernatant discarded; 200 µl of TE buffer was added, vortexed well, boiled for 10 min, and then put on ice immediately for 1 min; this was centrifuged again at 10,000 rpm (6,700 X g) for 10 min and 5 µl of supernatant was collected, which contains DNA for use as DNA template. 2) DNA template from frozen samples from Day 1 (three tubes): three frozen samples were thawed, vortexed well, boiled for 10 min, then put on ice immediately for 1 min, centrifuged at 10,000 rpm (6,700 X g) for 10 min, and 5 µl of supernatant was pipetted for use as DNA template. 3) DNA template from single isolated colonies that produce clear zones

from 18 colonies on replica plates from Day 3: Students randomly selected colonies resembling *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*, one colony each, resuspended the cells in 200 µl of TE buffer, boiled the mixture for 10 min, then put the same on ice immediately for 1 min, centrifuged at 10,000 rpm (6,700 X g) for 10 min, and pipetted 5 µl of supernatant for use as DNA template.

PCR Reactions – Students pipetted DNA template into PCR tubes as follows. Tube 1: positive control (DNA), 5 µl standard DNA was used (DNA mixture of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*); tubes 2–4 contain 5-µl supernatant of boiled culture broth of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* in separate tubes; tube 5 contains 5-µl supernatant of boiled Nham sample spiked with bacteriocin-producing bacteria; Tubes 6 and 7 contain 5-µl supernatant of the two food samples; tubes 8–10 contain the supernatant of three selected colonies that produced clear zones when overlaid with *Lb. plantarum*; tube 11: negative control contains 5 µl of sterilized 3A water instead of DNA template. Students pipetted 14.5 µl of solution I and 0.5 µl of solution II into each of the 11 PCR tubes, following the DNA templates, mixed each tube well, spinned down for 5 min, and then put all the tubes into the PCR machine.

PCR Conditions – Teaching assistants show the students how to adjust the PCR machine to achieve the following conditions: initial denaturation at 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 30 s, extension at 72˚C for 45 s, and a final extension at 72˚C for 5 min.

Day 5: Analysis of PCR Results – Students put the prepared 1% agarose gel into the electrophoresis chamber and poured 1X TBE buffer into the chamber to cover the gel. For sample preparation of the agarose gel, 2 µl aliquot of DNA loading dye was pipetted onto a piece of parafilm to make 11 spots, and 5 µl of each PCR product was added into one spot of loading dye (one product per spot) and mixed well. DNA ladder (5 µl) was loaded into the first well of the agarose gel and one by one into the remaining wells. The lid was placed on the chamber, and the power supply was connected. When the dye runs close to the bottom of the gel, the electricity was turned off and the gel for staining with ethidium bromide for 5 min was removed. The gel in water was destained for 10 min and the DNA bands were observed under UV transilluminator. PCR products can be seen as orange luminescent bands. Each group takes one photograph of their agarose gel. The specificity of bacteriocins in

killing closely related bacteria and specificity of PCR primers to detect bacteriocin genes were also discussed during this period.

Table 4. PCR reagents for detection of pediocin, enterocin, and nisin

Reagents	Stock concentration	Volume (µl)	Final concentration
Solution I			
Tris-KCl PCR buffer	100 mM Tris HCl pH 8.3, 500 mM KCl (10x)	200	10 mM Tris HCl pH 8.3, 50 mM KCl
$MgCl_2$	15 mM (10x)	200	1.5 mM
dNTPs (mixture)	10 mM	40	200 µM
Primers	20 µM pedF	40	0.4 µM
	20 µM pedR	40	0.4 µM
	20 µM entAF	40	0.4 µM
	20 µM entAR	40	0.4 µM
	20 µM nisRF	40	0.4 µM
	20 µM nisRR	40	0.4 µM
Sterilized 3A water		770	
Solution II			
Taq DNA polymerase	5U/µl	50	2.5/20µl

A single reaction comprises 14.5 µl of Solution I and 0.5 µl of Solution II.

2.2 Data collection

2.2.1 Triangulation technique

In this study, the triangulation technique (Cohen et al., 2000) was used to obtain data from a wide range of different and multiple sources to investigate the views of several sets of stakeholders. The triangulation used serves two main purposes which were confirmation and completeness. It can overcome problems of validity and bias. By collecting a diverse set of data derived from different methods there can be less chance of making errors, or drawing inappropriate conclusions than would be the case if relying on just one data set. Thus, the advantages of triangulation are to increase confidence in results, to strengthen the completeness of a study, to address different but complementary questions within a single study, and to enhance the interpretability which one set of data gives to understanding other set. For these reasons, in this biotechnology laboratory classes with 49 undergraduate students, the multiple methods for data collecting and assessment of this research were used. The four methods used for data collection were questionnaires, interviews, observations, and document analysis.

2.2.1.1 Questionnaire design

Questionnaires were designed (Appendix E). To check for the validity, reliability and practicability of the questions, the questionnaires were piloted with three of the fourth year undergraduate biotechnology students (These students performed screening of LAB for bacteriocins laboratory in the year 2006.).

Students were asked to consider their understanding of the methods and knowledge used in the laboratory, and skills they have developed after the final laboratory session. In addition, the students' attitudes towards biotechnology were elicited when answering the questionnaire.

The questionnaire contained a mix of closed and open-ended questions. Early in the questionnaire, the students were asked open-ended questions about general information about techniques used for bacterial isolation and detection they have learned before this experiment. In addition, students' perceptions about what and how they learn and who they learn from during classes were asked. In another part of the questionnaire, questions were asked using a 5-point Likert rating scale (strongly disagree to strongly agree) that provided some numerical data.

These questionnaires were used during the 2006 academic year, by the Department of Biotechnology, Faculty of Science, Mahidol University. They were given both before and after the experiment on "screening of LAB for bacteriocins" to evaluate the third year undergraduate students' pre-lab and post-lab understanding and attitudes.

2.2.1.2 Interview design

All stakeholders which were students, the teacher who taught in this experiment and teaching assistants were interviewed. The interview protocols are shown in Appendix F. All questions were formed in advance, and sequences of questions asked were predetermined. Before interviewing, all questions were sent to two undergraduate biotechnology students to check for the sense and validity of questions.

The interview for students was group interview (Arksey & Knight, 1999) expected to understand the dynamics of social relationships among group members where students can consider their own views in the context of the views of others (Patton, 1990), which relies on the systematic questioning of several individuals

simultaneously in an informal setting (Fontana & Frey, 2003). The focus group (Patton, 1990) interview was used to obtain some quality controls on data collection in those participants with a tendency to provide checks and balances on each other's false or extreme views. Only two focus groups of students which were the high and low achievement groups, were selected according to their achievement of previous laboratories. They were interviewed during the week after they had finished the last session of laboratory. Students heard each other's responses and made additional comments over their own original responses as they heard what other students said.

The students' interview was semi-structured, with questions probing students understanding of knowledge used in the two methods for screening of LAB for bacteriocins. In addition, students' attitudes to this biotechnology experiment and sociocultural view of learning were investigated. However, during the interviews, participants were also encouraged to have freedom of expression.

The teacher' s interview was to investigate her feeling about how the laboratory classes worked, what was different before laboratory classes started and after they finished. In addition, her attitudes to learning through sociocultural view of learning were investigated because stressing this perspective into laboratory classes by introducing more group activities is new in this context of research study.

All interviews were audio-taped and some notes were taken during interviews. After interviews, notes about non-verbal communication were made immediately after each interview. The tape was transcribed as soon as possible after interviewings. Participants were asked if they wanted to check and comment on the accuracy of the transcription and their comments are used in the part of data analysis. All participants were coded by their student IDs.

2.2.1.3 Observation design

The observation was also used in this biotechnology laboratory study. The observer-as-participant (Patton, 1990) method was conducted through all laboratory sessions. The main focus of observation was on students' engagement in laboratory work and learning through a sociocultural perspective of learning. A wide range of data were collected from a variety of situations, for example, when the teacher was teaching, when the students worked in groups, and when students tried to solve

problems. Field notes (about needed skills which students have and developed during this experiment, students' participation in group and class activities) were taken according to each activity students performed. The format of field notes consisted of three parts: verbal descriptions of the setting, the people, and the activities (Appendix G). Full field notes were made as soon as possible after each observation, often at the end of the day.

2.2.1.4 Documentary data collection design

The documentary data (Merriam, 1998) collecting was also used in this study to enhance trustworthiness of the data from questionnaires, interviews, and observations. The documentary data collected in this study was students' laboratory reports. Accordingly, the students were asked to make one report per group in the following topics, objective, introduction, materials, methods, results, discussion, conclusion, and answers the questions posed in the laboratory manual.

2.3 Data analysis

2.3.1 Questionnaire analysis

Content analysis (Bell, 1995) was used for open-ended questions. The students' responses were coded and categorized as qualitative data to be interpreted and presented as descriptive. For 5-point Likert rating scale part of the questionnaire, students were asked about how they feel about knowledge, skills, and attitudes they have gained or developed after performing this experiment. These responses were examined as quantitative data using average score ($\overline{X}$) ranging from strongly disagree (1) to strongly agree (5) ratings on the Likert scales. Standard deviation (S. D.) was also determined for each questionnaire item.

2.3.2 Interview analysis

A cross-case or cross-interview analysis (Patton, 1990) with a descriptive analytical framework was used for an analysis of interviews responses. In this study, the focus was on a sociocultural perspective of leaning for improvement of students' understanding of biotechnology and its application. The analysis began with a

description of variations in answers to common questions. Answers from different people were grouped by topics from the principles of intervention (PI3 and PI4).

2.3.3 Observation analysis

The observed activities were grouped by topics from the principle of interventions. The descriptive findings of people and processes (Patton, 1990) were presented.

2.3.4 Documentary data analysis

The students reports were analyzed followed the topics they were asked to present in their reports. The presentation of laboratory results compatible with discussion was emphasized. In addition, in order to investigate the students' understanding about the overall experiment on screening of LAB for bacteriocins, the answers from questions posed in the laboratory manual were examined.

CHAPTER V
RESULTS

1. Science

1.1 Experiment 1: Screening of LAB for bacteriocins

1.1.1 Bacteriocin activity

LABs (~1,800 strains) were isolated from Nham (fermented pork and fish sausage) samples and tested for their capability to kill shrimp pathogen by overlaying with an indicator strain, *Vibrio harveyi* 639. Only 11 colonies designated as F1, P1, P2, P3, P4, P5, P6, P7, P8, F103, and F141 produced clear halo zones. These 11 colonies were then tested further with more tester strains, *B. subtilis, S. aureus, Lb. plantarum* TISTR 050, *Ped. pentosaceus* TISTR 419, *E. coli, V. harveyi* 639, *V. harveyi* 1114, *V. cambelli, V. parahaemolyticus, Aeromonas* sp. 30-3c and *Aeromonas* sp. 30-3p. All, except *B. subtilis, S. aureus, Lb. plantarum* TISTR 050 and *Ped. pentosaceus* TISTR 419, are Gram-negative bacteria. *Vibrio* spp. and *Aeromonas* spp. are pathogens of shrimp and fish, respectively. The sensitivity of these bacteria to bacteriocin fractions, whether in culture broth (CB), culture broth boiled for 10 min (CBb10), filtered culture broth (F), and filtered culture broth boiled for 10 min (Fb10), was assayed and compared by agar well diffusion. All fractions of all 11 strains can inhibit growth of all tester strains, except *S. aureus*. Every fraction of P7 showed the highest inhibition activity against all sensitive tester strains, so the strain was selected for further study. The bacteriocin-containing fraction was usually localized in culture supernatant. However, clear inhibition was observed only against TISTR 050 and TISTR 419. Faint inhibition was observed against the rest of the tested strains which might cause by lactic acid produced by the 11 LABs or its broad host range activity. However, this observation was not tested.

Growth curve and pH change during cultivation were also studied with seven selected LAB strains showing highest bactericidal activity. P7 showed moderate growth and pH change during cultivation (Figures 4 and 5). Under the growth

conditions described in Materials and Methods, all selected strains grew very fast and reached stationary phase at 8-12 h (Figure 4). The pH of the culture broth were dropped from original pH around 5.7 to about 3.7 (P3, P4, P5, and P8) or around 4.2-4.5 (F1, P1, and P7) (Figure 5)

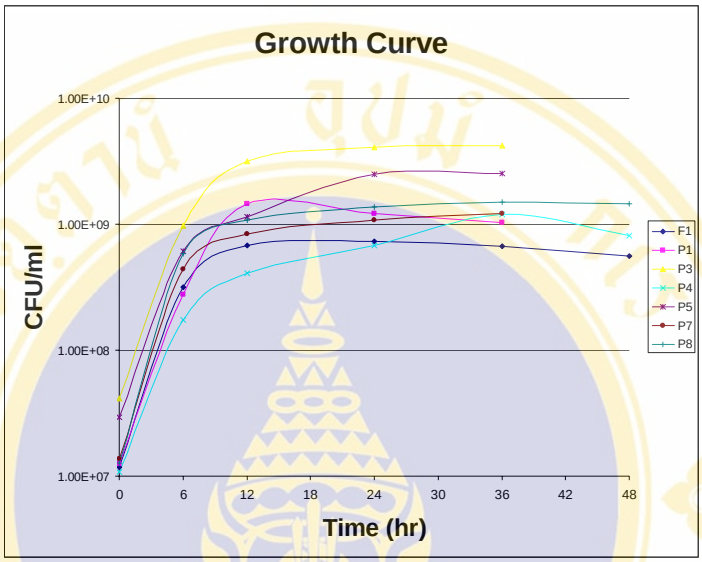

Figure 4. Growth curve of seven selected LAB strains

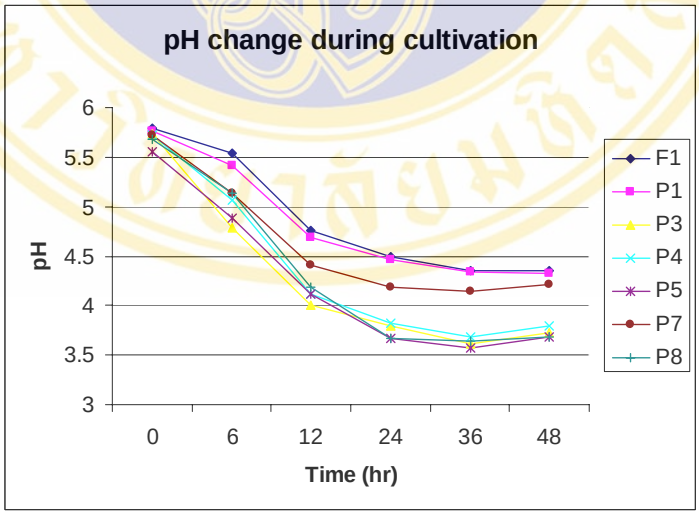

Figure 5. pH change during cultivation of seven selected LAB strains

1.1.2 Bacterial identification by 16S rDNA

The 500 bp of 16S rDNA PCR product (Figure 6) was amplified from chromosomal DNA using specific primers, UFUL and URUL and *Taq* DNA polymerase. Only single band of each strain was observed (Figure 6, lanes 1-11).

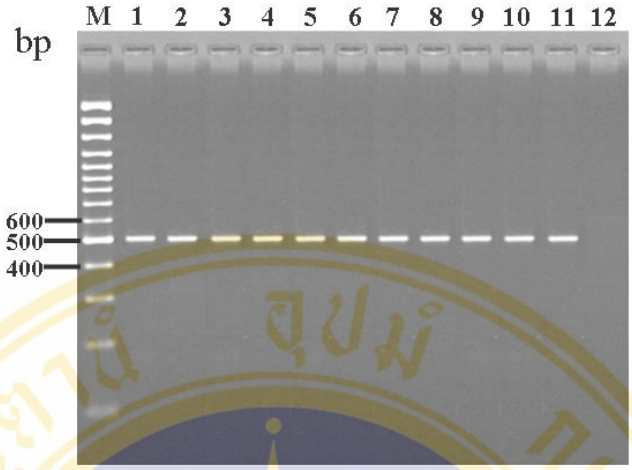

Figure 6. Agarose gel electrophoresis of 500 bp PCR amplified product of partial 16S rRNA gene. M, 100 bp DNA ladder; lanes 1-11 are F1, P1-P7, F103, and F141 isolates, respectively. Lane 12, negative control without any DNA template.

Nucleotide sequencing of amplified bands of 16S rRNA gene (500 bp) were performed by using UFUL primer. After obtaining nucleotide sequences, these sequences were analyzed using BLASTN. The similarities of these sequences with other sequences in the database are shown in Table 5.

Table 5. BLASTN results of LAB isolates producing bacteriocins

Isolates	BLASTN results	% Similarity
F1	*Enterococcus faecium*	100
P1	*Pediococcus pentosaceus*	100
P2	*Lactobacillus plantarum*	99
P3	*Lactobacillus plantarum*	99
P4	*Lactobacillus plantarum*	100
P5	*Lactobacillus plantarum*	99
P6	*Lactobacillus plantarum*	99
P7	*Pediococcus pentosaceus*	100
P8	*Lactobacillus faciminis*	98
F103	*Enterococcus faecium*	100
F141	*Lactococcus lactis*	99

1.2 Experiment 2: Amplification and sequencing of pediocin gene from *Ped. pentosaceus* P7

1.2.1 The amplification of the *ped* gene from *Ped. pentosaceus* P7

The pediocin A gene (*pedA*) from *Ped. pentosaceus* P7 was amplified by using Fppba and Rppba specific primers (Table 3) and *Taq* DNA polymerase. Only single band of approximately 812 bp which include the whole *pedA* gene was obtained (Figure 7, lane 1).

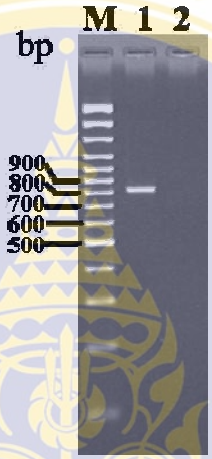

Figure 7. Agarose gel electrophoresis of 812 bp PCR amplified product of *pedA* gene from *Ped. pentosaseus* P7. M, 100 bp DNA ladder; lane 1, *ped* gene amplified product; lane 2, negative control without DNA template.

1.2.2 The nucleotide sequence of the *ped* gene from *Ped. pentosaceus* P7

Nucleotide sequencing of the amplified DNA fragment from *Ped. pentosacues* P7 was performed by using the designed Fppba and Rppba primers, as described in Materials and Methods. After sequence analysis (Appendix A), the open reading frame (ORF) of *pedA* with a 186 bp was found. Only nucleotide sequence of 720 bp of a 812 bp fragment was determined. The ORF of investigated sequences, homology and identity with the other *pedA* gene was analyzed by Vector NTI package. The *pedA* gene of *Ped. pentosaceus* P7 contained only one complete ORF which was *pedA*, encoding for pediocin A (Figure 8). The restriction site of various enzymes was determined. It revealed only two restriction sites of *Hpa*I and *Hind*III outside *pedA* ORF (Figure 8).

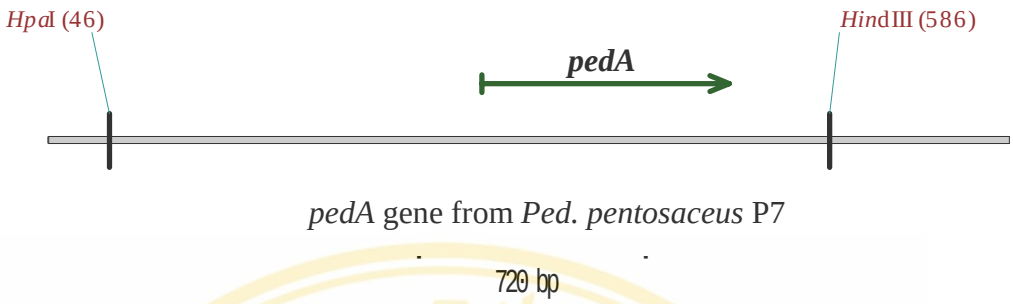

Figure 8. Restriction map of pediocin gene on a 720 bp DNA fragment including *pedA* which encoded for pediocin.

The nucleotide sequence of *pedA* from *Ped. pentosaceus* P7 was analyzed with those of pediocin-producing strains by using BLASTN analysis (www.ncbi.nlm.nih.gov). The sequence covered the region from start to stop codon of *pedA* was compared to those of other strains (Table 6)

Table 6. BLASTN analysis of *pedA* gene from start to stop codon

Strains	Accession nos.	Identities
Ped. acidilactici strain K10 pediocin operon	AY705375.1	100%
Ped. acidilactici H plasmid pSMB74	U02482.2	100%
Ped. acidilactici pediocin (*pedA*) gene	AY083244.3	100%
Lb. plantarum plasmid pWHE92 pediocin AcH	AY316526.1	100%
Ped. pentosaceus plasmid pS34 pediocin AcH	AY316525.1	100%
Ped. parvulus plasmid pAT077 pediocin AcH	AY316524.1	100%
Ped. acidilactici H plasmid pSMB74 pediocin AcH	M90679.1	100%
Ped. acidilactici bacteriocin operon genes	M83924.1	100%

1.3 Experiment 3: Screening of pediocin, enterocin, and nisin genes from lactic acid bacteria

The amplification of DNA template containing a mixture of pediocin, enterocin, and nisin genes of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* using three pairs of these three genes specific primers, pedF, pedR, entAF, entAR, nisRF and nisRR, the bands of 332, 412, and 608 bp, respectively, were obtained (Figure 9).

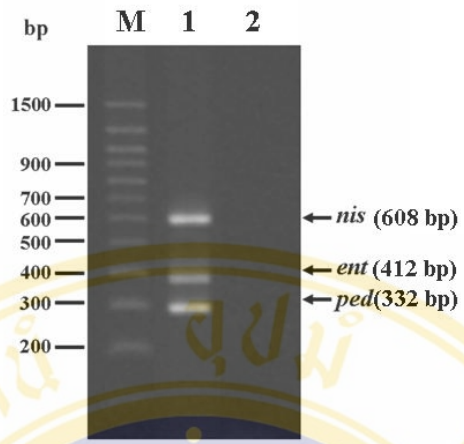

Figure 9. Agarose gel electrophoresis of amplified products from pediocin (*ped*), enterocin (*ent*), and nisin (*nis*) genes of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*. M, 100 bp DNA ladder; lane 1, pediocin, enterocin, and nisin genes amplified products; lane 2, negative control without DNA template.

1.4 Experiment 4: Application of multiplex-overlapping PCR method for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus*

1.4.1 The enumeration of LAB from fermented meat (Nham)

The total microflora count was approximately $2X10^9$ CFU/g for pork and beef Nham, and $3X10^8$ CFU/g for fish Nham (data not shown). Colonies (100 each from each Nham sample) were picked and purified by streaking on MRS plate for further analysis.

1.4.2 Differentiation of *Ped. acidilactici* and *Ped. pentosaceus*

Amplification of template DNA using four 16S rDNA specific primers resulted in a 500 and 248 PCR products for *Ped. acidilactici* and 500 plus 278 bp PCR fragments for *Ped. pentosaceus* (Figure 10). The two fragments of 278 and 248 bp could be differentiable in 2% agarose gel. PCR amplification of the reference isolates from TISTR showed that, four *Ped. acidilactici* and three *Ped. pentosaceus* gave the corresponding result on species identification, in which they were determined as *Ped. acidilactici* (Figure 10A, lanes 1-4) and *Ped. pentosaceus* (Figure 10A, lanes 6 and 7).

One strain TISTR 397 (NISL 7113) was designated as *Ped. acidilactici* but our PCR result identified the strain as *Ped. pentosaceus* (Figure 10A, lane 5). The result of 16S rDNA sequence also identified this strain as *Ped. pentosaceus* (GenBank accession no. EU131008). PCR amplified products of other *Pediococcus* species using our specific primers gave either only a single 278 bp fragment (Figure 10A, lanes 8-13 and Figure 10B, lane 3) or none (Figure 10A, lane 14). *Ped. dextrinicus* is the only *Pediococcus* that did not have any amplified band using our designed primers. In other LAB, a single 278 bp fragment was detected in *Tetragenococcus halophilus* TISTR 429, *Lactobacillus casei* TISTR 047, *Lb. delbrueckii* TISTR 326, *Leuconostoc dextranicum* TISTR 377, and *Leuc. mesenteroides* TISTR 053 (Figure 10B, lanes 3, 6, 10, 12, and 13, respectively). No amplicon was detected in *Aerococcus urinaeequi* TISTR 394, *Lb. plantarum* TISTR 050, *Lb. brevis* TISTR 860, *Lb. acidophilus* TISTR 1034, *Lb. bulgaricus* TISTR 451, *Enterococcus faecalis* TISTR 379, *Lc. lactis* TISTR 1401 (Figure 10B, lanes 4-5, 7-9, 11, and 14, respectively). In addition, the presence of pediocin gene could also be simultaneously detected as a 332 bp DNA fragment (Figure 10A, lanes 1, 5, 6). Thus, TISTR 051, TISTR 397, and TISTR 783 harbor pediocin gene. The microbiological assay by overlaying with *Lb. plantarum* TISTR 050 also showed a clear halo zone around the colonies of these three strains (data not shown), which implies functional pediocin.

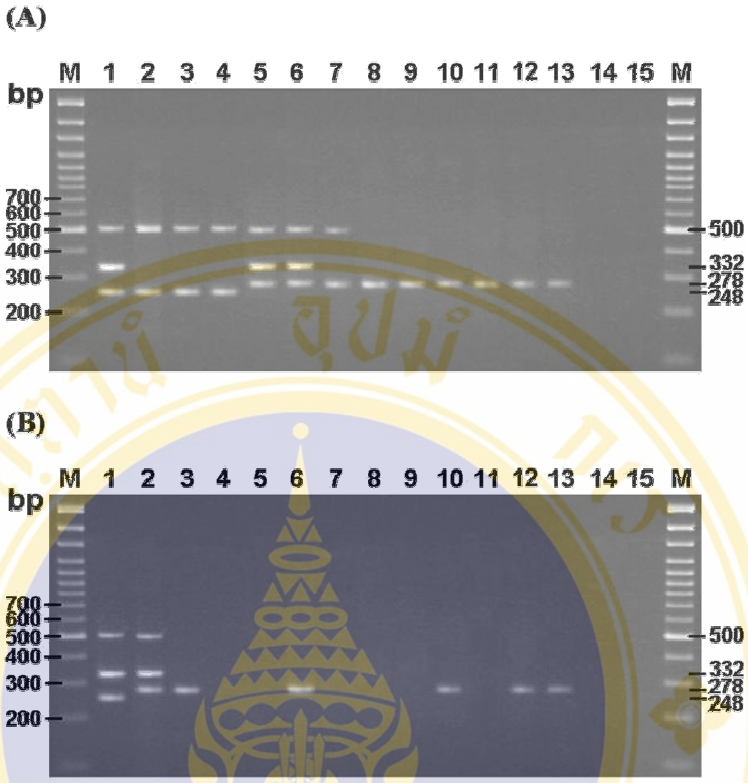

Figure 10. Agarose gel electrophoresis showing PCR amplification of *Pediococcus* genomic DNA. (A) lane M, 100 bp DNA ladder (GeneRulerTM 100bp DNA Ladder Plus, Fermentas); lane 1, *Ped. acidilactici* TISTR 051 (ATCC 8042); lane 2, *Ped. acidilactici* TISTR 952 (NRIC 0124); lane 3, *Ped. acidilactici* TISTR 953 (DSM 20284); lane 4, *Ped. acidilactici* TISTR 1117 (NRIC 1096); lane 5, *Ped. acidilactici* TISTR 397 (NISL 7113); lane 6, *Ped. pentosaceus* TISTR 783 (JCM 5885); lane 7, *Ped. pentosaceus* TISTR 419 (JCM 2027); lane 8, *Ped. claussenii* DSM 14800; lane 9, *Ped. cellicola* DSM 17757; lane 10, *Ped. stilesii* DSM 18001; lane 11, *Ped. inopinatus* DSM 20285; lane 12, *Ped. damnosus* DSM 20331; lane 13, *Ped. parvulus* DSM 20332; lane 14, *Ped. dextrinicus* DSM 20335; lane 15, negative control (no template DNA). (B) lane 1, *Ped. acidilactici* TISTR 051; lane 2, *Ped. pentosaceus* TISTR 783; lane 3, *Ped. halophilus* (*Tetragenococcus halophilus*) TISTR 429; lane 4, *Ped. urinaeequi* (*Aerococcus urinaeequi*) TISTR 394 (ATCC 29722); lane 5, *Lb. plantarum* TISTR 050 (ATCC 8014); lane 6, *Lb. casei* TISTR 047 (ATCC 7469); lane 7, *Lb. brevis* TISTR 860 (NRIC 0134); lane 8, *Lb. acidophilus* TISTR 1034 (ATCC 4355); lane 9, *Lb. bulgaricus* TISTR 451 (TUA 093L); lane 10, *Lb. delbrueckii* TISTR 326 (NRRL B-763); lane 11, *En. faecalis* TISTR 379 (ATCC 19433); lane 12, *Leuc. dextranicum* TISTR 377 (ATCC 8086); lane 13, *Leuc. mesenteroides* TISTR 053 (ATCC 10830); lane 14, *Lc. lactis* TISTR 1401 (JCM 7638); lane 15, negative control (no template DNA).

Table 7. Detection of pediocin (*ped*) gene and species identification of *Ped. acidilactici* and *Ped. pentosaceus* and other lactic acid bacteria from Nham samples by multiplex-overlapping PCR (mPCR) and 16S rDNA sequencing

Isolates or strains	Sources	GenBank accession no. of 16S rDNA gene	PCR fragment size [a] (bp)	Species identification [b] by		Presence [c] of *ped*
				mPCR	16S rDNA sequencing	
Pediococcus P1	Pork Nham	EU082180	500, 278	*P.p*	*P.p*	-
Pediococcus P7	Pork Nham	EU082178	500, 278	*P.p*	*P.p*	+
Pediococcus P127	Pork Nham	EU082182	500, 278	*P.p*	*P.p*	-
Pediococcus P132	Pork Nham	EU082183	500, 278	*P.p*	*P.p*	-
Pediococcus P144	Pork Nham	EU082184	500, 278	*P.p*	*P.p*	-
Pediococcus P146	Pork Nham	EU082185	500, 278	*P.p*	*P.p*	-
Pediococcus P150	Pork Nham	EU082186	500, 278	*P.p*	*P.p*	-
Pediococcus F328	Fish Nham	EU082179	500, 248	*P.a*	*P.a*	-
Pediococcus F58	Fish Nham	EU082181	500, 278	*P.p*	*P.p*	+
Pediococcus F201	Fish Nham	EU082187	500, 278	*P.p*	*P.p*	-
Pediococcus F342	Fish Nham	EU082190	500, 278	*P.p*	*P.p*	-
Pediococcus SF213	Fish Nham	EU082188	500, 278	*P.p*	*P.p*	-
Pediococcus SF216	Fish Nham	EU082189	500, 278	*P.p*	*P.p*	-
Pediococcus B348	Beef Nham	EU082191	500, 278	*P.p*	*P.p*	-
Pediococcus B362	Beef Nham	EU082192	500, 278	*P.p*	*P.p*	-
Pediococcus B363	Beef Nham	EU082193	500, 278	*P.p*	*P.p*	-
Pediococcus B364	Beef Nham	EU082194	500, 278	*P.p*	*P.p*	-
Pediococcus B366	Beef Nham	EU082195	500, 278	*P.p*	*P.p*	-
Pediococcus B300	Beef Nham	EU082196	500, 278	*P.p*	*P.p*	-
[d] *P. acidilactici* NISL 7113	TISTR 397	EU131008	500, 278	*P.p*	*P.p*	+

[a] *Ped. acidilactici* and *Ped. pentosaceus* gave amplified products of 500, 248 and 500, 278 bp, respectively.

[b] *P.a = Ped. acidilactici, P.p = Ped. Pentosaceus*

[c] + and -, presence and absence of *ped* gene, respectively

[d] NISL 7113 was designated as *Ped. acidilactici*, but from our PCR and 16S rDNA sequencing, it was identified as *Ped. pentosaceus*

The PCR amplification of a total of 300 LABs screened from Nham samples was performed and only 19 isolates were identified as either *Ped. acidilactici* or *Ped. pentosaceus*. Both multiplex-overlapping PCR and partial 16S rDNA sequencing (500 bp) showed that among those 19 isolates, one and 18 were identified as *Ped. acidilactici* and *Ped. pentosaceus*, respectively (Table 7). Identification of the 19 *Pediococcus* isolates by the mPCR technique showed the same results with the 16S rDNA sequence analysis.

Another 281 isolates were either other LAB or other bacteria since only one 278 bp DNA fragment was detected in two isolates and no fragment in 279 isolates (data not shown). Sixteen isolates were selected for 16S rDNA sequencing. Two isolates, which gave a single 278 bp amplified products were identified as *Ped. stilesii* by 16S rDNA sequencing (GenBank accession nos. EU161985 and EU161988).

Another set of 14 isolates without amplified products was identified as *Lb. plantarum* (GenBank accession nos. EU161986, EU161987, EU161989, EU167523, EU167524, EU167525, EU167526, and EU167527), *Lb. sakei* (GenBank accession nos. EU161984 and EU161990), *Lb. farciminis* (GenBank accession no. EU167528), *Leuc. pseudomesenteroides* (GenBank accession no. EU167529), *En. faecium* (GenBank accession no. EU082197), and *Lc. lactis* (GenBank accession no. EU082198).

2. Education

2.1 Laboratory design for students

2.1.1 Screening of LAB for bacteriocins by microbiological method

Bacterial colonies appearing on MRS agar were almost all LAB. Students could count the bacterial colonies at dilutions of 10^{-5} and 10^{-6} that should give well-isolated colonies in an appropriate quantity for overlaying with the tester strain. After overlaying with the tester strain, *Lb. plantarum*, every group should observe clear zones (Figure 11). Please be noted that in the preliminary screening, *V. harveyi* was used as the tester strain, but it was found later that the strain is sensitive to lactic acid and was not affected by pediocin. Hence, the tester strain was changed to *Lb. plantarum* which is killed by pediocin.

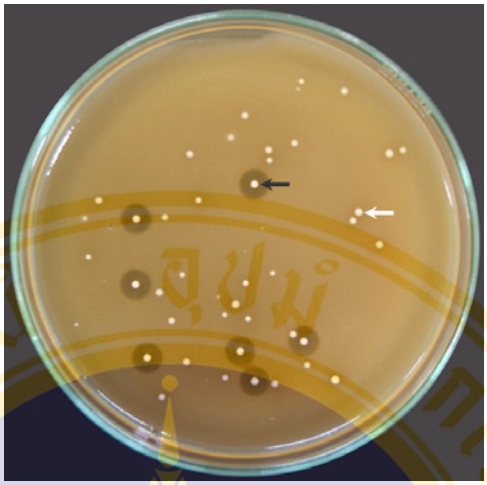

Figure 11. Screening of bacteriocin-producing LAB. The black and white arrows indicate examples of positive and negative colony with or without a clear halo zone, respectively.

2.1.2 Screening of LAB for bacteriocins by PCR method

A photograph of a gel (Figure 12) taken by one group of students shows the following: lane 1, the DNA ladder (Seegene, Korea) containing DNA fragments from 0.1–15.0 kb; lanes 2–6, PCR amplicons from positive controls; lane 2, PCR amplicons from the DNA mixture of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis* capable of producing pediocin, enterocin, and nisin, respectively; lanes 3–5, amplicons from boiled culture broth of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*, respectively; lane 6, amplicons from Nham sample spiked with all three bacteriocin-producing bacteria; lanes 7 and 8, PCR products of total DNA from fermented food samples; lanes 9–11, PCR products of selected colonies from overlay plates (i.e. colonies producing clear zones); lane 12, PCR products from the negative control that contained all reagents except DNA template.

The PCR product in Figure 12, lane 2 was derived from DNA template containing a mixture of pediocin, enterocin, and nisin genes, the students obtained bands of 332, 412, and 608 bp, respectively. Similarly, lanes 3–5 show the individual bands of 332, 412, and 608 bp, respectively, from the boiled culture broths of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*. In lane 6, the template from the Nham sample spiked with *Ped. pentosaceus*, *En. faecium*, and *Lc. Lactis* shows bands of

332, 412, and 608 bp, similar to lane 2. In lane 7, one of the selected food samples shows bands of 332 and 412 bp, indicating the presence of the pediocin and enterocin genes, respectively. In lane 8, a second food sample shows a single band of 332 bp indicating the presence of the pediocin gene. From selected colonies with clear zones, there are bands of 332 bp (pediocin) in lanes 9 and 10 and a band of 412 bp (enterocin) in lane 11.

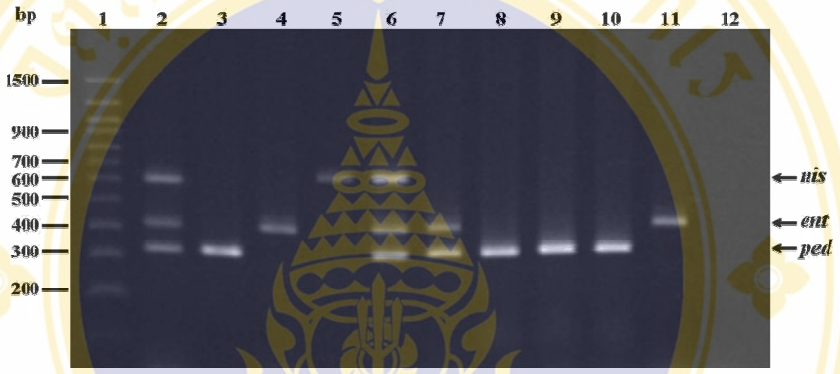

Figure 12. Agarose gel of PCR products from amplification of pediocin (*ped*), enterocin (*ent*), and nisin (*nis*) genes. lane 1, DNA ladder; lanes 2–11 amplicons from DNA isolated from cultured LAB or LAB in food samples; lane 12 is a negative control (without DNA template). DNA templates used in lanes 2–11 are DNA mixture of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis* cultures (lane 2); DNA from *Ped. pentosaceus*, *En. faecium* and *Lc. lactis* (lanes 3–5, respectively), DNA isolated from a Nham sample spiked with the three LABs (lane 6), DNA from food samples (lanes 7–8) and DNA from colonies producing clear halos in a *Lb. plantarum* overlay (lanes 9–11).

2.2 Evaluation

2.2.1 Questionnaire responses

The responses from open-ended questionnaires both before and after the experiment were presented as descriptive for each question item in the following section. In addition, proportion of students (N=49) who responded to understanding category is presented in Table 8. For the 5-point Likert rating scale questionnaires both before and after the experiment, average score ($\overline{X}$) ranging from strongly

disagree (1) to strongly agree (5) ratings on the Likert scales and standard deviation (S. D.) were determined for each questionnaire item and presented in Table 9.

Before performing the experiment, 37% of the students (18 of total 49 students) had ever heard of bacteriocins. Only 12 students answered the question “What do you think a bacteriocin is, and what does it do?” and only the three of them (6.12%) answered “substances/toxin produced by bacteria to kill other bacteria” which is the right and complete answer. Other nine students mentioned only “toxin produced by bacteria”. Some of these students added “secondary metabolites”, “substances produced by bacteria which inhibit/affect growth of other bacteria or other living organisms”, and “medicine to get rid of bacteria”. However, no one mentioned the nature of bacteriocin as a protein or peptide. When questioned “What do you think bacteriocins are used for what purpose in Thailand?” had been asked, only nine students answered the question. Five students mentioned bacteriocin which is used as “medicine”. Four students mentioned “insecticide”/”substances”/“antibiotics” which is used to kill/inhibit other undesirable bacteria.

After performing the experiment, 100% of the students had ever heard of bacteriocins. When questioned “What do you think a bacteriocin is, and what does it do?” had been asked again, 44 students (89.80%) gave the right answers. Twenty five students mentioned “protein that bacteria secrete to kill other closely-related bacteria” in which four of them added “protein produced by LAB”, one of them gave examples “nisin, pediocin” while 19 of them used the word “substance” instead of protein as they stated “substance that bacteria secrete to kill other closely-related bacteria” which one of them added “closely-related bacteria that need same ecological food niche” and one student stressed that bacteriocin is “not antibiotic”. Only five of them answered the question while missing important points as two of them stated “substance that bacteria produced to inhibit other bacteria”, one of them mentioned “metabolite produced by bacteria for get rid of bacteria which use the same food niche” two of them mentioned only “small protein produced by bacteria” which are not complete answers. When questioned “What do you think bacteriocins are used for what purpose in Thailand?” had been asked, a variety of answers were given by the students. As eight of them mentioned “food preservation”, 25 of them referred to “food industry” to yoghurt, fermented milk, and Nham in order to kill some spoilage,

contaminated, pathogenic bacteria. Two, four, three, four and six students mentioned the use of bacteriocins as “antibiotic”, “tooth paste/mouth cleaning solution”, “pathogenic control in shrimp”, “medicine/drug”, “the use in agriculture as insecticidal substances’, respectively.

Before performing the experiment, when questioned “A bio-active substance is produced by a living organism that can have an effect on or interact with other living organisms. Do you know any bio-active substances that can be used as food preservatives?” was asked, 28 students mentioned “antibiotic” which some of them added names of antibiotics such as “streptomycin”, “penicillin”, “chloramphenical”, “kanamycin”. Eight students mentioned “BT toxin”. Other five students mentioned “secondary metabolite”, “alkaloid” and “pheromone”. Only one student mentioned “bacteriocin”. Seven students left the spaces blank.

After performing the experiment, 35 students (71.43%) mentioned “bacteriocin” which 22 of them defined bactericin by adding “nisin, enterocin, pediocin”. Three students mentioned “antibiotic”, four mentioned “acid”, the rest mentioned “antifungal substance”.

Before performing the experiment, students were asked to “List any methods you know of which can be used to test the activity of bio-active substances”, three, seven, two, students stated “test with tester strains to look at inhibition”, “co-culture”, “disc soak with bioactive compound then put on lawn to observe clear zone”. Ten students mentioned “test with antibiotic”, four of them mentioned “biochemical test” without any detail. One student stated “bioassay: nutrient, drug/antibiotic resistant”, two students mentioned “in vivo test with living organism to look at changes”, one mentioned “selective media”. The rest of students left the spaces blank.

After performing the experiment when students were asked to “List any methods you know of which can be used to test the activity of bio-active substances” again, 21 students stated “overlay with tester strain to observe clear zone”, four students stated “co-culture with tester strain”, and two students stated “test with tester strain to observe inhibition”. One, ten, three, and three student mentioned “strain-strain inhibition”, “PCR and microbiological methods”, “selective medium”, “selectable markers”, respectively.

Before performing the experiment, when the students were asked to “Explain the DNA replication process”, 31 students mentioned “separation of double stranded DNA by enzyme helicase, 16 students mentioned “RNA primers binding at the single stranded DNA, and 20 students mentioned “DNA polymerase synthesizes new DNA”. Only ten students (20.41%) mentioned all of these three major steps. However, after performing the experiment, 30 students (61.22%) stated all of these three major steps.

Before performing the experiment, when the students were asked to “Explain the polymerase chain reaction (PCR)”, 25 students (51.02%) mentioned all three steps which were “denaturation”, “annealing”, and “extension”. After performing the experiment, 45 students (91.84%) gave the right answer stating all of the three major steps which were “denaturation”, “annealing”, and “extension”.

Before performing the experiment, when the students were asked “What can PCR be used for?”, 20 students mentioned “to increase amount of DNA of interest”, eight students stated “to detect GMOs/genetic disease/to use in forensic science”. However, after performing the experiment, 46 students mentioned “to increase amount of DNA of interest” and added some of PCR applications e.g. “to amplify DNA of interest”, “identify persons/strains”.

After performing the experiment, a question “Would you rather use the microbiological or PCR method to screen for bacteriocin-producing bacteria? Explain the reason for your answer.” was asked. Forty three students stated that they would rather “use PCR method because it is rapid, accurate and can be used to detect a small amount of DNA”. In addition, four students would rather “use both PCR and microbiological methods”, because “by using microbiological method, the bacteriocin activity can be observed”.

Before performing the experiment, when the students were asked “Please explain what you liked and/or didn’t like about working in groups”, 40 students answered that they “liked working in group because of e.g. co-operative working, sharing ideas” while 14 students “didn’t like working in group because of e.g., there was not enough work to do, some of them had to do the report alone”. There were five students who stated that they both liked and didn’t like working in group because of the above reasons.

After performing the experiment, when the students were asked “Please explain what you liked and/or didn’t like about working in groups in this experiment”, 37 students answered that they “liked working in group because of e.g. co-operative working, sharing ideas” while 12 students “didn’t like working in group because of e.g., there was not enough work to do, and some of them had to do the report alone”.

Before performing the experiment, a question “What will you do if you don’t know the reasons behind tasks in the biotechnology experiments” was asked. The students ranked these things in this order: asked my classmates, asked teaching assistants, asked the teacher, searched for them in books, searched on the internet, and ignored them, by the average score 1.68, 2.34, 2.44, 3.86, 4.97, and 5.48, respectively. This result indicated the students liked to ask classmates the most.

After performing the experiment, the question “What will you do if you don’t know the reasons behind tasks in the biotechnology experiments” was asked again. The students ranked these things in this order: asked my classmates, asked the teacher, asked teaching assistants, searched in books, searched on the internet, and ignored them by the average score 1.98, 2.13, 2.42, 3.83, 4.64, and 5.82, respectively.

Before performing the experiment, a question “What will you do if you don’t know how to do the tasks in the biotechnology experiments” was asked. The students ranked these things in this order: asked my classmates, asked teaching assistants, asked the teacher, searched in books, searched on the internet, and ignored them, by the average score 1.51, 2.56, 2.66, 3.51, 4.90, and 5.56, respectively.

After performing the experiment, a question “What will you do if you don’t know how to do the tasks in this experiment” was asked. The students ranked these things in this order: asked my classmates, asked teaching assistants, asked the teacher, searched in books, searched on the internet, and ignored them, by the average score 1.76, 2.38, 2.4, 3.68, 4.76, and 5.82, respectively.

Table 8. Proportion of students (N=49) who hold understanding response category in the open-ended questionnaires before and after the experiment

Items	Concepts	Response categories (understanding)	% of students holding response categories	
			before	after
1a	Bacteriocin is	substances/protein produced by bacteria to kill other bacteria	6.12	89.80
1b	Bacteriocins are used for	e.g. food preservation, to kill other undesirable bacteria	10.20	100.00
2	Bio-active substances	e.g. bacteriocin	2.04	71.43
3	Methods to test the activity	overlay with tester strains, co-culture to observe inhibition	24.49	57.14
4	DNA replication process	separation of double stranded DNA by enzyme helicase RNA primers binding at the single stranded DNA DNA polymerase synthesizes new DNA	20.41	61.22
5	PCR	denaturation: dDNA template becomes ssDNA by heating annealning: primers bind at ssDNA template extension: DNA polymerase synthesizes new DNA	51.02	91.84
6	PCR applications	to increase amount of DNA of interest to detect GMOs/genetic disease/to be used in forensic science	57.14	93.88
17	PCR method	rapid, accurate	-	95.92
	Microbiological method	to observe bacteriocin activity	-	8.16
25a/	Working in group	Like: e.g. co-operative working, sharing ideas	75.51	81.63
30a		Don’t like: not enough work to do, do report alone	28.57	24.49

Table 9. Students' evaluation of attitudes towards biotechnology before ([a]) and after ([b]) performing the "screening of LAB for bacteriocin" experiment (n=49)

Items	**Question** **Strongly Disagree 1 2 3 4 5 Strongly Agree**	Score ($\overline{X}$)[a]	S.D.[a]	Score ($\overline{X}$)[b]	S.D.[b]
9	DNA replication is difficult to understand.	3.06	0.83	2.70	0.76
10	PCR is an important tool for research in biotechnology.	4.18	0.63	4.20	1.02
11	PCR is difficult to understand.	2.90	0.68	2.67	0.84
12	PCR is relevant to my daily life.	3.04	0.80	3.52	0.75
13	After performing this experiment, I understand DNA replication better.	-	-	4.02	0.54
14	After performing this experiment, I understand the principles of PCR better.	-	-	4.09	0.51
15	Using PCR was faster than the microbiological method for detecting bacteriocin-producing bacteria.	-	-	4.11	0.64
16	Using PCR to detect bacteriocin-producing bacteria was less accurate than using the microbiological method.	-	-	2.5	1.05
13/18	Biotechnology laboratory classes/this experiment has given give me the skills I need to work in biotechnology.	4.12	0.56	4.16	0.47
14/19	I (usually) understand/understood all (the) instructions given in student work sheets/in this experiment.	2.76	0.95	3.18	0.65
15/20	I (usually) understand/understood all the reasons behind activities/the tasks I have done in biotechnology experiments/this experiment.	2.96	0.96	3.60	0.65
18/23	Lab sessions/This experiment helped me to better understand abstract concepts in biotechnology.	3.75	0.70	3.70	0.63
19/24	Lab sessions/This experiment helped me to better understand applications of biotechnology.	3.92	0.68	4.09	0.28
20/25	My biotechnology laboratory experiments are normally successful/My experiment was successful.	3.08	0.90	3.63	0.71
21/26	I liked to find out the reasons for any problems in my laboratory experiments/I had during this experiment.	3.33	0.69	3.67	0.63
22/27	I feel/felt confident in interpreting my laboratory results/in this experiment.	2.92	0.71	3.24	0.64
23/28	Teachers help/helped me to learn during biotechnology laboratory classes/this experiment.	4.15	0.46	4.17	0.61
24/29	Teaching assistants help/helped me to learn during biotechnology laboratory classes/this experiment.	4.00	0.55	4.07	0.65
25/30	I like working in a group of five in biotechnology laboratories/this experiment.	3.87	0.77	4.04	0.59
26/31	Working as part of a group in the biotechnology laboratories/this experiment helps/helped me to learn better than working as an individual.	4.04	0.72	4.00	0.78
27/32	As a group, everyone in the group helps/helped each other to learn.	4.04	0.66	4.18	0.66
28/33	I understand/understood every process I have been/was asked to do in the biotechnology laboratories/this experiment.	2.96	0.71	3.45	0.59
29/34	From my biotechnology laboratory experiences/after doing this experiment, I feel/felt confident in my ability to do biotechnology research.	3.13	0.79	3.57	0.55
30/35	I feel/felt I am/was a biotechnology researcher when I do/did the biotechnology laboratories/this experiment.	3.23	0.72	3.52	0.51
31/36	I enjoy/enjoyed doing biotechnology laboratory classes/this experiment.	3.60	0.79	3.73	0.54
32/37	Biotechnology laboratories motivate/doing this experiment has motivated me to work in the biotechnological area.	3.54	0.65	3.70	0.55

2.2.2 Interview responses

The interview responses from the two focus groups of students, the teacher, and two teaching assistants were grouped by following the principle of intervention.

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory.

Students: All of the interviewed students (two groups, nine students) felt that laboratory classes helped them in learning the abstract concepts in biotechnology. An example of statements is "in the laboratory, learning techniques lead to understanding abstract concepts". They preferred learning in biotechnology by listening to a lecture before doing experiment; in addition, they added that "reading about the experiment also helped". Students thought they might learn knowledge and skills "both technical and critical inquiry skills" in biotechnology laboratory classes. They learned by doing (first) and by group discussion (later). They stated that "group discussion is to share ideas and to gain more knowledge". The students added that "asking TA is also helpful in learning, in addition to group discussion".

The students thought that "the purpose of this experiment is to learn bacteriocin detection by microbiological and PCR methods". In addition, they learned about "bacteriocins, PCR and their applications". They learned "technical skills mostly about PCR". And they learned "critical inquiry skills by explaining their laboratory results". They knew "how to design specific primers from sequences available in database and they knew bacteriocins better". Knowledge that the students had gained in this experiment were "PCR procedure", "primer design", "using database, BLAST, alignment, bioinformatics", "T_a calculation", and critical inquiry explanation for laboratory results". This experiment helped them "to understand abstract concepts such as PCR principles, DNA structure and replication by doing the experiment". The value of biotechnological applications that is relevant to the students' daily lives were "using PCR to detect genes in food directly and rapidly, using bacteriocins in fermented foods is good and safe". All of the students in group 9 felt that they had been motivated a lot to do biotechnology research after performing this experiment because they "can see the applications" and they "can do it". They "would like to search for new substances from other food to be used in other products". For students in group 10, they felt that they haven't been motivated to do biotechnology research after performing this experiment because they "see the potential danger as

carcinogens from hazardous chemical reagents used in the experiment (ethidium bromide)".

Teacher: The teacher expected students to "have more understanding about fundamental genetics" in biotechnology laboratory classes. She felt that "PCR principle is very simple; most of students understand PCR procedure" and "students can learn abstract concepts such as PCR principles or DNA structure and replication in biotechnology laboratory classes". She added that "VDO clip with animation for explanation steps of doing PCR, bacteriocin testing results on killing bacteria" should be a better way to lead student to understand more about these abstract concepts. She expected students to better understand "microbiological and molecular biology methods which confirm each other" and to know "theories, meanings of the methods, and the results obtained" from doing the screening of LAB for bacteriocin experiment.

Learning outcomes that the teacher thought students have gained from this experiment were "LAB in fermented food and bacteriocin screening techniques by both microbiological and PCR methods". She thought that "the students gained a good understanding of this experiment by "reading lab manual and from the laboratory conference before, during, and after doing the experiment". She thought that "the students have gained and learned technical skills that they never had such as PCR and they learned the method for screening of LAB for bacteriocins" in this experiment.

Teaching assistants (TAs): TAs though that the students might learn "technical skills such as PCR, primers design" in this experiment. They felt that "students gained a good understanding of this experiment because they had a chance to do real experiment, discuss and conclude the laboratory results by themselves". TAs felt that "the students developed biotechnology practical skills in this experiment because the teacher and TAs helped this development". They added that another good way to help students to learn abstract concepts such as PCR principles or DNA structure and replication was "video presentation".

PI 3: Teaching and learning can be described by sociocultural views of learning.

Students: The students felt that they "liked working in groups" because e.g., "friends help in learning techniques and answering some questions". They described

their role in laboratory groups as “If there is a lot of work to do, one student will distribute the work and they will work separately, then they will “conclude laboratory results together”, but “if there is not much work to do and it is a new technique to learn” they will “take turns to allow every one in group to practice such a new technique. Students thought that “peers in group or classmates like and would be happy about working in groups”. They thought that “friends, TA, and teacher are important to their learning during laboratory classes”. In addition, “lab manual, introduction by the teacher, and laboratory instruments” helped them in learning in biotechnology laboratory classes. Students stressed that if they need help in the lab classes, they will “ask friends first because it is easy to understand each other” but if their friends don’t know, all of them will “go and ask the teacher or TA together”. The students stated that “TA and friends in other groups” have been helpful to their learning in the laboratory classes by “answering questions or training” them in “technical skills". The value that the students placed on their experience in this experiment was “technical skills and critical inquiry skills” that they “would not forget” and they “cannot find these by learning in a lecture setting”. The students felt that they “gained a quite good understanding of this experiment” even they “sometimes are confused because the lab was quite long”. However, “after completed the lab report”, they gained “a quite good understanding”. The students felt that their way of learning “has changed since their have done this experiment by discussing more questions with TAs”.

Teacher: The teacher thought that teaching/learning methods that help students learn in biotechnology laboratory classes were “to explain to the students until they understand before doing the experiment and during the experiment teacher should explain and answer their questions, or ask them to test whether they understand theories/principles or not, or by using quiz in order to ensure that the students read the lab manual before the experiment”. Working in groups in biotechnology laboratory classes and in this experiment helped the students to learn “co-operative working”. She described students’ roles in groups in biotechnology laboratory classes as “the students distribute the work and explain to each other and work together”. The role of the TAs in helping students in learning was to “suggest technical skills to students and answer students’ questions”. After performing the experiment, the teacher added that

"explanation, pictures, and doing the experiment could help students to learn". She explained more about students' roles in the groups in this experiment as "some students paid more attention and worked harder than others, however, most of students paid attention to the experiment". She saw group working in this experiment was "not different from other experiments". Questions that students asked were about "genetic principles, calculation, and techniques used in the experiment". She saw that "Teacher, TAs, and students themselves have been helpful to students learning in this experiment". The teacher thought that the students have been motivated in this experiment because "students can see inhibition zone, PCR product, use gel doc". She added that "the modern experiment could make students gain more interest about genetics".

Teaching assistants (TAs): The TAs saw their role in this experiment was "to help students with technical skills". They added that in this experiment "it was difficult" because "I don't have experiences in this field of study". This was their first year working as TAs. They hoped to help teacher to teach in this experiment about "planning the experiment, helping the students to work in group, and help them to understand steps, procedure of the experiment before doing". They felt "satisfied" about assisting teaching abstract concepts and assisting group work in this experiment especially "the way students planned and distributed tasks in this experiment for every one in the group that led to finish the experiment on time". They described the students' roles in the groups in this experiment as "the students helped each other on working systematically" which were not different from other laboratory. They thought the tool that helped students to learn in laboratory classes was "lab manual. They added that "the student should read lab manual and search for further information before doing the experiment". Questions the students asked TAs, teachers, or classmates, during this experiment were about "reason for each step of the experiment" and "technical skills used in the experiment". They also suggested that "if there were TAs who had experiences on screening of LAB for bacteriocins, the TAs could help students more e.g., about technical skills".

2.2.3 Observation results

PI 3: Teaching and learning can be described by sociocultural views of learning.

Group discussion: The students asked questions/make comments with peers, teacher, TAs very often. The students appeared to understand by explaining to peers, nods. They worked well together. The students wrote some notes during group discussion.

Class discussion led by the teacher: The teacher mainly gave explanation. Sometimes, she asked class questions to probes students' prior knowledge, made links to previous and future classes. She used practical examples, analogies/metaphors to explain and build concepts. She made links to research community, provided clear and detailed explanation. She provided relevant information and explained concepts. She checked class understanding, encouraged students to think. She usually wrote on the white board especially showing flow charts of every task in this experiment.

The students answered teacher's questions, asked questions and made comments when the teacher requested. Usually the students listened to the teacher carefully. They wrote notes and discussed with peers when requested.

Experiment on screening of LAB for bacteriocins: TAs corrected students' manipulation for students, asked questions/made comments to students, answered student's questions correctly, checked students' understanding, encouraged students to think, and supervised students how to use computer efficiently.

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory.

During screening of LAB for bacteriocins by microbiological method; collection of bacterial cells, performing serial dilution, plating and overlaying, the students manipulated pipette appropriately. They also learned more about aseptic techniques and skills in plating. The students developed skills in overlaying and interpreting laboratory results. They used computer efficiently and helped each other to use the computer. Almost every student in each group could use and understand the computers, appeared to understand nucleotide searching, gene alignment, and similarity searching by explaining to peers, and nods.

During screening of LAB for bacteriocins by PCR method; the students learned and practiced skills in DNA template preparation, preparation of PCR reactions, using the PCR machine, setting the electrophoresis chamber, loading PCR samples,

manipulated staining, and de-staning gel safely. The students also learned skills in using gel-doc for taking photos of gels.

2.2.4 Documentary data

The laboratory reports of each group were examined according to the following topics; objectives, introduction, materials, methods, results, discussion, conclusion, and answers of the questions posed in the laboratory manual.

Every group stated, in the laboratory report, the objectives of the experiment were “to study and compare screening methods for bacteriocin-producing bacteria using microbiological methods and polymerase chain reaction (PCR)”. Nine groups paraphrased the introduction from the laboratory manual into their laboratory reports. One group showed evidence that the introduction part revealed in the laboratory report was from the internet. The introduction part of this group was about the application of LAB in foods, including bacteriocins and probiotics. Every group presented the materials parts in the same way as provided in the laboratory manual. Nine groups presented their methods parts as flow charts and graphics. Only one group presented the methods parts as the same way as provided in the laboratory manual.

There were various food samples e.g., fermented pork and meat sausages (Nham samples), fermented fish, crab, bamboo shoot, and yoghurt that the students bought for the experiment. Every group presented rich data about feature of food samples. They wrote features of food such as color, smell etc. The students also noted about lipid contained in foods samples that they observed and estimated as high amount of lipid in pork Nham samples.

The students recorded the feature of bacterial colonies on MRS plates e.g., small, circular, convex, and entire margin. They can calculate CFU and CFU/g food correctly e.g., 1.2×10^8 CFU/g food. For the results from microbiological test, the students recorded diameter of clear zone they observed mainly from *Lb. plantarum* tester strain. The range of clear zone was about 6-10 mm. Features of colonies producing clear zone were also recorded, e.g., small, white, circular.

For screening of LAB for bacteriocins by PCR method, every group can observe all three bands from the amplification of the control DNA mixture. Four groups

obtained all three bands amplified from boiled cultured cells by themselves and from the provided Nham sample, spiked with all three positive strains. The remaining groups obtained some bands of nisin (608 bp), enterocin (412 bp), pediocin (332 bp) from boiled cultured cell and Nham sample. Students, who brought fermented fish, vegetables, crab, yakult and yoghurt, could not amplify any bands of bacteriocin genes from the samples. The selected colonies from Nham samples mostly harbored pediocin gene. Enterocin and nisin genes could also be detected but in a small proportion. The students observed no band from negative control, in which water was used instead of DNA template.

The students provided reasons from their laboratory results such as they could not observe clear zone by overlaying with *Lb. plantarum* because the tester strain died while inoculating into hot molten agar. Some groups who obtained more than one band from food sample discussed that "there might be more than one strain of bacteria-producing pediocin, enterocin, and/or nisin. Some groups, who could not obtain any band from food samples such as fermented fish, crab, and fermented milk discussed that there was no bacteria harboring some of these three bacteriocins. The students selected three different colonies to be tested by PCR because they wanted to observe different PCR products from the colonies. All ten groups noticed primer-dimer at the bottom of their gels.

The students discussed that they could not obtain any bacterial colony from yakult and yoghurt because the strains used in industrial company may require some additional nutrient for growth. For fermented fish and crab conditions were too salty, the bacteria could not grow, while in fermented bamboo shoot, it was too sour, bacteria could not grow as well. Some groups could not observe clear zones because there were too many bacterial colonies in the plates, after overlaid with the tester strains the clear zones fused together. Since after some of those colonies were selected for performing PCR, they can still obtain pediocin band.

Students' answers to the questions posed in the lab manual as follows.

1. In what kind of foods LABs can be found?

   Answers: sour fermented food e.g., Nham, yoghurt, fermented sausage
2. Give examples of bacteriocins produced by LABs.

   Answers: nisin, enterocin, pediocin

3. Why do LABs have to produce bacteriocins?

   Answers: to kill other genetically related strains in order to survive, to compete for growth

4. Why do we use LABs in biological control?

   Answers: because LAB produce bacteriocins to kill other genetically related strains, do not harm human and LAB produce lactic acid which inhibit growth of other bacteria

5. What mechanisms do bacteriocins use to kill other bacteria? Can bacteriocin kill other living organisms such as yeast or fungi? Why or why not? Do bacteriocins harm humans?

   Answers: Bactriocins bind specifically to receptor of other bacteria and destroy cell membrane of other bacteria, the sensitive cell lost proton motive force and ions, finally cell death. It does not kill yeasts or fungi because they don't have specific receptors.

6. What are the applications of bacteriocins?

   Answers: Bacteriocins can be used in fermented food industry to kill other contaminating bacteria which potentially cause food spoilage. It can also be used as medicine, in mouth cleaning solution, in tooth paste, and in agriculture.

7. Why are bacteriocins in subclass IIa heat stable, despite being protein?

   Answers: They are small proteins containing disulfide bonds.

8. What do you think about eating food contaminated with LABs or bacteriocins?

   Answers: It is not harmful to consumer because bacteriocins kill specifically other genetically related strains. It is safer because bacteriocin will kill some spoilage bacteria and food borne pathogens. It is beneficial to consumer's heath because LABs themselves are probiotics, they do not harm normal flora.

9. Explain the principles of PCR?

   Answers: There are 3 major steps in PCR reaction: denaturation, annealing, and extension.

   Denaturation: double stranded DNA denatures to become single stranded DNA by heating up to 90-95˚C for 30-60s.

   Annealing: primers bind to DNA template by decreasing temperature to 50-60˚C for 30-60s.

Extension: *Taq* DNA polymerase synthesizes new DNA strands by adjusting temperature to 70-75˚C for 60-120s.

10. If you did not obtain PCR products, what do you think could be the possible causes?

    Answers: Primers degraded, contamination with DNAase, contamination of lipid in food that can inhibit PCR, low quality of the DNA template prepared, lack of LAB harboring pediocin, enterocin, and nisin genes in their food samples, clear zones resulting from other bacteriocins or other antibiotics, etc.

11. How high is the annealing temperature used to affect this PCR reaction?
    Answers: It is highly specific for annealing to DNA template. But if it is too high, the primers cannot bind the DNA template.

12. How could contaminated DNA from other bacteria affect the PCR result in this study?

    Answers: Contaminating DNA would not interfere with the amplification of these bacteriocin genes.

13. Why are primers used in PCR?

    Answers: to initiate DNA synthesis

14. Can you use any other DNA polymerase, such as DNA polymerase from *E. coli*, in PCR?

    Answers: No, because *E. coli* DNA polymerase can not work at high temperature.

15. Compare microbiological and PCR methods to screen for bacterioicin-producing bacteria. Explain the disadvantages and advantages of each method.
    Answers: Microbiological: easier, inexpensive, and activity can be observed.

    PCR: rapid, accurate, convenient, DNA sequence can be specified. Small amount of DNA can be detected. The presence of gene can be detected.

16. Which PCR method will yield better results, isolated DNA or boiled cells? Why? Why not?

    Answers: Isolated DNA, because it is pure and good quality of DNA. Boiling may cause DNA fragmentation, contaminated from other substances inside cells which can inhibit PCR.

17. If you get unexpected PCR results, what are the probable causes?

Answers: Non-specific primers, forgot to add *Taq* DNA polymerase, or *Taq* DNA polymerase is expired, not appropriate condition.

18. If you get other DNA bands, not expected bands, what are the probable causes?
Answers: Non-specific primers can amplify other regions.

CHAPTER VI
DISCUSSION

1. Science

An approximately 1,800 LABs colonies were isolated from three Nham samples from primary screening for bacteriocin production against the indicator strain, *V. harveyi*. A total of 11 colonies were recorded as positive by observing an inhibition clear halo zones on agar media. The detection rate of the bacteriocin-producing strains from total number of colonies screened in this study was about 0.6%, which was higher than previous reports by Noonpakdee et al. (2003) and Rodriguez et al. (1995) which only 0.2% of 14,020 and less than 0.5 % of 4,608 of LAB examined was bacteriocin producers, respectively. Both results indicate that in meat Nham samples, bacteriocin-producing LAB may only be present in a low number. In this study, *V. harveyi* which is sensitive to lactic acid rather than bacteriocin may cause the higher percentage of positive strains.

The secondary screening against other 10 tester strains revealed that all fractions, which were culture broth (CB), culture broth boiled for 10 min (CBb10), filtered culture broth (F), and filtered culture broth boiled for 10 min (Fb10), can inhibit the tester strains, *B. subtilis*, *Lb. plantarum* TISTR 050, *Ped. pentosaceus* TISTR 419, *E. coli*, *V. harveyi* 639, *V. harveyi* 1114, *V. cambelli*, *V. parahaemolyticus*, *Aeromonas* sp. 30-3c and *Aeromonas* sp. 30-3p. These findings are the same as the previous reports by Jack et al. (1995) and Noonpakdee et al. (2003) that bacteriocin-producing LABs secreted inhibitory substance into culture broth and inhibitory activity was not destroyed by exposure to elevated temperature. In addition, the antibacterial spectrum of bacteriocins produced by LABs inhibited especially *B. subtilis*, *Lb. plantarum* TISTR 050, *Ped. pentosaceus* TISTR 419 in this study corresponded to the reports by Jack et al. (1995) and Noonpakdee et al. (2003) that the bacteriocins exhibited inhibitory activity against a broad range of closely related bacteria in the genera *Bacillus*, *Lactobacillus*, and *Pediococcus*. While the inhibition of Gram-negative

species in this study could be occurred by low pH or the presence of some chemical agents that weaken cell wall integrity (Jack et al., 1995).

Five, one, two, two, and one positive strains which were identified by 16S rRNA gene sequencing as *Lb. plantarum, Lb. faciminis, En. faecium, Ped. pentosaceus, Lc. lactis*, respectively, which was in agreement with the reports of the LABs species widely distributed in fermented Thai products by Paludan-Muller et al. (2002) and Tanasupawat and Daengsubha (1983). *Lb. plantarum* (Reenen et al., 2006), *En. faecium* (Cintas et al., 1997), *Ped. pentosaceus* (Miller et al., 2005) and *Lc. lactis* (Steen et al., 1991) have been reported to produce plantaricin, enterocin, pediocin, and nisin, respectively.

Ped. pentosaceus P7, *En. faecium* F103, and *Lc. lactis* F141 which were isolated from Nham samples produced substances that inhibit growth of various bacteria including shrimp pathogen, *V. harveyi*. However, it was later found that inhibitory effect of P7 and perhaps F103 and F141 against *V. harveyi* was due to the lactic acid it produced. *Ped. pentosaceus* P7 showed the highest inhibitory effect in every fraction. In addition, P7 showed good growth according to its growth curve, it can tolerate well to low pH from lactic acid it produced during cultivation. Thus, P7 was selected for further study to confirm the presence of pediocin gene.

Pediocin, inhibitory to a range of food pathogens, was produced by several strains in both *Ped. acidilactici* and *Ped. pentosaceus* (Nettles & Barefoot, 1993; Cheun et al., 2000). The same pediocin gene with 100% nucleotide identity was found in both *Ped. acidilactici* (Chikindas et al., 1993; Mora et al., 2000) and *Ped. pentosaceus* (Kantor et al., 1997). The results showed that *Ped. pentosaceus* P7 harbored *pedA* gene which is 100% identity with other pediocin genes deposited in database. The presence of *pedA* gene in P7 was also in accordance with the report of Albano et al. (2007) who have reported that various *Pediococcus* spp. produce pediocin, which has a potential for food application (Marrug, 1991). There was also a report that other LAB such as *Ped. parvulus* (Bennik et al., 1997) and *Lb. plantarum* (Ennahar et al., 1996) also harbor pediocin gene.

In addition, *En. faecium* F103 and *Lc. lactis* F141 can produce some substances which could kill the tester strains by their ability to produce enterocin and nisin, respectively. *En. faecium* has been reported to produce bacteriocin and it has been

used as co-culture in cheese manufacture (Moreno et al., 2003). *Lc. lactis* has been reported to produce nisin (Harris et al., 1992) which has been used for food application. The universal primers, UFUL and URUL (Nilsson et al., 2003) can be used to identify the LAB. The sequencing results identified the three selected strains P7, F103 and F141 as *Ped. pentosaceus*, *En. faecium* and *Lc. lactis*, respectively.

The similarities of the sequences of the 500 bp of 16S rDNA PCR product which was amplified from chromosomal DNA of isolated LABs using universal primers, UFUL and URUL and *Taq* DNA polymerase were above 97% considered to be the cutoff value indicating species identity (Stackebrandt & Goebel, 1994) with other sequences in the database. Thus, F1, P1, P2, P3, P4, P5, P6, P7, P8, F103, F141, which have 100, 100, 99, 99, 100, 99, 99, 100, 98, 100, 99 % identity with sequences in the database identified the strains as *En. fecium, Ped. pentosaceus, Lb. plantarum, Lb. plantarum, Lb. plantarum, Lb. plantarum, Lb. plantarum, Ped. pentosaceus, Lb. faciminis, En. faecium, Lc. lactis*, respectively.

The three pairs of newly designed primers could be used to detect pediocin (*pedA*), enterocin (*entA*) and nisin (*nisR*) genes. These specific primers can amplify DNA fragments from *Ped. pentosaceus* P7, *En. faecium* F103 and *Lc. lactis* F141, respectively. Thus, *Ped. pentosaceus* P7, *En. faecium* F103 and *Lc. lactis* F141 are presumably harboring pediocin, enterocin and nisin gens, respectively. Hence, the three strains were selected and used as positive controls in laboratory design for the students both for microbiological and PCR methods.

Eighteen and one isolates of *Ped. pentosaceus* and *Ped. acidilactici* were found among 300 LABs isolated from Nham samples. The predominance of *Ped. pentosaceus* over *Ped. acidilactici* found in fermented meat products in our study was in accordance with previous reports (Nigatu et al., 1998; Paludan-Muller et al., 2002; Tanasupawat & Daengsubha, 1983).

Various molecular genetic methods have been used to discriminate among species in the genus *Pediococcus* or subpopulations within a species. These include ribotyping (Satokari et al., 2000; Barney et al., 2001), 16S rRNA gene sequencing (Omar et al., 2000; Barney et al., 2001), randomly amplified polymorphic DNA (RAPD) (Nigatu et al., 1998; Mora et al., 2000) and pulsed-field gel electrophoresis (PFGE) in combination with other characterizations (Barros et al., 2001). A problem

with regard to reproducibility is known to occur with RAPD method (Tyler et al., 1997). The use of PFGE alone fails to distinguish between *Ped. acidilactici* and *Ped. pentosaceus* (Barros et al., 2001). In addition, PFGE protocols typically require six days to complete and are not practical for routine testing. Although 16S rRNA gene sequencing and ribotyping could be used to identify the genus and species of *Pediococcus*, the techniques are rather expensive and ribotyping requires the construction of a comprehensive fingerprint libraries (Satokari et al., 2000). Recently, a technique based on PCR-RFLP analysis of *rpoB* gene was used to differentiate rods and cocci of wine LAB species (Claisse et al., 2007). The technique efficiently identified several species of LAB but advanced software was required to analyze the lengths of gel bands since the sizes of restriction fragments of PCR amplicon in several LAB species were very similar.

Ped. acidilactici and *Ped. pentosaceus* show the 16S rRNA sequence homology of 98.3% as reported by Collins et al. (1990). Thus, for species differentiation by PCR, more than one specific target primer as recommended by Alm et al. (1996) should be used. In this study, the result clearly showed that the designed primers, PpF, PaR, and PdF (Table 3) on the 16S rRNA gene of both species could identify *Ped. acidilactici* and *Ped. pentosaceus* by a simple PCR technique without cross-reaction with other *Pediococcus* spp. and several other LABs. *Ped. dextrinicus* did not have any amplified band using our designed primers because there were two, one and one mismatched bases at 3' ends of the primers PpF, PaR, and PdF, respectively, to its 16S rRNA gene.

Identification of the 19 *Pediococcus* isolates by the multiplex-overlapping PCR technique showed the same results with the 16S rDNA sequence analysis. The 278 bp can be detected in some *Pediococcus* and *Lactobacillus* strains because from 16S rDNA alignment, there was only 2-3 bases difference within sequences of the PpF whereas URUL primer can anneal to all tested bacteria. However, the absence of 500 bp, clearly indicated that the strains were not either *Ped. acidilactici* and *Ped. pentosaceus*. Since the PdF primer was designed to be able to amplify 16S rRNA gene of only these two species.

As suggested by Mora et al. (1997), an assay based on 16S rRNA gene and *ldhD* gene-targeted multiplex PCR permitted a rapid and unambiguous identification of *Ped.*

acidilactici and *Ped. pentosaceus*. The approach should be an alternative to the use of more time-consuming techniques such as DNA-DNA similarity or the G+C content (Mora et al., 1997). In our study, species differentiation of *Ped. acidilactici* and *Ped. pentosaceus* obtained from a single colony within one day by this mPCR based on 16S rRNA gene was found to be very efficient and unambiguous in differentiating between both species as well as in distinguishing them from other LABs. This protocol represents a considerable reduction in the typical time required. This multiplex-overlapping PCR method gave the correct identification result as confirmed by 16S rDNA sequencing. The technique could be applied to identify other closely related species due to its simple and rapid method. In addition, detection of genes encoding bacteriocins such as pediocin could be performed simultaneously in a single PCR tube. The methods could also be used to detect other bacterocin genes such as enterocin and nisin by designing specific and appropriate primers.

2. Education

Psillos and Niedderer (2002b), discussing the nature of the laboratory, commented that: “in [the] standard laboratory students normally work in small groups, carry out hands-on experiments using conventional laboratory apparatus, and are engaged in a variety of complicated yet distinctive activities” (p. 49). And, as suggested by Hodson (1993) and Hegarty-Hazel (1990), this PCR laboratory unit was expected to provide for the development of conceptual biotecnology knowledge, motor and intellectual skills, and problem-solving and affective outcomes.

The 49 students, were in a class of a third year Biotechnology program, Mahidol University, academic year 2006. They had recently taken SCBT 302: Microbial Physiology & Genetics, and they completed their lecture classes in which PCR technique had already been introduced. Thus, this group of students had some knowledge of microbial physiology and bacterial genetics necessary for this newly introduced experiment.

The laboratory module included training in a microbiological method to screen Thai traditional fermented foods for the presence of LAB and the multiplex PCR to test the LAB for the presence of bacteriocins. The latter involves a simple DNA template isolation protocol and pairs of specific primers designed for detecting three

bacteriocin genes, which encode pediocin, enterocin, and nisin. The detection of bacteriocin genes in bacteria from fermented foods has been selected because Thailand has many traditional fermented foods, and because some bacteriocins (e.g. nisin and pediocin) are already used widely in the food industry (Jack et al., 1995; Klaenhammer, 1993). However, bacteriocin-producing bacteria can be found in other fermented foods such as cheeses, fermented sausages, and sauerkraut (Albano et al., 2007; Harris et al., 1992; Moreno et al., 2003), so the module could be easily adapted for use in other countries.

The previous screening of fermented food has shown that *Ped. pentosaceus* P7 produces a bacteriocin named pediocin that can be detected by employing a microbiological overlay technique. Using this technique, students can easily screen for bacteriocin activity and select specific bacterial colonies for further detection and differentiation of bacteriocin gene present. Fortunately, both chromosomal and plasmid DNA can be released from bacteria simply by boiling in buffer solution. This is how students prepare DNA from their selected LAB to carry out a multiplex PCR test for pediocin, enterocin, and nisin genes.

The overlay method by other tester bacterium was selected for detection of bacteriocin in order to show the students that bacteriocin activity in LABs can be observed by using this simple method. Selecting the most appropriate tester strain such as *Lb. plantarum*, enabled the students to observe the activity clearly. However, in order to confirm the presence of bacteriocin genes, PCR method was exploited to detect pediocin, enterocin, and nisin genes.

The occurrence of clear zones around colonies grown on MRS means that the bacterial colonies can produce some active substance(s) capable of preventing the growth of the tester strain. There is a possibility that some of these bacterial colonies are producing either bacteriocins or other types of antibiotics. To verify the presence of bacteriocins, colonies need to be probed by multiplex PCR.

By the multiplex PCR, if the DNA templates isolated from foods or selected LAB colonies contain gene that encode pediocin, enterocin, and nisin, students should observe 332, 412, and 608-bp DNA bands, respectively. The students can observed the DNA bands from DNA template containing a mixture of pediocin, enterocin, and nisin genes, the boiled culture broths of *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*,

and the template from the Nham sample spiked with *Ped. pentosaceus*, *En. faecium*, and *Lc. lactis*. This result indicates that the bacteriocin genes can be directly amplified from a food sample by the multiplex method using the three pairs of primers. One of the selected food samples of group of students showed bands of 332 and 412 bp, indicating the presence of the pediocin and enterocin genes, respectively. The result implies that this group's food sample probably contained the LAB *Ped. pentosaceus* and *En. faecium* harboring pediocin and enterocin genes, respectively.

As many researchers (Baker et al., 2002; Bowlus & Grether, 1996; Brinegar & Levee, 2004) suggested the use of PCR methodology in exploratory student laboratory exercises that it allows the students to generate their own meaningful data for the analysis and also provides them with essential laboratory skills in the modern laboratory methods. In addition, the positive laboratory experience will clearly show how such methods can be applied to their daily lives, and it may also stimulate some students to continue their education in molecular biology. In this study, an evaluation questionnaire given to students at the completion of the laboratory work showed that they appreciated this laboratory unit because they could see the relevance of the molecular technique in biotechnology research and its application in detecting bacteriocin genes in LAB present in fermented foods. PCR which has previously been shown to be useful in student laboratory practice (Brandner, 2002; Holst-Jensen et al., 2003; Thion et al. 2002) is also useful in this study because after performing this work in laboratory, students felt that they understood PCR principles better and that they had gained skills in using PCR. Finally, the students felt that the laboratory module motivated them for further study and research in the field of biotechnology.

The students completed every required table correctly and appropriately. It indicated that they understood the objectives of each task and the command used. The student laboratory reports showed that the students gained an understanding of bacteriocins and the principles of PCR and that they understood the relevance of this knowledge to their daily lives (Baker et al., 2002; Bowlus & Grether, 1996; Brinegar & Levee, 2004). The limitations of the microbiological and molecular methods for the detection of bacteriocin activity and bacteriocin genes were discussed. In addition, the students were able to discuss their laboratory results based on their empirical data. As examples of study questions posed to the students and example

answers from their laboratory reports described below showed students understanding of the principles of PCR.

Item 10. If you did not obtain PCR products, what do you think could be the possible causes? The students mentioned the degradation of primers, contamination with DNAase, contamination of lipid in food that can inhibit PCR, low quality of the DNA template prepared, lack of LAB harboring pediocin, enterocin, and nisin genes in their food samples, clear zones resulting from other bacteriocins or other antibiotics, etc. These answers indicated that students realized that bacteriocin from LAB could kill only closely related bacteria (i.e. in the same Gram-positive bacteria) and that the primers designed for each bacteriocin gene will be able to detect only the corresponding bacteriocin genes.

Item 12. How could contaminated DNA from other bacteria affect the PCR result in this study? As the students understand that this PCR method was designed to detect the presence of bacteriocin genes from DNA directly isolated from food samples, the students understood that the three pairs of primers were specific to only pediocin, enterocin, and nisin genes. Hence, students answered that contaminating DNA would not interfere with the amplification of these bacteriocin genes.

This laboratory exercise, thus, could be adapted to study bacteriocins and bacteriocin genes in LAB that are commonly found in foods such as cheese (Moreno et al., 2003), fermented sausage (Albano et al., 2007), dairy products (Olasupo et al., 1994), and sauerkraut (Harris et al., 1992) from other countries.

CHAPTER VII
CONCLUSION

The scientific study showed that microbiological and PCR methods can be used efficiently to screen for bacteriocin-producing LAB in Thai fermented foods. Using microbiological method by overlaying the isolated LABs by the appropriate tester strain, clear halo zones can be observed for the first determination of bacteriocins activity. By using PCR methods, the bacteriocin genes can be detected. In this study, the three bacteriocin genes specific primers for pediocin (*pedA*), enterocin (*entA*), nisin (*nisR*), were used to detect these three bacteriocin genes in *Ped. pentosaceus* P7, *En. faecium* F103, and *Lc. lactis* F141, respectively.

The further study revealed that, *Ped. pentosaceus* P7 harbored *pedA* gene which is 100% identity with other pediocin genes from several LABs strains available in database.

Both *Ped. acidilactici* and *Ped. pentosaceus* are closely similar and both are found in fermented meat. By conventional method, it is difficult to differentiate between the two species. The multiplex-overlapping PCR (mPCR) method can be applied for simultaneous detection of pediocin gene and species differentiation between *Ped. acidilactici* and *Ped. pentosaceus*. It was proven to be a simple, rapid determination and reliable method for differentiation of both pediococcal species as well as determination of the ability to produce pediocin in a single PCR reaction.

Species differentiation of *Ped. acidilactici* and *Ped. pentosaceus* obtained from a single colony within one day by the mPCR was found to be very efficient and unambiguous in differentiating between both species as well as in distinguishing them from other LABs. This protocol represents a considerable reduction in the typical time required. This mPCR method gave the correct identification result as confirmed by 16S rDNA sequencing. The technique could be applied to identify other closely related species due to its simple and rapid method. In addition, detection of genes encoding bacteriocins such as pediocin could be performed simultaneously in a single

PCR tube. The methods could also be used to detect other bacterocin genes such as enterocin and nisin by designing specific and appropriate primers.

The science education study results showed that the students' experiment for screening of LAB for bacteriocins by microbiological and PCR methods enhanced four learning outcomes which are scientific knowledge, technical skills, critical inquiry skills, and attitude to science.

The development of scientific knowledge was considered to be important learning outcome according to the biotechnology program at Mahidol University, Thailand. The program aims to produce graduates who have basic knowledge in biotechnology, including PCR can be emphasized at stages during the laboratory sessions. In this screening of LAB for bacteriocins by microbiological and PCR methods, the principle of the two methods and their applications can be emphasized at stages during the laboratory session both in the underlying theory and during practice. Thus, carrying out the screening of LAB for bacteriocins experiment improved students' understanding of PCR and its applications in biotechnology.

The development of technical skills which was considered to be an important learning outcome, since the biotechnology program at Mahidol University, Thailand, aims to produce graduates who are well equipped with such skills, can be developed in this study. The laboratory on screening of LAB for bacteriocins by microbiological and PCR methods, provided the basic task design e.g., PCR skills and research skills needed for future research relevant to PCR can be used effectly for students to learn technical skills. Thus, carrying out the screening of LAB for bacteriocins helped students to learn practical biotechnology skills.

In the laboratory unit for screening of LAB for bacteriocins by microbiological and PCR methods, the tasks which focused on the importance of the development of critical inquiry skills could be emphasized at several stages of the laboratory such as during the discussion sessions. After students got the PCR results, they can interpret their data. Even some groups cannot obtain the PCR results; they can propose empirical and logical explanations for the lack of results. These mean the students developed critical inquiry skills by doing the screening of LAB for bacteriocins experiment.

The laboratory design can also be used to enhance attitudes to science. Because the students can see the relevance of PCR to their daily lives. This also motivated them to know more about application of PCR in various fields of study that are enormously available.

In conclusion, the laboratory module provided the students with experiences on the use of microbiological and multiplex PCR methods and showed them how the molecular biology techniques can be related and applied to their daily lives. In addition, it promoted students to feel that they have learned practical biotechnology skills. In consideration of the third science education research question, this study provided a better understanding of learning outcomes in the biotechnology laboratory.

From a sociocultural perspective of learning, carrying out the experiment on screening of LAB for bacteriocins by microbiological and PCR methods helped students to learn how to screen LAB for bacteriocins by microbiological and PCR methods, how to work in groups of five and engaged in group discussion; and how they can learn from teachers, teaching assistants, and peers. This practical work took place in a group was a social activity. The experiences of individuals could be shared and learned together.

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APPENDIX

APPENDIX A

Nucleotide sequences (720 bp) of a DNA fragment harboring pediocin gene from *Pediococcus pentosaceus* P7

Note: 1. Start and stop codons are in bold letters

2. Arrow indicates the signal peptide cleavage sites

APPENDIX B

Pediocin A (*pedA*) gene sequences from various lactic acid bacteria which all show 100 % identity at both nucleotide and amino acid levels.

Organism	*Ped A* gene accession No.
Ped. pentosaceus P7	EU082178
Ped. acidilactici K10	AY705375.1
Ped. acidilactici H pSMB74	U02482.2
Ped. acidilactici	AY083244.3
Lb. plantarum plasmid pWHE92	AY316526.1
Ped. pentosaceus plasmid pS34	AY316525.1
Ped. parvulus plasmid pAT077	AY316524.1
Ped. acidilactici H plasmid pSMB74	M90679.1
Ped. acidilactici	M83924.1

APPENDIX C

Manual for Experiment 4

Screening of bacteriocin-producing bacteria

Objective

To study and compare screening methods for bacteriocin-producing bacteria using microbiological methods and Polymerase Chain Reaction (PCR).

Introduction

Several bacteria produce and secrete substances to kill other bacteria, especially closely related species or species that require the same ecological niches, for example, food or habitat, in order to compete for growth and survival of the producing bacteria. An important substance that some bacteria produce, which is not an antibiotic, is a protein called a bacteriocin. This protein is active on specific target bacteria which are similar to the producing strain. Bacteria used in food fermentation (producing the sour taste which is common in Thai foods) are usually Lactic Acid Bacteria (LAB). Several LABs produce bacteriocins which can be used in biological control of other bacteria, and there is an application of bacteriocin in the food industry. By adding bacteriocins or bacteriocin-producing bacteria to the food, other contaminating bacteria which can cause rotten food or food poisoning will be killed. The bactericidal action appears to be membrane active, resulting in ion leakage, loss of proton motive force, and ultimately, cell death (Jack, Tagg, & Ray, 1995). For example, nisin, a bacteriocin produced by *Lactococcus lactis*, is added to food to kill *Listeria monocytogenes*. Bacteriocin-producing bacteria are found in several genera of LABs such as *Lactobacillus*, *Pediococcus*, *Lactococcus*, and *Enterococcus*.

Bacteriocins have been classified, depending on structures, into three classes (Klaenhammer, 1993; Nes *et al*., 1996):

Class I, Lantibiotics are small (<5 kDa), heat-stable proteins. Bacteriocins in this class have post-translational modification by dehydration of serine and threonine molecules to form didehydroalanine (Dha) and didehydrobutyrine (Dhb). Then the SH group of the cysteine molecule is added at C=C of Dha or Dhb to form a thioether

amino acid, called lanthionine, which is acid tolerant. One example of a bacteriocin in this class is nisin, which is produced by *Lactococcus lactis*.

Class II are small (<10 kDa), heat-stable bacteriocins, but there is no post-translational modification. Class II bacteriocins are further classified into three subclasses.

Subclass IIa bacteriocins possess a conserved N-terminus which has the YGNGV amino acid sequence. An example is pediocin, produced by *Ped. pentosaceus* and *Ped. acidilactici.* In addition, there are other bacteriocins such as plantaricin 423 which is produced by *Lb. plantarum* 423.

Subclass IIb bacteriocins are activated when there is complementation of two bacteriocin chains of completely different structure. Examples of bacteriocins in this class are plantaricin EF and JK produced by *Lb. plantarum* C11.

Subclass IIc bacteriocins possess a secondary-independent leader peptide in which there is an ATP-binding cassette at the C terminal. This sec-independent leader peptide functions in transportation of bacteriocins out of the cell. Examples of bacteriocin in this subclass are lactococcin A produced by *Lactococcus lactis,* and plantaricin A produced by *Lb. plantarum* C11.

Class III bacteriocins are large (>30 kDa), heat sensitive proteins. One example of a bacteriocin in this subclass is helveticin J, produced by *Lc. helveticus* 481.

One bacterium can produce more than one bacteriocin, such as *Lb. plantarum*, and *Lc. lactis*. Different bacteria can also produce the same bacteriocin, such as *Ped. pentosaceus* and *Ped. acidilactici*. However, most bacteria cannot produce bacteriocins.

In this experiment, students will screen for bacteriocin-producing bacteria by a microbiological method so as to study their ability to kill *Lb. plantarum*. This method will be compared to the use of PCR, a molecular method used to detect bacteriocin genes in bacteria in fermented food rapidly by amplification of bacteriocin genes by primers specific to those genes. Table 4.1 lists the guiding questions for this experiment.

Table 4.1 Concept for the experiment

	Questions	Procedures
1.	What are types and sources of fermented foods expected to contain lactic acid bacteria?	Information searching
2.	What kinds of fermented food and samples are needed?	Choose types of sour food in which the presence of LAB has been reported.
3.	What tester strains should be used?	Choose *Lb. plantarum* which is a Gram positive bacteria.
4.	What types of bacteriocin will be detected?	Choose class I and IIa bacteriocins
5.	How would we design primers?	Perform sequence alignment and select primers from conserved region

The following reading is assigned and the questions below are discussed before the first session.

1. Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., & Panyim, S. (2003). Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology, 81*, 137-145.
2. Moreno, M. R. F., Rea, M. C., Cogan, T. M., & Vuyst, L. D. (2003). Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. *International Journal of Food Microbiology, 81*, 73- 84.
3. Moreno, M. R. F., Sarantinopoulos, P., Tsakalidou, E., & Vuyst, L. D. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology, 106*, 1-24.

Focus questions to think about for discussion in this experiment:

1. What is a bacteriocin?
2. Where can bacteriocin-producing bacteria be found?
3. What are the applications of bacteriocins?

Experiment 4.1

Screening of bacteriocin-producing bacteria by microbiology method

Day 1

From students' reading provided before this class, students should know what are bacteriocins, the food sources of bacteriocin-producing bacteria, and the applications of bacteriocins. In this experiment, students will isolate LAB from foods by using a specific media, de Man Rogosa Sharpe (MRS) media, that has been used for the recovery of LABs from various food products.

Expected outcomes for Day 1 session

1. Students know about bacteriocins.
2. Students know some food sources of bacteriocin-producing bacteria.
3. Students know about the applications of bacteriocins.
4. Students have developed skills in LAB isolation and have isolated LABs from their food samples.
5. Students have prepared bacterial cell samples for PCR work on Day 4.

Materials

(For one group of five students)

1	Food samples which students should bring for screening of bacteriocin-producing bacteria (Each group of students choose and bring two food samples.)	2 samples
2	Nham sample contaminated by bacteriocin-producing bacteria	1 g
3	MRS broth (5 ml)	3 tubes
4	0.85% NaCl (4.5 ml)	18 tubes
5	MRS agar	18 plates
6	TE buffer (2.5 ml)	1 tube
7	Sterilized pipette (10 ml)	3 pipettes
8	Sterilized pipette (1 ml)	18 pipettes
9	Microcentrifuge tube (Eppendorf)	3 tubes

10	Glass spreader	3 spreaders
11	Forceps	1pr
12	Beaker 100 ml with 95% Alcohol 30 ml	1 beaker
13	Microcentrifuge tube (Eppendorf) rack	1 rack
14	Test tube rack	1 rack
15	Centrifuge	1 machine

(For all of students in class of 50 students)

1	Laminar air flow	1 laminar
2	Incubator shaker 30°C	1 shaker
3	Incubator 30°C	1 incubator
4	Sterilized papers	30 pieces
5	Balance	2 balances

Method

1 Record details of your food samples in Table 4.2

2 Weigh 1g of food samples and suspend into MRS broth (5 ml), one sample per one tube (by using sterilized forceps or spoon), mix well, and shake at 30°C for 1 hr. Students need to use a specific media, MRS, because students need to isolate LABs and MRS has been used for the recovery of LABs from various food products. MRS is an enrichment media containing glucose to promote the growth of LABs and sodium acetate to inhibit the growth of other bacteria.

3 To prepare bacterial cells for the PCR template on Day 4, leave the tubes to stand still for 1 min for the precipitation of food pellets onto the bottom of the tubes, then pipette 1.5 ml of upper phase of culture broth of each sample - bacterial cells are in this upper phase - into microcentrifuge tubes, then centrifuge at 8,000 rpm for 5 min and carefully discard the supernatant. By collecting the pellets, you are collecting bacterial cells.

4 To wash the cell pellet, add 200 µl TE buffer, mix well, then centrifuge at 8,000 rpm for 5 min and discard the supernatant. Students need to wash the cell pellet to get rid of oil from the food, because oil might inhibit the PCR that students will

conduct on Day 4. Add another 200 µl TE buffer, mix well, and then store at -20 °C for the experiment on Day 4. Students need to store cell samples at -20 °C to prevent DNA degradation. At this temperature, all enzymes are inactivated, so you can store the cell samples for a long period of time.

5 Use the each samples remained from step 2 to perform 10-fold serial dilution to 10^{-6} (pipette 0.5 ml into 0.85% NaCl 4.5 ml), use a sterilized glass spreader to spread 0.1 ml of dilution 10^{-4}, 10^{-5} and 10^{-6} onto separate MRS plates (each dilution on 2 plates for replicates).

6 Incubate plates at 30 °C for 24 hrs, for inspection on Day 2.

Table 4.2 Feature of food samples

Food samples	**Date of manufacture**	**Sources (manufacturers /places)**	**Features (color/smell/others)**

Day 2

From the previous lab session, you should have isolated LABs on the MRS plates. In this experiment, you will screen these LABs for bacteriocin-producing bacteria by a microbiological method, an overlay method, so as to study their ability to kill *Vibrio harveyi*, a shrimp pathogen, and *Lb. plantarum* which is a Gram-positive bacteria.

Expected outcomes for Day 2 session

1. You know about methods for testing bacteriocin activities, including their advantages and disadvantages.
2. You gain skills in testing bacteriocin activity by a microbiological method.
3. You learn how to read journal articles and to critique the methods through discussion with your peers and teacher.

Materials

(For one group of five students)

1	*V. harveyi*, cultured in Tryptic Soy Broth (TSB) 3% NaCl (5 ml), prepared from *V. harveyi* overnight cultured in TSB 3% NaCl, shaken at 30°C, then 1% subcultured in TSB 3% NaCl for 4-5 hrs further incubation with shaking before the experiment	1 tube
2	*Lb. plantarum*, cultured in MRS broth (5 ml), prepared from *Lb. plantarum* overnight cultured in MRS broth shaken at 30°C, then 1% subcultured in MRS broth for 4-5 hrs further incubation with shaking before the experiment	1 tube
3	TSA 3% NaCl + 0.8% agar in 60°C water bath	3 tubes
4	MRS 0.8% agar in 60°C water bath	3 tubes
5	Sterilized pipette (1 ml)	3 pipettes

(For all of students in class of 50 students)

1	Incubator shaker 30°C	1 shaker
2	Incubator 30°C	1 incubator

Note: *V. harveyi* is a Gram negative bacteria and it is very heat sensitive.

: We need to add 3% of NaCl into TSB, because *V. harveyi* usually lives in the sea.

: We usually test bacteriocin produced by Gram positive bacteria against Gram positive tester strains. We are using *Lb. plantarum* to test bacteriocin activity because it is a Gram positive bacteria like the producer strains and it is easy to grow and manipulate in the laboratory.

Method

1 Examine plates from the last session, and record results in Table 4.3.

2 Select two plates of each sample containing 30-300 colonies for overlay with tester strains (*V. harveyi* and *Lb. plantarum*), one per plate.

3 Overlay by pipetting *V. harveyi* cultured in TSB 3% NaCl 1 ml into tube containing warm TSA 3% NaCl + 0.8% agar (5 ml). For *Lb. plantarum*, pipette *Lb. plantarum* in MRS broth 1 ml into tube containing warm MRS broth 0.8% agar (5 ml) (Do not use hot molten agar, leave it to cool to about 45 °C to protect the heat sensitive strain), use vortex mixer or shake by hands to mix well, then overlay on each sample selected plate (one agar tube per one 1 plate).

4 Wait until overlay agar solidifies, turn the six plates upside down, then incubate plates in incubator at 30°C for 24 hrs, for inspection on Day 3.

Table 4.3 Feature of colonies on MRS and number of colonies

Food samples	Feature of colonies	Dilution	CFU	CFU/g food
Nham sample (provided)				
Food sample 1 ……...				
Food sample 2 ……...				

Day 3

On examining the plates from Day 2, students should be able to observe clear zones on the tested MRS plates. This means that the bacterial colony with the clear zone produces some kind of active substances that can kill tester strains. These substances are likely to be bacteriocins. In addition, before the next laboratory session on PCR, students have an opportunity to review the principles of PCR and the application of PCR to detect bacteriocin genes. In this laboratory session, students also have an opportunity to perform sequence alignment and similarity searching, and design of primers. These processes are important and necessary for PCR-based research in biotechnology.

Expected outcomes for Day 3 session

1. Students obtain microbiological data about LABs from foods.
2. Students know about principles of PCR and the application of PCR to detect bacteriocin genes.
3. Students develop an improved understanding of the structure of DNA and how that can be applied to PCR.
4. Students know that in PCR research you need to perform sequence alignment and similarity searching, and design of primers.
5. Students understand the methods used and develop some skills in sequence alignment and similarity searching, and primer design.

Materials

(For one group of five students)

1	MRS agar	4 plates
2	Sterilised toothpick	1 plate

(For all of students in class of 50 students)

1	Incubator 30°C	1 incubator

Method

1 Observe clear zones on the six plates from Day 2, and record results in Table 4.4.

2 For students' first food sample, use a sterilized toothpick to pick colonies that produce a clear zone (bacterial colonies will be under overlaid agar) and transfer to 2 MRS plates (9 colonies per plate). Repeat for another food sample onto 2 further MRS plates.

3 Turn the plates upside down, then incubate plates in incubator at 30°C for 24 hrs. Students will use these bacterial colonies for DNA template preparation on Day 4.

Table 4.4 Results from microbiological tests

Food samples	**Dilution**	**Tested bacteria**	**n^1/N^2**	**Diameter of clear zone (mm)[3]**	**Features of colonies producing clear zone[4]**
Nham sample (provided)		*V. harveyi*			
		Lb. plantarum			
Food sample 1 ……...		*V. harveyi*			
		Lb. plantarum			
Food sample 2 ……...		*V. harveyi*			
		Lb. plantarum			

Note : *1* n = number of colonies that produce a clear zone

2 N = total number of colonies on plate

3 = range of lowest diameter – highest diameter

4 = for colony that produces the highest diameter of clear zone on plate

In your group, you will now design primers that could be used in the PCR screening method for bacteriocins. The first step is nucleotide searching.

Nucleotide searching (You should spend 30 min on nucleotide searching)

The first step of primer design is to look for the nucleotide sequence of the gene of interest. In this study the genes of interest code for pediocin, nisin, and enterocin. Nisin has been allowed to be used as a food preservative, and pediocin and enterocin have a potential to be used as food preservatives. Thus, students have to search for these gene sequences in the database. This database is a national resource for molecular biology information, NCBI. It creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and disseminates biomedical information - all for the better understanding of molecular processes affecting human health and disease. It is recommended that within students' group, primers for each bacteriocin are designed by 1-2 students, so that everyone has an opportunity to do it.

1 Login to the web page www.ncbi.nlm.nih.gov
2 Click the "search" button, and select "nucleotide".
3 Type "pediocin" in the box.
4 Click "go".
5 Select and copy 3-5 complete sequences of pediocin, then save these five sequences in a word document. Name each sequence according to the accession numbers revealed before each sequence, such as "pediocin AY316524" where AY316524 is its accession number. The definition of this sequence is "*Pediococcus parvulus* plasmid pAT077 pediocin AcH production operon, complete sequence".
6 Repeat steps 3-6 for nisin and enterocin nucleotide sequences, but use the key terms "nisin R" and "enterocin A" for searching instead.

Sequence alignment (You should spend 30 min on sequence alignment)

After you have obtained gene sequences from the database, you have to identify conserved regions in order to design primers specific to those genes. There are a number of programs available for sequence alignment. In this study, students will use an online program for sequence alignment to find conserved regions.

1 Login to the web page www.ebi.ac.uk/clustalw/

2 Paste all five sequences of pediocin in the box "Enter or Paste a set of Sequences in any supported format:" Follow this example:

>sequence 1

ATGAAGGATGAGGAGAAGATGGAGATTCAGGAGATGCAGCTCAAAGAGG

AAGCACATTATGAAGGATGAGGAGAAGATGGAGATTCAGGAGATGCAGC

>sequence 2

GCAGACGACGCANAGGATCGCGCGCAAGGCCTGCAGCGCGAACTGGATG

GCGGACGAGGCAGAGGATCGCGCGCAGGGCCTGCAGCGGGAGCTGGACG

>sequence 3

GCGGAAGAGGCTGACCGCAAATACGAGGAGGTAGCTCGTAAGCTGGTCA

GCGGACGAGGCCGAAGAGCGGGCCGAGATCCTGCAGAGGGAGGTGGACG

3 Click the "Run" button.

4 Wait until completed.

5 Click the button "Start Jalview" to see the highlighted conserved region.

6 Select and copy a conserved region (from the same page as you click "Start Jalview") suitable for primer design i.e. 20 bases in length, 50 % GC content.

7 Copy the selected region and paste it into a word document and name it "pediocin forward primer"

8 Select and copy another conserved region suitable for primers design i.e. 20 bases in length, 50 % GC content.

9 Copy the second selected region and paste it into the word document and name it "pediocin reverse primer" Note: The two forward and reverse primers should flank 200-1000 bp to be PCR products which can be observed in gel.

10 Repeat steps 2-10 with the nisin and enterocin sequences instead.

Similarity searching (You should spend 30 min on similarity searching)

After you have designed primers, in order to make sure that these primers can be used to amplify only these gene targets, you need to search for the similarity of the designed primers and other gene sequences in the database. There is an online program, BLAST, available for researchers to perform similarity searching.

1 Students again log in to the web page, www.ncbi.nlm.nih.gov.
2 Click on "BLAST".
3 Click on "Nucleotide-nucleotide BLAST (blastn)"
4 Copy and paste pediocin forward primer into the search box.
5 Click on "BLAST" button.
6 Wait until completed, then click on "Format" button
7 Wait until completed, then look at significant similarity found. Is there 100% similarity only with pediocin nucleotide sequences? Or with other sequences in the database? Please note that good primers should have 100% similarity with only pediocin nucleotide sequences or target sequences, because, if it has 100% similarity with other nucleotide sequences, it means that this primer can amplify those genes too. It will produce more than one band of PCR products.
8 Repeat steps 2-7 for pediocin reverse primer, nisin forward primer, nisin reverse primer, enterocin forward primer, and enterocin reverse primer.
9 Check the primers you have found with ones below that will be used in the PCR solution:

pedF 5'-GGTAAGGCTACCACTTGCAT-3'
pedR 5'-CTACTAACGCTTGGCTGGCA-3'
entAF 5'-GGGTACCACTCATAGTGGAA-3'
entAR 5'-CCAGCAGTTCTTCCAATTTCA-3'
nisRF 5'-CTATGAAGTTGCGACGCATCA-3'
nisRR 5'-CATGCCACTGATACCCAAGT-3'

If students' sequences are not the same as the above sequences, repeat steps 2-7 to find them.

You should discuss the following questions with your peers in your group, then answer them in your laboratory report.

Questions

1. In what kind of foods can LABs be found?
2. Give examples of bacteriocins produced by LABs.
3. Why do LABs have to produce bacteriocins?
4. Why do we use LABs in biological control?
5. What mechanisms do bacteriocins use to kill other bacteria? Can bacteriocin kill other living organisms such as yeast or fungi? Why or why not? Do bacteriocins harm humans?
6. What are the applications of bacteriocins?
7. Why are bacteriocins in subclass IIa heat stable, despite being protein?
8. What do you think about eating food contaminated with LABs or bacteriocins?

Experiment 4.2

Screening of bacteriocin-producing bacteria by PCR method

In the experiment 4.1 you have isolated LABs from foods, and screened them for bacteriocin-production using a microbiological testing method. There is a molecular technique (PCR) which can also be used to screen for bacteriocin-producing bacteria by the detection of the presence of the genes. In this experiment, your purpose now is to use PCR to detect the presence of pediocin, enterocin, and nisin genes, and to then compare the PCR method with the microbiological method.

PCR is a molecular technique used to amplify a specific region of template DNA by using a heat tolerant enzyme (*Taq* DNA polymerase) to amplify that specific region. In the PCR reaction, DNA template, *Taq*, dNTPs (dATP, dCTP, dGTP, dTTP), $MgCl_2$, PCR buffer and primers are needed. These are the initiation factors for new DNA synthesis. We have to design primers specific to the two ends of the template DNA that we want to amplify.

The PCR reaction begins by heating DNA to 94°C to destroy the hydrogen bonds holding the complementary strands together, to denature the double stranded DNA template into single stranded DNA. Then, the temperature decreases to 50-60°C for annealing of primers to the complementary regions. And finally, it increases the temperature to 72°C, the appropriate temperature for *Taq* polymerase to synthesize new DNA extended from primers from 5' to 3' end.

PCR can be used to amplified a very small amount of DNA template because in the reaction, the number of DNA strands will be increased exponentially, i.e. from 1 to 2 in the first round, from 2 to 4 in the second round, and from 4 to 8 in the third round and so on, i.e. exponentially (2^n). For this reason, if we use 30 cycles of PCR, theoretically we will get 2^{30} DNA fragments.

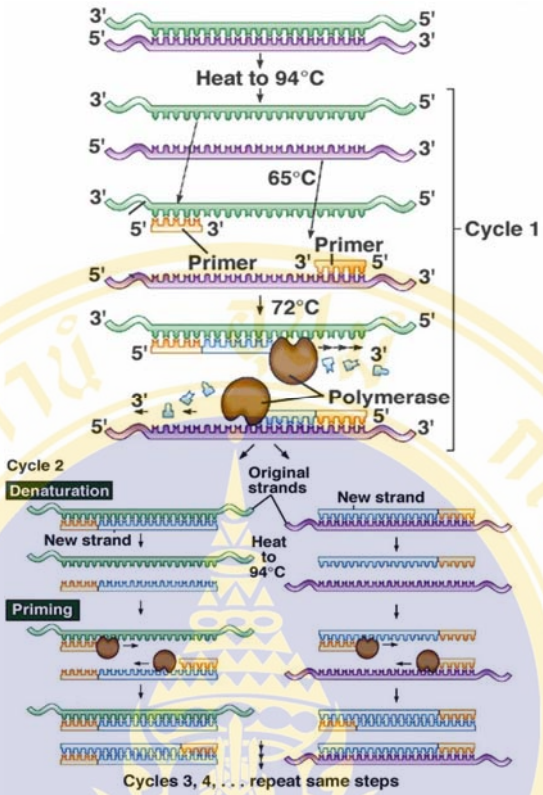


Figure 4.1 DNA amplification by PCR

Day 4

In this experiment, you will screen for bacteriocin-producing bacteria using a PCR method. PCR will be used to detect bacteriocin genes in bacteria in fermented foods. You will be provided with a PCR solution which includes the three pairs of primers to detect nisin, enterocin, and pediocin genes, as identified in the primer design exercise on the previous day. The PCR will rapidly amplify these three bacteriocin genes, if they are present in the food samples, using the three pairs of primers specific to those genes.

Expected outcomes for Day 4 session

1. Students clarify your knowledge about PCR.
2. Students learn how to prepare DNA, prepare PCR reactions, run PCR and obtain samples of amplified LAB DNA.
3. Students gain skills in operating PCR instruments.
4. Students learn how to conduct practical work cooperatively as a team.

Materials

(For one group of five students)

1	PCR reagents, consist of:	
	• Solution I (see component in Table 4.6)	174 µl
	• Solution II (5U/µl *Taq* DNA polymerase)	6 µl
	• Standard DNA (DNA mixture of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis*)	5 µl
	• 3A water	5 µl
2	Cultured *Ped. pentosaceus* in MRS broth (5 ml)	1 tube
3	Cultured *En. faecium* in MRS broth (5 ml)	1 tube
4	Cultured *Lc. lactis* in MRS broth (5 ml)	1 tube

The *Ped. pentosaceus*, *En. faecium* and *Lc. lactis* have been prepared before the experiment. They have been cultured overnight in MRS and shaken at 30°C, then 1% has been subcultured in MRS for 4-5 hrs further incubation and shaking.

5	TE buffer (1.5 ml)	1 tube
6	PCR tubes (0.2 ml)	11 tubes
7	Microcentrifuge tube (Eppendorf)	15 tubes
8	Automatic pipette (20 µl and 2 µl) each	1 pipette
9	Sterilised pipette tip (200 µl)	1 box
10	Sterilised pipette tip (20 µl)	1 box
11	Sterilised pipette (5 ml)	3 pipettes
12	PCR rack	1 rack
13	Microcentrifuge tube (Eppendorf) rack	1 rack
14	Boiling Rack for Microcentrifuge tube (Eppendorf)	1 rack
15	Ice box	1 box
16	Sterilized toothpick	1 plate
17	Centrifuge	1

(For all of students in class of 50 students)

1	PCR machine (96 wells)	2
2	Mini spin	1

3	Hot plates	2
4	Boiling pod	2

Method

1. DNA Template Preparation

1.1 DNA template from LAB strains

1.1.1 Pipette 1.5 ml culture broth of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis* into an Eppendorf; one tube per sample

1.1.2 Centrifuge at 8,000 rpm for 5 min, then discard supernatant, collect cell pellet

1.1.3 Add 200 µl TE buffer, vortex well

1.1.4 Boil for 10 min to break the cells, then put on ice immediately for 1 min to precipitate some proteins

1.1.5 Centrifuge at 10,000 rpm for 10 min

1.1.6 Pipette 5 µl supernatant of each sample into PCR tubes number 2-4 for use as DNA template

1.2 DNA template from frozen samples from Day 1 (3 tubes)

1.2.1 Thaw 3 frozen samples, vortex well

1.2.2 Boil for10 min, then put on ice immediately for 1 min

1.2.3 Centrifuge at 10,000 rpm for 10 min

1.2.4 Pipette 5 µl supernatant of each sample into PCR tubes number 5-7 for use as DNA template

1.3 DNA template from colonies that produce clear zone from 28 colonies in replica plates, select colonies resembling *Ped. pentosaceus*, *En. faecium* and *Lc. lactis*, one colony for each

1.3.1 Re-suspend cells in 200 µl TE buffer

1.3.2 Boil for 10 min, then put on ice immediately for 1 min

1.3.3 Centrifuge at 10,000 rpm for 10 min

1.3.4 Pipette 5 µl supernatant of each sample into PCR tubes number 8-10 for use as DNA template

Q: Why do you need to use TE as a buffer in the steps of DNA template preparation?

Discuss this answer in your group and check your answer with your TA.

2. PCR reactions

2.1 Pipette DNA template into PCR tubes as following:

- Tube 1: Positive control (DNA): Use 5 µl standard DNA (DNA mixture of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis*)
- Tubes 2-4: Use 5 µl supernatant of boiled culture broth of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis* each tube
- Tube 5: Use 5 µl supernatant of boiled Nham sample contaminated with bacteriocin-producing bacteria
- Tubes 6-7: Use 5 µl supernatant of two food samples
- Tubes 8-10; Use supernatant of three selected colonies
- Tube 11: Negative control: Use 5 µl sterilized 3A water instead of DNA template

2.2 Now pipette Solution I and Solution II as stated in Table 4.5 into each of the 11 PCR tubes

2.3 Mix each tube well, spin down for 5 min, then put all tubes into the PCR machine.

Table 4.5 Volume of solution for PCR (total volume 20 µl)

Solution	Volume (µl)
Solution I	14.5
Solution II	0.5
DNA template	5.0
Total	**20.0**

1. The composition of solution I is shown in Table 4.6.
2. Solution II is 5U/µl *Taq* DNA polymerase
3. Standard DNA (DNA mixture of *Ped. pentosaceus*, *En. faecium* and *Lc. lactis*) (20 ng/µl)

Table 4.6 Composition of solution I (100 reactions)

Reagents	Stock concentration	Volume (µl)	Final concentration
Tris-KCl PCR buffer	100 mM Tris HCl pH 8.3, 500 mM KCl (10x)	200	10 mM Tris HCl pH 8.3, 50 mM KCl
$MgCl_2$	15 mM (10x)	200	1.5 mM
dNTPs (mixture)	10 mM	40	200 µM
Primers	20 µM Ped F	40	0.4 µM
	20 µM Ped R	40	0.4 µM
	20 µM EntA forward	40	0.4 µM
	20 µM EntA reverse	40	0.4 µM
	20 µM NisR forward	40	0.4 µM
	20 µM NisR reverse	40	0.4 µM
Sterilised 3A water		770	

3. PCR conditions

Initial Denaturation	94 ºC, 5 min	
Denaturation	94 ºC, 1 min	30 cycles
Annealing	55 ºC, 30 sec	
Extension	72 ºC, 45 sec	
Final Extension	72 ºC, 5 min	

The initial denaturation step which results in the complete denaturation of the DNA template at the start of the PCR reaction is of key importance. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and in a poor yield of PCR product. The initial denaturation should be performed over an interval of 1-5 min at 94-95°C if the GC content is 50% or less. This interval should be extended up to 10min for GC-rich templates.

Denaturation step: Usually 0.5-2min at 94-95°C is sufficient, since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and is completely denatured under these conditions. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 min.

Annealing step: Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2 min is

usually sufficient. However, if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature should be optimized by increasing it stepwise by 1-2°C.

Extension step: Usually the extending step is performed at 70-75°C. The rate of DNA synthesis by *Taq* DNA Polymerase is highest at this temperature. The extending time of DNA amplification is usually 1min for each 1000 bp.

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient.

Final Extension: After the last cycle, the samples are usually incubated at 72°C for 5-7 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA Polymerase adds extra A nucleotides to the 3'-ends of PCR products.

After PCR amplification was completed, all PCR samples will be stored at -20°C until use on Day 5.

Day 5

Having performed PCR on your samples in the last laboratory session, you now need to visualize and analyze the PCR results. In this experiment, you will perform agarose gel electrophoresis which is a method for the interpretation of PCR results.

Agarose gel electrophoresis is a method used in molecular biology to separate DNA strands by size, and to estimate the size of the separated strands by comparison to known fragments (DNA ladder). This is achieved by pulling negatively charged DNA molecules through an agarose matrix with an electric field. Shorter molecules move faster than longer ones.

Expected outcomes for Day 5 session

1. Students have run an agarose gel using your PCR product.
2. Students have critical inquiry skills to interpret your laboratory results.
3. Students have skills in PCR result interpretation and gel electrophoresis.
4. Students may have enhanced positive attitudes towards biotechnology and its application to their lives.
5. Students have an understanding of how PCR can be used in a biotechnological application.

Materials

(For one group of five students)

1	Parafilm sheet 2x2 cm	1 piece
2	Automatic pipette (20 µl)	1 pipette
3	Sterilised pipette tip (200 µl)	1 box
4	Electrophoresis chamber	1 chamber
5	Power supply	1
6	λ DNA marker (cut with *Pst*I) (5 µl)	1 tube
7	TE buffer (2.5 ml)	1 tube
8	Loading dye	1 tube
9	Agarose gel	1 piece

(For all of students in class of 50 students)

1	Ethidium Bromide Solution (1µg/ml) (300 ml) in tray	2 trays
2	Destaining water (500 ml) in tray	2 trays
3	TBE for run gel	5 liters
4	UV transilluminator	1
5	Gloves	1 box

Method

1. Prepare 1% agarose gel as described in the appendix
2. Method for PCR results analysis
    - 2.1 Put the prepared 1% agarose gel into the electrophoresis chamber
    - 2.2 Pour 1X TBE buffer into the chamber to cover the gel
    - 2.3 Pipette 11 drops of 2 µl DNA loading dye onto parafilm
    - 2.4 Pipette 5 µl PCR product, mix (by pipetting) with dropped loading dye, one drop for each sample
    - 2.5 Load 5 µl λ*Pst*I DNA marker into the first well of the agarose gel and load samples into one well each.
    - 2.6 Cover the lid of the chamber and connect the power supply and switch on. The electric current will run from + pole to – pole, use 100V.
    - 2.7 When the dye has run to the other end of gel, switch off the power supply, remove gel and stain in Ethidium Bromide for 5 min
    - 2.8 Destain gel in water for 10 min
    - 2.9 Observe DNA bands under UV transilluminator. Students will see orange luminescent bands marking the position of DNA fragments.

Note: Ethidium Bromide is a mutagenic substance. Students MUST wear gloves during the experiment and be careful not to spread this substance to other places.

Result Analysis: If the foods are contaminated with bacteria harboring nisin, enterocin and pediocin genes, you will observe 608, 412 and 332 bp DNA bands, respectively. (See Figure. 4.2)

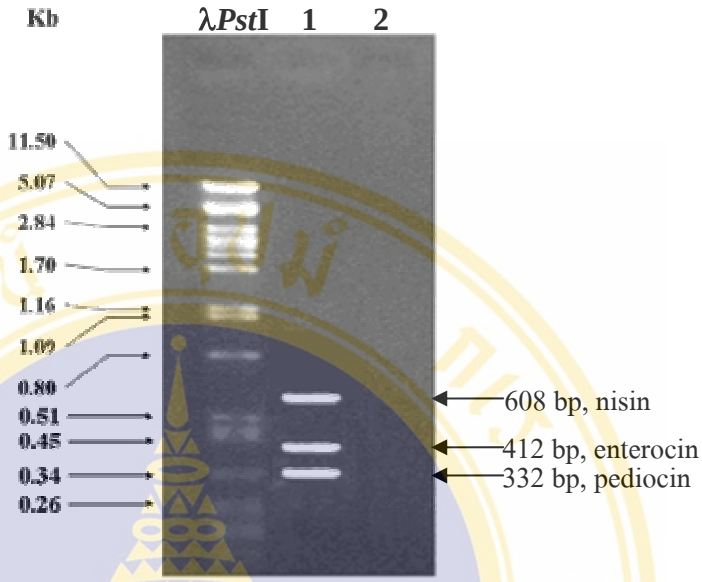


Figure 4.2 Agarose gel showing DNA marker (λ*Pst*I) and positive control DNA, for nisin (608 bp), enterocin (412 bp) and pediocin (332 bp) genes (lane 1); DNA mixture of and *Lc. lactis*, *En. faecium* and *Ped. pentosaceus* negative control (without DNA template) (lane 2)

Table 4.7 PCR results

Tubes	**DNA template from**	**PCR results** (+ =PCR product obtained, - = PCR product not obtained)		
		nisin (608 bp)	**enterocin (412 bp)**	**pediocin (332 bp)**
1	DNA mixture			
2	*Ped. pentosaceus*			
3	*En. faecium*			
4	*Lc. lactis*			
5	Nham sample			
6	Food sample 1			
7	Food sample 2			
8	Selected colony 1			
9	Selected colony 2			
10	Selected colony 3			
11	Negative control (water)			

Students should discuss these questions with your peers in your group, then answer them in your laboratory report.

Questions

9. Explain the principles of PCR? .
10. If you did not obtain PCR products, what do you think could be the cause?
11. How high is the annealing temperature used to affect this PCR reaction?
12. How does contamination with DNA from other bacteria affect the PCR result?
13. Why are primers used in PCR?
14. Can you use any other DNA polymerase, such as DNA polymerase from *E. coli*, in PCR?
15. Compare using microbiological and PCR methods to screen for bacterioicin-producing bacteria. Explain the disadvantages and advantages of each method.
16. Which PCR method will yield better results, isolated DNA or boiled cell? Why? Why not?
17. If you get unexpected PCR results, what are the probable causes?
18. If you get other DNA bands, not expected bands, what are the probable causes?

Report

Your laboratory report should include:

1. Objective
2. Introduction
3. Materials
4. Methods
5. Results
6. Discussion
7. Conclusion
8. Answers of the questions 1-18 on pages 12 and 26 in the Student Work Sheet

Appendix

Media and solutions preparation

1. Tryptic Soy Broth/Agar

TSA broth (synthetic medium of Difco) 1 litre consists of:

1	Pancreatic digest of casein	17.0g
2	Enzymatic digest of soybean meal	3.0g
3	Sodium chloride	5.0g
4	Dipotassium phosphate	2.5g
5	Dextrose	2.5g

Prepare TSA 3% NaCl by weighing TSB powder 15 g, NaCl 12.5 g (2.5%) and agar 7.5 g (1.5%) in 500 ml of water in 1000 ml flask. Cover with silicone, then autoclave at 121 ºC, 15 Pa/I^2 for 15 min, leave it warm, pour into plates, 20-25 ml per plate (approximately 22 plates), leave until agar solidifies, and keep plates in refrigerator.

For TSB 3% NaCl, prepare by the same method as TSA but without addition of agar and should stir well before pipetting into tubes, 5 ml for each tube. Cover tubes by using silicone, autoclave at 121 ºC, 15 Pa/I^2 for 15 min, then keep until use.

2. de Man, Rogosa, Sharpe (MRS) Broth/Agar

MRS broth (synthetic medium of Oxoid) 1 liter consists of

1	Peptone	10.0g
2	‘Lab-Lemco’ Powder	8.0g
3	Yeast extract	4.0g
4	Glucose	20.0g
5	‘Tween’ 80	1 ml
6	Di-potassium hydrogen phosphate	2.0g
7	Sodium acetate $3H_2O$	5.0g
8	Tri-ammonium citrate	2.0g
9	Magnesium sulphate $7H_2O$	0.2g
10	Manganese sulphate $4H_2O$	0.05g

Prepare MRS agar by weighing MRS powder 26.4 g and agar 7.5 g (1.5%) in 500 ml of water in 1000 ml flask. Cover with silicone, then autoclave at 121 ºC, 15 Pa/I^2 for 15 min, leave it warm, pour into plates, 20-25 ml per plate (approximately 22 plates), leave until agar solidifies, keep plates in refrigerator.

For MRS broth, prepare by the same method as MRS agar, but without the addition of agar, and stir well before pipetting into tubes, 5 ml for each tube. Cover tubes by using silicone, autoclave at 121 ºC, 15 Pa/I^2 for 15 min, then keep until use.

3. Preparation of Agarose gel

1. Prepare 1% agarose gel by weighing 1 g of agarose in 100 ml 1X TBE buffer solution, heat in microwave oven until agarose melts. Leave until it becomes warm.
2. Pour agarose gel into a tray with comb, gel thickness should be 3-5 mm without bubbles. Leave it for solidification, about 25-30 min.
3. When agarose solidifies, remove comb carefully, put the gel in electrophoresis chamber by putting well side at the - pole
4. Pour 1X TBE buffer into electrophoresis chamber to cover the gel.

4. TE buffer

Prepare by mixing Tris-HCl and EDTA to become 10 mM Tris-HCl, 1 mM EDTA. Adjust pH to 8.0, autoclave at 121 ºC, 15 Pa/I^2 for 15 min.

5. TBE (Tris-Borate-EDTA) buffer

1X TBE buffer consists of 90 mM Tris-borate, 2 mM EDTA pH 8.0.

Prepare by weighing 17 g TBE powder (Amresco), dissolve 1 l in water

1X TBE buffer for agarose gel preparation. Filter by 0.45 µM membrane before use.

References

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of Gram-positive bacteria. *Microbiological Reviews, 59*(2), 171-200.

Moreno, M. R. F., Rea, M. C., Cogan, T. M., & Vuyst, L. D. (2003). Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. *International Journal of Food Microbiology, 81*, 73- 84.

Moreno, M. R. F., Sarantinopoulos, P., Tsakalidou, E., & Vuyst, L. D. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology, 106*, 1-24.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., & Panyim, S. (2003). Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology, 81*, 137-145.

APPENDIX D

Teacher instructions

	A week before laboratory session I
Activities	Topics about bacteriocins from journal articles are assigned to each group to read for the discussion and the selection of foods to be tested. The topics are: 1) What are bacteriocins? 2) Where can bacteriocin-producing bacteria be found? 3) What are the applications of bacteriocins?
Principles of Intervention	2. Students need to know bacteriocin and its application. 2.1 Students understand the nature and structure of bacteriocin. 2.2 Bacteriocins are used as biological control 2.3 Bacteriocins are used in food preservations 2.4 Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method. 3.4 An application of bacteriocins is indirectly used in many kinds of fermented food in Thailand. Students need to know about these applications because they can be used instead of food preservatives.
Expected Outcomes	1. Students know about bacteriocins. 2. Students know some food sources of bacteriocin-producing bacteria. 3. Students know about the applications of bacteriocins.

	Day 1
Activities	
First 15 min	Each group discusses and identifies the important ideas in the assigned readings.
Next 15 min	A whole class discussion is led by the teacher according to those topics the students were assigned to read. Each group will be asked randomly: What are bacteriocins? Where can bacteriocin-producing bacteria be found? What are the applications of bacteriocins? (Guided answers are shown in Table 1 in Teacher Instructions.) Teacher briefs the lab students that today's tasks are the isolation of LAB and preparation of bacterial cells for PCR. If students want to exchange their food samples with other groups after this discussion, the teacher allows them to do that. This may need to happen if students in each group bring the same samples, so that they have at least two different samples per group, or they bring sterilized samples, as students cannot grow bacteria from the sterilized samples.
Next 2.30 h	Students follow their lab instruction in Student Work Sheets. • 30 min for the first step of LAB isolation; students weigh food samples into tubes containing liquid media and enrich them (see p. 4-5 of Student Work Sheets for procedure). • During 1 h waiting time, students use: o 15 min for a written quiz, set by instructor, relevant to their reading and discussion. o 15 min to complete Table 4.2, p.5 in Student Work Sheets • 45 min for collection of bacterial cells for PCR on the Day 4 (see steps 3-4, p.5 of Student Work Sheets for procedure). • 45 min for doing serial dilution and plating (see step 5, p.5 of Student Work Sheets for procedure).

Principles of Intervention	2. Students need to know the applications of bacteriocins. 2.1 Students understand the nature and structure of bacteriocin. 2.2 Bacteriocins are used as biological control 2.3 Bacteriocins are used in food preservations 2.4 Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method. 3.1 Learning in biotechnology is enhanced through social interaction such as group work. 3.4 An application of bacteriocins is indirectly used in many kinds of fermented food in Thailand. Students need to know about these applications because they can be used instead of food preservatives.
Expected Outcomes	1. Students know about bacteriocins. 2. Students know some food sources of bacteriocin-producing bacteria. 3. Students know about the applications of bacteriocins. 4. Students have developed skills in LAB isolation and have isolated LABs from their food samples. 5. Students have prepared bacterial cell samples for PCR work on Day 4.

	Day 2
Activities First 1 h	Students read about testing bacteriocin activity by microbiological methods from selected journal articles, then discuss these in their groups. Each group identifies advantages and disadvantages of each microbiological method. (One TA supervises two groups. Expected answers are shown in Table 2 in Teacher Instructions)
Next 30 min	The teacher leads in drawing comparisons, identifying advantages and disadvantages among methods and explains why the students should perform a microbiological method, an overlay method, for this screening of bacteriocin-producing bacteria and why this method has been chosen over the others.
Next 30 min	Students follow the lab instructions to examine plates from the last session, and record results in Table 4.3, p.7 in Student Work Sheets and to select plates containing 30-300 bacterial colonies for overlay with tester strains (*V. harveyi* and *Lb. plantarum*) (see p.6-7 of Student Work Sheets for procedure).
Next 45 min	Students follow the lab instructions to overlay the selected plates with the tester strains (see p.7 of Student Work Sheets for procedure) Note: Students should be aware not to overlay melted agar that is too hot because it may damage or kill the heat sensitive strains. Students wait until overlay agar solidifies, then incubate plates in incubator at 30°C for 24 hrs.
Principles of Intervention	2.4 Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method. 3. Teaching and learning can be described by sociocultural views of learning 3.1 Learning in Biotechnology is enhanced through social interaction such as

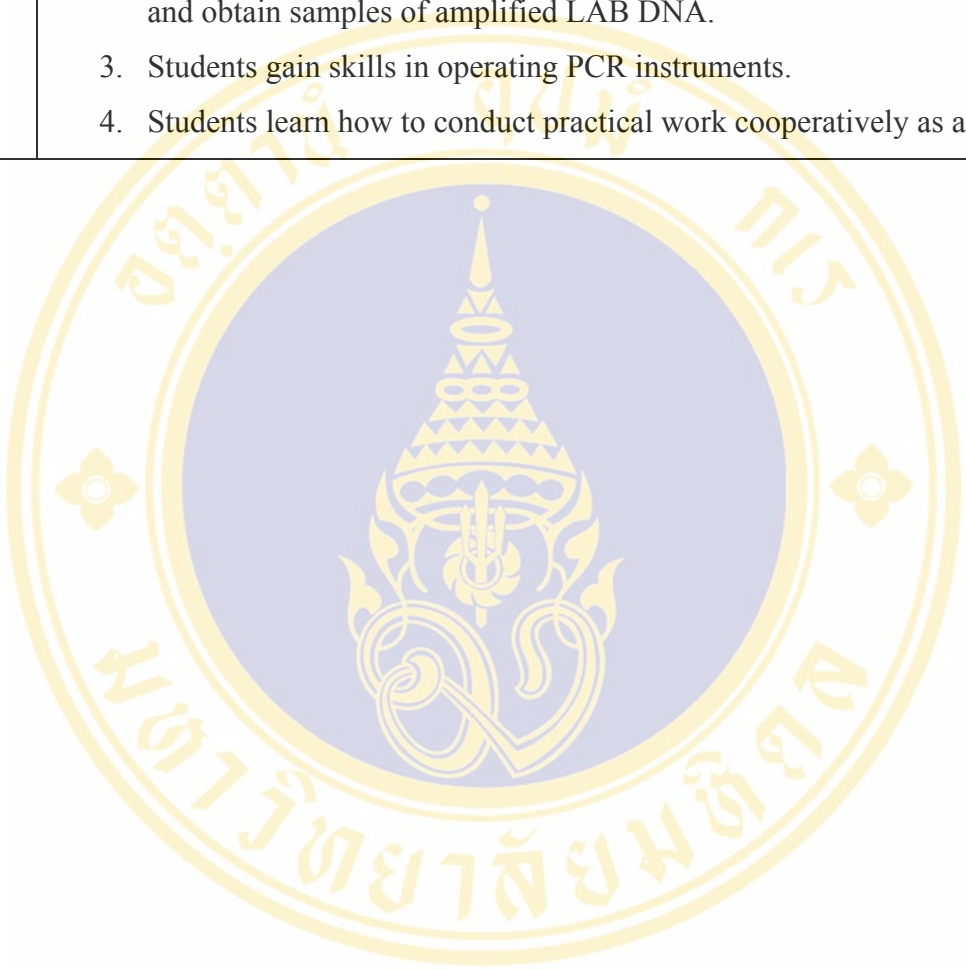
	group work. 3.2 Teachers and teaching assistants, and well-designed laboratory protocols with clear instructions, provide scaffolding for students.
Expected Outcomes	1. Students know about methods for testing for bacteriocin activity, including their advantages and disadvantages. 2. Students have skills in testing for bacteriocin activity by a microbiological method. 3. Students learn how to read journal articles and to critique them though discussion with their peers and teacher.

	Day 3
Activities	
First 30 min	Students follow the lab instructions. They observe clear zones on the six plates from Day 2, and record results in Table 4.4, p.9, in Student Work Sheets, then pick colonies that produce clear zones (see p. 8 of Student Work Sheets for procedure).
Next 15 min	The teacher leads the whole class discussion by using a PCR diagram (p. 14 of Student Work Sheets). She/he stresses how PCR works, etc.
Next 45 min	Each group reads and discusses a paper (Noonpakdee, Santivarangkna, Jumriangrit, Sonomoto, & Panyim, 2003) about using PCR to amplify bacteriocin genes for the next lab session. TAs guide each group to make a short summary as suggested in Table 3 in Teacher Instructions. TAs also directs students to take a closer look at Fig.3 p. 142 in the provided article to see nisin gene sequence and annealing site of primers. For the following performance, students work in ones or twos in their group to take responsibility for each gene and each pair of primers to make sure that everyone in each group will go though every process.
Next 30 min	Students use a Web page, www.ncbi.nlm.nih.gov, to search for pediocin, enterocin, and nisin genes by using nucleotide search (see p.10 of Student Work Sheets for ***Nucleotide searching*** procedure).
Next 30 min	Then, the students use a CLUSTALW program (available online in the page www.ebi.ac.uk/clustalw/) for sequence alignment. Students will see a conserved region we used for primer design to detect each gene (see p.10-11 of Student Work Sheets for ***Sequence alignment*** procedure).
Next 30 min	Then, students use the BLAST program available online in the page www.ncbi.nlm.nih.gov to search for the similarities of the designed primers and

	sequences in GENBANK. Students will see that the designed primers have a 100% similarity only with those specific nucleotides of pediocin, enterocin, and nisin (see p. 11-12 of Student Work Sheets for ***Similarity searching*** procedure).
Principles of Intervention	1. Students studying Biotechnology need to learn the principle of PCR and how to use PCR machine (PI1). 1.1 Students understand the principle of PCR. 1.2 PCR is an important tool for research in Biotechnology. 1.3 PCR can help students to understand DNA structure and replication. 2.4 Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method. 3.2 Teachers and teaching assistants, and well-designed laboratory protocols with clear instructions, provide scaffolding for students. 4.1 Knowledge: PCR principles and the applications of bacteriocins can be emphasized during the lab sessions.
Expected Outcomes	1. Students obtain microbiological data about LABs from foods. 2. Students know about principles of PCR and the application of PCR to detect bacteriocin genes. 3. Students develop an improved understanding of the structure of DNA and how that can be applied to PCR. 4. Students know that in PCR research they need to perform sequence alignment and similarity searching, and design of primers. 5. Students understand the methods used and develop some skills in sequence alignment and similarity searching, and primer design.

	Day 4
Activities First 15 min	Quiz: a written quiz, set by instructor, relevant to knowledge students gained from Day 3. The teacher stresses how PCR works, etc. Then, she/he provides an introduction to the lab.
Next 2.45 h	Students follow the lab instructions. • 1.30 h for DNA template preparation (see p.16 of Student Work Sheets for procedure) • 45 min for preparation of PCR reactions (see p.17 of Student Work Sheets for procedure) • 15 min for using the PCR machine according to PCR conditions provided by TAs (see p.17 of Student Work Sheets for procedure)
Principles of Intervention	2. Students need to know the applications of bacteriocins. 2.1 Students understand the nature and structure of bacteriocin. 2.2 Bacteriocins are used as biological control 2.3 Bacteriocins are used in food preservations 2.4 Bacteriocin genes can be detected by PCR, in addition to testing the bacteriocin activity by a microbiological method. 3.2 Teachers and teaching assistants, and well-designed laboratory protocols with clear instructions, provide scaffolding for students. 3.3 Using PCR to detect bacteriocin genes in foods in the laboratory can lead students into the biotechnological research culture. 4.1 Knowledge: PCR principles and the applications of bacteriocin can be emphasized during the lab sessions. 4.2 Skills: PCR skills for bacteriocin screening should be used as a model for student's development of practical skills (training students to use

	PCR machine, and biological screening method).
Expected Outcomes	1. Students clarify their knowledge about PCR. 2. Students learn how to prepare DNA, prepare PCR reactions, run PCR and obtain samples of amplified LAB DNA. 3. Students gain skills in operating PCR instruments. 4. Students learn how to conduct practical work cooperatively as a team.



	Day 5
Activities	
First 30 min	Students follow the lab instructions beginning with the step 2 (see p. 21 of Student Work Sheets for procedure) • Students set the electrophoresis chamber and power supply • Students load PCR samples into each well of agarose gel. Note: Do not forget to load 5 µl λ*Pst*I DNA marker into the first well
Next 45 min	While students are waiting for gel electrophoresis to run, they should study and discuss information from Figure 4.2 on p. 22 of the Student Work Sheets. From Figure 4.2, students should know that nisin, enterocin, and pediocin genes amplified by these primers will result in 608, 412, and 332 bp DNA bands on the gel, respectively. They should also review process needed to do gel electrophoresis and principles of gel electrophoresis. That is, agarose gel electrophoresis is a method used in molecular biology to separate DNA strands by size, and to estimate the size of the separated strands by comparison to known fragments (DNA ladder). This is achieved by pulling negatively charged DNA molecules through an agarose matrix with an electric field. Shorter molecules move faster than longer ones. Thus, students should understand why the three bands are expected to appear in the position as shown in Figure 4.2.
Next 30 min	Students stain and de-stain their gels, then they take a photo of each gel.
Next 15 min	After students observe PCR results in the gel, they complete Table 4.7, p.23 in the Student Work Sheets and discuss it in their groups. If the foods were contaminated with bacteria harbouring nisin, enterocin, and pediocin genes, students will observe 608, 412, and 332 bp DNA bands, respectively (see Figure 4.2, p. 22 of Student Work Sheets).
Next 30 min	Then the teacher leads a discussion about the PCR results, including any unexpected outcomes. The teacher also asks students to compare the advantages

	and disadvantages between the microbiological method and PCR method. The teacher asks whether students prefer the microbiological method or PCR method. After the labs have finished, each group writes their lab report and answers questions 1-20 posed in the Student Work Sheets, then sends it in next week. This write up will be done outside class time.
Principles of Intervention	3.3 Using PCR to detect bacteriocin genes in foods in the laboratory can lead students into the biotechnological research culture. 4.3 Critical inquiry: Encourage students to interpret their laboratory results such as giving reason to explain their empirical data when they can not obtain the expected PCR results, etc. In addition, bioinformatics information can be learned trough internet access. 4.4 Scientific attitude: Students can see PCR as relevant to their daily lives. This should bring students motivation and enjoyment.
Expected Outcomes	1. Students have PCR data on an agarose gel. 2. Students have critical inquiry skills to interpret their laboratory results. 3. Students have skills in PCR results interpretation and gel electrophoresis. 4. Students may have enhanced positive attitudes towards biotechnology and its application to their lives. 5. Students have an understanding of how PCR can be used in a biotechnological application.

Table 1 Questions and guided answers from assigned readings

Questions	Guided answer
What are bacteriocins?	Bacteriocins are ribosomally synthesised, extracellularly released, antibacterial peptides. They display a limited inhibitory spectrum. They kill, particularly, closely related strains. Large numbers of bacteriocins are produced by all genera of the Lactic Acid Bacteria (LAB) (Jack, Tagg, & Ray, 1995; Moreno, Rea, Cogan, & Vuyst, 2003).
Where can the bacteriocin-producing bacteria be found?	o Fermented vegetables; e.g. Kimji o Fermented fish and meat products; e.g. fish Nham, pork Nham, beef Nham o Cheese
What are the applications of bacteriocins?	Bacteriocins are used in food technology as food preservatives against food borne pathogens. LABs, e.g. lactobacilli, pediococci, have been used as starter cultures for fermentation. But in many fermented meat products in Thailand, the technology of fermentation is still mediated by indigenous bacteria rather than added starter cultures. The students will analyse some food samples for presence of indigenous LABs.

Table 2 The comparison among bacteriocin testing methods

Methods	What to do	Advantages	Disadvantages
Overlay	1. Spread the bacteriocin-producing strains on a plate and incubate overnight. 2. Culture the tester strain in liquid media overnight (We can do this on the same day as No.1). 3. On the next day, mix the culture of tester strain with soft agar media, then overlay on top of the bacteriocin plate, incubate overnight. 4. Observe for clear zones the next day, showing where the LAB has produced bacteriocin.	1. It takes a short period of time for testing (3 days).	1. We have to be very careful about the death of heat sensitive tester strains when adding to the soft agar at 45^0C.
Co-culture	1. Culture the purified bacteriocin-producing and tester strains separately in liquid media overnight. 2. Culture the two strains together in the same flask/test tube overnight. 3. Perform serial dilutions and spread plates, incubate overnight. 4. Perform plate count, compare the number of colonies between the two strains.	1. We can follow the changes of number of both strains during co-cultivation by taking samples and plates several times.	1. It takes a longer period of time for testing (4 days). 2. We have to be accurate in identifying the differences in colonies of both strains. 3. We can test only two strains each time, one bacteriocin-producing strain and against

			one tester strain. 4. We cannot know exactly that each culture started with the same number of cells, even if we measure turbidity of culture by spectrophotometer or perform plate count before co-culture.
Agar-well diffusion	1. Culture a tester strain in liquid media overnight. 2. Spread the tester strain on a plate and incubate overnight. 2. Culture the bacteriocin-producing strain in liquid media overnight (We can do this on the same day as No.1). 3. Make wells in the plate from No. 1. 4. Fill each well with filtrate of culture from No. 2 5. Incubate overnight, and then observe clear zones on the next day.	1. We can measure concentration of active substance we tested.	1. It takes a longer period of time for testing (4 days). 2. We can test only two strains each time.

Table 3 Guide for short summary from a paper assigned on Day 3

Questions	Answers
What kind of food did the researchers use?	Thai fermented sausage, Nham
What kind of bacteria were they looking for?	LAB, *Lactococcus lactis*
What method did they use to screen for bacteriocin-producing bacteria?	A microbiological method, overlay method
Why did they use PCR?	To confirm that this strain is nisin-producing bacteria and harbours nisin gene
What genes had been detected?	The gene encoding nisin
What are the primers?	The primers were designed from nisin A structural gene, which were complementary to regions 17 bp upstream and 2 bp downstream of the coding region. primer 1: 5' CCG GAATTC ATA AGG AGG CAC TCA AAA TG 3' primer 2: 5' CGG GGT ACC TAC TAT CCT TTG ATT TGG TT 3'
What are the PCR conditions?	30 cycles of 90 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min
What is the size of PCR product?	227-bp
What is the importance of this work?	The bacteriocin produced by *Lc. lactis* WNC 20 may be useful in improving the food safety of the fermented product.

References

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of Gram-positive bacteria. *Microbiological Reviews, 59*(2), 171-200.

Moreno, M. R. F., Rea, M. C., Cogan, T. M., & Vuyst, L. D. (2003). Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. *International Journal of Food Microbiology, 81*, 73- 84.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., & Panyim, S. (2003). Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology, 81*, 137-145.

APPENDIX E

Questionnaire (before the laboratory)
Screening of bacteriocin-producing bacteria

This survey is a part of my PhD research study. I am interested in your ideas and attitudes towards biotechnology prior to doing the "Screening of Bacteriocin-Producing Bacteria" experiment. Please answer the following questions as honestly as possible.

Your responses will only be seen by the researcher, and not by any member of the teaching staff.

Student ID……..

1. Have you ever heard of bacteriocins?

YES / NO (circle one)

1a. If you answered NO, go to Question 2

Or: If you answered YES, What do you think a bacteriocin is, and what does it do?

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1b. What do you think bacteriocins are used for in Thailand?

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2. A bio-active substance is one produced by a living organism that can have an effect on or interact with other living organisms. What bio-active substances do you know of that can be used as food preservatives?

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3. List any methods you know of which can be used to test the activity of bio-active substances.

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4. Explain the DNA replication process.

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5. Explain the Polymerase Chain Reaction (PCR).

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6. What can PCR be used for?

……..

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7. Do you know how to perform PCR in the laboratory?

YES / NO (circle one)

8. Have you ever used PCR?

YES / NO (circle one)

(Tick the one that applies)	Strongly Disagree 1	Disagree 2	Neither 3	Agree 4	Strongly Agree 5
9. DNA replication is difficult to understand.	☐	☐	☐	☐	☐
10. PCR is an important tool for research in biotechnology.	☐	☐	☐	☐	☐
11. PCR is difficult to understand.	☐	☐	☐	☐	☐
12. PCR is relevant to my daily life.	☐	☐	☐	☐	☐
13. Biotechnology laboratory classes give me the skills I need to work in biotechnology.	☐	☐	☐	☐	☐
14. I usually understand all instructions given in student work sheets.	☐	☐	☐	☐	☐
15. I usually understand all the reasons behind activities I have done in biotechnology experiments.	☐	☐	☐	☐	☐

16. If I don't know the reasons behind tasks in the biotechnology experiments, I do these things in this order:

☐ ask my classmates ☐ search in books

☐ ask the teacher ☐ search on the internet

☐ ask teaching assistants ☐ ignore them

☐ other (specify)……

17. If I don't know how to do in tasks in the biotechnology experiments, I do these things in this order:

☐ ask my classmates ☐ search in books

☐ ask the teacher ☐ search on the internet

☐ ask teaching assistants ☐ ignore them

☐ other (specify)……

(Tick the one that applies)	Strongly Disagree 1	Disagree 2	Neither 3	Agree 4	Strongly Agree 5
18. Lab sessions help me to better understand abstract concepts in biotechnology.	☐	☐	☐	☐	☐
19. Lab sessions help me to better understand applications of biotechnology.	☐	☐	☐	☐	☐
20. My biotechnology laboratory experiments are normally successful.	☐	☐	☐	☐	☐
21. I like to find out the reasons for any problems in my laboratory experiments.	☐	☐	☐	☐	☐
22. I feel confident in interpreting my laboratory results.	☐	☐	☐	☐	☐
23. Teachers help me to learn during biotechnology laboratory classes.	☐	☐	☐	☐	☐
24. Teaching assistants help me to learn during biotechnology laboratory classes.	☐	☐	☐	☐	☐
25. I like working in a group of five in biotechnology laboratories.	☐	☐	☐	☐	☐

25a. Please explain what you like and/or don't like about working in groups.

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		Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
	(Tick the one that applies)	1	2	3	4	5
26.	Working as part of a group in the biotechnology laboratories helps me to learn better than working as an individual.	☐	☐	☐	☐	☐
27.	As a group, everyone in the group helps each other to learn.	☐	☐	☐	☐	☐
28.	I understand every process I have been asked to do in the biotechnology laboratories.	☐	☐	☐	☐	☐
29.	From my biotechnology laboratory experiences, I feel confident in my ability to do biotechnology research.	☐	☐	☐	☐	☐
30.	I feel I am a biotechnology researcher when I do the biotechnology laboratories.	☐	☐	☐	☐	☐
31.	I enjoy doing biotechnology laboratory classes.	☐	☐	☐	☐	☐
32.	Biotechnology laboratories motivate me to work in the biotechnological area.	☐	☐	☐	☐	☐

Thank you very much

for taking the time to complete this questionnaire.

Questionnaire (after the laboratory)

Screening of Bacteriocin-Producing Bacteria

This survey is a part of my PhD research study. I am interested in what you have learned and about your experiences, in the "Screening of Bacteriocin-Producing Bacteria" laboratory classes.

Your responses will only be seen by the researcher, and not by any member of the teaching staff.

Student ID……..

1. Have you ever heard of bacteriocins?

YES / NO (circle one)

1a. If you answered NO, go to Question 2

Or: If you answered YES, What do you think a bacteriocin is, and what does it do?

……..

……..

……..

1b. What do you think bacteriocins are used for in Thailand?

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2. A bio-active substance is one produced by a living organism that can have an effect on or interact with other living organisms. What bio-active substances do you know of that can be used as food preservatives?

……..

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3. List any methods you know of which can be used to test the activity of bio-active substances.

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4. Explain the DNA replication process.

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5. Explain the Polymerase Chain Reaction (PCR).

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6. What can PCR be used for?

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7. Do you know how to perform PCR in the laboratory?

YES / NO (circle one)

8. Have you ever used PCR?

YES / NO (circle one)

		Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
	(Tick the one that applies)	1	2	3	4	5
9.	DNA replication is difficult to understand.	☐	☐	☐	☐	☐
10.	PCR is an important tool for research in biotechnology.	☐	☐	☐	☐	☐
11.	PCR is difficult to understand.	☐	☐	☐	☐	☐
12.	PCR is relevant to my daily life.	☐	☐	☐	☐	☐

The following questions relate to the Screening of Bacteriocin-Producing Bacteria experiment that you have just completed.

		Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
	(Tick the one that applies)	1	2	3	4	5
13.	After performing this experiment, I understand DNA replication better.	☐	☐	☐	☐	☐
14.	After performing this experiment, I understand the principles of PCR better.	☐	☐	☐	☐	☐
15.	Using PCR was faster than the microbiological method for detecting bacteriocin-producing bacteria.	☐	☐	☐	☐	☐
16.	Using PCR to detect bacteriocin-producing bacteria was less accurate than using the microbiological method.	☐	☐	☐	☐	☐

17. Would you rather use the microbiological or PCR method to screen for bacteriocin-producing bacteria? Explain the reason for your answer.

……..

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……..

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……..

	Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
(Tick the one that applies)	1	2	3	4	5
18. This experiment has given me the skills I need to work in biotechnology.	☐	☐	☐	☐	☐
19. I understood all the instructions given in student work sheets in this experiment.	☐	☐	☐	☐	☐
20. I understood all the reasons behind the tasks I have done in this experiment.	☐	☐	☐	☐	☐

21. When I didn't know the reasons behind tasks in this experiment, I did these things in this order:

☐ asked my classmates ☐ searched in books

☐ asked the teacher ☐ searched on the internet

☐ asked teaching assistants ☐ ignored them

☐ other (specify)……

22. When I didn't know how to do in the tasks in this experiment, I did these things in this order:

☐ asked my classmates ☐ searched in books

☐ asked the teacher ☐ searched on the internet

☐ asked teaching assistants ☐ ignored them

☐ other (specify)……

	Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
(Tick the one that applies)	1	2	3	4	5
23. This experiment helped me to better understand abstract concepts in biotechnology.	☐	☐	☐	☐	☐
24. This experiment helped me to better understand applications of biotechnology.	☐	☐	☐	☐	☐
25. My experiment was successful.	☐	☐	☐	☐	☐
26. I liked to find out the reasons for any problems I had during this experiment.	☐	☐	☐	☐	☐
27. I felt confident in interpreting my results in this experiment.	☐	☐	☐	☐	☐
28. Teachers helped me to learn during this experiment.	☐	☐	☐	☐	☐
29. Teaching assistants helped me to learn during this experiment.	☐	☐	☐	☐	☐
30. I liked working in a group of five in this experiment.	☐	☐	☐	☐	☐

30a. Please explain what you liked and/or didn't like about working in groups in this experiment.

……..

……..

……..

		Strongly Disagree	Disagree	Neither	Agree	Strongly Agree
	(Tick the one that applies)	1	2	3	4	5
31.	Working as part of a group in this experiment helped me to learn better than working as an individual.	☐	☐	☐	☐	☐
32.	As a group, everyone in the group helped each other to learn.	☐	☐	☐	☐	☐
33.	I understood every process I was asked to do in this experiment.	☐	☐	☐	☐	☐
34.	After doing this experiment I felt confident in my ability to do biotechnology research.	☐	☐	☐	☐	☐
35.	I felt I was a biotechnology researcher when I did this experiment.	☐	☐	☐	☐	☐
36.	I enjoyed doing this experiment.	☐	☐	☐	☐	☐
37.	Doing this experiment has motivated me to work in the biotechnological area.	☐	☐	☐	☐	☐

Thank you very much

for taking the time to complete this questionnaire.

APPENDIX F

Interview questions
(two groups students after performing the "screening of bacteriocin-producing bacteria" experiment)

General questions

Firstly I would like to find out a little about you.

1. Tell me about why you decided to come to university to study Biotechnology?
2. Which topics in biotechnology are most interesting to you?
3. Why you are interested in these topics?

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory (in general)

4. How do you feel about learning abstract concepts in biotechnology? (ScEd1)
    a. Do lecture classes help in this learning?
    b. Do laboratory classes help in this learning?
5. Do you prefer learning in biotechnology by doing labs, or reading about it, or listening to a lecture? (ScEd1,2,3)
6. What do you think you might learn in biotechnology laboratory classes? (ScEd1,2)
    a. Knowledge, skills, others?
7. How do you like to learn in biotechnology laboratory classes? (ScEd3)
    a. By doing? By group discussion?

PI 3: Teaching and learning can be described by sociocultural views of learning (in general)

8. Have you been working mainly individually or in a group in biotechnology laboratory classes? (ScEd3)
    a. Normally, how many students in a group?
    b. How do you feel about this way of working?
    c. How would you describe your role in laboratory groups?

d. Do you know how your peers in your group or your classmates feel about working in groups?

9. Who do you think is important to your learning during laboratory classes? (ScEd3)
10. What kind of tools help you learn in biotechnology laboratory classes? (ScEd3)
11. If you need help in the lab classes, who do you ask? Why? (ScEd3)
12. Has anyone else been helpful to your learning in the laboratory classes? How have they helped you learn? (ScEd3)

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory (focused on the "Screening of bacteriocin-producing bacteria" experiment)

13. What do you think was the purpose of this experiment? (ScEd1,2,3)
14. What did you learn from doing this experiment? (ScEd1,2,3)
15. What knowledge have you gained in this experiment? How have you learned it? (ScEd1,3)
16. What skills have you learned in this experiment? How have you learned them? (ScEd2,3)
17. Has this experiment helped you to understand abstract concepts such as PCR principles or DNA structure and replication? How? (ScEd1,3)
18. What value do you think biotechnological applications have to your daily life? (ScEd1)
19. Have you felt motivated to do biotechnology research after performing this experiment? (ScEd1,2,3)

    If yes, what motivates you to do so?

PI 3: Teaching and learning can be described by sociocultural views of learning (focused on the "Screening of bacteriocin-producing bacteria" experiment)

20. What value do you place on your experience in this experiment? (ScEd1,2,3)
21. Do you feel that you gained a good understanding of this experiment? How did you gain this understanding? (ScEd1,2,3)

22. Do you feel your way of learning has changed since you have done this experiment? How? (ScEd3)
23. Do you have any suggestions about how to improve this laboratory class?
24. Tell me about your expected future career.
25. Has this experiment changed your feelings about your future career? How?

Interview questions (for TAs after the lab)

General questions

Firstly I would like to find out a little about you.

1. How long have you been working as a TA in this biotechnology laboratory course?
2. What are your roles in this biotechnology laboratory class? (ScEd3)
3. What are your views of teaching and learning that can be used in a biotechnology laboratory class? How can these methods be used to improve learning? (ScEd3)

PI 3: Teaching and learning can be described by sociocultural views of learning (focused on the "Screening of bacteriocin-producing bacteria" experiment)

4. What were your roles in this experiment? (ScEd3)

   Were they difficult?
5. What did you hope to help teach in this experiment? Could you please compare to other biotechnology experiments? (ScEd1,2,3)

   How do you feel about assisting teaching abstract concepts in biotechnology laboratory classes?
6. How did you feel about assisting group work in this experiment? (ScEd3)

   How would you describe the students' roles in the groups in this experiment?

   Could you please compare the group work in this experiment with what you have seen in other biotechnology experiments?
7. What kind of tools (techniques, teaching/learning methods) help students learn in laboratory classes? (ScEd3)
8. What kind of questions did the students ask of you (or teachers, classmates) during a session of this experiment? Could you please compare this to other biotechnology experiments? (ScEd1,2,3)
9. Has anyone else been helpful to students learning in this experiment? How have they helped them learn? (ScEd3)

10. What do you think could help you to better assist teaching in this experiment?

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory (focused on the "Screening of bacteriocin-producing bacteria" experiment)

11. What do you think students might learn in this experiment? Could you please compare this to other biotechnology experiments? (ScEd1,2,3)
12. Do you feel that students gained a good understanding of this experiment? How did they gain this understanding? (ScEd1)
13. Do you feel students developed biotechnology practical skills in this experiment? What helped or hindered this development? (ScEd2,3)
14. Are there any better ways to help students learn abstract concepts such as PCR principles or DNA structure and replication? What are they? How do they help the students? (ScEd1)
15. Are there any other outcomes you think students could learn in this experiment? Could you please compare these to other biotechnology experiments? (ScEd1,2,3)
16. Please give me any suggestions for improving this experiment.

Interview questions (for the teacher before the lab)

Firstly I would like to find out a little about you.

1. How long have you been teaching in this biotechnology laboratory course?
2. What is your role in this biotechnology laboratory class? (ScEd3)
3. What are your views of teaching and learning that can be used in a biotechnology laboratory class? How can these methods be used to improve learning? (ScEd3)

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory

4. What would you expect students to learn in biotechnology laboratory classes? (ScEd1,2,3)
5. Do you feel that students can learn abstract concepts such as PCR principles or DNA structure and replication in biotechnology laboratory classes? (ScEd1)
6. Are there any better ways to learn these abstract concepts? What are they? (ScEd1,2,3)
7. What would you expect students to learn from doing the "Screening of bacteriocin-producing bacteria" experiment? (ScEd1,2,3)

PI 3: Teaching and learning can be described by sociocultural views of learning

8. What kind of tools (techniques, teaching/learning methods) help students learn in biotechnology laboratory classes? (ScEd3)
9. How do you feel about students working in groups in biotechnology laboratory classes? (ScEd3)
10. How would you describe the students' roles in groups in biotechnology laboratory classes? (ScEd3)
11. What do you think is the role of the TAs in helping students learn? (ScEd3)

Interview questions (for the teacher after the lab)

1. How did you feel about teaching in this experiment?

PI 4: Four learning outcomes can be enhanced in the biotechnology laboratory (focused on the "Screening of bacteriocin-producing bacteria" experiment)

2. What kind of learning outcomes do you think students have gained from this experiment? Knowledge, skills, other? (ScEd1,2,3)
3. Do you feel that students gained a good understanding of this experiment? How did they gain this understanding? (ScEd1,3)
4. Do you feel students developed biotechnology practical skills in this experiment? What helped or hindered this development? (ScEd2,3)
5. Are there any better ways to help students learn abstract concepts such as PCR principles or DNA structure and replication? What are they? How do they help the students? (ScEd1,2,3)
6. Do you think there any other outcomes for students and their learning in this experiment? Could you please compare these to other biotechnology experiments? (ScEd1,2,3)

PI 3: Teaching and learning can be described by sociocultural views of learning (focused on the "Screening of bacteriocin-producing bacteria" experiment)

7. What kind of tools helped students learn in this experiment? (ScEd3)
8. How did you feel about students working in groups in this experiment? (ScEd3)
9. How would you describe the students' roles in the groups in this experiment? (ScEd3).

   Could you please compare the group work in this experiment to what you have seen in other biotechnology experiments?

10. What kind of questions did the students ask of you (or TAs, classmates) during a session of this experiment? Could you please compare to other biotechnology experiments? (ScEd1,2,3)
11. Has anyone else been helpful to students learning in this experiment? How have they helped them learn? (ScEd3)
12. Do you think the students have been motivated in this experiment? Could you please compare to other biotechnology experiments? (ScEd1,2,3)
13. Has your experience in teaching this experiment been the same as your expectations? (ScEd1,2,3)
14. Is there anything that you think could help students learn more in this experiment? (ScEd1,2,3)
15. Please give me any suggestions to improve this experiment.

APPENDIX G

Example of observation sheet for laboratory designer

Task on Day 4	**Performance' rating scales***		**Research question**
Task1 Introduction to the lab (15 min)			
Teacher			
Lectures	1 – 2 – 3 – 4 – 5		ScEd3
Asks class questions	1 – 2 – 3 – 4 – 5		ScEd3
Probes students' prior knowledge	1 – 2 – 3 – 4 – 5		ScEd3
Makes links to previous or future classes	1 – 2 – 3 – 4 – 5		ScEd3
Uses practical examples to explain and contextualise concepts	1 – 2 – 3 – 4 – 5		ScEd3
Uses analogies/metaphors to explain and build concepts	1 – 2 – 3 – 4 – 5		ScEd3
Connects to research community	1 – 2 – 3 – 4 – 5		ScEd3
Clear detailed explanation	1 – 2 – 3 – 4 – 5		ScEd3
Provides relevant information and explains concepts	1 – 2 – 3 – 4 – 5		ScEd3
Checks class understanding	1 – 2 – 3 – 4 – 5		ScEd3
Encourages students to think	1 – 2 – 3 – 4 – 5		ScEd3
Writes on a white board	1 – 2 – 3 – 4 – 5		ScEd3
Other			
Students' Behaviours			
Appear Engaged			
Ask questions/make comments	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Answer teacher' questions correctly	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd1
Listen carefully	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Write notes	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Appear understanding (explain to peers, nods etc)	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd1

Example of observation sheet for laboratory designer (continued)

Task on Day 4	**Performance' rating scales***		**Research question**
Students' Behaviours			
Appear Engaged			
Discuss with peers when requested	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Other response to teacher (smiles, nods etc)			
Appear disengaged			
Talk to classmate when teacher talking			
Distracted (asleep, dreaming)			
Appear stressed/rushed			
Task2-4	**Group1**	**Group2**	
• DNA template preparation (1.30 h)			
• Preparation of PCR reactions (45 min)			
• Using the PCR machine (15 min)			
Students' Behaviours			
Appear Engaged			
Manipulate pipette appropriately	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd2
Skills in DNA template preparation	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd2
Skills in preparation of PCR reactions	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd2
Skills in using the PCR machine	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd2
Ask questions/make comments with peers	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Ask questions/make comments with TAs	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Ask questions/make comments with the teacher	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Appear understanding (explain to peers, nods etc)	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd1
Share ideas	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Help classmates with techniques	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Write notes	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3

Example of observation sheet for laboratory designer (continued)

Task on Day 4	**Performance' rating scales***		**Research question**
Students' Behaviours			
Appear disengaged			
Distracted (asleep, dreaming)	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	
Time not on task	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	
Appear stressed/rushed	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	
TAs' Behaviours			
Appear Engaged			
Correct manipulative skills for students	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Ask questions/make comments with students	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Answer student's questions correctly	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Check students' understanding	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd1
Encourage students to think	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Provide time for students to discuss with each other	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	ScEd3
Other			
Appear disengaged			
Distracted (asleep, dreaming)	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	
Talk amongst themselves	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	
Appear stressed/rushed	1 – 2 – 3 – 4 – 5	1 – 2 – 3 – 4 – 5	

For scale 1 - 5, 1 = low and 5 = high

BIOGRAPHY

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