Antimicrobial Peptide from Lactic Acid Bacteria and Development of Antimicrobial Film for Food Packaging

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ เปปไทด์ที่มีฤทธิ์ในการยับยั้งจุลินทรีย์จากแบคทีเรียแลคติกและการพัฒนาฟิล์มต้านจุลินทรีย์เพื่อใช้เป็นบรรจุภัณฑ์สำหรับอาหาร
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บทคัดย่อ

เนื่องจากกระแสความนิยมบรรจุภัณฑ์แอคทีฟที่เป็นบรรจุภัณฑ์สีเขียวที่เป็นมิตรต่อสิ่งแวดล้อม ร่วมกับความต้องการใช้สารปฏิชีวนะจากธรรมชาติ ที่ปลอดภัย ออกฤทธิ์อย่างจำเพาะกับแบคทีเรียเป้าหมาย การพัฒนาแผ่นฟิล์มย่อยสลายได้ทางชีวภาพชนิดพอลิแลคติกแอซิดที่มีฤทธิ์ยับยั้งการเจริญของจุลินทรีย์จากเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์จากเชื้อแบคทีเรียแลคติก สำหรับใช้เป็นบรรจุภัณฑ์อาหาร จึงได้รับความสนใจอย่างมาก อย่างไรก็ตามการผสมเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ที่มีความชอบน้ำเข้าสู่ฟิล์มพอลิเมอร์พอลิแลคติกแอซิดโดยตรง ยังถูกจำกัดด้วยคุณสมบัติความไม่ชอบน้ำของพอลิเมอร์พอลิแลคติกแอซิด ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ที่จะพัฒนาวิธีการผสมเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์จากแบคทีเรียแลคติกเข้ากับฟิล์มพอลิแลคติกแอซิดเพื่อผลิตเป็นบรรจุภัณฑ์อาหารที่มีความสามารถในการยับยั้งจุลินทรีย์และย่อยสลายได้ตามธรรมชาติ

ในขั้นต้น ได้ทำการศึกษาสภาวะที่เหมาะสมสำหรับผลิตเพดิโอซิน พีเอวัน-เอซีเอช (pediocin PA-1/AcH หรือ Ped) ซึ่งเป็นเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ที่ผลิตจากเชื้อ *Pediococcus pentosaceus* BCC 3772 เพื่อปรับปรุงประสิทธิภาพในการผลิตและลดค่าดัชนีสีน้ำตาลของสารละลาย Ped ที่ได้ พบว่า การเลี้ยงเชื้อในอาหารที่เหมาะสมที่ประกอบด้วย โซเดียมเคซีนเนต ร้อยละ 1.5 โดยมวลต่อปริมาตร ยีสต์สกัด ร้อยละ 1.5 โดยมวลต่อปริมาตร และน้ำตาลกลูโคส ร้อยละ 0.5 โดยมวลต่อปริมาตร ปรับค่าตวามเป็นกรดด่างเริ่มต้นให้มีค่าเท่ากับ 6.5 และทำการบ่มที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง นอกจากจะทำให้ได้ผลผลิตเพดดิโอซินเพิ่มขึ้น คิดเป็น 16 และ 256 เท่าเมื่อเทียบกับผลผลิตที่ได้ในอาหาร TGE และ MRS ตามลำดับ แล้วยังสามารถลดค่าดัชนีสีน้ำตาลของสารละลายเพดดิโอซินที่เตรียมได้ประมาณ 2 เท่า เมื่อเทียบกับ MRS ทำให้ได้เพดดิโอซินที่มีปริมาณและสีเหมาะสมสำหรับนำไปใช้ในการพัฒนาบรรจุภัณฑ์ต่อไป

ได้ทำการพัฒนาวิธีสำหรับผสมเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ที่มีความชอบน้ำเข้ากับฟิล์มที่มีความไม่ชอบน้ำ โดยใช้ Ped และ ฟิล์มพอลิแลคติกแอซิดเป็นต้นแบบ พบว่า สามารถทำให้เพดดิโอซิน พีเอวัน-เอซีเอช เข้ากับฟิล์มคอมโพสิทพอลิแลคติกแอซิดกับผงขี้เลื่อย (PLA/SP) ได้ด้วยเทคนิคการเคลือบแบบแพร่ซึม (diffusion coating technique) โดยผงขี้เลื่อยทำหน้าที่ในการกักเก็บ Ped ให้อยู่ในฟิล์มพอลิแลคติกแอซิด การเตรียมฟิล์มด้วยการอบด้วยความร้อนแห้งก่อนการเคลือบ ทำให้ฟิล์ม PLA/SP สามารถดูดซับ Ped ได้เพิ่มขึ้น โดยสามารถดูดซับได้สูงสุด 11.63 ± 3.07 ไมโครกรัมโปรตีนต่อตารางเซนติเมตร การใช้ฟิล์มพอลิแลคติกแอซิดผสมผงขี้เลื่อยเคลือบเพดดิโอซิน (PLA/SP + Ped) เป็นบรรจุภัณฑ์สัมผัสอาหารสำหรับเนื้อสุกรสด สามารถลดจำนวนเชื้อ *Listeria monocytogenes* ได้ร้อยละ 99 ของจำนวน *Listeria* ทั้งหมด ในระหว่างการเก็บรักษาแบบแช่เย็น

นอกจากนี้ การใช้ฟิล์ม PLA/SP + Ped สามารถยืดอายุการเก็บรักษาเนื้อสุกรสดที่เก็บรักษาที่อุณหภูมิ 4 ± 2 องศาเซลเซียส ได้อย่างมีนัยสำคัญ จากการประเมินด้วยคุณลักษณะทางประสาทสัมผัสของเนื้อสุกรที่บรรจุในบรรจุภัณฑ์ PLA/SP + Ped พบว่า สามารถยืดอายุการเก็บรักษาเนื้อสุกรสดในสภาวะแช่เย็นได้นานถึง 7 วัน มากกว่าเนื้อสุกรที่ไม่ได้บรรจุในฟิล์มอย่างน้อย 3 วัน

เพื่อเพิ่มประสิทธิภาพในการยับยั้งเชื้อจุลินทรีย์ ในการศึกษานี้ ได้ค้นพบ แบคเทอริโอซินซึ่งคาดว่าเป็นเปปไทด์ชนิดใหม่ 2 ชนิด คือ แบคเทอริโอซิน 7293 เอ และ แบคเทอริโอซิน 7293 บี ที่ผลิตโดยเชื้อ *Weissella hellenica* BCC 7293 ที่มีฤทธิ์ยับยั้งได้ทั้งแบคทีเรียแกรมบวกและแกรมลบที่เป็นเชื้อก่อโรคและ/หรือการเสื่อมเสียของอาหาร เช่น *L. monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Escherichia coli* และ *Salmonella* Typhimurium จากการทำให้บริสุทธิ์ด้วยเทคนิคโครมาโตรกราฟี ซึ่งประกอบด้วย Hydrophobic interaction chromatography, ion exchange chromatography และ reversed-phase HPLC พบว่า แบคเทอริโอซิน 7293 เอ และ แบคเทอริโอซิน 7293 บี มีน้ำหนักโมเลกุล 6249.302 และ 6489.716 ดาลตัน ตามลำดับ แบคเทอริโอซินทั้งสองชนิดออกฤทธิ์ฆ่าแบคทีเรียทั้งแกรมบวกและแกรมลบ แต่ไม่ทำให้เซลล์แตก ฤทธิ์ยับยั้งจุลินทรีย์มีความคงตัวในสภาวะที่มีค่าความเป็นกรดด่างและอุณหภูมิในช่วงกว้าง สามารถทำลายได้โดยโปรตีเอส แต่ไม่ถูกยับยั้งโดยไลเปส อะไมเลส ตัวทำละลายอินทรีย์ และสารลดแรงตึงผิวชนิดต่างๆ

เมื่อทำการทดลองผสมแบคเทอริโอซิน 7293 ที่ทำให้บริสุทธิ์บางส่วนเข้ากับฟิล์มคอมโพสิทพอลิแลคติกแอซิดกับผงขี้เลื่อย (PLA/SP) เพื่อตรวจสอบประสิทธิภาพของวิธีการผสมเปปไทด์กับฟิล์มที่ได้พัฒนาขึ้นสำหรับ Ped พบว่า ฟิล์มคอมโพสิทพอลิแลคติกแอซิดกับผงขี้เลื่อยที่เคลือบด้วยแบคเทอริโอซิน 7293 มีประสิทธิภาพในการยับยั้งเชื้อแบคทีเรียก่อโรคหลายชนิดที่มีการปนเปื้อนในเนื้อปลาแพงกาเซียสแล่ ได้แก่ *L. monocytogene, S. aureus, P. aeruginosa, A. hydrophila, E. coli* และ *S.* Typhimurium ด้วยการทดสอบทั้งในหลอดทดลองและในตัวอย่างเนื้อปลาแล่ที่ทำให้เกิดการปนเปื้อนด้วยวิธีการทดสอบแบบกระตุ้น (challenge test) โดยพบว่า ฟิล์มคอมโพสิทพอลิแลคติกแอซิดกับผงขี้เลื่อยที่เคลือบด้วยแบคเทอริโอซิน 7293 สามารถลดจำนวนเชื้อแบคทีเรียที่ทดสอบได้สูงสุดถึง 5 log CFU ต่อตารางเซนติเมตรขึ้นอยู่กับชนิดของจุลินทรีย์ ซึ่งแสดงให้เห็นว่าวิธีการผสมเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์เข้ากับฟิล์มพอลิแลคติกแอซิดที่ได้พัฒนาขึ้นสามารถใช้ได้กับเปปไทด์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ชนิดอื่นๆ ได้ ทำให้ได้ฟิล์มพอลิแลคติกแอซิดที่มีฤทธิ์ยับยั้งจุลินทรีย์ ที่มีศักยภาพในการใช้เป็นบรรจุภัณฑ์ยับยั้งจุลินทรีย์ในอาหาร

Thesis Title Antimicrobial Peptide from Lactic Acid Bacteria and Development of Antimicrobial Film for Food Packaging
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ABSTRACT

Due to the trend toward active and green packaging and the need for safe and natural antimicrobials with specific target, development of biodegradable film, especially poly(lactic acid) (PLA) enriched with antimicrobial peptides from lactic acid bacteria for food packaging has been received a consideration attention. However, direct incorporation of hydrophilic antimicrobial peptide to PLA film was limited by the hydrophobic characteristics of PLA. Therefore, this study aims to develop the method for incorporation of antimicrobial peptides from lactic acid bacteria to PLA film to produce an antimicrobial biodegradable food packaging.

Initially, the production of pediocin PA-1/AcH (Ped), an antimicrobial peptide produced by *Pediococcus pentosaceus* BCC 3772 was firstly optimized in order to improve the productivity and to reduce the browning index of pediocin preparation. The production of pediocin in the optimal medium containing 1.5% (w/v) of sodium caseinate, 1.5% (w/v) of yeast extract and 0.5% (w/v) of glucose at the initial pH of 6.5, temperature at 30 °C for 12 h not only improved the pediocin yields (16-fold and 256-fold higher than those in TGE and MRS broths, respectively) but also significantly reduced browning index about 2-fold lower than that of MRS broth, thus providing suitable quantity and color of pediocin preparation for use in food packaging development.

A method for incorporating hydrophilic antimicrobial peptide into hydrophobic film by using pediocin PA-1/AcH and PLA film as a prototype was developed. Pediocin PA-1/AcH was successfully impregnated into PLA/sawdust particle (SP) biocomposite film (or PLA/SP) using diffusion coating technique. Sawdust particle played an important role in embedding Ped in PLA film. Dry-heat treatment of PLA/SP composite film before coating with Ped maximized Ped

adsorption with the highest amount of 11.63 ± 3.07 μg protein/cm^2. A model study of PLA/SP + Ped as a food-contact antimicrobial packaging on raw pork revealed a potential inhibition of *Listeria monocytogenes* (99%-reduction of total listerial population) on raw pork during the chilled storage. In addition, PLA/SP + Ped significantly improved the quality and shelf life of packaged fresh pork meat stored at 4 ± 2 °C. Based on sensory characteristics of packaged pork, PLA/SP + Ped could maintain the quality of fresh pork meat up to 7 days under chilled condition, which was at least 3 days longer than the unpackaged meat.

In order to broaden the antimicrobial spectrum of the packaging, putatively novel bacteriocin 7293A and bacteriocin 7293B from *Weissella hellenica* BCC 7293, which active against both Gram-positive and Gram-negative food-borne pathogenic and spoilage bacteria including *L. monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Escherichia coli,* and *Salmonella* Typhimurium were discovered. Through a series of chromatographic purification including hydrophobic interaction chromatography, ion exchange chromatography and reversed-phase HPLC, bacteriocins 7293A and 7293B were purified to homogeneity and revealed the molecular weight of 6249.302 and 6489.716 Da, respectively. Both bacteriocins exhibited bactericidal effect against both Gram-positive and Gram-negative indicators without cell-lysis. Bacteriocin 7293A and 7293B were stable in wide range of pH and temperature. Antimicrobial activity of both peptides was inactivated by proteolytic enzymes but was not inactivated by lipase, amylase, organic solvents and surfactants.

Lastly, to validate the incorporation technique previously developed for Ped, a partially purified bacteriocin 7293 was tested with PLA/SP film. The PLA/SP film coated with Bacteriocin 7293 effectively inhibited several important bacteria reported to contaminate on Pangasius fish fillet (*L. Monocytogene, S. Aureus, P. Aeruginosa, A. Hydrophila, E. coli* and *S.* Typhimurium) both in *vitro* and challenge testing with sliced fish fillet. The result indicated that PLA/SP + Bac7293 could reduce the growth of target microorganisms up to 5 log CFU/cm^2. The results validated the efficiency of the developed technique for incorporating other antimicrobial peptides into PLA film for an antimicrobial PLA-based film production.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Antimicrobial peptides (AMPs) from lactic acid bacteria (LAB) are the peptides that ribosomally synthesized by lactic acid bacteria and exhibit the antimicrobial activity against other bacteria, especially bacteria which are closely related to the antimicrobial peptide producer (Klaenhammer, 1993). AMPs from LAB have been used as food bio-preservatives because of their desirable properties such as: i) generally recognized as safe, ii) inactive and nontoxic on eukaryotic cells, iii) easily deactivated by digestive protease, thus having little influence on the gut microbiota, iv) stable in wide range of pH and heat treatment v) relatively broad antimicrobial spectrum against food-borne pathogenic and spoilage bacteria and vi) possess no cross resistance with other antibiotics and hardly cause resistant bacteria (Helander *et al*., 1997; Cleveland *et al*., 2001; Topissirovic *et al*., 2006; Gálvez *et al*., 2007; Hwanhlem *et al*., 2014).

The application of AMPs from LAB in food packaging is a promising way to control the growth of spoilage and pathogenic microorganisms in food since the packaging could act as a reservoir and diffuser of the concentrated AMPs molecules to the food ensuring a gradient-dependent continuous supply of AMPs. Moreover, packaging could protect AMPs from deactivation by interaction with food components such as lipid and enzyme deactivation. The application of AMPs on food packaging required lower amounts of AMPs compared to direct application in the whole food volume, decreasing the production cost and could avoid the addition of AMPs directly to foods, attemping to actual tendency of consumers of searching for healthier foods and free of additives (Appendini and Hotchkiss, 2002).

However, because of the opposite hydrophobicity of AMPs and packaging materials, only few studies have achieved to apply AMPs in food packaging to produce active packaging with high antimicrobial effectiveness and desirable packaging property (Daesche *et al*., 1992; Ming *et al*., 1997; Natrajan and Sheldon, 2000; Sebti *et al*., 2003; Sebti *et al*., 2007; Iseppi *et al*., 2008; Ye *et al*.,

2008a; Jin *et al*., 2009; Imran *et al*., 2010). In this study, the emerging technology to incorporate hydrophilic AMPs to hydrophobic food packaging material was developed using pediocin and poly(lactic acid) (PLA) as a model. Sawdust particle, a low-water solubility hydrophilic particle, was incorporated into PLA film to enhance the adsorption of pediocin using diffusion coating technique.

Pediocin is one of only two AMPs from LAB that have been received attention to be used in food and food packaging (Rodriguez *et al*., 2002). According to Kingcha *et al*. (2012), pediocin PA-1/AcH from *Pediococcus pentosaceus* BCC 3772 has high efficiency in the inhibition of the growth of *Listeria. monocytogenes*, an important food-borne pathogen that causes a severe disease called listeriosis (Alves *et al*., 2006; Gialamas *et al*., 2010). In contrast to other antimicrobial peptides, pediocin PA-1/AcH has potential to inhibit *Listeria* without disturbing other bacteria exceptionally beneficial ones (Blay et al., 2007). In addition, pediocin PA-1/AcH was heat-stable and active in wide range of pH (pH 2-10) which showed high potential to be used as natural antimicrobial agent in food packaging and food application for various types of food including low-acid and high-acid food (Degnan *et al*., 1993; Nieto-Lozano *et al*., 2010: Kingcha *et al*., 2012).

Although pediocin shows high potential to be used as food bio-preservatives, the high cost of pediocin production and high browning index of pediocin preparation,which could affect color characteristics of food and/or food packaging may limit its uses in food industry (Arokiyamary *et al*., 2011). To overcome these limitations, optimization of pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 was performed. Previous studies showed that culture medium compositions especially nitrogen and carbon sources significantly influence both of AMP productivity and production cost (Guerra *et al*., 2001; Halami and Chandrashekar, 2005). Moreover, initial pH of culture medium and incubation temperature also significantly affect the production of AMP (Biswas *et al*., 1991; Altuntas *et al*., 2010). In this study, we focused on the use of low cost, food grade materials instead of the commercial media with optimized incubation condition in order to reduce the production cost, improve productivity, and reduce the browning index, and thus increasing the potential use of pediocin PA-1/AcH in food and food packaging applications.

Poly(lactic acid) (PLA) is recognized as compostable biopolymer that attracts the interest for the food packaging industry because of its outstanding properties and environmental-friendly biodegradability (Jin *et al*., 2009; Theinsathid *et al*., 2012). PLA packaging exhibits many properties that are equivalent to or better than many petroleum-based packaging (Liu *et al*., 2009) and could be produced by many manufacturing processes, such as film blowing, injection molding, sheet extrusion, blow molding and thermoforming (Imam *et al*., 2008; Jamshidian *et al*., 2010). The combination of biodegradability of PLA with antimicrobial property of pediocin against an important food-borne pathogen will be of full benefit as the active packaging. As a consequence, health-risk of consumers can be reduced. Shelf-life can be extended, thereby lowering the economic loss. Importantly, the waste of post use of this packaging will be decomposed through compostable system without causing environmental waste problems.

Before being introduced to the market, the developed antimicrobial packaging was characterized for physical, mechanical and antimicrobial properties. Moreover, effect of the packaging on shelf life and quality of packaged food was investigated in order to ensure the potential use of the produced film in real food system.

In order to broaden the antimicrobial spectrum of the packaging rather than *Listeria monocytogenes*, new AMPs with wide spectra of inhibition against other food spoilage and pathogenic microorganisms have been explored. Some characteristics including molecular masses, spectra of inhibition, some factors affecting antimicrobial activity and mode of action of putatively novel antimicrobial peptides were studied since this information will be benefit for the selection of appropriate application. The new found AMPs were applied to the packaging system which has been developed in this study in order to validate the developed incorporating technology and to produce a novel type of antimicrobial food packaging with broader antimicrobial spectrum.

1.2 Review of Literature

1.2.1 Antimicrobial peptides from lactic acid bacteria (LAB)

The lactic acid bacteria (LAB) comprise a clade of Gram-positive, acid-tolerant, generally non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcu*s, *Lactococcus* and *Streptococcus* as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*; these belong to the order Lactobacillales. LAB play an important role in the fermentation process, contributing to the organoleptic and textural profile of fermented foods. During fermentation LAB do not produce only lactic acid but they are also known to produce and excrete antimicrobial substances, including antimicrobial peptides (AMPs) or bacteriocins that exhibit a great potential as food bio-preservatives (Ray, 1992; Hwanhlem *et al*., 2014).

Several desirable properties that make AMPs from LAB suitable for food preservation are (i) are generally recognized as safe (GRAS) substances, (ii) are inactive and nontoxic on eukaryotic cells, (iii) become inactivated by digestive protease, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria, (vi) show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Helander *et al*., 1997; Cleveland *et al*., 2001; Topissirovic *et al*., 2006; Gálvez *et al*., 2007; Hwanhlem *et al*., 2014).

1.2.1.1 Sources of antimicrobial peptide producing LAB

LAB found in numerous foods and non-foods sources produce a high diversity of different AMPs (Cleveland *et al*., 2001). According to Klaenhammer (1988), 99% of all bacteria may make at least one AMP and the only reason more have not been isolated is that very few researchers have looked for them. On the

screening of AMP producing LAB, it is very important what materials are used as the isolation sources. The efficiency of isolating LAB strains is easily influenced by the isolation sources. The materials associated with lactic acid fermentation such as natural cheese, natural fermented milk products, fermented vegetables, should be used. Until now, many European groups have used natural cheese, fermented milk products, fermented sausage, etc, originated from animal products as the isolation sources (Ennahar *et al*., 1996). AMP producing LAB have been discovered from many animal-origin foods such as fermented sausages (Garriga *et al*., 1993), Spanish raw meat (Nieto-Lozano *et al*., 2006), traditionally Tunisian fermented meat called Gueddid (Belgacem *et al*., 2008), Xuan-Wei Ham, a traditional Chinese fermented meat product (Liu *et al*., 2008), Alheira, a fermented meat sausage (Albano *et al*., 2009), artisanal dry sausages (Castro *et al*., 2011), Thai shrimp paste (Kapi) (Kaewklom *et al*., 2013), charqui, a Brazillian fermented, salted and dried meat product (Biscola *et al*., 2013).

In addition, it is proposed that AMP originated from LAB isolated from local fermented vegetables have the advantage of obtaining permission for the use as food additives. AMP producing LAB have been successfully isolated from many plant-origin foods such as fermented mixed salad and fermented carrot (Uhlman *et al*., 1992), Spanish-style table olives fermentation (Maldonado *et al*., 2002), poto poto, a Congolese fermented maize product (Omar *et al*., 2008), traditional Chinese fermented cabbage (Gao *et al*., 2010), cereal based fermented beverage from Bulgaria (Todorov, 2010), traditional Chinese fermented radish (Jiang *et al*., 2012), fermented baobab seed (maari) (Kaboré *et al*., 2013.). These materials should be used for the isolation sources of LAB strains to find new AMP because there are few reports using these materials. Namely, the use of these materials will promise us to find new AMP with high frequency. However, Ohmomo *et al*. (2003) reported that no LAB strain was isolated from the fermented vegetables (Pak Dong) purchased at the urban area market in Bangkok, Thailand. The authors suggested that this vegetable should be soaked in lactic acid solution instead of fermentation. Namely this is a fermented-like product, not real fermented product. If these sources are used for isolation, it will be very hard to isolate LAB. So, the use of hand-made fermented vegetables purchased at the rural markets is better way for the isolation of LAB.

Non-foods are also the interested sources for isolation of AMP producing LAB. Several AMP producing LAB have been isolated from this kind of sources including oral cavity of human (Lima *et al*., 2002), soil in Japan (Chen and Yanagida, 2006), mangrove forests in southern Thailand (Hwanhlem *et al*., 2014). However, AMP from LAB isolated from these sources have not been accepted for use in food and food packaging application yet since both of these AMP and LAB have not been approved to be safely used.

1.2.1.2 Some characteristics of antimicrobial peptides

As mentioned above, the AMP from LAB exhibited high potential to be used as a natural food preservative. Understanding the characteristics of these AMP would be useful in designing the technology for their application in food and food packaging systems. Some characteristics of AMP always studied are molecular weight, amino acid sequence, antimicrobial spectrum, and some factors affecting the antimicrobial activity of peptides.

1.2.1.2.1 Molecular mass and amino acid sequence of antimicrobial peptides

The amino acid sequence of AMP could be useful to predict the secondary structure, antimicrobial activity and physicochemical characteristic when applied in food or food packaging systems. Moreover, molecular mass and amino acid sequencing of antimicrobial peptides were used to indentify or classify the antimicrobial peptides. Base on molecular mass (size) and amino acid sequence with other properties of AMP, Klaenhammer (1993) classified AMPs from LAB into four groups as shown in Table 1.

The molecular mass of purified antimicrobial peptides generally ranged from 2.5 kDa to 6.5 kDa (Elegado *et al*., 1997; Ennahar *et al*., 2000). Molecular mass and amino acid sequence of some antimicrobial peptides were presented in Table 2. Many antimicrobial peptides shown in Table 2 have the same amino acid sequence on the basis of the N-terminal YGNGV so these were classified to the class IIa bacteriocins.

Table 1. Classification of bacteriocins

Class	General characteristics
Class I	Lanthionine-containing bacteriocin, lantibiotics
Class II	Heat-stable, non-lanthionine-containing bacteriocins with molecular mass of not more than 10 kDa. This class is further divided into four groups.
Class IIa	Listeria-active bacteriocins with a consensus sequence in the N-terminal of YGNGVXC
Class IIb	Two-peptide bacteriocins
Class IIc	Cyclic bacteriocins
Class IId	Other class II bacteriocins
Class III	Heat-labile bacteriocins with molecular mass larger than 30 kDa
Class IV	Bacteriocins which require non-protein moieties for the antimicrobial acitivty such as glycoproteins and lipoproteins

Table 2. Molecular mass and amino acid sequence of some antimicrobial peptides

Bacteriocin	Producing strain	Molecular mass (kDa)	Amino acid sequence	Reference
Bac 31	*Enterococcus faecalis*	5.0	ATYYGNGLYCNK QKCWVDWNKASR EIGKIIVNGWVQH GPWAPR	Tomita *et al*., 1996
Bavaricin A	*Lactobacillus sake* MI401	3.5	KYYGNGVHCGKH SCTVDWGTAIGNI GNNAAANXATGX NAGG	Larsen *et al*., 1993
Bavaricin MN	*Lactobacillus bavaricus* MN	4.8	TKYYGNGVYCNS KKCWVDWGQAA GGIGQTVVXGWL GGAIPGK	Kaiser and Montville, 1996
Bifidocin B	*Bifidobacterium bifidum* NCFB 1454	3.3	KYYGNGVTCGLH DCRVDRGKATCGI INNGGMWGDIG	Yildirim *et al*., 1999
Carnobacteriocin B2	*Carnobacterium piscicola* LV17B	5.0	VNYGNGVSCSKT KCSVNWGQAFQE RYTAGINSFVSGV ASGASIGRRP	McCormick *et al*., 1996

Table 2. Molecular mass and amino acid sequence of some antimicrobial peptides (cont.)

Bacteriocin	Producing strain	Molecular mass (kDa)	Amino acid sequence	Reference
Coagulin	*Bacillus coagulans* I4	3.4	KYYGNGVTCGKH SCSVDWGKATTCI INNGAMAWATGG HQGTHKC	Le Marrec *et al*., 2000
Curvacin A	*Lactobacillus curvatus* LTH1174	4.3	ARSYGNGVYCNN KKCWVNRGEATQ SIIGGMISGWASGL AGM	Tichaczek *et al*., 1992
Divercin V41	*Carnobacterium divergens* V41	4.5	TKYYGNGVYCNS KKCWVDWGQAS GCIGQTVVGGWL GGAIIPGKC	Metivier *et al*., 1998
Enterocin A	*Enterococcus faecium*	4.8	TTHSGKYYGNGV YCTKNKCTVDWA KATTCIAGMSIGG FLGGAIPGKC	Aymercih *et al*., 1996

Table 2. Molecular mass and amino acid sequence of some antimicrobial peptides (cont.)

Bacteriocin	Producing strain	Molecular mass (kDa)	Amino acid sequence	Reference
Enterocin P	*Enterococcus faecium* P13	4.4	ATRSYGNGVYCN NSKCWVNWGEAK ENIAGIVISGWASG LAGMGH	Cintas *et al*, 1997
Leucocin A-UAL187	*Leuconostoc gelidum*	3.9	KYYGNGVHCTKS GCSVNWGEAFSA GVHRLANGGNGF W	Hastings *et al*., 1991
Mesentericin Y105	*Leuconostoc mesenteroides* Y105	3.7	KYYGNGVHCTKS GCSVNWGEAASA GIHLANGGNGFW	Fleury *et al*., 1996
Mundticin	*Enterococcus mundtii*	4.3	KYYGNGVSCNKK GCSVDWGKAIGII GNNSAANLATGG AAGWSK	Bennik *et al*., 1998
Pediocin PA-1	*Pediococcus acidilactici* PAC-1.0	4.6	KYYGNGVTCGKH SCSVDWGKATTCI INNGAMAWATGG HQGNHKC	Henderson *et al*., 1992

Table 2. Molecular mass and amino acid sequence of some antimicrobial peptides (cont.)

Bacteriocin	Producing strain	Molecular mass (kDa)	Amino acid sequence	Reference
Piscicocin V1a	*Carnobacterium pisdicola* V1	4.4	KYYGNGVSCNKN GCTVDWSKAIGIIG NNAAANLTTGGA AGWNKG	Bhugaloo-Vial *et al*., 1996
Piscicolin 126	*Carnobacterium maltaromaticum* UAL307	4.4	KYYGNGVSCNKN GCTVDWSKAIGIIG NNAAANLTTGGA AGWNKG	Jack *et al*., 1996
Sakacin A	*Lactobacillus sake* LB706	4.3	ARSYGNGVYCNN KKCWVNRGEATQ SIIGGMISGWASGL AGM	Holck *et al*., 1992
Sakacin G	*Lactobacillus sake* 2512	3.8	KYYGNGVSCNSH GCSWNWGQAWT CGVNHLANGGHG VC	Simon *et al*., 1992

Table 2. Molecular mass and amino acid sequence of some antimicrobial peptides (cont.)

Bacteriocin	Producing strain	Molecular mass (kDa)	Amino acid sequence	Reference
Sakacin P	*Lactobacillus sake* LTH673	4.4	KYYGNGVHCGKH SCTVDWGTAIGNI GNNAAANWATGG NAGWNK	Tichaczek *et al*., 1992
Weissellicin M	*Weissella hellenica* QU13	4.9	MVSAAKVALKVG WGLVKKYYTKVM QFIGEGWSVDQIA DWLKRH	Masuda *et al*., 2011
Weissellicin Y	*Weissella hellenica* QU13	4.9	MANIVLRVGSVAY NYAPKIFKWIGEG VSYNQIIKWGHNK GWW	Masuda *et al*., 2011
Weissellicin 110	*Weissella cibaria* 110	3.4	SDKNNVFFQIGKR YVAPVLYXFGKX AE	Srionnual *et al*., 2007
Weissellicin A	*Weissella paramescenteroid es* DX	4.4	KNYGNGVYCNKH KCSVDWATFSANI ANNSVAMAGLTG GNAGNK	Papagianni and Papamichael, 2011

1.2.1.2.2 Antimicrobial spectra of antimicrobial peptides

The antimicrobial activity of antimicrobial peptides is commonly directed against several Gram-positive bacteria such as *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Carnobacterium, Enterococcus, Micrococuccus, Staphylococcus, Strephylococcus, Clostridium, Bacillus,* and *Brochothrix* (Ennahar *et al.*, 2000; Anastasiadou *et al*., 2008a, b). In the case of class IIa bacteriocin, its antimicrobial activity seems to be targeting primarily *Listeria* strains. Furthermore, some class IIa bacteriocins have been shown to prevent the outgrowth of spores and vegetative cells of *Clostridium* spp. (Ennahar *et al*., 2000). Pinto *et al*. (2009) investigated that bacteriocins produced by *Enterococcus faecium* ALP7 and *Pediococcus pentosaceus* ALP57 have an antimicrobial activity against the gram-positive strains, especially for *Listeria* species, but have no antimicrobial activity against gram-negative indicator strains. The antimicrobial spectra of some AMPs produced by LAB were shown in Table 3.

Table 3. Antimicrobial spectra of some AMPs produced by LAB

Bacteriocin	Producing strains	Spectrum of action	Reference
Acidicin A	*Lactobacillus acidophilus TK9201*	*Enterococcus* *Lactobacillus* *Pediococcus* *Streptococcus* *Listeria monocytogenes*	Kanatani *et al*., 1995
Acidocin J1132	*Lactobacillus acidophilus* JCM 1132	*Gram-positive bacteria* *Lactobacillus* *Lactobacillus fermentum* *Enterococcus faecalis*	Tahara *et al*., 1996

Table 3. Antimicrobial spectra of some AMPs produced by LAB (cont.)

Bacteriocin	Producing strains	Spectrum of action	Reference
Bavaricin A	*Lactobacillus sake MI401*	*Enterococcus* *Lactobacillus* *Lactococcus* *Leuconostoc* *Pediococcus* *Listeria monocytogenes*	Larsen *et al*., 1993
Curvacin A	*Lactobacillus curvatus LTH1174*	*Listeria monocytogenes* *Listeria innocua* *Listeria ivanovi* *Pediococcus* *Enterococcus*	Eijsink *et al.*,1998
Divercin V41	*Carnobacterium divergens* V41	*Enterococcus* *Lactobacillus* *Pediococcus* *Listeria monocytogenes* *Listeria innocua* *Listeria ivanovi*	Métivier *et al*., 1998
Enterocin A	*Enterococcus faecium CTC492*	*Listeria monocytogenes* *Listeria innocua* *Pediococcus* *Enterococcus* *Lactobacillus*	Aymerich *et al*., 1996
Helveticin J	*Lactobacillus helveticus*	*Lactobacillus bulgaricus* *Lactococcus lactis*	Parada *et al*., 2007
Lactacin B	*Lactobacillus acidophilus*	*Lactobacillus delbrueckii* *Lactobacillus helveticus* *Lactobacillus debrweckii* *Lactobacillus helveticus*	Parada *et al*., 2007

Table 3. Antimicrobial spectra of some AMPs produced by LAB (cont.)

Bacteriocin	Producing strains	Spectrum of action	Reference
Lactacin F	*Lactobacillus acidophilus*	*Lactobacillus fermentum* *Enterococcus faecalis*	Parada *et al*., 2007
Lacticin 3147	*Lactococcus lactis DPC3147*	*Enterococcus* *Lactobacillus* *Lactococcus* *Leuconostoc* *Pediococcus* *Streptococcus* *Listeria monocytogenes*	Ryan *et al*., 1996
Lacticin 3147	*Lactococcus lactis DPC3147*	*Listeria innocua* *Staphylococcus aureus* *Bacillus* spp. *Clostridium* spp.	Ryan *et al*., 1996
Lactocin 705	*Lactobacillus casei*	*Listeria monocytogenes* *Lactobacillus plantarum*	Parada *et al*., 2007
Lactocin A	*Lactobacillus amylovorus*	*Lactobacillus delbrueckii*	Parada *et al*., 2007
Lactocin S	*Lactobacillus sake L45*	*Lactobacillus* *Lactococcus* *Leuconostoc* *Enterococcus* *Pediococcus* *Listeria monocytogenes* *Listeria innocua* *Staphylococcus* *Bacillus cereus* *Clostridium* spp.	Cintas *et al*., 1998

Table 3. Antimicrobial spectra of some AMPs produced by LAB (cont.)

Bacteriocin	Producing strains	Spectrum of action	Reference
Leucocin A	*Leuconostoc gelidum*	*Lactobacillus* *Enterococcus faecalis* *Listeria monocytogenes*	Hastings *et al*., 1991
Mesentericin Y105	*Leuconostoc mesenteroides Y105*	*Enterococcus faecalis* *Pediococcus* *Leuconostoc* *Lactobacillus* *Listeria monocytogenes* *Listeria innocua* *Listeria ivanovi*	Fleury *et al*., 1996
Nisin	*Lactococcus lactis subsp. Lactis*	*Enterococcus* *Lactobacillus*	Cintas *et al*., 1998
Nisin	*Lactococcus lactis subsp. Lactis*	*Lactococcus* *Leuconostoc* *Pediococcus* *Listeria monocytogenes* *Listeria innocua* *Listeria ivanovii* *Listeria murrayi* *Listeria seeligeri* *Listeria welchimeri* *Staphylococcus* spp. *Bacillus* spp. *Clostridium* spp.	Cintas *et al*., 1998
Pediocin A	*Pediococus pentosaceous*	*Lactobacillus ,Lactococcus, Leuconostoc ,Pediococcus, Staphylococcus,Enterococcus, Listeria, Clostridium*	Parada *et al*., 2007

Table 3. Antimicrobial spectra of some AMPs produced by LAB (cont.)

Bacteriocin	Producing strains	Spectrum of action	Reference
Pediocin F Pediocin PA-1 Pediocin AcH	*Pediococcus acidilactici*	*Gram-positive bacteria* *Listeria monocytogenes* *Gram-positive and Gram-negative bacteria under stressing situations*	Parada *et al*., 2007
Plantaricin C	*Lactobacillus plantarum LL441*	*Enterococcus* *Lactobacillus* *Lactococcus* *Leuconostoc*	Gonzalez *et al*.,1994
Plantaricin C	*Lactobacillus plantarum LL441*	*Pediococcus* *Stroptococcus* *Staphylococcus carnosus* *Bacillus* spp. *Clostridium* spp.	Gonzalez *et al*.,1994
Sakacin A	*Lactobacillus sake LB706*	*Enterococcus* *Lactobacillus* *Pediococcus* *Listeria monocytogenes* *Listeria innocua*	Holck *et al*., 1992
Sakacin P	*Lactobacillus sake LB674*	*Enterococcus* *Lactobacillus* *Pediococcus* *Listeria monocytogenes* *Listeria innocua*	Guyonnet *et al*., 2000
Weissellicin A	*Weissella paramensenteroides* DX	*Lactobacillus* *Bacillus cereus* *Listeria monocytogenes* *Micrococcus luteus*	Papagianni and Papamichael, 2011

Table 3. Antimicrobial spectra of some AMPs produced by LAB (cont.)

Bacteriocin	Producing strains	Spectrum of action	Reference
Weissellicin D	*Weissella hellenica* D1501	*Streptococcus thermophilus* *Lactobacillus* *Staphylococcus aureus* *Escherichia coli* *Bacillus* *Pseudomonas aeruginosa* *Listeria monocytogenes* *Candida albicans* *Mucor*	Chen *et al*., 2014
Weissellicin L	*Weissella hellenica* 4-7	*Streptococcus thermophilus* *Lactobacillus* *Listeria monocytogenes*	Leong *et al*., 2013
Weissellicin M and Y	*Weissella hellenica* QU 13	*Lactobacillus* *Pediococcus pentosaceus* *Bacillus*	Masuda *et al*., 2013
Weissellicin 110	*Weissella cibaria* 110	*Lactobacillus sake*	Srionnual *et al*., 2007

1.2.1.2.3 Some factors affecting the antimicrobial activity of antimicrobial peptides

A. Temperature

The effect of temperature on antimicrobial activity of AMPs was investigated in several studies. The resistance to heat treatment seemed to be AMP-dependent. Anastasiadou *et al*., (2008b) tested the antimicrobial activity of pediocin SA-1 produced by *Pediococcus acidilactici* NRRL B5627 after heat treatments at 40, 60, 80, and 100°C for 10, 30, and 60 min, 121°Cfor 10 and 20 min, and -80, -20, 4, and 30°C for 4 weeks. They found that treatment for 60 min at 121°C and storage for

4 weeks at -80, -20, 4, and 30 °C did not affect the antimicrobial activity, similarly to pediocin PA-1 produced by *Pediococcus pentosaceus* ALP57 that remained fully active after treatments at 4, 10, 25, and 30 °C (Pinto *et al*., 2009). The activity of pediocin PA-1 was reduced when subjected to 121 °C for 20 min, in contrast to pediocin SM-1, pediocin SA-1 (Anastasiadou *et al*., 2008b), pediocin ACCEL (Wu *et al*., 2004), pediocin PD-1 (Green *et al*., 1997), pediocin SJ-1 (Schved *et al*., 1993), pediocin N5p (Strässer de Saad *et al*., 1995), pediocin AcH (Bhunia *et al*., 1987), and pediocin PA-1 (Gonzales and Kunka, 1987) in which their antibacterial activity remained after subjected to 121 °C for 60 min. The antimicrobial activity of weissellicins was stable even incubated at high (121 °C) or low (-20 °C) temperature (Srionnual *et al*., 2007; Papagianni and Papamichael, 2011; Leong *et al*., 2013; Masuda *et al*., 2013; Chen *et al*., 2014).

B. pH

The pH value was investigated as a factor affecting the antimicrobial activity of AMPs. Generally, the antimicrobial activity of AMPs was stable in wide range of pH (pH of 2.0-10.0). At the pH value of lower than 2.0 and higher than 10.0, the antimicrobial activity of AMPs was decreased (Bhunia *et al*., 1987; Gonzales and Kunka, 1987; Green *et al*., 1997; Wu *et al*., 2004; Anastasiadou *et al*., 2008a; Anastasiadou *et al*., 2008b). In the case of class I and class IIa bacteriocins, the antimicrobial activity are usually very stable at acidic pH (Chen and Hoover, 2003).

C. Enzymes

Since the AMPs are proteinaceous in nature, they could be digested by proteolytic enzymes and consequently the antimicrobial activity was inactivated. The sensitivity of antimicrobial peptides to many enzymes such as lipases, amylase, and proteases are usually studied. Pinto *et al.* (2009) reported that pediocin PA-1, an antimicrobial peptide, produced by *Pediococcus pentosaceus* ALP57 were completely inactivated after treatment with the proteolytic enzymes trypsin, proteinase K, pronase E and papain, in contrast to pediocin SA-1 produced by *Pediococcus acidilactici* NRRL B5627 which is resistant to treatment with trypsin, pepsin, and papain, but not

to proteinase K (Anastasiadou *et al*., 2008b). Lipase and α-amylase had no effect on antimicrobial activity of pediocin SA-1, confirmed the proteinaceous nature of this AMP. (Pinto *et al*., 2009).

D. Chemicals

In many cases, purified or crude AMPs were mixed or used with chemicals in food matrix, film forming solutions, or film coating solutions. Understanding the effect of chemicals always used in food systems or food processing on the antimicrobial activity of AMPs may help us to design the application technologies. Pinto *et al*. (2009) studied the effect of some chemicals on the antilisterial activity of pediocin PA-1, an antimicrobial peptide produced by *Pediococcus pentosaceus* ALP57, used *Listeria monocytogenes* ESB54 and *Listeria innocua* 2030c as the indicator strains. From this study, they found that the treatments of cell free supernatant containing pediocin PA-1 with 1% NaCl, Triton X-100, Tween 20, Tween 80, and EDTA did not affect the antilisterial activity of pediocin as reported similarly for enterocin EJ97 produced by *Enterococcus faecalis* (Gláves *et al*., 2005), pediocin HA-6111-2 produced by *Pediococcus acidilactici* (Albano *et al*., 2007) and bacteriocin ST15 produced by *Enterococcusmundtii* (De Kwaasdsteniet *et al*., 2005), while the treatments with ox-bile and urea (in the case of *Listeria innocua* 2030c) reduced the antilisterial activity of pediocin. The antimicrobial activity of Weissellicin D was also not affected by tested chemical including 25% methanol, 25% ethanol, 1% Tween 80, SDS and Urea (Chen *et al*., 2014), whilst EDTA exhibited the synergistic effect with AMPs to inhibit the growth of target microorganisms (Cutter and Siragusa, 1995: Lappe *et al*., 2009; Martin-Visscher *et al*., 2010; Chen *et al*., 2014).

1.2.2 *Listeria monocytogenes*

Listeria monocytogenes is a small non-spore forming gram-positive and catalase positive rod shape bacterium, difficult to identify in old cultures because of coccoidal appearance (BAM, 1995). It can grow under anaerobic or microaerophilic conditions and under a wide temperatures range (0 - 45°C) with an

optimum range of 30-37 °C. Because it can grow at low temperatures, it is considered as psychrotrophic microorganism that can easily adapt and grow under conditions of most foods. Its capacity to grow at refrigeration temperatures can be one of the most important factors that make them present at the end of the shelf life of non-sterile refrigerated products. The limiting growth conditions for *Listeria monocytog*enes are summarized in Table 4.

Table 4. Limiting growth factors for *Listeria monocytogenes*

Parameter	Range
A_w	> 0.92
Temperature (°C)	-0.4 – 45 (optimum 30-37)
pH	4.5 - 9.6
NaCl	< 0.5 - 10

Sources: Donnelly *et al*. (1992) and Huss *et al*. (1997)

Increased attention had been paid to *Listeria monocytogenes* since it was recognized as a food-borne pathogen being responsible for human listeriosis (Jemmi and Keusch, 1994). Listeriosis is a severe invasive illness in humans, which may result in death. The clinical syndromes associated with adult listeriosis include mainly central nervous system infections and primary bacteremia. The risk of contracting listeriosis is high in immune-compromised persons, the elderly, pregnant women and neonates. More recently, *Listeria monocytogenes* has been implicated in a new form of disease, causing mild gastrointestinal symptoms (Thévenot *et al*., 2006).

The human population responses to exposures to a food-borne pathogen are highly variable. Disease incidence is dependent on a variety of factors,

including virulence of the pathogen, dose (the number of pathogens ingested), the general health and immune status of the host and the attributes of the food matrix that alter microbial or host status (Thévenot *et al*., 2006). However, the incidence of *Listeria monocytogenes* in meat products is generally low, even if the pathogen is present at low or moderate levels (Encinas *et al*., 1999). Even if a single bacterial cell has the potential to cause disease, epidemiological data indicate that foods involved in listeriosis outbreaks are those in which the organism has multiplied and in general have reached levels significantly >1,000 CFU/g (Ross *et al*., 2002).

Criteria or recommendations for tolerable levels of *Listeria monocytogenes* in processed foods have been established in some countries. For example, the USA practices 'zero tolerance' or no organisms found in 25 g of a food product (Shank *et al*., 1996). The probability of contracting listeriosis is thought to be very low when food contamination was below 100 CFU/g. Therefore, a level of not more than 100 CFU/g is tolerated in certain foodstuffs, while zero tolerance is applied to foods which support *Listeria monocytogenes* growth and have extended shelf life (Thévenot *et al*., 2006).

In order to reduce the contamination of *Listeria monocytogenes* in foodstuffs, many types of technology and antimicrobial agents have been used. Pork meat products which are cooked at very high temperatures are quite likely free of live *Listeria monocytogenes* cells. However, products such as 'rillettes', which are cooked at fairly low temperatures (50 - 60 °C) may not be *Listeria monocytogenes* free even after if cooking times are long (Thévenot *et al*., 2006). Addition of salt to foodstuffs which reduces the water activity (A_w) of food can inhibit the growth of *Listeria monocytogenes* (Lücke, 1985). After drying, the water activity is < 0.90, which also inhibits bacterial growth (Tyopponen *et al*., 2003) Smoke, which contains phenols, carbonyls and different organic acids, may inhibit different bacteria including *Listeria monocytogenes* on the surfaces of cured pork products. According to Encinas *et al*. (1999), the manufacturing process and smoking significantly reduced *Lisetria* counts in sausages and ham. Encinas *et al*. (1999) also found significant differences in numbers of *Listeria* between chorizos formulated with hot and mild paprika. Other spices, such as pepper, cardamom and garlic may also have antimicrobial properties (Tyopponen *et al*., 2003). Other preservatives used to inhibit the growth of *Listeria*

monocytogenes are lactic acid, acetic acid, sodium chloride (NaCl), sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), potassium nitrate (KNO_3) and potassium nitrite (KNO_2). In recent years, antimicrobial peptides (AMPs) such as pediocin which was produced by lactic acid bacteria have already received wide attention to be used as a natural anti-listeria agent in food.

1.2.3 Pediocin PA-1/AcH

1.2.3.1 Characteristics

Pediocin PA-1/AcH is an antimicrobial peptide (AMP) ribosomally synthesized by some strains of Pediococci. Several strains and several species of *Pediococcus* such as *Pediococcus acidilactici* and *Pediococcus pentosaceus* were found to produce pediocins (Anastasiadou *et al*., 2008a, b). According to Kingcha *et al*. (2012), *Pediococcus pentosaceus* BCC 3772 produced pediocin PA-1/AcH with high antimicrobial efficiency against *Listeria monoc*ytogens. Moreover, pediocin PA-1/AcH from *Pediococcus pentosaceus* BCC 3772 has an antimicrobial activity against *Listeria innocua*, *Enterococcus faecalis*, *Lactobacillus sakei* and *Bacillus coagulans*. The outstanding properties of pediocin PA-1/AcH are i) heat stable, ii) active in wide range of pH, iii) digested by proteolytic enzyme occured in digestive tract of human, iv) safe to consume as it was certified as Generally Recognized As Safe (GRAS), v) environmental friendly and vi) high effective in inhibiting the growth of *Listeria monocytogenes*. For all of these characteristics, pediocin PA-1/AcH has been one of the interesting groups of AMP for use in food preservation (Cleveland *et al*., 2001).

Pediocin PA-1/AcH is composed of 44 amino acid residues (NH_2-Lys-Tyr-Tyr-Gly-Asn^{5}-Gly-Val-Thr-Cys-Gly^{10}-Lys-His-Ser-Cys-Ser^{15}-Val-Asp-Trp-Gly-Lys^{20}-Ala-Thr-Thr-Cys-Ile^{25}-Ile-Asn-Asn-Gly-Ala^{30}-Met-Ala-Trp-Ala-Thr^{35}-Gly-Gly-His-Gln-Gly^{40}-Asn-His-Lys-Cys-COOH), has two-disulfide bridges (Ray, 1992). It has a molecular mass of 4623 Da, pI of 8.6 (Ennahar *et al*., 2000). Similar to other pediocin-like bacteriocins, the N-terminal 20 amino acids are mainly polar, cationic, and highly conserved with a consensus sequence –Tyr^{3}-Gly^{4}-Asn^{5}-Gly^{6}-Val^{7}-. Its C-terminal region (residues Ala^{20} to Cys^{44}) is much less polar and less conserved containing a hypothetical hydrophobic membrane interacting domain. The

antimicrobial activity of this AMP was retained after treatment with many chemical agents, but was destroyed by proteolytic enzymes. Retention of antimicrobial activity following treatment with lipase, ribonuclease, lysozyme and organic solvents indicates that it is a pure protein. Bhunia *et al*. (1991) first reported on the mode of action of pediocin PA-1/AcH. The authors described that treatment with pediocin PA-1/AcH results in the leakage of K^+ and some UV adsorbing materials. Pediocin PA-1/AcH was shown to dissipate the membrane potential ($\Delta\Psi$) of *Pediococcus pentosaceus* and to cause release of amino acids accumulated either in a proton motive force (PMF)-dependent or –independent manner. Furthermore, pediocin PA-1/AcH induces the release of amino acids and other low molecular weight compounds from the vesicles of sensitive cells while it hardly induces carboxyfluoresceine (CF) efflux from liposomes, suggesting that a target protein may be required for AMP activity. Chen *et al*. (1997) reported that pediocin PA-1/AcH induces CF efflux in a concentration dependent manner from liposomes, suggesting that a protein receptor is not required. They also indicated that the binding of pediocin PA-1/AcH to liposomes is dependent on electrostatic interactions and not on the YGNGV consensus motif. It is finally proposed that pediocin PA-1/AcH might modify the permeability of sensitive cells likely by forming pores in the cytoplasmic membrane and that it needs a specific target molecule at the surface of the sensitive cells.

1.2.3.2 Optimum conditions for pediocin production

The production of pediocin by *Pediococcus* spp. is normally performed in complex growth media such as de Man, Rogosa and Sharpe (MRS) and Tryptone Glucose Yeast Extract (TGE). Biswas *et al*. (1991) studied the production of pediocin PA-1/AcH by *Pediococcus acidilactici* H in MRS and TGE (1% tryptone, 1% glucose, 1% yeast extract, 0.2% Tween 80, 0.005% $MgSO_4$ and 0.005% $MnSO_4$) broth. They found that this strain could produce relatively much higher levels of pediocin in TGE broth than in MRS, several modifications of MRS and buffered TGE broth. The authors suggested that buffered system in MRS and buffered TGE broth exhibited the negative effect on the production of pediocin by this producing strain. In contrast, *Lactobacillus plantarum* WHE92 produced pediocin at a higher level in

MRS broth than in TGE broth indicated that the optimum culture medium for pediocin production is dependent on the producer strain.

Carbon source, nitrogen source, vitamins and minerals were found to influence the pediocin production. Generally, glucose was the optimal carbon source for pediocin production by several producer strains (Anastasiadou *et al*., 2008b, Papagianni and Anastasiadou, 2009), whilst organic nitrogen source was more suitable for pediocin production than inorganic nitrogen source (Guerra and Pastrana, 2001; Vázquez et al., 2006). The addition of NH_4PO_4, $CaCl_2$ and KH_2PO_4 in culture medium decreased pediocin production, whilst $MnSO_4.H_2O$ increased pediocin production as reviewed by Papagianni and Anastasiadou (2009).

Incubation temperature and pH of culture medium also affected pediocin production (Biswas *et al*. 1991; Papagianni and Anastasiadou, 2009; Altuntas *et al*. 2010). The optimum temperature for pediocin production was in the range of 30-37 °C (Yin et al., 2003; Wu et al., 2004; Todorov and dicks., 2005; Anastasiadou et al., 2008; Altuntas *et al*. 2010). The optimal pH of culture medium was initially between 6.0 and 6.5 and declined to reach a value of 3.7 to 3.5 after 25 hours of incubation (Anastasiadou et al., 2008). The low pH value at the end of fermentation is required for the posttranslational processing and secretion of pediocin. This could explain why the buffer system in culture medium was not suitable for pediocin production.

1.2.3.3 Control of *Listeria* sp. in food by pediocin PA-1/AcH

The use of pediocin PA-1/AcH and other variants of pediocin to control the growth of *Listeria* sp. in food is increasing. ALTA^{TM} 2351 is an example of pediocin that has already marketed as a food additive. As a role of food bio-preservative, pediocin could be applied by two way including direct application into food matrix (Schlyter *et al*., 1993; Huang *et al*., 1994; Garriga *et al*., 2002; Chen et al., 2004; Mattila *et al*., 2003; Grosulescu *et al*., 2011) and indirect application by coated or immobilized on food packaging (Santiago-Silva et al., 2009). Both of these techniques exhibited high anti-listeria efficiency. The 0.9 log reduction of *Listeria monocytogenes* in turkey slurries was observed after pediocin (5000 arbitrary units/ml) was applied (Schlyter *et al*., 1993). Huang *et al*. (1994) reported that the

addition of pediocin 5 to partly skimmed milk containing 1% and 3.25% milk fat led to a sharp reduction in viable *Listeria* by approximately 3.0 log cycle during the first day. At the moderate contamination level of about 3 log CFU/ml, the survival of *Listeria* in milk was below the detection limit suggested the potential of its use as anti-listeria agent in milk. The hurdle of high pressure technology with chilled refrigeration and application of pediocin could reduce the Listerial population contaminated in meat model system for more than 2 log CFU/g (Garriga *et al*., 2002). Mattial *et al*. (2003) found that application of pediocin AcH on the surface of sausages could decrease the number of *L. monocytogenes* for more than 1 log CFU/g.

Using a packaging as a carrier of pediocin could be advantage for its anti-listeria activity on food. The packaging could act as a reservoir and diffuser of the concentrated pediocin molecules to the food ensuring a gradient-dependent continuous supply of bacteriocin. It could protect pediocin from inactivation by interaction with food components such as lipid and enzyme inactivation. In addition, the application of pediocin on food-contact packaging required lower amounts of pediocin compared to application in the whole food volume, decreasing the production cost. Moreover, the application of pediocin via antimicrobial packaging could avoid the addition of pediocin, a preservative, directly to foods, attemping to actual tendency of consumers of searching for healthier foods and free of additives. However, for our knowledge, only few researches have studied on the application of pediocin to food packaging. Santiago-Silva *et al*. (2009) developed cellulose-based packaging incorporated with pediocin (ALTA® 2351) to control the growth of *Listeria innocua* on sliced ham. It was found that the viable count of *Listeria innocua* on sliced ham was reduced for 2 log cycle after being contacted with the film.

1.2.4 Application of antimicrobial peptides in food packaging

The use of antimicrobial peptides in food packaging can provide the antimicrobial packaging that act to reduce, inhibit or retard the growth of microorganism that may be present in the packaged food or packaging material itself (Appendini and Hotchkiss, 2002), and/or improve microbial food safety and/or improve sensorial properties (Vermeiren *et a*l., 2002; Devlieghere *et al*., 2004). Two methods of incorporation of antimicrobial peptides into food packaging materials

usually used are (i) direct incorporation of antimicrobial peptides into film matrix, (ii) indirect incorporation by coating the antimicrobial peptide onto the film surface.

1.2.4.1 Direct incorporation of antimicrobial peptides into film matrix

Incorporation of bioactive agents including antimicrobials into polymers has been commercially applied in drug and pesticide delivery, household goods, textiles, surgical implants and other biomedical devices. The number of recently published articles and patents suggest that research on the incorporation of antimicrobials into packaging for food applications has more than doubled in the past 5 years. GRAS, non-GRAS and 'natural' antimicrobials have been incorporated into paper, plastics, biopolymers, and have been tested against a variety of microorganisms including *Listeria monocytogenes*, pathogenic *E. coli*, and spoilage organisms (Han and Floros, 1997; Siragusa *et al*., 1999; Han, 2000;Appendini and Hotchkiss, 2002; Dawson *et al*., 2002;Dawson *et al*., 2003;Lee *et al*., 2003;Mauriello *et al*., 2004; Mauriello *et al*., 2005; Liu *et al*., 2007; Marcos *et al*., 2007; Millette *et al*. 2007; COMA, 2008; Iseppi *et al*., 2008; Jin *et al*., 2009; Santiago-Silva *et al*., 2009). Many antimicrobial peptides have the potential to be incorporated into polymers such as magainins, cecropins, and defensins (Hotchkiss, 1997). Other examples included: the polyethylene-based plastic film incorporated with nisin in liquid form that was used to vacuum-pack beef carcasses (Siragusa *et al*., 1999).The rationale for incorporating antimicrobial peptides into the packaging is to prevent surface growth in foods where a large portion of spoilage and contamination occurs.

Many antimicrobials are incorporated at 0.1-5% w/w of the packaging material, particularly films. Antimicrobials may be incorporated into polymers in the melt or by solvent compounding (Dawson *et al*., 2003). For antimicrobial peptides, the heat-sensitive compounds (Appendini and Hotchkiss, 2001), solvent compounding may be a more suitable method for their incorporation into polymers. Although some bacteriocins and antimicrobial peptides are relatively heat-resistant (Appendini and Hotchkiss, 2001), their antimicrobial activity may be higher when heat is not used in the process. Studies on nisin showed that the activity of the bacteriocin in cast films was three times greater than that of heat-pressed films (Dawson *et al*., 2003).

Dawson *et al*. (2003) studied the effect of film-forming method (casting or heat-pressing) on the retention of biologically active nisin (Nisaplin®) and release of activity into water at four different temperatures (5, 25, 35 and 45 °C). Nisin activity was measured using the agar diffusion method against *Lactobacillus plantarum* 1752. The authors reported that film-formation method had an effect on retention of nisin activity ($p < 0.05$) with casting retaining greater activity than heat-pressing. Higher temperatures used to form heat-pressed films may have attributed to the greater loss of activity although the higher temperature used to form heat-pressed wheat gluten films (140 °C) compared to that used for heat-pressed corn zein (125 °C) did not result in a significant difference in activity retention.

The release of nisin activity into water was also affected by heat-pressed processing. The result showed that higher amounts of activity were released from casted wheat gluten (CWG) films at all times compared to other film types. The higher nisin release from CWG films was due in part to the higher retention of activity through the film-formation step. However, both CWG and heat-pressed wheat gluten (HPWG) films released approximately 1% of retained nisin activity. The casted corn zein (CCZ) and heat-pressed corn zein (HPCZ) films released about 0.7% of retained activity. At 45 °C as with all other temperatures, CWG released more nisin activity than all other film types. At 45 ºC, more nisin activity migrated into water from HPWG than either corn zein film type. Between the corn zein films, the CCZ film released more activity than the HPCZ after 4 h of exposure to water. Although the net release of nisin activity into water was greatest from CWG films, the percentage of activity released (based on activity that was left available after film production) did not differ between CCZ and CWG films ($p > 0.05$) with both cast films having greater % release rates than heat-pressed films. This trend held for all exposure temperatures but differences between cast and heat-pressed release was greater at higher temperatures compared to 5 °C.

This study supported the hypothesis of casting or solvent compounding processing is more suitable for used to incorporate antimicrobial peptide into food packaging when compared to heat-pressed processing. Therefore, the later researches and developments in technique to incorporate antimicrobial peptide into food packaging have been focused on casting or solvent compounding process.

In solvent compounding, both the antimicrobial and the polymer need to be soluble in the same solvent. Biopolymers are good candidates for this type of film forming process, due to the wide variety of proteins, carbohydrates and lipids which act as plasticizers that form films and coatings. These polymers as well as their combinations are soluble in water, ethanol and many other solvents compatible with antimicrobial peptides (Dawson *et al*., 2003). There are many studies using biopolymers as film forming materials in casting process to produce the antimicrobial packaging incorporated with antimicrobial peptide such as incorporation of enterocin produced by *Enterococcus faecium* CTC492 into alginate, zein and polyvinyl alcohol film (Marcos *et al*., 2007; Marcos *et al.*, 2008), incorporation of nisin into modified alginate film (Millette *et al*., 2007) and incorporation of pediocin ALTA® 2351 into cellulose acetate film (Santiago-Silva *et al*., 2009).

Santiago-Silva *et al*. (2009) investigated the antimicrobial efficiency of cellulose acetate film incorporated with Pediocin ALTA®2351 on preservation of sliced ham. 25% and 50% of Pediocin ALTA®2351 was added directly into cellulosic base film solution and then cast to produce the film. The antimicrobial efficiency of the films against *Listeria innocua* ATCC 33090 and *Salmonella* sp. ATCC 6539 on sliced ham was tested by means of a challenge test, in which the slices were immersed in 0.1% peptone solution containing about 10^6 CFU/mL of *Listeria innocua* or *Salmonella* sp. The experiment was set up overlapping the slices of ham with the films (control, 25% and 50% of pediocin). These systems were packaged under vacuum and stored at 12 ºC. The slices of ham were analyzed for *Listeria innocua* and *Salmonella sp*. counts at 0, 3, 6, 9, 12 and 15 storage days.

The result showed that the film with 50% pediocin promoted an inhibition on *L. innocua* counting, while films with 25% pediocin and control allowed the microorganism growth, increasing, on average, 0.8 and 1.4 logarithmic cycles, respectively, during storage period. At the end of storage period was found significant difference ($p < 0.05$) on *L. innocua* counting among treatments. It was observed for the ham slices separated with the films: control, 25% and 50% groups, counts around to 7.60, 6.80 and 5.60 logCFU/g, respectively, demonstrating reduction of 2 log cycles for the product in contact with film 50%, and less than 1 log cycle for the film 25%, in relation to the control.

In the case of *Salmonella* sp., it can be noted a small reduction in *Salmonella* sp. counting in the ham slices in contact with film 25% and 50% pediocin in relation to the control. The results confirm the in vitro studies, in which ALTA®2351 pediocin was slightly effective against *Salmonella* sp. a gram-negative microorganism. In this study, it was detected greater efficiency of pediocin on the inhibition of *L. innocua* growth compared to *Salmonella* sp. This can be attributed to the fact that pediocin has specificity against species of Listeria, and also due to the preference of this microorganism to grow at the surface of the food. Katz (1999) emphasizes that the pediocin acts particularly against *Listeria* sp., on its inhibition or elimination.

The conditions used in this assay were drastic. The storage temperature used in this work (12 °C) is not adequate for storage under refrigeration, but this is a reality commonly found in the retail market product. Besides the abusive temperature in the storage period, a high amount of microorganism (10^6 CFU/mL) was inoculated in the slices of ham. Therefore, these factors must be taken in consideration in the analysis of the performance of the films.

This study is a case that shows the potential of direct incorporation technique to incorporate the antimicrobial peptide directly into food packaging matrix to produce the antimicrobial food packaging with high antimicrobial efficiency. The morphological characteristic of film produced by this technique in this study, however, was poorer when compared with the non-pediocin contained film. The alteration of film morphology was increased when pediocin concentration increased. The control film was visually the most transparent and the most homogeneous one. The film surfaces containing pediocin became rougher as the antimicrobial concentration increased and, consequently increased the quantity of granules dispersed in the matrix, due to the lack of antimicrobial compound solubility. Moreover, direct incorporation of pediocin into film matrix showed the negative effect on the film thickness and some mechanical properties of produced films such as force in rupture and deformation in rupture.

To improve the physico-chemical, especially film transparency and thickness, and antimicrobial properties of film incorporated with antimicrobial peptide, Imran *et al*. (2010) added hydrophilic plasticizer into cellulosic matrix of film

incorporated with commercial antimicrobial peptide. In this study, the antimicrobial film was produced using casting process with direct incorporation of antimicrobial peptide into film forming solution. Nisaplin®, commercial nisin, acted as the antimicrobial peptide when glycerol was used as hydrophilic plasticizer.

The authors suggested that film thickness depended greatly on film nature and composition as suggested by Mali *et al*. (2004) and Sebti *et al*. (2007). The addition of plasticizer alone (10-50% w/w) produced the HPMC films with thickness statistically indifferent ($p > 0.05$). The narrow range variation in thickness might be there because elevated glycerol content maintained higher moisture content at the end of film drying (Chen and Lai, 2008). However, the incorporation of Nisaplin® radically increased the film thickness (70 ± 12 µm) due to the formation of salt crystals (salt present in Nisaplin® formulation) in course of drying and the film was non-homogenous. In this study, the composite films of Nisaplin® with 30 and 50% glycerol normalized the crystals effect by homogenous dispersibility because plasticizer could reduce the intermolecular forces and increase the mobility of polymer chains. The tensile strength of composite films decreased, while ultimate elongation was increased significantly.

The transparency of antimicrobial film with glycerol (10-30% glycerol) as a plasticizer was not significantly different from control film (without nisin and glycerol); however, the film incorporated with nisin without glycerol reduced the film transparency. The result indicated that Nisaplin® was the more important factor which reduces the film transparency compared with glycerol. This attribute of the HPMC edible film justified its use as edible film biopolymer primarily to fulfill the consumer eagerness to see food through packaging. Transmission percentage had depended on the concentration of plasticizer (glycerol) and addition of active agent Nisaplin®. The transparency of HPMC film was inversely proportional to the Nisaplin® addition and glycerol concentration as an effect individual.

As suggested by the authors glycerol played a positive role to improve the transparency of films containing Nisaplin® due to improved dispersibility of Nisaplin® in network of HPMC film and thus provided homogenous film which had higher transparency values. HPMC film containing Nisaplin® only had given transmission value close to bottom which was improved with the addition of glycerol.

But higher addition of plasticizer decreased the transparency, as HPMC films containing both Nisaplin® (104 IU) and glycerol (50%, db) showed minimum transmission value second to Nisaplin® addition alone.

This study demonstrated that the addition of glycerol as a plasticizer can improve stretch-ability, transparency and film homogeneity. In addition, the antimicrobial efficiency of active film was high. The film had an ability to inhibit the growth of 16 strains of indicators (*Listeria, Staphylococcus, Bacillus* and *Enterococcus*). Glycerol found to have no effect on the release of nisin from film and thus the inhibition zone from active film was similar to the plasticized active film. However, plasticizer generally causes the increased water permeability so it must be added at a certain amount to obtain the films with improved flexibility, thickness and transparency without significant decrease of mechanical strength and barrier property to mass transfer (Möller *et al*, 2004; Brindle and Krochta, 2008).

1.2.4.2 Indirect incorporation by coating the antimicrobial peptides onto film surface

In some cases, the antimicrobial peptide was incorporated into plastic film which had preferable properties such as water solubility, tensile strength, flexibility, water and gas permeability, color and transparency when compared to biopolymer films. The problems occurred during incorporating the antimicrobial peptide directly into plastic polymer was the incompatibility of antimicrobial peptide with polymers, caused the poor distribution of antimicrobial peptide in film matrix and made film opaque which lowering the consumer acceptance (Santiago-Silva *et al*., 2009). To solve this problem, the indirect incorporation by coating the antimicrobial peptide onto plastic film surface was used (Iseppi *et al*., 2008). Cast edible films, for example, have been used as carriers for antimicrobial peptides and applied as coatings onto packaging materials and/or foods. Examples include, pediocin-containing milk-based powder adsorbed onto cellulose casings and barrier bags (Ming *et al*., 1997), nisin adsorbed onto the silicon surface (Daesche *et al*., 1992), nisin/EDTA/citric solutions coated onto PVC, nylon and LLDPE films (Natrajan and Sheldon, 2000), nisin in methylcellulose coating (Sebti *et al*., 2003; Sebti *et al*., 2007), nisin coated onto the surface of polyethylene, ethylene vinyl

acetate, polypropylene, polyamide, polyester, acrylics and polyvinyl chloride films (COMA, 2008) and plastic film coated with nisin-chitosan solution to use as anti-listerial packaging for cold-smoked salmon products (Ye *et al.*, 2008a).

Iseppi *et al*. (2008) studied the anti-listerial activity of a polymeric film coated with hybrid coatings doped with Enterocin 416K1 for use as bioactive food packaging. The antimicrobial film was produced by entrapping the Enteriocin 416K1, produced by *Enterococcus casseliflavus* IM416K1, in an organic-inorganic hybrid coating and then was coated onto a low-density polyethylene (LDPE) film surface. The antimicrobial activity of the coated film was evaluated against *Listeria monocytogenes* NCTC 10888 by qualitative modified agar diffusion assay, quantitative determination in listeria saline solution suspension and direct contact with artificially contaminated food samples.

The result of qualitative evaluation showed that the enterocin doped coated film showed clear zone of inhibition in the indicator lawn in contact with and around (about 2 mm). No activity against the indicator strain was observed for the undoped coated film. This result suggested that the manipulations required to prepare the coated films do not affect the anti-listerial activity of bacteriocin.

For the quantitative antibacterial evaluation, after 24 h of exposure, the viable counts of *Listeria monocytogenes* NCTC 10888 in the solutions in contact with the enterocin-activated coated films decreased by about 0.5 log units in comparison with the results observed for undoped control films. Listeria enumeration was further reduced to a difference of 1.5 log units after 72 h ($p = 0.0096$). This further reduction of the listeria counts suggested that there was a progressive release of enterocin and ruled out an effect due to the enterocin present only on the surface of the coating. In this latter case, a decrease of the listeria viable counts would be observed exclusively over a short time of contact. Similar rates of inhibition have been reported in other studies on plastic films containing different antimicrobial compounds (Scannell *et al*., 2000; Mauriello *et al*., 2005; Han *et al*., 2007).

Listeria monocytogenes NCTC 10888 viable counts, observed in the contaminated frankfurters samples packed in enterocin-doped and undoped coated films stored at 4 and 22 °C, were also reported. The *Listeria monocytogenes* population in the frankfurters samples maintained at 4 °C in presence of enterocin

doping, rapidly decreases during the first 24 h (> 1 log units) compared to the control. This level of decreased of *Listeria monocytogenes* population was maintained until the 14th day, and only after 28 days there was a reduced difference (< 0.5 log units) (p = 0.0127) with respect to the control. For the samples stored at 22 °C, a similar trend was observed after one day and the difference with respect to the control remains significant (p = 0.0475) at longer times, even if it decreased with time (Iseppi *et al*., 2008).

By observing, it was noteworthy that after a first significant decrease of the bacteria population over the first 24 h, the curve profiles of both undoped and enterocin-doped films were very similar for frankfurters. This suggested that the diffusion of enterocin out of the coating was fast for the enterocin contained within the first layers of the coating, but was slower than the bacteria growth rate for the enterocin contained in the deep layers of the coating. In this respect, we had to consider that the slow diffusion of enterocin, with molecular weights of about 3–4 kDa, was not surprising and that coatings with different morphologies and compositions had to be developed in order to achieve higher diffusion rates. This data were in agreement with those of other researchers who had observed prompting results in reducing bacteria populations within food samples wrapped up in packaging materials containing bacteriocins (Ming *et al*., 1997; Scannell *et al*., 2000; Dawson *et al*., 2002; Lee *et al*., 2003; Mauriello *et al*., 2004).

In conclusion of this study, the effects of the bioactive packaging demonstrated that the Enterocin 416K1 retained a good antimicrobial activity when trapped in a hybrid coating applied to plastic film for food packaging, as it was previously reported for other systems (Ming *et al*., 1997; Siragusa *et al*., 1999; Scannel *et al*., 2000;Mauriello *et al*., 2005). A further advantage of the approach presented in this paper in point of potential applications, was that the coated films showed the same transparency of the uncoated ones, and that the bioactive coating has good adhesion to the plastic films even without any preliminary treatment of the plastic film surface. All these results, along with the relatively low cost of reactants and the easy application to plastic substrates, confirmed the possible industrial use of this technology in the food packaging field.

1.2.5 Some properties of antimicrobial films

When antimicrobial peptides are added to packaging materials to reduce microbial spoilage, it may affect general physical properties and processing ability/machinability of the packaging materials. General properties of packaging materials include mechanical properties such as tensile strength, elongation, stiffness, and physical properties such as oxygen (and other gas) permeability, water vapor permeability, wettability, water absorptiveness, grease resistance, brightness, haze, gloss, transparency, and others (Han, 2000).

1.2.5.1 Mechanical properties of film

The performance of the packaging materials must be maintained with the addition of the active substances, even though the materials contain more heterogeneous formulations. In the case of plastics, the antimicrobial peptides are very-low-molecular-weight chemicals compared to the size of the polymeric structure and are added in small amounts. In properly designed antimicrobial packaging system, the chemicals will position themselves in the amorphous structural regions of the polymeric structure and may not affect the mechanical strength of the polymeric packaging materials (Han, 2000; Pérez- Pérez *et al*., 2006).

Considering the huge size of the amorphous area of the polymeric materials (or porous area of papers) to the relatively small size of the antimicrobial peptide, a large amount of the antimicrobial peptides may be needed to show any effect on the tensile strength of the packaging materials. However, after the amorphous area of the polymers and the porous area of the papers are saturated by a high concentration or large powder particle of antimicrobial peptides, the tensile strength of the antimicrobial materials could be adversely affected. Santiago-Silva *et al*. (2009) investigated that the maximum force in rupture of the cellulose acetate film incorporated with 25% (w/w of cellulose weight) of pediocin indicated that it presented greater resistance ($p < 0.05$) in relation to the control and 50% films, which did not differ between each other. However, it was not found significant difference ($p < 0.05$) among the films for deformation in rupture. The addition of pediocin in the concentration of 25% increased the force required to film rupture in relation to

control, which contained 0% pediocin, indicating a possible interaction between the pediocin and the cellulosic matrix which became more rigid. However, at the concentration of 50%, it possibly had an excess of pediocin incorporated thus weakening the cellulosic chains. On the other hand, Pires (2006 in Santiago-Silva *et al*.,2009) when evaluated the addition of nisin and natamycin, isolated or combined into cellulosic base films observed reduction of force at rupture with the increase of substances concentration in the matrix. The control film (without antimicrobial) was the most resistant compared to those incorporated with 50% nisin + 8% natamycin and 50% nisin. Similar results were obtained by Kristo *et al*. (2008) when investigated the impact of antimicrobial compounds on mechanical properties of sodium caseinate films. They verified that the addition of increasing sodium lactate and potassium sorbate concentration resulted in reduction of maximum force and increasing of elongation at break, suggesting that they act as plasticizers for the films. Cha *et al.* (2002) when evaluated the effects of antimicrobial incorporation on mechanical properties of films also verified that films containing antimicrobial compounds were weaker compared to those without antimicrobials. Sivarooban *et al.* (2008) investigated that the tensile strength of soy protein edible film decreased when nisin was incorporated into the film. They described that when the hydrophobic nisin incorported into soy protein film solution which had both hydrophilic and hydrophobic properties, those hydrophobic nisin molecules were tent to form larger clusters in film matrix and may weaken the strong interactions between protein molecules.

In the case of coated film, the study of Chollet *et al.* (2009) when investigated the difference in the tensile strength of Poly Ethylene (PE) film coated and uncoated with nisin loaded Hydroxy Propyl Methyl Cellulose (HPMC). The tensile strength of plastic films were 31 ± 4 MPa for the control and 28 ± 3 MPa for the multi-layer PE film coated with nisin loaded HPMC film. This absence of difference could be due to a lack of interactions or low interactions between the hydrophilic HPMC coating polymers and hydrophobic PE films. The possibility of adherence of HPMC and PE films was obtained by PE film corona surface treatment at a potency of 5 kW before coating. Similarly, Grower *et al*. (2004) reported that

nisin addition made no difference in the mechanical properties of LDPE films coated with a nisin-containing cellulose-based coating.

1.2.5.2 Optical properties of film

Besides mechanical strength changes, incorporation of antimicrobial peptides usually reduces optical properties of plastic films such as transparency. This may result in serious disadvantage in using this antimicrobial plastic film for see-through packaging. In the study of Chollet *et al.* (2009), there was no change in opacity of plastic film coated with nisin loaded HPMC film. Therefore, the films maintained a good transparency which is essential for consumers, who wish to see foods through the packaging before purchase.

1.2.5.3 Effect of antimicrobial packaging on quality and shelf life of packaged food

A little research has been conducted to investigate the effect of antimicrobial packaging on quality of packaged food. However, effect of modified atmosphere packaging and vacuum packaging on the quality of packaged food was widely studied. Lee *et al*. (2003) studied the effects of micro-perforated film on the quality and shelf life improvements of pork loins during chilled storage. The results showed that the total aerobic counts of control sample were significantly lower than those from micro-perforated film treatment. At 7 days of storage at 0 °C, total color difference of control meat sample was significantly higher than other samples. Moreover, sensory evaluation analyses showed that micro-perforated film treated samples had generally better in all evaluated parameter (color, odour, outer appearance and overall acceptability) over the storage time. Lee and Yoon (2001) studied on the quality changes and shelf life of imported vacuum-packaged beef chuck during storage at 0 °C. The result showed that the off-odour of vacuum-packaged sample was detected after 66 days of storage and colour deterioration was detected at day 76 of storage. It means the vacumm-packaging can maintain the quality of beef upto 66-76 days at 0 °C. Park *et al.* (2010) studied on the application

of chitosan-incorporated LDPE film, as an antimicrobial packaging, to sliced fresh red meat for shelf life extension. Parameters investigated in this study were microbial counts, color and total color different of packaged meats.

In conclusion, food packaging affects the quality and shelf life of packaged foods. Quality attributes of food that affected by packaging are color, pH, microbial growth, biogenic amines, volatile compounds, as well as, sensory characteristic. The effective antimicrobial packaging should inhibit the microbial growth and improve the quality of packaged foods in order to prolong shelf life and obtain the consumer acceptance. Previous literatures reviewed that the parameters that should be concerned when study on the effect of antimicrobial packaging on quality and shelf life of packaged food are pH of food, color, microbial growth, biogenic amines formation. All of these parameters relate to sensory characteristics (color, odor and outer appearance) which directly relate to consumer acceptability.

1.2.6 Polylactic acid (PLA) in the role of antimicrobial food packaging

1.2.6.1 Introduction to PLA

Polylactic acid (PLA) is a biodegradable polymer derived from lactic acid. It is a highly versatile material and is made from 100% renewable resources like corn, sugar beets, wheat and other starch-rich products (Krishnamurthy *et al*., 2004). Polylactic acid exhibits many properties that are equivalent to or better than many petroleum-based plastics, which makes it suitable for a variety of applications (Auras *et al*., 2003). It is important that PLA compares well with other popular plastics already used for packaging. It is clear and naturally glossy like the polystyrene used in "blister packs" for products such as batteries, toys, and many others.

PLA is resistant to moisture and grease. It has flavor and odor barrier characteristics similar to the popular plastic polyethylene terephthalate (PET) used for soft drinks and many other food products (Ruban, 2009). Tensile strength and modulus of elasticity of PLA is also comparable to PET. It can be formulated to be either rigid or flexible and can be copolymerized with other materials. Polylactic acid can be made with different mechanical properties suitable for specific manufacturing processes, such as injection molding, sheet extrusion, blow molding, thermoforming,

film forming and fiber spinning using most conventional techniques and equipment (Jamshidian *et al*., 2010).

PLA is a nonvolatile, odorless polymer and is classified as GRAS (generally recognized as safe) by the Food and Drug Administration in the United States (Jamshidian *et al*., 2010). For all of its outstanding properties and earth-friendly biodegradability, PLA is a material that is creating a lot of interest in the packaging industry.

1.2.6.2 The uses of PLA as food packaging material

As review by Jamshidian *et al*. (2010), PLA has potential for use in a wide range of applications including the use of PLA as a food packaging materials. PLA food packaging applications are ideal for fresh products and those whose quality is not damaged by PLA oxygen permeability. PLA is a growing alternative as a "green" food packaging polymer. New applications have been claimed in the field of fresh products, where thermoformed PLA containers are used in retail markets for fruits, vegetables, and salads. The market capacity of these products packaged in PLA is unlimited.

The major PLA application today is in packaging (nearly 70%). In the field of packaging, two specific areas have received close attention, namely high-value films and rigid-thermoformed containers. PLA brings a new combination of attributes to packaging, including stiffness, clarity, deadfold and twist retention, low-temperature heat sealability, as well as an interesting combination of barrier properties including flavor, and aroma barrier characteristics. Moreover PLA is good resistant to oils and terpens, excellent printabiliby, metalizable and antifogging ability. Commercialized PLA products demonstrate this fact that PLA is not being used solely because of its degradability, nor because it is made from renewable resources; it is being used because it functions very well and provides excellent properties at a competitive price. As reviewed by Jamshidian *et al*. (2010), there are many commercialized PLA products in today's market and their variety and consumption are increasing rapidly (Table 5).

Table 5. Some commercialized PLA products

Product	Company name
Film and trays for biscuits, fruit, vegetables and meat	Treophan, Natura, IPER, Sainsburys, Sulzer, Ecoproducts, RPC
Yogurt cup	Cristallina/Cargill Dow
Trays and bowls for fast food	McDonalds
Envelope with transparent window, paper bag for bread with transparent window	Mitsui, Ecocard

Source: Jamshidian *et al*. (2010)

1.2.6.3 Development of PLA film incorporated with antimicrobial peptides for uses as antimicrobial food packaging

The innovative strength of PLA antimicrobial packaging has a direct impact on consumer health by creating safer and more wholesome packaged foods. Active packaging realizes certain extraordinary and vital functions other than providing an inert barrier between product and external conditions. Active substances that are important and considered for novel bioactive packaging include antimicrobials, vitamins, phytochemicals, prebiotics, marine oils, and immobilized enzymes (Lopez-Rubio *et al*., 2006). A whole range of active additives, including silver-substituted zeolite, organic acids and their salts, bacteriocins such as nisin and pediocin, enzymes such as lysozyme, a chelator like ethylenediaminetetraacetic acid (EDTA), lactoferrin, and plant extracts have already been successfully incorporated in antimicrobial active packaging (Joerger 2007).

Currently, there are very little research exists to address the incorporation of antimicrobial peptide to PLA film for use as antimicrobial food packaging. An example of development of PLA film incorporated with antimicrobial peptide for use as antimicrobial food packaging is the direct incorporation of nisin to PLA film (Jin and Zhang, 2008). The PLA film containing nisin inhibited the growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in simulated food including orange juice and liquid egg white. The developed film showed high potential for use

in antimicrobial food packaging. Jin *et al*. (2009) developed the PLA film coated with nisin solution. The authors suggested that neat PLA had no capability to be coated with nisin because of the opposite hydrophobic property of PLA and nisin solution. In order to improve the capability to be coated with nisin solution, the authors incorporated pectin with hydrophilic property into PLA film before coated with nisin solution. Pectin added into PLA film play an important role in embedding nisin into the film. The result from this study indicated that PLA/pectin incorporated with nisin has a great potential in antimicrobial food packaging to reduce post-process growth of food pathogens.

1.3 Objectives of study

1. To optimize culture medium and incubation conditions for the production of pediocin PA-1/AcH, an antimicrobial peptide produced by *Pediococcus pentosaceus* BCC 3772.
2. To develop a method for incorporating hydrophilic antimicrobial peptide into hydrophobic film by using pediocin PA-1/AcH and poly(lactic acid) film as a model and to characterize the physical, mechanical and antimicrobial properties of the produced film.
3. To study the effect of the PLA/SP film incorporated with pediocin PA-1/AcH on quality and shelf life of packaged food.
4. To screen, purify and characterize the putatively novel antimicrobial peptide produced by lactic acid bacteria which exhibits wide spectrum of inhibition.
5. To validate the incorporation technique developed in 3. using a putatively novel antimicrobial peptide as an active compound and poly(lactic acid) as a packaging polymer.

CHAPTER 2

OPTIMIZATION OF PEDIOCIN PA-1/ACH PRODUCTION BY *PEDIOCOCCUS PENTOSACEUS* BCC 3772

2.1 Abstract

A sequential optimization based on Plackett-Burman design (PBD), central composite design and one-factor-at-a-time (OFAT) method was employed to optimize the production of pediocin PA-1/AcH by *Pediococcus pentosaceus* BCC 3772. Tryptone Glucose yeast Extract (TGE) broth (1% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) glucose, 0.2% (w/v) Tween80, 0.03 mM $MnSO_4$ and 0.02 mM $MgSO_4$) was used as basal medium. The analysis from PBD indicated that sodium caseinate and yeast extract had significant positive effects on pediocin PA-1/AcH production. According to the response surface methodology (RSM) analysis, the optimal culture medium contained 1.5% (w/v) of sodium caseinate, 1.5% (w/v) of yeast extract and 0.5% (w/v) of glucose. Based on OFAT, the optimal initial pH and incubation temperature were at 6.5 and 30 °C, respectively. The production of pediocin PA-1/AcH under the optimal condition was about 16-fold and 256-fold higher than cultivations in the un-optimized TGE and MRS broths, respectively. In addition, browning index of pediocin PA-1/AcH preparation was significantly lower, which overcomes the limitations and increases the possibility for use in food packaging.

2.2 Introduction

Pediococcus pentosaceus BCC 3772 produced the anti-listeria substance called pediocin PA-1/AcH (Kingcha *et al*., 2012). Pediocin PA-1/AcH has received a considerable attention because of its outstanding properties (Degnan *et al*., 1993; Gálvez *et al*., 2007; Bernbom *et al*., 2009; Nieto-Lozano *et al*., 2010; Devi *et al*., 2011; Naghmouchi *et al*., 2013; Woraprayote *et al*., 2013). Pediocin PA-1/AcH has been approved for use in food. Pediocin is likely to has higher activity and acts more specifically against *L. monocytogenes* than nisin (Cintas *et al*., 1998; Rodriguez *et al*., 2002). According to the study of Kingcha *et al.* (2012), pediocin PA-1/AcH from *P. pentosaceus* BCC3772 had a molecular mass of 4622.35 Da with N-terminal amino acid sequence of KYYGNGVTXGKHSXSVDWGKATTXIINNGA (X represents an undetermined amino acid residue). From the antimicrobial property point of view, pediocin PA-1/AcH inhibited the growth of *Listeria monocytogenes* (Kingcha *et al*., 2012), an important food borne pathogen always found to be out break in ready to eat (RTE) meat products in many countries (Ye *et al*., 2008), and other pathogens including *Listeria innocua*, *Enterococcus faecalis* and *Bacillus coagulans*. In contrast to nisin, pediocin PA-1/AcH has potential to inhibit Listeria without disturbing other beneficial bacteria (Blay *et al*., 2007). Moreover, pediocin PA-1/AcH was heat-stable and active in wide range of pH (pH 2-10) which showed high potential to be used as natural antimicrobial agent in food packaging (Woraprayote *et al*., 2013) and food application for wide types of food including low-acid and high-acid food (Degnan *et al*., 1993; Nieto-Lozano *et al*., 2010; Kingcha *et al*., 2012).

The production of pediocin PA-1/AcH by *P. pentosaceus* BCC 3772 and other pediococci strains is normally performed in complex growth media such as de Man, Rogosa and Sharpe (MRS) broth which makes the high yield of production (Arokiyamary and Sivakumaar, 2011). However, there were some limitations of the use of MRS broth as a culture medium for a large-scale or industrial-scale production such as the high cost of culture medium, the complexity of medium composition and high browning index which interferes pediocin purification and clarification for

further applications. To overcome these limitations, the simple culture medium with low browning index and low cost for pediocin production should be developed.

Biswas *et al*. (1991) reported that *Pediococcus acidilactici* H in Tryptone Glucose Yeast Extract (TGE) broth (1% tryptone, 1% yeast extract, 1% glucose, 0.2% Tween80, 0.03 mM Mn^{2+} and 0.02 mM Mg^{2+}) yielded the higher production of pediocin AcH compared to MRS and modified MRS broths. The authors suggested that TGE broth was not only effective for pediocin production but also inexpensive. The alternative low cost nitrogen and carbon sources for pediocin production were optimized in several studies. Whey protein, yeast extract and glucose, sucrose and lactose were always used as nitrogen and carbon sources respectively (Guerra *et al*., 2001; Halami and Chandrashekar, 2005). Initial pH of culture medium and incubation temperature were found to play an important role in pediocin production by LAB and were also optimized (Biswas *et al*., 1991; Altuntas *et al*., 2010). To our knowledge, sequential OFAT, Plackett-Burman design and RSM have not been followed in optimization study for the improvement of pediocin production.

The objective of this study was to optimize the culture medium and incubation conditions for the production of pediocin PA-1/AcH by *P. pentosaceus* BCC 3772 using sequential statistical experimental design. To achieve the optimum conditions, the present study was carried out in five stages; firstly, the basal medium was selected from the culture media usually used in pediocin production including TGE and MRS broths. Secondly, optimal food grade nitrogen and carbon sources were screened using OFAT method. Thirdly, the Plackett-Burman design was applied to address the most significant medium components which affect pediocin production. Then, response surface methodology (RSM) based on central composite design (CCD) was employed to determine the optimal concentration of each medium component. Finally, optimal pH and incubation temperature was studied to maximize the pediocin PA-1/AcH production.

2.3 Materials and Methods

2.3.1 Cultures and conditions

Pediococcus pentosaceus BCC 3772 was obtained from BIOTEC culture collection (BCC), Thailand. Prior to experimentation, *P. pentosaceus* BCC 3772 was streaked on de Man Rogosa and Sharpe (MRS, Merck, Germany) agar and grown at 30 °C for 24 h. A single colony obtained on MRS agar was grown twice in MRS broth and incubated at 30 °C for 24 h without shaking. For use as an inoculum in optimization study, the cells of *P. pentosaceus* BCC 3772 was harvested from overnight culture by centrifugation (8000 × g, 10 min, 4 °C) and the pellet was resuspended in one volume of sterile 0.1% peptone water. *Listeria monocytogenes* ATCC 19115 was used as an indicator strain. Prior to use, indicator was streaked on TSB agar (Tryptic Soy Broth, Merck, Germany) and incubated at 37 °C for 24 h. Before used in the experiment, the single colony obtained on TSB agar was grown twice in TSB broth at 37 °C for 24 h.

2.3.2 Assay for anti-listeria activity

The anti-listeria activity was evaluated using spot-on-lawn assay (Ennahar *et al*., 2001) with *Listeria monocytogenes* ATCC 19115 as indicator. Prior to assay, pH of culture supernatants was adjusted to 6.5 and the supernatants were filtered through sterile cellulose acetate filters, 0.2 μm pore size (ADVANTEC®, Japan). For qualitative assay, 10 μl of aliquot of cell-free culture supernatant was spotted directly onto the prepared indicator lawn (in TSB with 1% (w/v) agar). For semi-quantitative assay, two-fold serial dilutions of 10 μl of cell-free culture supernatant were prepared in sterile distilled water in a 96-well microtiter plate prior to spotting on the indicator lawn. Anti-listeria activity is defined as the reciprocal of the highest two-fold serial dilution showing zone of growth inhibition and is expressed as arbitrary unit (AU) per ml (Kwaadsteniet *et al*., 2006).

2.3.3 Optimization procedure and experimental design

2.3.3.1 Effect of culture medium

To select the most suitable culture medium for the production of pediocin PA-1/AcH by *Pediococcus pentosaceus* BCC 3772, MRS broth and TGE broth (Biswas *et al*., 1991) were compared. The medium were sterile by autoclaving at 121 °C for 15 min. An 1% (v/v) inoculum of *Pediococcus pentosaceus* BCC 3772 prepared as in 2.3.1 was added to 50 ml of culture medium in 125 ml Erlenmeyer flask. The samples were incubated at 30 °C without shaking. The samples were taken at different time intervals to determine the viable count of producing strain. The changes in pH were measured using pH meter (Mettler-Teledo, Switzerland). The anti-listeria activity (AU/ml) produced was determined using critical dilution spot-on-lawn method as descirbed in 2.3.2 using *Listeria monocytogenes* ATCC 19115 as the indicator strain. All parameters were measured at the same time intervals for both culture media.

2.3.3.2 Screening for the optimal nitrogen sources

To investigate the effect of nitrogen sources on the pediocin PA-1/AcH production, tryptone and yeast extract in the original TGE broth were replaced with different nitrogen sources at the equivalent nitrogen content. Nitrogen sources and the corresponding concentrations were shown in Figure 2. The production of anti-listeria activity of the samples was performed as described 2.3.2.

2.3.3.3 Screening for the optimal carbon sources

To evaluate the effect of carbon sources on pediocin PA-1/AcH production, glucose in the original TGE broth was replaced with 1% (w/v) of various carbon sources including D-glucose, D-sucrose, D-fructose, D-galactose, D-lactose, D-maltose, D-mannose, and D-xylose. The production of pediocin PA-1/AcH and anti-listeria activity assay of the samples were performed as described in 2.3.2.

2.3.3.4 Screening for the medium components having significant effect on the production of pediocin PA-1/AcH

To identify the significance of culture medium components for the production of pediocin PA-1/AcH, Plackett and Burman (1944) statistical experimental design was performed. Six factors were tested in two level; + (high level) and – (low level). The components tested were, Yeast extract, Sodium caseinate, Glucose, Tween 80, $MgSO_4$ and $MnSO_4.7H_2O$. PB design is a fraction of a two level factorial design and this design offers the screening of a large number of independent factors (N) in a small number of (N + 1) experiments. The main effect of each variables was calculated as the difference between the average of measurements made at the high value (+) and at the low value (–) of that factor. In this study, PB design gave an output of 13 experimental runs (combinations) with 6 independent variables as shown in Table 6. All the experiments were performed in duplicate and the average of anti-listeria activity produced by *P. pentosaceus* BCC 3772 was used as the response (dependent variable). The Plackett-Burman experimental design is based on the first-order model which is as follows:

$$Z = b_0 + \sum b_i x_i \quad (1)$$

Where Z is the response (anti-listerial activity), b_o is the model intercept and b_i is the linear coefficient, and x_i is the level of the independent variable.

The program Design Expert® was used to analyze the experimental Plackett-Burman design. In this experiment, the components having confidence levels above 95% were considered as significant components for the production of pediocin PA-1/AcH and were taken up for further optimization studies keeping the insignificant components at the constant level.

2.3.3.5 Optimization of the screened medium components for pediocin PA-1/AcH production

Response surface methodology (RSM), which has been extensively applied in optimization of fermentation medium composition for bacteriocin production (Li *et al*., 2002; Lee *et al*., 2012), enzymes production (Rajendran and Thangavelu, 2007; Rajendran and Thangavelu, 2009; Natarajan and Rajendran, 2012)

and drug production (Seraman *et al*., 2010), is a collection of statistical techniques for experiment designing, model developing, evaluating factors and searching optimum conditions. In this study, RSM using three-level CCD was used to optimize the screened components for enhance pediocin PA-1/AcH production. A total of 13 experimental run was employed with duplicates at the center point and an axial point located at a specified distance α from the design center in each direction on each axis. The coded and actual values of variables at various levels are given in Table 7.

Upon completion of experimental, pediocin PA-1/AcH production was taken as dependent variable as response (*Y*). The independent variables were coded for statistical calculation according to the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x_i} \qquad (2)$$

When X_i is the dimensionless coded value of the independent variable x_i, x_i is the real value of that independent variable, x_0 is the real value of the independent variable x_i at the center point, Δx_i is the step change. The role of each variable, their interactions, and statistical analysis to obtain predicted yields is explained by applying following quadratic equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad (3)$$

When *Y* is predicted response, β_0 is effect term, β_i is linear effect, β_{ii} is squared effect, β_{ij} is interaction effect, x_i and x_j are the levels of the independent variables.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the promotion of variance explained by the model was given by the multiple coefficient of determination, *R* squared (R^2) value. For each variable, the quadratic models were represented as counter plots (2D) and response surface curves were generated. The software Design Expert® was used in this investigation.

2.3.3.6 Effect of initial pH of culture medium on pediocin PA-1/AcH production

Samples of the optimized medium were prepared by adjusting pH to 6.0, 6.5, 7.0, 7.5 and 8.0 with 0.1 N or 1.0 N NaOH and 0.1 N or 1.0 N HCl before sterilization. The production of anti-listeria activity of the samples was performed as described in 2.3.2.

2.3.3.7 Effect of incubation temperature on pediocin PA-1/AcH production

To find the optimum temperature for the production of anti-listeria activity, the cultivation was performed at 25, 30, 37 and 40 °C in the optimal medium as described above.

2.3.3.8 Time course of pediocin PA-1/AcH production

Pediocin PA-1/AcH production and microbial growth were compared in original (un-optimized) TGE broth (Biswas *et al*., 1991), and optimized media. Cultivations were conducted at 30 °C for 24 h without shaking. Sample were taken at different time intervals to determine the bacterial growth by plating method, pH changes, and the anti-listeria activity (AU/ml) produced was determined using a critical dilution spot-on-lawn method as described above using *L. monocytogenes* ATCC 19115 as indicator strain.

2.3.4 Color characteristics of culture supernatants

The color characteristics (*L*, a*, b**) of culture supernatants were assessed using a Lamda950 UV/Vis spectrophotometer (PerkinElmer, USA). The presence of browning products in culture supernatants was determined by measuring the absorbance at 420 nm and browning index (*BI*) was calculated according to the method of Palou *et al*. (1999) as follows:

$$BI = [100\ (x - 0.31)]/10.72$$

where, $x = (a^* + 1.75L^*)/(5.645L^* + a^* - 3.012b^*)$

2.3.5 Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 17.0 software unless otherwise mentioned. Duncan's Multiple Range Test (DMRT) was used for comparison of mean values at a significant level of 0.05.

2.4 Results and Discussion

2.4.1 Pediocin PA-1/AcH production by *Pediococcus pentosaceus* BCC 3772 in MRS and TGE broths

Growth and the pediocin PA-1/AcH production of *P. pentosaceus* BCC 3772 in MRS and TGE broths were compared (Figure 1). The maximum amount of pediocin PA-1/AcH was produced after 12 h of incubation in both MRS and TGE broths. After 12 h, the anti-listeria activity of cell-free supernatant produced in TGE broth was 16-fold higher than that produced in MRS broth. This result indicated that TGE medium was more suitable for pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 when compared with MRS broth and was further used as the basal medium in optimization study.

As reported by Biswas *et al*. (1991), TGE broth was found to yield the maximum amounts of pediocin AcH, an anti-listeria bacteriocin produced by *P. acidilactici* H, compared with original and modified MRS broths. Pediocin production might be dramatically inhibited by buffering system and phosphate ions present in MRS broth. At pH above 4, pediocin synthesis was suppressed (Guerra, 2001). The need of a final pH of 3.6-3.7 for post-translational processing of pediocin to active pediocin has been also reported (Biswas *et al*., 1991).

From the culture medium compositions, higher amount of glucose in MRS compared with that in TGE broth could decrease the pediocin production. As reported by Guerra *et al*. (2007), the increase in glucose concentration inhibited both the growth and pediocin production by *P. acidilactici* NRRL B-5627. The inhibitory effect of increasing glucose concentrations on pediocin production could be due to

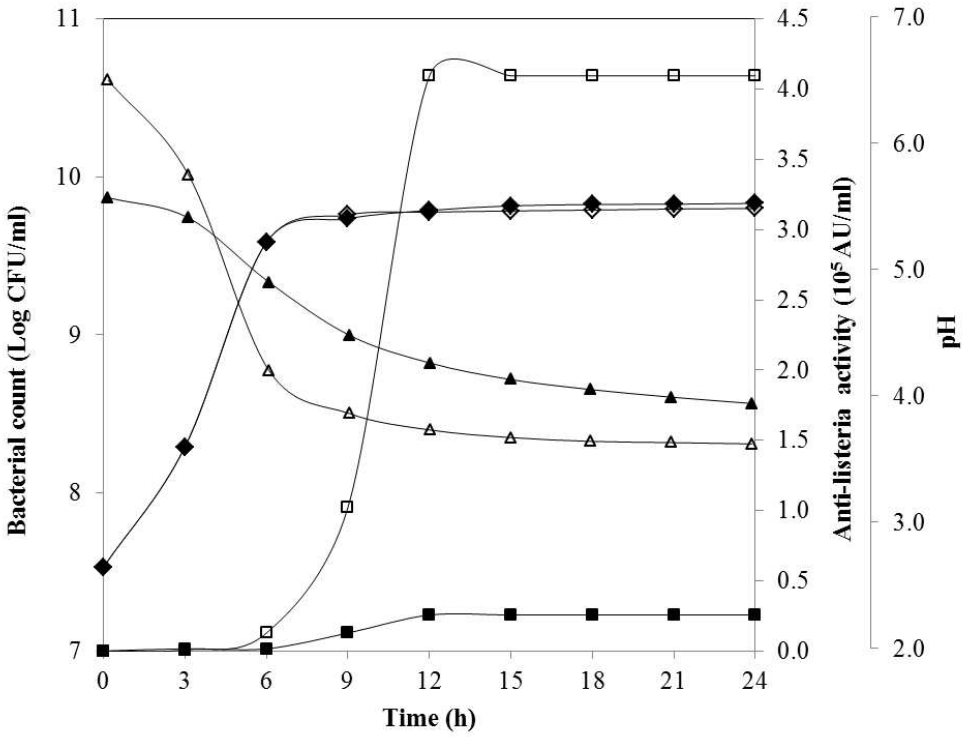

Figure 1. Comparison of bacterial growth (◆,◇), anti-listeria substance production (■,□), and pH changes (▲,△) profiles for *P. pentosaceus* BCC 3772 in MRS medium (closed symbols) and TGE medium (opened symbols). Cells were grown in 50 ml of MRS medium or TGE medium in 125 ml Erlenmeyer flask at 30 °C without shaking; the initial pH of both culture medium was adjusted to 6.5. The anti-listeria activity of the cell-free supernatants was tested against *L. monocytogenes* ATCC 19115. Data are means of duplicate determinations from two independent experiments.

substrate inhibition (Vázquez *et al*., 2003) or due to the carbon source regulation involved in the bacteriocin biosynthesis (de Vuyst *et al*., 1989). In addition, high content of $MgSO_4$ in MRS broth (50-fold of $MgSO_4$ in MRS broth when compared with that in TGE broth) may repress the pediocin production. Jack and Tagg (1992) reported that the reduction of antimicrobial peptide production by *Streptococcus pyogenes* FF22 was found when the excess magnesium was added into culture medium. The same authors explained that the reduction of the anti-listeria activity might be caused by the repression of the growth of the producing strain by excess

magnesium and/or the increasing of growth of the indicator strain by excess magnesium in tested cell-free supernatant.

2.4.2 Screening for the optimal nitrogen sources

The effect of nitrogen sources was determined using TGE broth as a basal medium. Organic nitrogen sources such as tryptone and yeast extract yielded a higher amount of pediocin PA-1/AcH than inorganic nitrogen sources such as ammonium sulfate and monosodium glutamate (Figure 2). Jensen and Hammer (1993) and de Vuyst (1995) suggested that the most important feature of nitrogen sources is the content of peptides and amino acids that can act as inducers or precursors of bacteriocin biosynthesis. In addition, yeast extract may contain various types of minerals, and multi-vitamins that are essential for the growth and bacteriocin production. Compared to hydrolyzed protein sources, utilization of sodium caseinate, protein isolates from whey and soy as nitrogen sources may be restricted by their limited solubility in acidic pH.

Figure 2 indicates that the highest amount of anti-listeria activity was produced in TGE basal medium containing 2% yeast extract and TGE basal medium containing the combination of 1% yeast extract and 1% sodium caseinate (8.2×10^5 AU/ml). Due to the medium cost, the combination of 1% yeast extract with 1% sodium caseinate was selected as the optimal nitrogen source for pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772. In addition, the browning index of culture medium containing this combination was lower than other formulations which produced the equal amount of pediocin (data not shown). Colorless bacteriocin preparation or bacteriocin preparation with low browning index could be directly applied in food and food packaging without any effect on color characteristic of food or food packaging. Combination of casein and yeast extract was also used as nitrogen source for pediocin production by *P. acidilactici* (Bhunia *et al*., 1988, 1991).

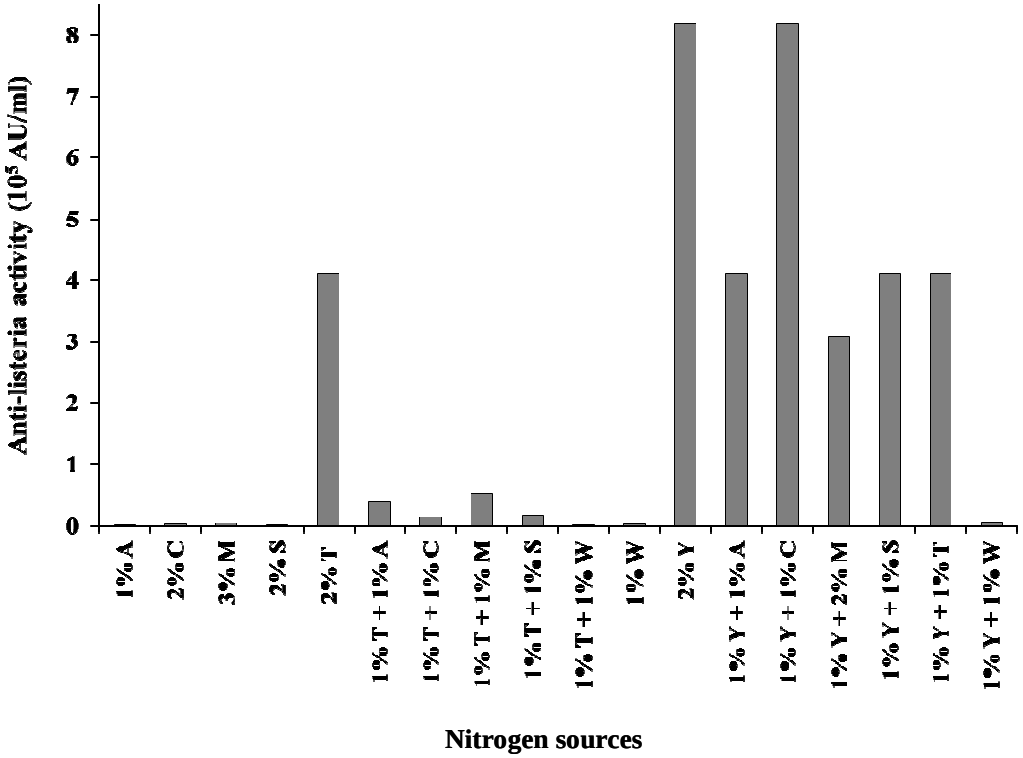

Figure 2. Effect of nitrogen sources on the pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 in static flask culture after 12 h incubation. (A, ammonium sulfate; C, sodium caseinate; M, monosodium glutamate; S, soy protein isolate; T, tryptone; W, whey protein isolate; Y, yeast extract). Results are means of duplicate determinations from two independent experiments.

2.4.3 Screening for the optimal carbon sources

The optimal carbon sources for the pediocin PA-1/AcH was screened in TGE basal medium with 1% yeast extract and 1% sodium caseinate as nitrogen source. Glucose, the carbon source in TGE basal medium, was replaced by sucrose, fructose, galactose, lactose, maltose, mannose and xylose in the equal concentration. Glucose was shown to be the most suitable carbon source for pediocin PA-1/AcH production (Figure 3). Glucose was also reported to be optimal carbon source for the production of streptococcin A-FF22 (John and Ingrid, 1991; Jack and Tagg, 1992), nisin Z (Matsusaki *et al*., 1996) and pediocin (Anastasiadou *et al*., 2008b; Papagianni

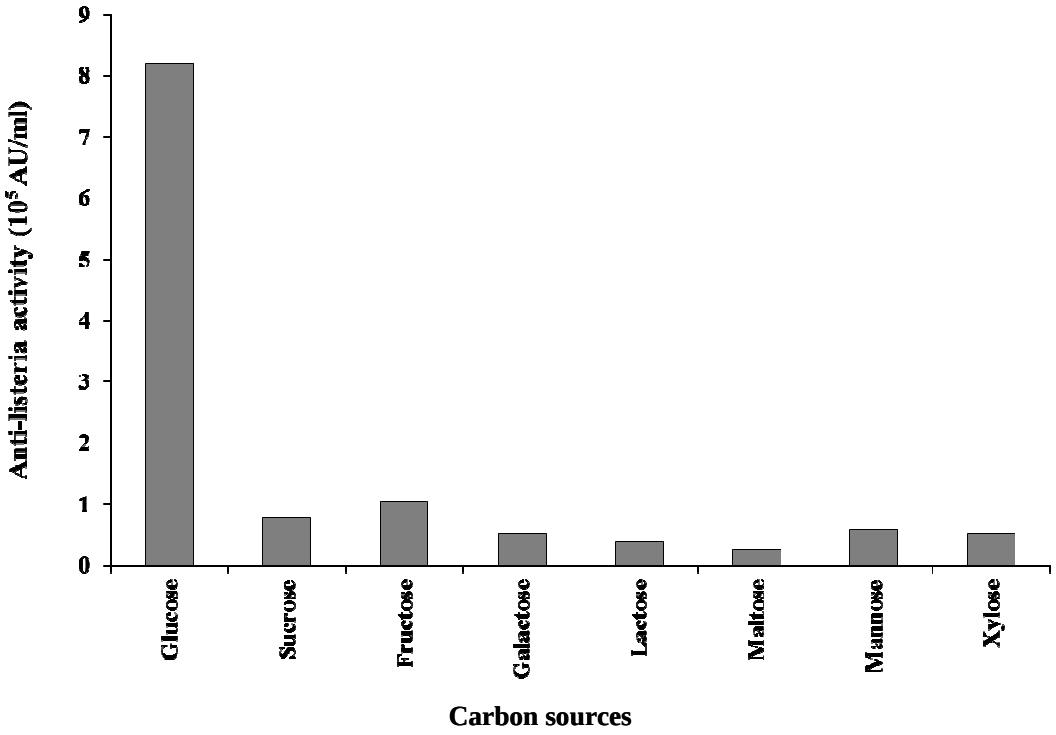

Figure 3. Effect of carbon sources on the pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 in static flask culture after 12 h incubation. Results are means of duplicate determinations from two independent experiments.

and Anastasiadou, 2009) since it could be used instantly for bacterial metabolism and bacteriocin production.

2.4.4 Evaluation of medium components by Plackett-Burman design (PBD)

PBD was used to evaluate the significance of medium components on bacteriocin production. Anti-listeria activity of 1.3×10^5 to 12.3×10^5 AU/ml was observed in the thirteen runs conducted based on PB experimental design (Table 6). The main effect of each medium component was statistically analyzed by PB experimental design and presented in Figure 4.

The analysis indicated that sodium caseinate, yeast extract and glucose had the positive effect, increasing the bacteriocin production when the concentration of these variables were increased in the tested range and vice versa for the other three

Table 6. Plackett-Burman design for the screening of the medium compositions

Run	Yeast extract (%)	Sodium caseinate (%)	Glucose (%)	Tween80 (%)	$MgSO_4$ (mM)	$MnSO_4.7H_2O$ (mM)	Response Anti-listeria activity (10^5 AU/ml)
1	1.50	0.50	1.50	0.40	0.00	0.07	4.10
2	0.50	0.50	0.50	0.40	0.00	0.07	4.10
3	1.50	1.50	0.50	0.00	0.00	0.07	8.19
4	1.50	0.50	1.50	0.40	0.04	0.00	1.28
5	1.00	1.00	1.00	0.20	0.02	0.03	8.19
6	1.50	1.50	1.50	0.00	0.00	0.00	12.29
7	0.50	1.50	1.50	0.00	0.04	0.07	4.10
8	0.50	0.50	0.50	0.00	0.00	0.00	2.56
9	0.50	1.50	0.50	0.40	0.04	0.00	3.07
10	1.50	1.50	0.50	0.40	0.04	0.07	8.19
11	1.50	0.50	0.50	0.00	0.04	0.00	4.10
12	0.50	0.50	1.50	0.00	0.04	0.07	1.54
13	0.50	1.50	1.50	0.40	0.00	0.00	8.19

Data are means of duplicate determinations.

variables (Tween80, $MgSO_4$ and $MnSO_4.7H_2O$) which showed negative effects. Among the six variables tested, sodium caseinate, yeast extract and $MgSO_4$ had the confidence level of 95% or more which suggested that these variables had significant influence on pediocin PA-1/AcH production. Sodium caseinate and yeast extract had significant positive effect on pediocin production but $MgSO_4$ was found to have significant negative effect in the tested range of composition. As discussed above, $MgSO_4$ and nitrogen sources played an important role in the regulation of pediocin production. The analysis also indicated that the increasing of sodium caseinate and yeast extract concentration range from 0.5 to 1.5% significantly increased pediocin production.

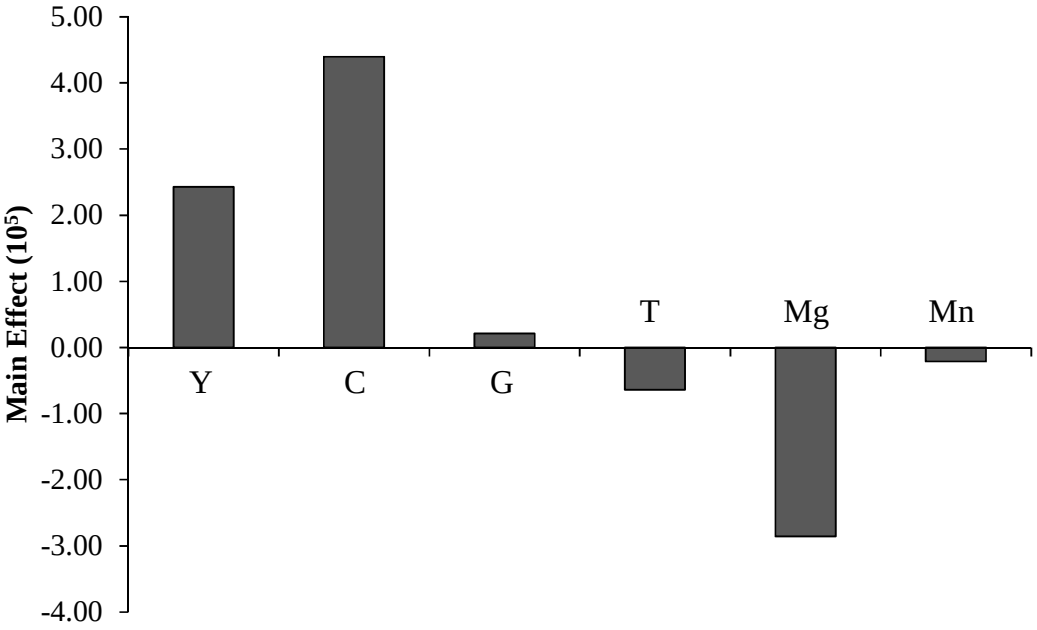

Figure 4. Main effects of the medium compositions on the anti-listeria substance production to the Plackett-Burman experimental results (Y, yeast extract; C, sodium caseinate; G, glucose; T, Tween80; Mg, $MgSO_4$; Mn, $MnSO_4.7H_2O$).

2.4.5 Optimization of the screened medium components for pediocin PA-1/AcH

Response surface methodology was used to determine the optimal concentration of significant variables (yeast extract and sodium caseinate) evaluated by PB screening. Each variable was studied at five concentration levels (Table 7). The concentrations of non-significant components as evaluated by PB design were maintained at their low levels for further optimization procedures.

The average anti-listeria activity (AU/ml) of cell-free supernatant obtained from the culture was used as response or dependent variable. For predicting the optimal value of pediocin PA-1/AcH produced within the experiment, a second order polynomial model was fitted to the experimental results for pediocin PA-1/AcH yield by the Design Expert software. An empirical relationship between the response and variables was expressed by the following equation:

Table 7. Experimental design using CCD showing coded and actual values along with the experimental and predicted (using model equation) values of the anti-listerial substance production

Run	Yeast extract (%)	Sodium caseinate (%)	Anti-listerial activity (10^5 AU/ml)	
			Experimental	Predicted
1	2.59 (+α)	2.00 (0)	4.10	0.71
2	2.00 (0)	2.00 (0)	8.19	4.40
3	2.00 (0)	2.00 (0)	8.19	4.40
4	2.50 (+1)	2.50 (+1)	8.19	6.38
5	2.00 (0)	2.00 (0)	8.19	4.40
6	2.00 (0)	2.00 (0)	8.19	4.40
7	2.00 (0)	1.41 (-α)	65.54	60.17
8	1.50 (-1)	2.50 (+1)	16.38	11.08
9	2.00 (0)	2.00 (0)	8.19	4.40
10	2.50 (+1)	1.50 (-1)	16.38	37.68
11	2.00 (0)	2.59 (+α)	4.10	8.72
12	1.41 (-α)	2.00 (0)	16.38	20.77
13	1.50 (-1)	1.50 (-1)	65.54	66.98

Data are means of duplicate determinations.

$$Y = (6.42 \times 10^7) - [1.39 \times 10^7(A)] - [4.38 \times 10^7(B)] + [2.46 \times 10^6(AB)] + [1.82 \times 10^6(A^2)] + [8.63 \times 10^6(B^2)] \quad (4)$$

Where, Y was the predicted response of pedicoin PA-1/AcH production (AU/ml), A and B were yeast extract and sodium caseinate, respectively.

At the model level, the proportion of variance explained by the model was given by the multiple coefficient of determination (R^2). In this experiment, the coefficient of determination R^2 was 0.97, which implied that pediocin production was attributed to the given independent variables (Wang *et al*. 2008). The R^2 indicated that only 3% of the total variations were not explained by the model. The value of the adjusted determination of coefficient (adjusted R^2 = 0.9563) was also high to indicate

a high significance of the model. These measures indicated that the accuracy and general ability of the polynomial model was good and the analysis of the response trends using this model was reasonable.

Statistical significance and adequacy of the model was determined by Fisher's test value (*F*-value). The greater the *F*-value indicates that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real (Bari *et al*., 2009). The corresponding analysis of variance (ANOVA) is presented in Table 8. The quadratic regression model was highly significant, as an evident from the Fisher's *F*-test with a very low probability value [($p_{\text{model}} > F$) = 0.0001]. The analysis of variance (Table 8) showed that the variables that were highly significant were the linear effect of sodium caseinate (*B*) and the square term of sodium caseinate (B^2). Yeast extract (*A*) was significant at the level of $p < 0.01$ and the interactive term between yeast extract and sodium caseinate (*AB*) was significant at the level of $p < 0.05$. The square term of yeast extract (A^2) was not significant ($p > 0.05$). It means that yeast extract and sodium caseinate played a significant role in the higher production of pediocin PA-1/AcH ($p < 0.01$). Linear and quadratic effects of parameters were significant, meaning that they can act as limiting nutrient and little variation in their concentration would alter the production (Imandi*et al*., 2008; Bari *et al*., 2009). However, as it was a hierarchical model, the insignificant coefficients were not omitted from the equation (4) (Wang and Lu, 2004).

The 3D and 2D contour plots, the representative of the interactive effect of yeast extract and sodium caseinate on pediocin, were presented in Figure 5. It could be seen that the pediocin PA-1/AcH production increased upon decreasing the concentration of yeast extract and sodium caseinate from 2.5 to 1.5% (w/v). Based on the regression equation [equation (4)] and contour plots, the optimum concentration of both yeast extract and sodium caseinate were at 1.5% (w/v) (Figure 5).

The model predicted that the maximum pediocin PA-1/AcH production could be obtained by using above optimum concentrations of the variables was 6.7×10^6 AU/ml. The verification of the results using the optimized culture medium was accomplished. The maximum pediocin production obtained experimentally was found to be 6.6×10^6 AU/ml. This was obviously in close agreement with the model prediction and validation. After optimization of culture medium, the pediocin

Table 8. Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of squares	*F*-value	*p*-value >*F*
Model	6.18×10^{13}	53.53	< 0.0001
Yeast extract, *A*	4.93×10^{12}	21.33	0.0024**
Sodium caseinate, *B*	3.32×10^{13}	143.47	< 0.0001**
AB	1.51×10^{12}	6.54	0.0377*
A^2	9.06×10^{11}	3.92	0.0881
B^2	2.04×10^{13}	88.08	< 0.0001**

*$p < 0.05$ indicates that the model terms are significant.

**p < 0.01 indicates that the model terms are highly significant.

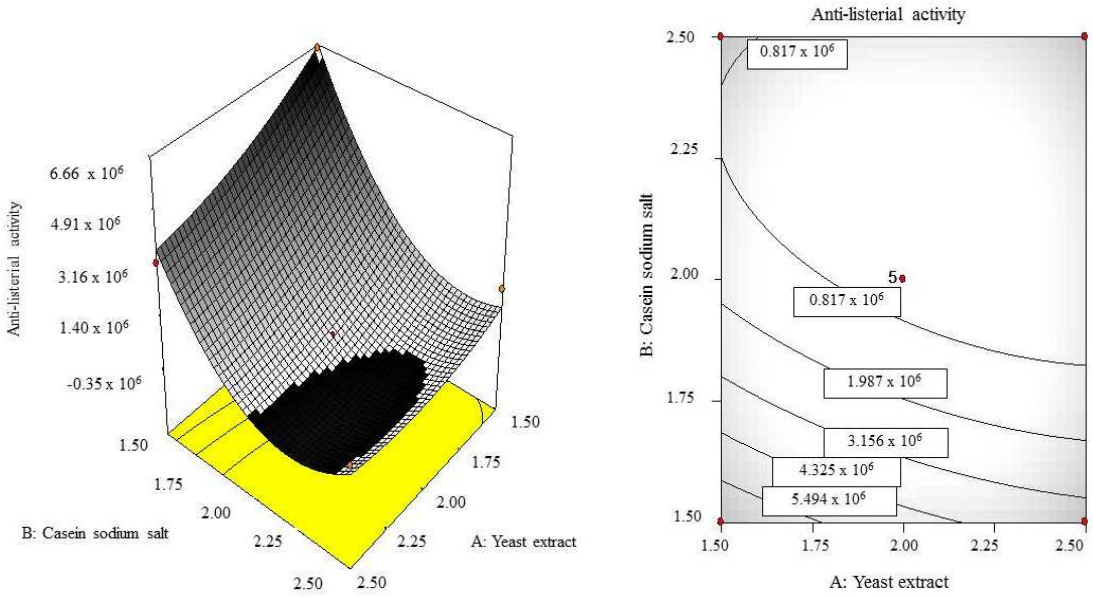

Figure 5. 3D response surface and 2D contour plots show the effect of yeast extract concentration (%) and sodium caseinate concentration (%) on the production of the anti-listeria substance (AU/ml). Inoculum concentration was 1% (v/v).

production was improved by 16-fold, compared with the un-optimized TGE medium.

According to CCD analysis, the increasing of sodium caseinate and yeast extract concentration range from 1.5 to 2.5% significantly decreased pediocin production. This result may be explained by the fact that the high content of sodium caseinate (2 to 2.5%) in culture medium could form high-strength gel (data not shown) which could not be utilized by the producer strain. Moreover, the gel could entrap pediocin in its structure that interfered pediocin extraction and antimicrobial activity assay. Since yeast extract used in this study contained $MgSO_4$, the increasing of yeast extract concentration from 1.5 to 2.5% is the increasing of $MgSO_4$ concentration which caused the inhibition of pediocin production. As reported above, the optimal concentration of each component for pediocin production was 1.5% sodium caseinate, 1.5% yeast extract and 0.5% glucose.

2.4.6 Effect of initial pH of culture medium on pediocin PA-1/AcH production

The effect of initial pH of culture medium on pediocin production was determined using 50 ml of the optimized culture medium in 125 ml Erlenmeyer flask incubated at 30 °C for 12 hours without shaking. Figure 6 shows that pH 6.5 was the optimum pH for *P. pentosaceus* BCC 3772 to produce pediocin PA-1/AcH with the anti-listeria activity against *L. monocytogenes* ATCC 19115 of 6.6×10^6 AU/ml. In general, pH is known to be important to cell growth as well as to bacteriocin production due to aggregation, adsorption of bacteriocin onto the cells, and/or proteolytic degradation (Kim *et al*., 2006). Moreover, pH of culture medium played an important role in the expression of the biosynthetic genes (Mataragas *et al*., 2003) and post-translational processing of pediocin to produce active pediocin (Biswas *et al*., 1991). Bacteriocin production is generally optimal at suboptimal pH for growth since higher amount of nutrients and energy are available for bacteriocin biosynthesis (Møretrø *et al*., 2000). However, the decrease or increase in pH to below or above the threshold value of bacterial growth could be resulted in repression of bacteriocin synthesis due to the increased energy demand for cell maintenance (Mataragas *et al*., 2003; Messens *et al*., 2003). The optimal initial pH of culture medium for pediocin

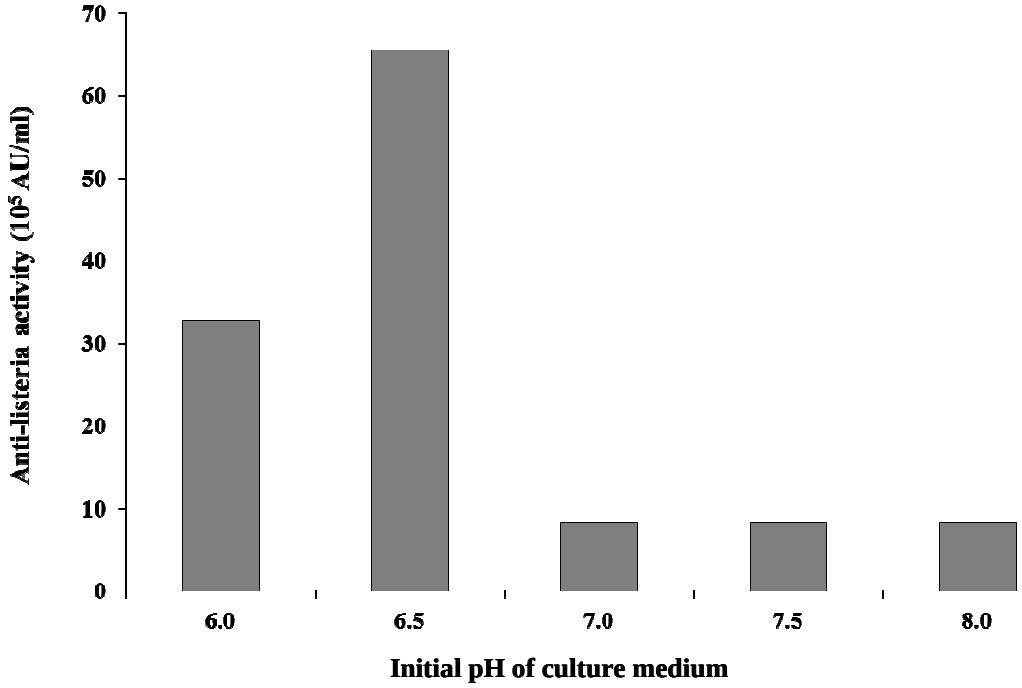

Figure 6. Effect of initial pH of culuture medium on the pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 in static flask culture after 12 h incubation. Results are means of duplicate determinations from two independent experiments.

production by *P. pentosaceus* BCC 3772 was pH 6.5 which was the same as pediocin AcH produced by *P. acidilactici* H (Biswas *et al*., 1991).

2.4.7 Effect of incubation temperature on pediocin PA-1/AcH production

In order to investigate the effect of incubation temperature on pediocin production, the initial pH of the optimized culture medium was adjusted to 6.5 and incubated at four different temperatures of 25, 30, 37 and 40 °C for 12 h without shaking. Among tested temperatures, 30 °C was found to be an optimum temperature for the production of pediocin PA-1/AcH by *P. pentosacues* BCC 3772 with anti-listeria activity of 6.6× 10^6 AU/ml (Figure 7). The result was in agreement with *P. pentosaceus* ST18 (Todorov and Dicks, 2005), *P. pentosaceus* SM-1 (Anastasiadou *et al*., 2008a), *P. acidilactici* H (Biswas *et al*., 1991; Ray, 1995),

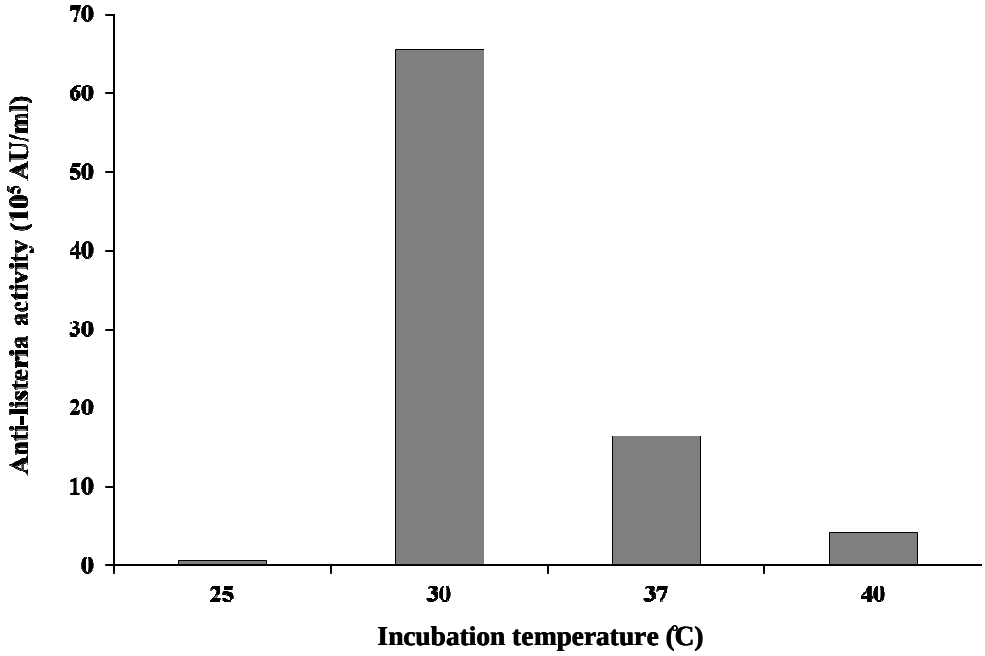

Figure 7. Effect of incubation temperature on the pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 in static flask culture after 12 h incubation. Results are means of duplicate determinations from two independent experiments.

P. acidilactici NRRL B5627 (Anastasiadou *et al*., 2008b) and *P. damnosus* NCFB 1832 (Bauer *et al*., 2005). The production of bacteriocin has been known to be regulated by temperature (Yusuf *et al*., 2012). In a similar way to the effect of pH on bacteriocin production, temperatures below that of optimal growth may increase pools of energy and nutrients for bacteriocin production (Møretrø *et al*., 2000). However, decrease or increase in temperature to below or above the threshold of bacterial growth may cause the reduction of bacteriocin synthesis (Mataragas *et al*., 2003). The increased temperature may also cause the loss in bacteriocin activity due to higher protease activity or a more pronounced cell-bacteriocin or bacteriocin-bacteriocin interaction (Messens *et al*., 2003).

2.4.8 Time course of pediocin PA-1/AcH production

Time course of bacterial growth and pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 in TGE and optimized medium broth were investigated. In

both culture media, *P. pentosaceus* BCC 3772 produced pediocin during early logarithmic growth phase and the maximum antimicrobial activity against *L. monocytogenes* ATCC 19115 was detected at 12 h of growth (Figure 8). No changes in anti-listeria activity were found during 12-24 h at the stationary phase. After 12 h of incubation, the production of pediocin in the optimized medium was 16-fold higher than that in TGE basal medium. The viable cell count of the producer strain in optimized medium was also higher than that in TGE basal medium. Using the optimal culture medium and cultivation condition, pediocin PA-1/AcH production by *P. pentosaceus* BCC 3772 displays primary metabolite kinetics with the rate of production paralleled the growth rate, so it shows similarity to other bacteriocins (Altuntas *et al*., 2010). Higher pediocin production in the optimized culture medium may be explained by the elimination of some limiting agents, including Tween80, magnesium sulfate and manganese sulfate, which were presented in TGE basal medium. Therefore, optimal nitrogen and carbon sources at the optimal concentration and optimal pH and incubation temperature enhanced both cell growth and pediocin production.

2.4.9 Color characteristics of culture supernatants

Browning index and absorbance at 420 nm, which indicates the occurrence of products from browning reaction, of culture supernatant of the optimal medium were lower than that of MRS medium ($p < 0.05$) (Table 9). Compared with MRS broth, the optimal culture medium contained much lower amount of glucose, (Table 10) which can react by caramelization and maillard reaction with amino acid of nitrogen sources to produce browning products, thus provide culture supernatant with lower browning index. Formation of browning products indicated the loss of nutrients and may directly exert anti-microbial effect on bacterial growth and bacteriocin biosynthesis (Sant'Anna *et al*., 2011).

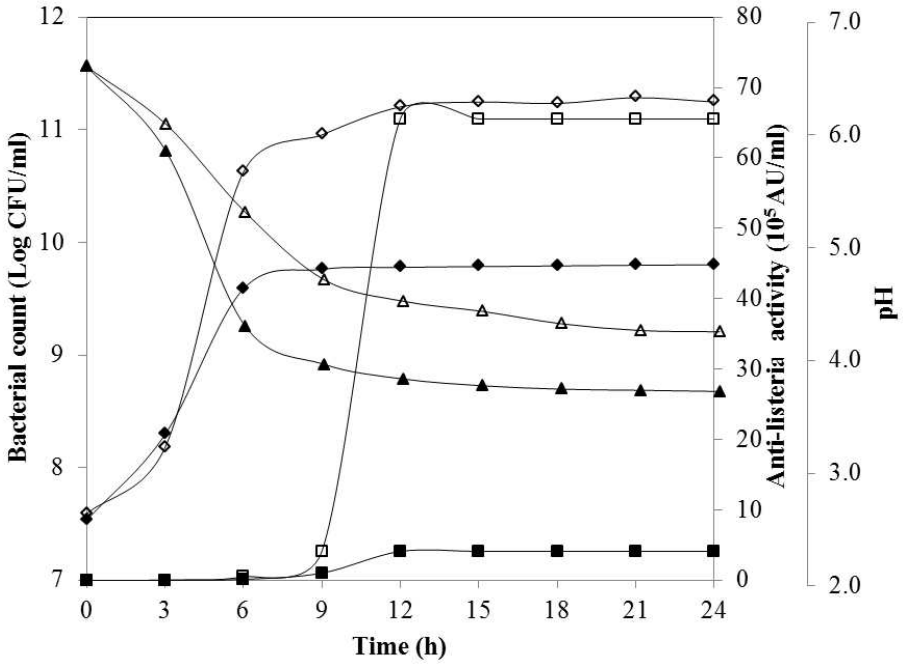

Figure 8. Comparison of bacterial growth (◆,◇), anti-listeria substance production (■,□), and pH changes (▲,△) profiles for *P. pentosaceus* BCC 3772 in TGE basal medium (closed symbols) and optimized medium (opened symbols). Cells were grown in 50 ml of TGE basal medium or optimized medium in 125 ml Erlenmeyer flask at 30°C without shaking; the initial pH of both culture medium were adjusted to 6.5. The anti-listeria activity of the cell-free supernatants was tested against *L. monocytogenes* ATCC19115. Data are means of duplicate determinations from two independent experiments.

Table 9. Color characteristics of culture supernatants

Media	MRS	TGE	Optimized medium
*L**	69.13 ± 0.02a	76.58 ± 0.06b	76.73 ± 0.01b
*a**	7.75 ± 0.00b	2.53 ± 0.01a	2.60 ± 0.01a
*b**	46.75 ± 0.03b	32.80 ± 0.02a	32.52 ± 0.00a
Browning index	110.76 ± 0.52b	56.03 ± 0.02a	55.35 ± 0.02a
$OD_{420\ nm}$	1.15 ± 0.00b	0.71 ± 0.00a	0.61 ± 0.00a

Data are means ± SD of triplicate determinations.

Different lower case letters in the same row indicate significant difference ($p < 0.05$).

Table 10. Composition of MRS, TGE and optimized media

Ingredients	Culture media		
	MRS	TGE	Optimized
Sodium caseinate	-	-	15 g/L
Tryptone	10 g/L	10 g/L	-
Beef extract	10 g/L	-	-
Yeast extract	5 g/L	10 g/L	15 g/L
Glucose	20 g/L	10 g/L	5 g/L
Tween80	1 g/L	2 g/L	-
Ammonium citrate	2 g/L	-	-
Sodium acetate	5 g/L	-	-
Magnesium sulfate	0.1 g/L	0.002 g/L	-
Manganese sulfate	0.05 g/L	0.005 g/L	-
Di-potassium phosphate	2 g/L	-	-

2.5 Conclusion

Using TGE as a basal medium, combination of sodium caseinate with yeast extract and glucose were the optimal nitrogen and carbon sources, respectively. Plackett-Burman analysis indicated that sodium caseinate and yeast extract had the significant positive effect on pediocin production. According to the analysis of CCD and RSM, the optimal medium contained 1.5% (w/v) of sodium caseinate, 1.5% (w/v) of yeast extract and 0.5% (w/v) of glucose. The production of pediocin in the optimal medium with optimal pH of 6.5 and optimal incubation temperature of 30 °C not only improved pediocin production but also decreased the browning index of pediocin preparation, which is more suitable for use in food packaging.

CHAPTER 3

DEVELOPMENT OF POLY(LACTIC ACID)/SAWDUST PARTICLE BIOCOMPOSITE FILM IMPREGNATED WITH PEDIOCIN PA-1/ACH AND APPLICATION IN RAW SLICED PORK

3.1 Abstract

A novel poly(lactic acid) (PLA)/sawdust particle (SP) biocomposite film with anti-listeria activity was developed by incorporation of pediocin PA-1/AcH (Ped) using diffusion coating method. Sawdust particle played an important role in embedding pediocin into the hydrophobic PLA film. The anti-listeria activity of the PLA/SP biocomposite film incorporated with Ped (PLA/SP + Ped) was detected, whilst no activity against the tested pathogen was observed for the control PLA films (without SP and/or Ped). Dry-heat treatment of film before coating with Ped resulted in the highest Ped adsorption (11.63 ± 3.07 μg protein/cm^2) and the highest anti-listeria activity. A model study of PLA/SP + Ped as a food-contact antimicrobial packaging on raw sliced pork suggests a potential inhibition of *Listeria monocytogenes* (99% of total listerial population) on raw sliced pork during the chilled storage. This study supports the feasibility of using PLA/SP + Ped film to reduce the initial load of *L. monocytogenes* on the surface of raw pork.

3.2 Introduction

Listeria monocytogenes is a food-borne pathogen that causes a severe disease called listeriosis (Alves *et al*., 2006; Gialamas *et al*., 2010). *L. monocytogenes* has been listed in the top five highest ranking pathogens with respect to the total case of food-borne illness in the United States (Theinsathid *et al*., 2012) and is a major concern in food industries (Farber and Peterkin, 1991). This pathogenic bacterium is of major concern in a wide variety of foods, especially in chilled meat and ready-to-eat (RTE) meat products due to its ability to survive and grow at refrigeration temperatures (Schlech, 2000; Ye *et al*., 2008). In order to reduce healthy risk for the consumer from *L. monocytogenes*, criteria or recommendations for tolerable levels of *L. monocytogenes* in processed foods have been established. The USA and Thailand practices “zero tolerance”, while Canada and France apply different norms according to the foodstuff (Thévenot *et al*., 2006).

To guarantee food safety through the inhibition of *L. monocytogenes*, the use of bacteriocins and other biologically derived antimicrobials with anti-listeria activity in packaging material have received a considerable attention. Thus, during the last decades, innovative bioactive films enriched with bacteriocins have been developed (Coma *et al*., 2008; Jin *et al*., 2009; Cao-Hoang *et al*., 2010; Theinsathid *et al*., 2012). The most-commonly studied antimicrobial agents for applications in this sense are nisin (Hoffman *et al*., 2001; Ko *et al*., 2001; McCormick *et al*., 2005; Neetoo *et al*., 2008; Jin *et al*., 2009; Cao-Hoang *et al*., 2010; Guiga *et al*., 2010), pediocin PA-1/AcH (Ming *et al*., 1997; Santiago-silva *et al*., 2009), and enterocin (Iseppi *et al*., 2008; La Storia *et al*., 2012). Among these bacteriocins, nisin and pediocin PA-1/AcH are the only bacteriocins, to date, that have been approved for use in food. Although anti-listeria efficiency of nisin and pediocin significantly differed depending on the producing or indicator strains, the sample preparation method, and the bacteriocin assay conditions, pediocin is likely to have higher activity and acts more specifically against *L. monocytogenes* than nisin (Cintas *et al*., 1998; Rodriguez *et al*., 2002). In addition, pediocin PA-1, in contrast to both nisins A and Z, has potential to inhibit *Listeria* without disturbing other bacteria including beneficial ones (Blay *et al*., 2007).

Due to a trend toward active and green packaging, the use of bio-materials including cellulose, starch, pectin and poly(lactic acid) (PLA) have been more emphasized (Rodriguez *et al*., 2006; Liu *et al*., 2009; Kuorwel *et al*., 2011). PLA is recognized as compostible biopolymer that attracts the interest for the packaging industry because of its outstanding properties and earth-friendly biodegradability. PLA exhibits many properties that are equivalent to or better than many petroleum-based plastics (Liu *et al*., 2009). Importantly, PLA packaging can be produced by many manufacturing processes, such as film blowing, injection molding, sheet extrusion, blow molding and thermoforming (Imam *et al*., 2008; Jamshidian *et al*., 2010). The combination of biodegradability of PLA with antimicrobial property of pediocin against a wide broad spectrum of food pathogen will be of full benefit as the active packaging. As a consequence, health-risk of consumers can be reduced. Shelf-life can be extended, thereby lowering the economic loss. Importantly, the waste of post use of this packaging will be decomposed through compostable system without causing environmental waste problems.

However, direct incorporation of antimicrobial peptide to PLA film has been limited by the hydrophobic characteristics of PLA. The incompatibility of bacteriocin in hydrophobic polymers caused the phase separation in film, leading to poor antimicrobial activity and mechanical properties. To solve this problem, sawdust particle (SP), a low-water solubility hydrophilic particle, was incorporated in PLA film to enhance adsorption of pediocin using diffusion coating technique. In contrast to the large amount of information on the antimicrobial activity of packaging films containing antimicrobials, to our knowledge no information is available about using natural fiber as carrier of pediocin PA-1/AcH in PLA film. Research on the possibility of using sawdust particle would lead to an alternative natural preservation method, easily applicable and of low cost. In addition, effect of pre-conditioning methods was also investigated to enhance the pediocin adsorption. Finally, anti-listeria activity of the PLA/SP biocomposite film toward a model pork system was determined in order to ensure the potential use of the film in real food system.

3.3 Materials and Methods

3.3.1 PLA, saw dust, pediocin and other chemicals

PLA polymer 4042D was purchased from NatureWorks®. To prepare sawdust particle, wood sawdust was subjected to a cutter-mill (Wonder Blender,WB-1,Waring Products, Inc., Connecticut, USA) to obtain particles in the range of 100–300 μm and dried at 70 °C for 24 h in air circulating oven before further pretreatment. Dried wood sawdust was stirred with absolute ethanol at room temperature for 1 h in order to eliminate the impurities on the surface of sawdust. Treated sawdust was dried at 70 °C for 3 h to evaporate the solvent and then soaked into 10% sodium hydroxide at 40 °C for 3 h to remove natural impurities including pectin, lignin, waxy substances and natural oils. Finally, processed wood sawdust was rinsed with distilled water to remove sodium hydroxide and dried at 60 °C for 24 h. The obtained powder was referred to as sawdust particle or SP.

Partially purified pediocin from *Pediococcus pentosaceus* BCC 3772 (Kingcha *et al*., 2012) was prepared by the method of Zendo *et al*. (2003). *P. pentosaceus* BCC 3772 was obtained from BIOTEC culture collection (BCC), National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand. Culture was maintained as frozen stock held in −80 °C in culture broth containing 15% (w/v) glycerol. Prior to use, culture was streaked on medium agar (1.5% casein sodium salt, 1.5% yeast extract and 0.5% glucose) and grown at 30 °C for 24 h. A single colony obtained from the plate was grown twice in culture broth and incubated at 30 °C for 24 h without shaking. The cell-free supernatant was obtained by centrifugation at 7500 ×g for 15 min at 4 °C using a Hi-speed centrifuge (Beckman: Avanti J-E). The anti-listeria substance was concentrated at 1000 ml of cell-free supernatant by hydrophobic interaction chromatography using an Amberlite XAD-16 polymeric resin (Sigma, USA). In brief, a 20 g of the resin was activated in 50% (v/v) isopropanol at 4 °C for 24 h. Thereafter, isopropanol was completely removed from the resin by washing with one volume of deionized water. The activated resin was added into a 1000 ml of the culture supernatant with gently mixed and kept at 4 °C for 24 h. The resin was loaded into the Econo fast flow column 2.5 × 30 cm (Bio-rad, USA) and washed with 100 ml of deionized water, followed by 100 ml of 40% (v/v)

ethanol in deionized water. The anti-listeria substance was eluted with 100 ml of 70% (v/v) isopropanol in deionized water containing 0.1% (v/v) trifluoroacetic acid (TFA). The eluent was then evaporated to get rid of isopropanol using rotary evaporator (40 °C for 30 min) and then freeze dried at −60 °C, 0.1 mBar and kept at 4 °C prior to use. The powder was referred to as partially purified pediocin.

3.3.2 Elaboration of PLA/SP biocomposite film

PLA/SP biocomposite film was fabricated using a blown film extrusion. Firstly, 5% (w/w) sawdust was mixed with PLA resin using treated sawdust, and subjected to a twin screw extruder (SHJ-36 twin screw extruder product line, Nanjing Chengke Machinery, China). Then, the composite film was fabricated via blown film technique. For PLA film without SP, the PLA resin was subjected to a blown film extruder to fabricate the control film without pre-compounding with SP. The thickness of the film was adjusted to 400–500 μm by controlling the take up speed.

3.3.3 Film pretreatment and coating procedure

PLA/SP biocomposite films were pretreated with three methods including dry-heat treatment (heating the film samples at 90 °C for 2 h in air-circulating oven), dry-heat treatment followed by acid treatment (soaking in 2% acetic acid for 30 min) and moist-heat treatment (heating the film samples at 90 °C, 90% RH for 2 h). Partially purified pediocin was loaded into pretreated films by a diffusion coating method according to Liu *et al*. (2007) and Jin *et al*. (2009) with some modifications. Briefly, eight (2 × 2 cm^2) film samples were soaked in a beaker containing 5 ml of 0.2% (w/v) partially purified pediocin solution for 30 min at room temperature. After pediocin adsorbed onto the film surface, the films were removed from the pediocin solution and washed three times with 5 ml of deionized water by shaking in the solution for 1 min for each time in order to eliminate the un-adsorbed pediocin.

The washed films were dried under laminar flow for 30–60 min and stored at 4 ± 2 °C in a refrigerator prior to bacterial inhibition tests. To quantitatively

determine the pediocin adsorption on composite film, the amount of pediocin retained in the solution was analyzed as the total protein content according to the method of Lowry *et al*. (1951). Pediocin adsorption on the film was calculated by the following equation:

$$\text{Pediocin adsorbed } (\mu g/cm^2) = [P1\text{-}P2]/A$$

where *P1* and *P2* represent the total protein (μg) of pediocin solution before and after adsorption, respectively and *A* represents the total surface area (cm^2) of the films used in the experiment.

3.3.4 Anti-listeria activity of film

Anti-listeria activity of film was evaluated using *L. monocytogenes* ATCC 19115 as the indicator strain. Culture was maintained as frozen stock held at −80 °C in tryptic soy broth (TSB, Merck, Germany) containing 15% (w/v) glycerol. The indicator was streaked on TSB with 1.5% agar and incubated at 37 °C for 24 h. A single colony obtained on TSB agar was grown twice in TSB broth and incubated at 37 °C for 24 h. The inhibition of *L. monocytogenes* was evaluated using an agar diffusion method as described by Appendini and Hotchkiss (2002) and Jin and Zhang (2008) with some modifications. Film sample (2 × 2 cm^2) was placed on the surface of TSB agar plate. The plate was overlaid with 5.0 ml of the semi-soft TSB agar (1.0% (w/v) agar) containing approximately 10^6 CFU/ml of *L. monocytogenes* ATCC 19115. After the plates were incubated at 37 °C for 24 h, inhibition zones were calculated using the following equation (Erdem and Yurudu, 2008):

$$H = [D\text{-}d]/2$$

where *H* is the inhibition zone or clear zone width in mm; *D* is the total width of specimen and inhibition zone in mm; and *d* is the width of specimen in mm.

Pretreated and un-pretreated films coated with pediocin were evaluated for anti-listeria activity. Film uncoated with pediocin was also assayed as negative control.

3.3.5 Determination of film properties

3.3.5.1 Film thickness

Thickness of the film sample was measured with a thickness gauge (Mitutoyo Absolute, Model ID-C112BS, Japan) to the nearest 0.0001 mm at five random locations on the films. Average thickness was evaluated and used in the calculation of tensile strength and film transparency.

3.3.5.2 Tensile strength and elongation at break

A universal testing machine (Instron, Model 55R4502, Canton, MA, USA) was used to determine tensile strength and elongation at break. Five specimens (10 × 10 mm^2) cut from each coated and uncoated films were tested for tensile properties. Initial grip separation and cross-head speed were set at 10 mm and 10 mm/min, respectively. Tensile strength value were calculated by dividing the maximum stress by cross sections area of the specimen, and elongation values were expressed as percent units, with the ratio of extended length at break point of initial length.

3.3.5.3 Film solubility in water

Film solubility in water was determined as the percentage of sample dry matter solubilized after 24 h immersion in distilled water at pH 6 (Pereda *et al*., 2011). Film samples (2 × 2 cm^2) were dried in an air circulating oven at 105 °C for 24 h and weighed for initial dry mass (60-90 mg/piece). Films were directly immersed in a 50 ml screw cap tube containing 30 ml of distilled water with 0.02% sodium azide to prevent microbial growth. After 24 h of storage in a shaking water bath at 25 ± 2 °C, the specimens were recovered, gently rinsing with distilled water and the oven dried at 105 °C for 24 h, to determine the weight of dry matter not dissolved in water. Three measurements were taken for each treatment. Film solubility was calculated from the initial dry mass and the final dry mass using the following equation:

$$\text{Film solubility (\%)} = [(W1-W2)/W1] \times 100$$

where *W1* and *W2* represent the film mass before and after solubility test, respectively.

3.3.5.4 Film color

The color of film samples was assessed using a colorimeter (Minolta, CR-300, Tokyo, Japan) with an 8 mm diameter measuring area. A white standard color plate (L^*= 97.26, a^*= +0.34, b^*= +1.84) for instrument calibration was used as a background for color measurement of films (Lee *et al*., 2008). Five measurements at different locations on each specimen were conducted on a 10 × 10 cm^2 segment of film (Chana-Thaworn *et al*., 2011a). The total color difference (ΔE) and whiteness index (*WI*), were calculated by following equation (Pereda *et al*., 2011):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

3.3.5.5 Film transparency

The transparency of films was determined using a Lamda950 UV/Vis spectrophotometer (PerkinElmer, USA). The film samples were cut into rectangles and placed on the internal side of the spectrophotometer cell. The percent transmittance (%T) was determined at wavelength of 600 nm and the transparency was calculated from the following equation (Han and Floros, 1997):

$$\text{Transparency} = [\text{Log \%T}]/x, \text{ where } x \text{ is film thickness (mm).}$$

3.3.6 Determination of the activity of the antimicrobial-coated PLA/SP biocomposite films against *L. monocytogenes* on raw sliced pork

The antimicrobial effectiveness of the coated PLA/SP biocomposite films was evaluated using raw sliced pork. According to Siragusa *et al*. (1999), sections (5 × 5 cm^2) of pork loin were UV-sterilized under a biosafety hood for 15

min. Sterilized meat sections were then inoculated with 250 µl of an overnight culture of *L. monocytogenes* ATCC 19115 to obtain the final bacterial density on meat surface of 10^4 CFU/cm^2. The inoculated pork samples were then packed in direct contact with the pediocin coated films, placed on the tray and stored at 4 ± 2 °C. Samples packed with uncoated film and unpacked meats were used as negative controls. After 0, 1, 2, 3, 4, 5, 6, 7 and 14 days of storage, selective viable counts of listeria were performed. For the analysis, the samples were placed in sterile plastic bags, 10 ml of sterile 0.1% (w/v) peptone solution were added, and then the samples were homogenized for 1 min in a Stomacher Lab Blender (Seward; UK). PALCAM agar (Merck) with *L. monocytogenes* selective supplement was used to allow the growth of *L. monocytogenes*. Each plate was incubated at 37 °C for 48 h, and the colonies were enumerated. The experiments were performed in triplicate and the results were expressed as CFU/cm^2.

3.3.7 Statistical analysis

Antimicrobial experiments were conducted in triplicate, with two observations per film treatment for each replicate. For physical and mechanical testing, all measurements were performed on five samples. Data value was expressed as the mean ± SD. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 17.0 software. Duncan's Multiple Range Test (DMRT) was used for comparison of mean values at a significant level of 0.05.

3.4 Results and Discussion

3.4.1 Anti-listeria activity of composite film and effect of pre-conditioning methods

PLA/SP + Ped films showed the inhibition zone against growth of *L. monocytogenes* ATCC 19115 as shown in Figure 9. The average clear zone width of these films ranged from 2.75 ± 0.52 to 3.88 ± 1.02 mm. The result indicated that sawdust particles in the composite film not only played the role in embedding pediocin but also allowed the adsorbed pediocin to release to the environment and act as antimicrobial agent. In contrast, PLA/SP biocomposite film without pediocin and

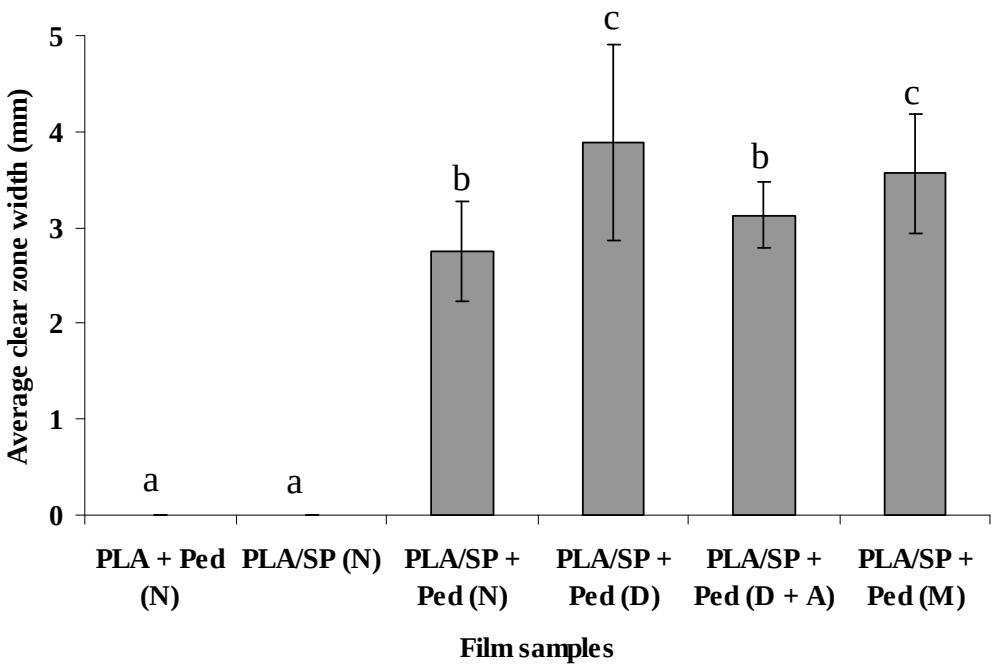

Figure 9. Anti-listeria activity of PLA and PLA/SP biocomposite film pretreated with different treatments (N: no pretreatment, D: dry-heat treatment, D + A: dry-heat treatment followed by acid treatment, M: moist-heat treatment). Bars represent the standard deviation of duplicate determinations from three independent experiments. Different letters on the bars denote the significant difference ($p < 0.05$).

PLA film coated with pediocin showed no zone of inhibition, suggesting no anti-listeria activity. Hydrophobic PLA film might not able to adsorb the hydrophilic pediocin. As reported by Jin *et al*. (2009), PLA film coated with nisin showed a small antimicrobial effect against *L. monocytogenes*.

After soaking, PLA/SP biocomposite film adsorbed pediocin at a level of 2.75 ± 0.52 μg protein/cm^2 of film surface area, whilst no protein was adsorbed on the PLA film surface (Figure 10). The result suggested that the sawdust particle in PLA/SP biocomposite film played the role in embedding pediocin during the diffusion coating process. Due to hydrophobic characteristic and the smooth surface

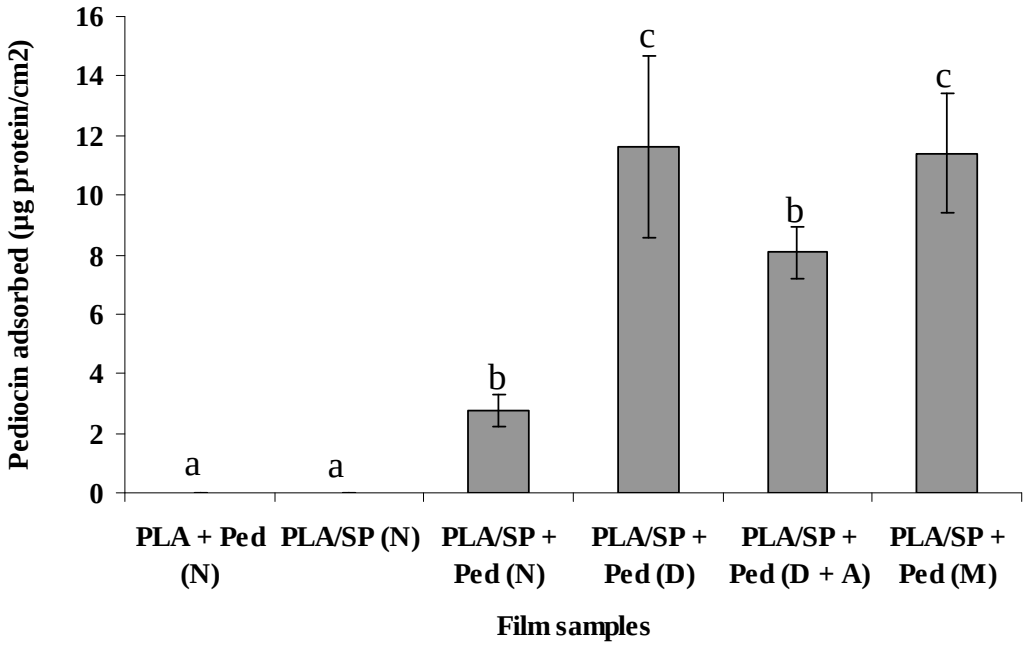

Figure 10. Protein adsorption of PLA and PLA/SP biocomposite film pretreated with different treatments (N: no pretreatment, D: dry-heat treatment, D + A: dry-heat treatment followed by acid treatment, M: moist-heat treatment). Bars represent the standard deviation of duplicate determinations from three independent experiments. Different letters on the bars denote the significant difference ($p < 0.05$).

of PLA film, the capability to incorporate the hydrophilic pediocin onto the film surface was limited. After washing, pediocin was easily removed. On the contrary, the incorporation of hydrophilic sawdust particle into PLA film not only increased the surface hydrophilicity but also facilitated the adsorption of pediocin.

Bower *et al*. (1995) reported that higher nisin content was retained when it was embedded to less hydrophobic surfaces. Increasing amount of chitosan, a hydrophilic molecule, into PLA/chitosan films increased the availability of hydroxyl group of chitosan in film, thus increasing the adsorbent character of the films (Sébastien *et al*., 2006). As reported by Jin *et al*. (2009), the addition of pectin, as a hydrophilic particle into PLA film created a rough and hydrophilic surface which improved the adsorption of nisin onto the film.

In order to improve pediocin adsorption onto PLA/SP film, the films were treated with three different pre-conditioning methods prior to be coated with pediocin. Dry-heat treatment was hypothesized to enhance the adsorption by enlarging polymer entanglement thus expose a number of SP inside the film matrix to adsorb more amount of pediocin. Whilst, moist-heat and acid treatments were hypothesize to disclose and enlarge the absorption surface by swelling SP thus increase capability of SP to embed pediocin molecules into the films. Regarding these film pre-conditioning methods, heat treatments by either dry-heat or moist-heat were found to enhance the anti-listeria activity of resulting film and pediocin adsorption (Figure 9 and 10). Based on specific activity, pediocin adsorbed in all pretreated films could be estimated to be 0.63-0.67 AU/µg of total protein/cm^2 which was the maximum amount of pediocin adsorbed on PLA/SP film. In this study, pediocin adsorption was limited by the amount of SP impregnated in film. Our preliminary study showed that addition of SP of higher than 5% (w/w) seriously affected the film formation. Therefore, the amount of SP used in this study was the highest possible. Since the maximum pediocin adsorption of 0.63-0.67 AU/µg of total protein/cm^2 was still observed even the concentration of pediocin soaking solution was increased higher than 0.2% (w/v), the concentration of pediocin in soaking solution was used at the lowest possible (0.2% (w/v)) in this study.

The effect of heat treatment on the incorporation of pediocin into PLA/SP biocomposite film is proposed in Figure 11. It is postulated that during pretreatment of PLA/SP biocomposite film by heating at 90 °C, the PLA chains can readily move apart from each other which in turn induce numbers of void formation. This phenomenon would enable pediocin to be trapped by the soaked film. Moreover, pediocin not only diffused rapidly into the loosening entanglement of molecular chain of the soaked film but also attached to natural sawdust particles. Consequently, the small amount of pediocin was gradually released from the composite film. When the film was cooled down, the entangled molecular chains of the polymer moved closer and become stiff and well pack again, leading to the difficulty to adsorb or release the pediocin molecule from the film. For untreated film, this process did not occur. While, for acid treated film, the exposed SP was re-enclosed by PLA chain after treated with acid solution. Therefore, pediocin could attach only on the film surface

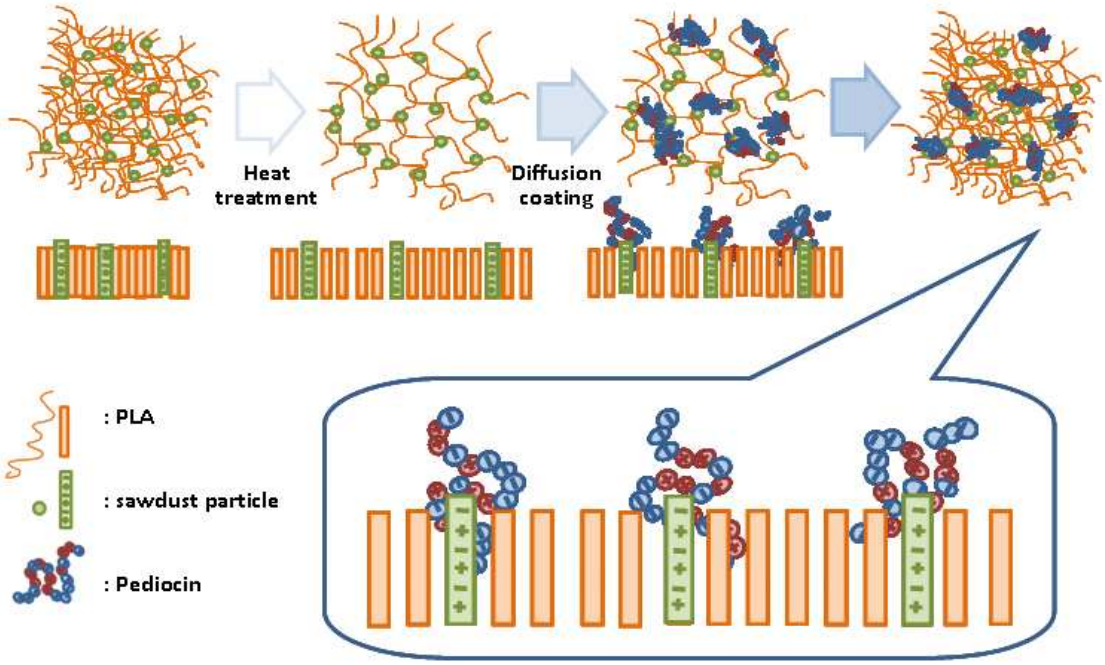

Figure 11. Proposed effect of heat treatment on the incorporation of pediocin into PLA/SP biocomposite film

and could not penetrate into the film matrix. The pediocin adsorption and anti-listeria activity of untreated and acid treated films were also lower than those of heat treated films ($P < 0.05$).

3.4.2 Film properties

3.4.2.1 Thickness

The thickness of PLA/SP biocomposite film was around 0.4 mm. The thickness of films with and without pediocin coating were not significantly different and were not affected by pre-conditioning methods (Table 11). This result was similar to the study of Jin *et al*. (2009) who reported that the addition of nisin to PLA and pectin/PLA films by diffusion coating had no effect on the film thickness.

3.4.2.2 Tensile strength and elongation at break

Tensile strength of films was found to be 7-10 MPa (Table 11). Dry-heat treatment of film prior to coating with pediocin solution increased the tensile

Table 11. Properties of PLA/SP and PLA/SP + Ped films pretreated with different treatments (N: no pretreatment, D: dry-heat treatment, D + A: dry-heat treatment followed by acid treatment, M: moist-heat treatment)

Treatments	Thickness (mm)	Tensile strength (MPa)	Elongation at break (%)	Solubility (%)
PLA/SP (N)	0.42 ± 0.03a	8.32 ± 0.87ab	4.83 ± 2.37a	1.61 ± 0.39a
PLA/SP + Ped (N)	0.41 ± 0.01a	7.38 ± 1.40a	3.92 ± 1.06a	1.64 ± 0.28a
PLA/SP + Ped (D)	0.42 ± 0.03a	10.06 ± 0.96c	4.53 ± 0.97a	2.01 ± 0.18a
PLA/SP + Ped (D + A)	0.41 ± 0.01a	9.77 ± 0.73bc	4.92 ± 1.21a	1.65 ± 0.22a
PLA/SP + Ped (M)	0.43 ± 0.09a	7.92 ± 1.91a	3.86 ± 1.41a	1.85 ± 0.17a

Data shown are means ± SD of five determinations.

Different lower case letters in the same column indicate significant difference ($p < 0.05$).

strength of PLA/SP biocomposite film. The increase in tensile strength of the film with dry-heat treatment might be explained by the reorientation or reorganization of the polymer chain. By slow cooling after dry-heat treatment of the PLA/SP biocomposite film, there was a formation of numbers of small stacks of crystalline phase which led to higher mechanical strength. This reordering of new formed crystal stacks was known as densification which caused a considerable decrease in the free volume and molecular mobility, resulting in a more packed structure. Consequently, the stress required for establishing yielding during tensile testing was greater in dry-heat treated film. Zilberman *et al.* (2001) reported that heat treatment at $T > T_g$ resulted in the increase in strength of the PLA film due to further crystallization and evaporation of solvent residues and moisture.

In the case of moist-heat treated PLA/SP film, although the same temperature as those of dry-heat treatment was used, the tensile strength was lower

than those of dry-heat treated one. Degradation or hydrolysis of PLA by high temperature and high humidity during moist-heating might take place, resulting in the decrease in the film strength (Weir *et al*., 2004).

Elongation at break of films with all treatments were not significantly different ($p < 0.05$), all around 4-5% (Table 11), indicating that pre-conditioning and coating technique did not affect film elongation. However, elongation at break of PLA/SP biocomposite film developed in this study was higher than those of PLA/pectin film developed by Jin *et al*. (2009) and Liu *et al*. (2009) which was around 1 to 3%. Dorgan *et al*. (2000) reported that the elongation at break of commercial-grade PLA films was nearly 4% that is higher than that of Poly styrene (PS).

3.4.2.3 Film solubility in water

Film solubility in water is an important property, which governs potential applications of these materials to food preservation. Films with low water solubility are necessary for the protection of food stuffs with high or intermediate water activity (a_w). The very low water solubility of PLA/SP biocomposite film was observed in this study (Table 11). The solubility in water of PLA/SP biocomposite film was in the range of 1.61 to 2.01%. It was lower than those of PLA film with 5 to 16.6% PEG400 as plasticizer and PLA/chitosan composite film which had about 5% and 35% solubility, respectively (Sébastien *et al*., 2006). Normally, PLA and its composites are found to have very low water solubility compared with other bio-based materials especially protein- and polysaccharide-based materials which have water solubility around 5-50% (Padgett *et al*., 1998; Hoffman *et al*., 2001; Ko *et al*., 2001; Chana-Thaworn *et al*., 2011a, 2011b). The high water solubility of the film made from bio-based polymers remained a major problem. These films adsorb water and swell over the course of contacting with packaged foods (Millette *et al*., 2007), thus degrading the films, causing a rapid release of the antimicrobials, decreasing physical and mechanical property of packaging, increasing the contamination of packaging materials into packaged food and lowering consumer acceptability. The incorporation of water-soluble materials such as pectin (Jin *et al*., 2009) into PLA composite film may cause the same problems as described above.

The PLA/SP biocomposite films developed in this study overcame these problems since they had very low water solubility. The very low water solubility of the resultant films may be explained by the low water solubility of film formulation which mainly consisted of PLA and sawdust particle. The solubility in water of PLA/SP biocomposite film was not significantly affected by film pre-conditioning methods although it was slightly increased by dry- and moist-heat treatment. Based on solubility characteristic, PLA/SP biocomposite film developed in this study can be expected to be mechanically stable when packaging moist foods at chilled to ambient temperatures.

3.4.3 Film color and transparency

Appearance of PLA/SP biocomposite film pre-conditioned with different methods was not visually different. The films were quite brown, opaque and appeared to have particulates. The resulting films were similar to PLA/pectin composite film developed by Jin *et al*. (2009) and Liu *et al*. (2009) and Hydroxy Propyl Methyl Cellulose (HPMC) film developed by Chana-Thaworn *et al*. (2011a, 2011b). Color characteristics of film determined instrumentally are shown in Table 12. The result showed that pre-conditioning methods at high temperature (including dry-heat treatment, dry-heat treatment followed by acid treatment and moist-heat treatment) affected color characteristic of films. Whiteness index (*WI*) of pre-conditioned films decreased, whilst redness (*a**) and yellowness (*b**) values of films increased. The increased redness (*a**) and yellowness (*b**) value may be caused by the discoloration of sawdust particle during high temperature conditioning. However, pre-conditioning methods did not affect film transparency and total color difference (ΔE) between the films coated and uncoated with pediocin (data not shown). ΔE values of all treatments of film were not more than 2.3 which corresponded to non-noticeable difference or not different when observed by naked eyes (Sharma, 1997). The addition of pediocin into film by diffusion coating did not affect both color characteristic (*L**, *a** and *b**) and transparency of film (data not shown). Compared with synthetic or petroleum based polymer films, PLA/SP biocompostie film was more opaque or had lower transparency. However, this film had higher transparency when compared with other bio-based packagings. Edible HPMC films incorporated with Kiam wood

Table 12. Color characteristics of PLA/SP and PLA/SP + Ped films pretreated with different treatments (N: no pretreatment, D: dry-heat treatment, D + A: dry-heat treatment followed by acid treatment, M: moist-heat treatment)

Treatments	Transparency	L*	a*	b*	WI
PLA/SP (N)	4.53 ± 0.36a	88.07 ± 0.90a	0.94 ± 0.17a	12.44 ± 0.56a	82.73 ± 1.00c
PLA/SP + Ped (N)	4.55 ± 0.11a	87.87 ± 0.75a	0.94 ± 0.15a	12.53 ± 0.64a	82.53 ± 0.94bc
PLA/SP + Ped (D)	4.44 ± 0.33a	87.62 ± 1.14a	1.12 ± 0.18b	13.83 ± 0.95b	81.39 ± 1.40a
PLA/SP + Ped (D + A)	4.48 ± 0.15a	87.86 ± 0.93a	1.09 ± 0.20b	13.68 ± 0.92b	81.68 ± 1.29ab
PLA/SP + Ped (M)	4.31 ± 0.84a	87.72 ± 1.05a	1.16 ± 0.19b	13.52 ± 0.88b	81.70 ± 1.35ab

Data shown are means ± SD of five determinations.

Different lower case letters in the same column indicate significant difference ($p < 0.05$).

extracts had the transparency range from about 1.4 to 1.8 (Chana-Thaworn *et al*., 2011b) which lower than those of PLA/SP biocomposite film. The optical characteristics of PLA/SP film may be improved by addition of natural sawdust particle subjected to clarification or bleaching step before compounding with PLA. This step could reduce the effect of the color of the sawdust on the yellowness of the film. Moreover, the particle size of natural sawdust could be reduced in order to reduce light scattering of the produced film. However, PLA/SP biocomposite film developed in this study can be used as food packaging which see-through characteristic of products was not required.

3.4.4 Antimicrobial activity of coated PLA/SP biocomposite films against *L. monocytogenes* on raw sliced pork

The anti-listeria activity of film in raw sliced pork inoculated with *L. monocytogenes* ATCC 19115 and stored at 4 ± 2 °C in a retail refrigerator for 14 days is shown in Figure 12. Among four treatments of PLA/SP + Ped, film pre-conditioned

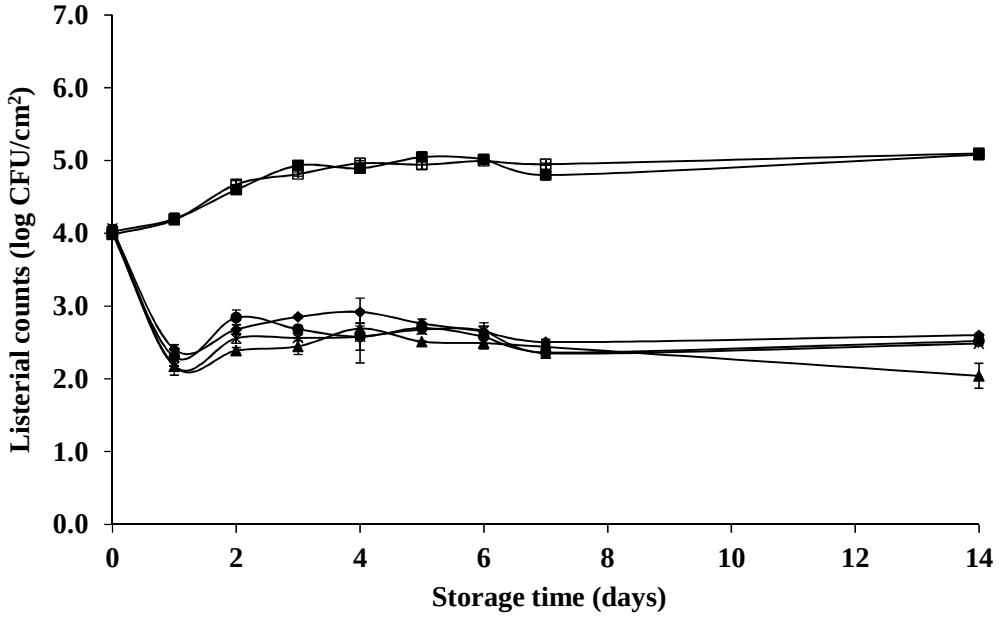

Figure 12. Challenge test of control of *Listeria monocytogenes* on pork meat surface (□: no film, ■: PLA/SP, ♦: PLA/SP + Ped (no pretreated), ▲: PLA/SP+ Ped (pretreated with dry-heat treatment), ×: PLA/SP + Ped (pretreated with moist-heat treatment) and •: PLA/SP + Ped pretreated with dry-heat treatment followed by acid treatment). Bars represent the standard deviation of duplicate determinations from three independent experiments.

by dry-heat treatment showed the highest anti-listeria activity (2 log cycles or 99% reduction of listerial counts compared with those of control was investigated at day 14 of storage). However, all treatments of PLA/SP + Ped significantly reduced the listerial population by about 1.5-2 log cycles from 1 to 14 days. As reported by previous studies, antimicrobial film incorporated with pediocin reduced the listerial population on meat surface by about 1-3 log cycles. Santiago-Silva *et al*. (2009) reported that the reduction of 2 log cycles of listeria on sliced ham contacted with cellulose-based film incorporated with 50% pediocin was observed during 15 days of storage at 12 °C. Ming *et al*. (1997) studied the anti-listeria activity of cellulosic casing sprayed with pediocin and observed that the film completely inhibited the

listerial growth in meat samples during 12 weeks of storage at 4 °C. Santiago-Silva *et al*. (2009) reported the behavior of film incorporated with 60% pediocin over sliced mortadella previously inoculated with *L. monocytogenes* and found that the antimicrobial film reduced 1.18 log cycles of listeria after 15 days of storage.

Normally, raw meats and other raw foods are contaminated with a moderate level of *L. monocytogenes* (not more than 100 CFU/g) (Inoue *et al*., 2000). If the film developed in this study is applied, the contaminated bacterial can be reduced to 1 CFU/g or not found in 25 g which is the acceptable levels of contamination and below the dose necessary to make healthy people ill.

The present study demonstrated that PLA/SP + Ped is an effective approach not only to reduce the population of *L. monocytogenes* on pork during refrigerated storage, but also to reduce the health risk of consumer from listeriosis.

3.5 Conclusion

Natural sawdust particle (SP) played an important role in embedding pediocin into the PLA film. Pre-conditioning of the PLA/SP biocomposite film by dry-heat treatment not only improved anti-listeria activity but also increased tensile strength with no effect on film color, transparency and solubility in water. PLA/SP biocomposite film coated with pediocin can be used as a good anti-listeria biodegradable packaging for pork and other high-moisture foods.

CHAPTER 4

QUALITY AND SHELF LIFE OF CHILLED PORK PACKAGED WITH POLY(LACTIC ACID)/SAWDUST PARTICLE BIOCOMPOSITE FILM INCORPORATED WITH PEDIOCIN

4.1 Abstract

Quality and shelf life of chilled pork were improved by packaging with poly(lactic acid)/sawdust particle biocomposite film incorporated with pedicoin (PLA/SP + Ped). The listerial counts of pork packaged with PLA/SP + Ped declined to below the detection limit (10 CFU/g) and were significantly lower than those of pork unpackaged (control) and packaged with PLA/SP uncoated with pediocin. According to the biogenic amine index (BAI), PLA/SP + Ped maintained the acceptable quality of packaged pork up to 7 days. Sensory evaluation showed that pork packaged with PLA/SP + Ped gained higher score in all attributes tested over the storage time. Based on sensory characteristics, PLA/SP + Ped could extend the shelf life of fresh pork meat by maintaining consumer acceptance of the products up to 7 days in retail chilled condition, which is at least 3 days longer than those observed for the unpackaged one. It could be concluded that PLA/SP + Ped could be used as an effective anti-listeria biodegradable packaging for preserving the fresh pork loin during chilled storage.

4.2 Introduction

Pork meat and pork products have been the world's most consumed meat for decades. Worldwide pork meat consumption of more than 104 million tons achieved very high market value in 2012 (USDA, 2013). *Listeria monocytogenes* is an important food-borne pathogen always found to contaminate in pork meat and its products. The contamination of this pathogenic microorganism in pork has been concerned since it can survive and multiply in pork meat during chilled storage (Hugas *et al*., 1998; Schlech, 2000; Ye *et al*., 2008b). During storage at refrigeration temperature, the contamination level of *Listeria monocytogenes* may reach from the moderate level (> 100 CFU/g) (Inoue *et al*., 2000) to higher level, which can causes a severe disease called listeriosis in the consumers (Alves *et al*., 2006; Gialamas *et al*., 2010).

Antimicrobial packaging is one of promising approaches that is used to control the microbial contamination in food (Roever, 1998; Appendini and Hotchkiss, 2002; Aymerich *et al*., 2008; De Ma *et al*., 2013; Rhim *et al*., 2013; Salarbashi *et al*., 2013). Antimicrobial packaging with biodegradable property has been received great attention to be used in food application according to the trend toward environmental-friendly packaging and global warming problem (Suppakul *et al*., 2003; Cutter, 2006; Peelman *et al*., 2013). To date, the use of antimicrobial biodegradable packaging in meat products has been reported in various studies (Ouattara *et al*., 2000; Millette *et al*., 2007; Santiago-Silva *et al*., 2009; Zinoviadou *et al*., 2009; Emiroğlu *et al*., 2010; Qin *et al*., 2013; Bonilla *et al*., 2014).

According to our previous study, we succeeded in controlling the contamination level of *Listeria monocytogenes* in chilled pork meat using a novel type of anti-listeria biodegradable packaging (Woraprayote *et al*., 2013). The developed poly(lactic acid)/sawdust particle biocomposite film incorporated with pediocin (PLA/SP + Ped) exhibited high potential to reduce the population of *Listeria monocytogenes* (99% of total listerial population) contaminated in raw sliced pork. Because of high anti-listeria efficiency of this film, PLA/SP + Ped seem to be an effective antimicrobial packaging for pork meat. In order to ensure the potential use of PLA/SP + Ped as a pork packaging, effect of PLA/SP + Ped on quality and shelf life

of packaged pork was investigated. The effect on the changes in microbial load, pH, color, biogenic amines content, and consumer acceptance of packaged pork samples were evaluated during chilled storage.

4.3 Materials and Methods

4.3.1 Elaboration of PLA/SP + Ped

PLA/SP biocomoposite film was fabricated by blown film extrusion technique as previously described by Woraprayote *et al*. (2013). Briefly, 5% (w/w) treated sawdust was mixed with PLA resin and subjected to a twin screw extruder (SHJ-36 twin screw extruder product line, Nanjing Chengke Machinery, China). Then, the composite film was fabricated via blown film technique. The thickness of composite film was adjusted to 400-500 µm by controlling the take up speed. The produced film was designated as PLA/SP and used as control film. PLA/SP + Ped was elaborated by coating partially purified pediocin solution onto PLA/SP film pre-treated with dry-heat treatment. Briefly, PLA/SP film was pre-heated at 90 °C for 2 h in air-circulating oven. The pre-heated film was removed from the oven and immediately placed into 0.2% (w/v) partially purified pediocin solution prepared from culture supernatant of *Pediococcus pentosaceus* BCC 3772 for 30 min at room temperature. Then the films were removed from pediocin solution and washed three times by shaking in deionized water for 1 min for each time in order to eliminate the un-adsorbed pediocin. Pediocin adsorbed on PLA/SP + Ped was 2.75 ± 0.52 µg protein/cm^2 with anti-listeria activity against *Listeria monocytogenes* ATCC 19115 of 0.63-0.67 AU/µg of total protein/cm^2. Film coated with pediocin was then dried under laminar flow for 60 min, sterilized under UV lamp for 15 min each side and stored at room temperature prior to use.

4.3.2 Preparation and treatment of meat samples

Pork loin labeled as no preservatives was purchased from local market in Pathum Thani, Thailand. The loin thickness was approximately 1.5 cm. Meat sections were cut into 5 cm × 5 cm squares. These samples were put on the table covered with a plastic bag that had been previously disinfected with 70% ethanol

solution for 30 min and turned inside out. Then, the samples were sufficiently mixed with inherent microorganisms by hand wearing sterile plastic gloves for ensuring the homogeneous contamination of the sample surface (Lee *et al*., 2003). Samples were randomly separated into three groups. The first group was untreated and used as the control. Each pork piece of the second group was packaged in direct contact with PLA/SP. The pork pieces of the third group were packaged in direct contact with PLA/SP + Ped. All samples were then placed in polystyrene trays, wrapped with polyethylene film and stored in a retail chilled refrigerator that was maintained at 4 ± 2 °C until testing. Two samples for each treatment were randomly selected for analysis.

4.3.3 Microbiological analysis

Pork samples were aseptically removed from each package, placed in a sterilized stomacher bag and homogenized with 80 ml of 0.1% sterile peptone water for 2 min in a stomacher (Seward model 400, West Sussex, United Kingdom). Decimal dilutions of the samples were prepared in the same diluent and plated onto appropriate media in triplicate. Viable counts of total aerobic bacteria, psychrophilic bacteria, Staphylococci, lactic acid bacteria, Enterobacteriaceae, and Listeria were carried out. Total aerobic bacteria and psychrophilic bacteria were determined by incubating on Plate Count Agar (PCA; Merck, Darmstadt, Germany) at 30 °C for 2 days and 7 °C for 10 days, respectively. Staphylococci count was determined using Baird-Parker agar supplemented with Egg-yolk Tellurite Emulsion 20% (Merck, Darmstadt, Germany) as a medium after incubation at 35 °C for 24-48 h. Staphylococci was recognized as black colony surrounded by clear zone on this medium. Lactic acid bacteria (LAB) were enumerated by incubation at 30 °C for 48-72 h on de Man Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) agar plate containing 0.5% calcium carbonate. The colony surrounded by clear zone was recognized as LAB. Determination of Enterobacteriaceae was carried out on Violet Red Bile Agar (VRBA; BD Difco^{TM}, Becton, Dickinson and Company, NJ, USA) supplemented with 1% glucose after incubation at 37 °C for 18-24 h. Enterobacteriaceae was recognized as purple-red colony, 0.5 mm in diameter,

surrounded by a zone of precipitated. Listeria were enumerated by plating 0.1 ml of serial dilutions and 0.1 to 1 ml of pork homogenate on PALCAM agar plates supplemented with PALCAM antimicrobic supplement (BD Difco™, Becton, Dickinson and Company, NJ, USA) after incubation at 37 °C for 24-48 h. On PALCAM agar, Listeria was recognized as grey-green colony with black precipitate. In addition, the selectivity of each medium was checked routinely by Gram staining and microscopic examination of smears prepared from randomly selected colonies from all the media. Microbial colonies were counted and expressed as logCFU (colony forming unit)/g of pork sample. The microbiological analysis was carried out twice on two different batches.

4.3.4 Determination of biogenic amines

Biogenic amines were determined by HPLC method according to Tosukhowong *et al*. (2011). Biogenic amines were extracted with 0.4 M perchloric acid and then derivertized with dansyl chloride. The derivertives were separated on Hypersil BDS C18 column, 4.6 x 200 mm, 5 μm (Thermo, Bellefonte, PA, USA) and the analyte was quantified on an Alliance 2690 HPLC system (Waters, Milford, MA, USA). The column temperature was set at 40 °C. A 20 μl aliquot of standard or derivatized sample was injected. The mobile phase was composed of 0.1% acetic acid as solvent A and 0.1% acetic acid in acetonitrile as solvent B. The flow rate was 1 ml/min. The gradient program started at 50% A and 50% B and then solvent B was raised to 90% within 25 min, after that the gradient was switched to 50% A and 50% B within 10 min and held for 5 min before starting the next run. Biogenic amines were detected at wavelength 254 nm by a Photo diode array detector model 996 (Waters, MA, USA). The concentration of biogenic amine in pork meat was calculated by comparing with the concentration of standards.

4.3.5 Color evaluation

Color characteristics of packaged pork were determined by a colorimeter (CR-300, Minolta, Japan). Color was measured 30 min after packaging opening as described by Djenane *et al*. (2001), in order to allow color stabilization on

air exposure. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were measured and thereafter the total color difference (ΔE) with respect to the pork sample stored for 0 day was calculated as: $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Redness index ($a^*/b^*$) was also calculated according to method of Chen et al. (1997).

4.3.6 pH

The pH of all pork samples were directly measured at five different locations on pork samples using an Inlab® pH combination puncture electrodes meter equipped with a SevenEasy™ pH meter S20 (Mettler Toledo, Schwerzenbach, Switzerland). The pH values were reported as average of five replicates.

4.3.7 Sensory evaluation

The pork samples were subjected to sensory evaluation in order to ascertain whether there were differences between control (unpackaged), PLA/SP and PLA/SP + Ped samples over the storage time. The 15-member-trained panel was formed for the evaluation of the samples. The panelists were selected on the basis of their sensory skills (ability to accurately determine and communicate the sensory attributes, color, odor, appearance of fresh pork). Prior to testing pork samples, the panelists were trained in sensory vocabulary and identification of particular attributes in order to let them familiarize with samples objected of investigation. Panelists were asked to evaluate color, odor, appearance and overall acceptability of pork samples using five-point scale ranging from 1 to 5 (Lee *et al*., 2003). The scale points were: excellent, 5; good, 4; acceptable limit of market ability, 3; poor, 2 and extremely poor, 1. Shelf life criteria assumed that rejection would occur when the sensory attributes declined below 3. During the test sessions, the samples were served randomly, identified by three random digit codes. Fresh pork loin was provided as reference sample at different stage of storage. In order to avoid any logical error, the odor attributes were evaluated under blinded condition. The color of packaged pork samples was assessed 30 min after package opening in order to stabilize the color of sample.

4.3.8 Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 17.0 software. Duncan's Multiple Range Test (DMRT) was used for comparison of mean values at a significant level of 0.05.

4.4 Results and discussion

4.4.1 Changes in microbial counts

The changes in microbial counts of pork loin as a function of storage time were shown in Figure 13. PLA/SP + Ped effectively controlled the growth of *Listeria* in chilled pork meat during storage at 4 ± 2 °C. Listerial count of PLA/SP + Ped sample decreased initially from 1.96 ± 0.08 logCFU/g to below the detection limit and was lower than those of control and PLA/SP sample ($P < 0.05$) after 1 day storage (Figure 13a). Pediocin coated on PLA/SP film acted as the *Listeria* growth inhibitor (Kingcha *et al*., 2012). In agreement with our previous study (Woraprayote *et al*., 2013), this anti-listeria compound could release from the film matrix to reduce the listerial counts in pork meat up to 99%. For pork meat with moderate level of *Listeria* contamination (>100 CFU/g), this anti-listeria film could reduce the contamination to the level which is safe to consume.

In contrast, PLA/SP + Ped could not reduce the growths of total aerobic bacteria (Figure 13b), Enterobacteriaceae (Figure 13c), LAB (Figure 13d) and psychrophilic bacteria (Figure 13e) whilst it could delay the growth of Staphylococci. During storage time, the significant increases in total aerobic bacteria, Enterobacteriaceae and LAB of all samples was observed. After 14 days, the growth of these microorganisms was more pronounced in packaged samples. This result may be explained by the fact that the condensation of water vapor inside packaging accelerated the microbial growth, whilst the unpackaged one was getting dry during storage and thereby the microbial growth was relatively retarded compared with packaged groups as reported by Marriot *et al*. (1997) and Rea *et al*. (1972). The viable counts of these microorganisms in PLA/SP and PLA/SP + Ped were not significantly

different, indicating that pediocin in PLA/SP + Ped could not inhibit the growth of these microorganisms.

The increase in psychrophilic count was also detected in all pork samples throughout the storage time (Figure 13e). PLA/SP + Ped could retard but could not reduce the growth of psychrophile due to the fact that pediocin had no inhibitory activity against the member of psychrophilic bacteria including *Pseudomonas* (Kingcha *et al*., 2012).

The Staphylococcus count of packaged samples decreased with an increasing time, whilst the count of control (unpackaged) sample increased (Figure 13f). It is assumed that the growth of Staphylococci of packaged samples was inhibited by low oxygen and/or high carbon dioxide concentrations created in the package. Acid and antimicrobial substances, produced by LAB which rapidly grew during storage time, also suppressed the growth of staphylococci (Newton and Gill, 1978). Compared with PLA/SP samples, PLA/SP + Ped could not reduce the number of Staphylococci due to the fact that pediocin from *Pediococcus pentosaseus* BCC 3772 has no antimicrobial activity against Staphyloccoci (Kingcha *et al*., 2012).

4.4.2 Changes in biogenic amines in pork meat

The changes in biogenic amine content in control and packaged samples as a function of storage time are shown in Table 13. Spermine of control sample was significantly decreased whilst those of PLA/SP and PLA/SP + Ped samples was constant throughout the storage period. Bardócz (1995) and Hernández-Jover *et al*. (1996) pointed out that spermine content often decreased during food spoilage since it could be used as a nitrogen source by some microorganisms. This result is in agreement with our study that the spermine content decreased along with the increase of total aerobic bacteria and the progression of spoilage.

In this study, putrescine, cadaverine and tyramine were not found at time zero. As reviewed by Hernández-Jover *et al*. (1996), putrescine, cadaverine and tyramine did not occur in fresh meat. However, the formation of tyramine, cadaverine and putrescine was observed in all meat samples after 1, 4 and 14 days of storage, respectively. A continuous increase in these biogenic amines along with the storage period was observed. Putrescine, cadaverine and tyramine content were more

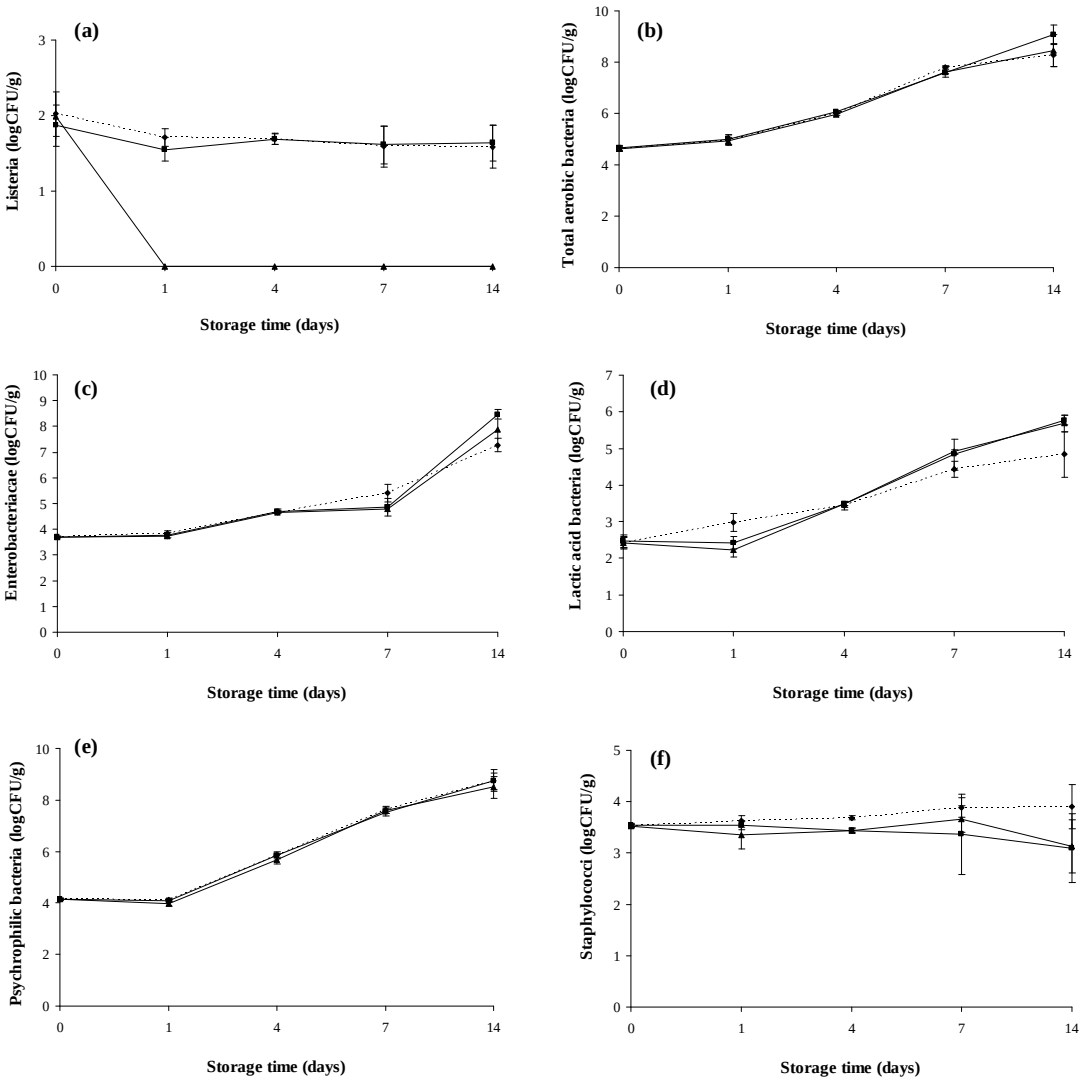

Figure 13. Changes in microbial counts on pork meats during chilled storage (♦: control, unpackaged, ■: PLA/SP, ▲: PLA/SP + Ped). Bars represent the standard deviation of duplicate determinations from two independent experiments.

pronounced in PLA/SP and control samples than in PLA/SP + Ped. This result suggested that PLA/SP + Ped could decrease the formation of some biogenic amines, such as putrescine, cadaverine and tyramine in meat samples. It was noticed that the formation of these biogenic amines was related to the increasing of bacterial load in pork samples. This relationship may be explained by the fact that the biogenic amines in foods are produced by the breakdown of amino acids due to the action of

decarboxylases of microbial origin (Chander *et al*., 1989). As previously reported, biogenic amine production becomes evident when bacterial load approach 6 logCFU/cm^2 (Dainty *et al*., 1986; Smith *et al*., 1993; Lee and Yoon, 2001).

In this study, the histamine content in all samples was not detected until the end of storage time (data not shown). As reported by Ruiz-Capillas and Jimènez-Colmenero (2004), the concentration of histamine in meat and meat products are usually quantitatively lower than those found in other foods (e.g., fish).

The presence of biogenic amines in pork meat is of interest for two reasons. Firstly, the intake of foods containing high concentrations of biogenic amines can present at health hazard through the direct toxic effect of these compounds and their interaction with some medicaments. Secondly, they may have a role as indicators of quality and/or acceptability of food (Ruiz-Capillas and Jimènez-Colmenero, 2004).

The biogenic amine index (BAI) consisting of the sum of putrescine, cadaverine, histamine and tyramine was calculated and used as a meat quality index with the following limits: BAI < 5 mg/kg for good quality fresh meat, between 5 and 20 mg/kg for acceptable meat but with initial spoilage signs, between 20 and 50 mg/kg for low meat quality, > 50 mg/kg for spoiled meat (Hernández-Jover *et al*., 1996). According to these values, pork meat packaged with PLA/SP + Ped was designated as low quality pork after 14 days of storage, whilst both unpackaged and PLA/SP packaged samples were designated as spoiled pork after 14 days (Table 13).

4.4.3 Changes in pork color

The color characteristic of packaged samples compared with that of the control is shown in Table 14. The L^* value of all samples did not change during storage period. PLA/SP and PLA/SP + Ped did not affect the L^* value of packaged pork.

During storage, a^* value gradually increased followed by bending down. The peak point of a^* value was reached at day 1 for all of samples. After 7 days, a^* value of all samples decreased. It was found that a^* value of packaged samples was significantly lower compared with that of control samples ($P < 0.05$). It can be assumed that the condensed water vapor and drip loss from meat samples inside the package extracted the water-soluble meat pigment, myoglobin, which

ultimately led to a pale surface color. Lee *et al*. (2003) observed with naked eye that the pork meat surface discolored palely and light red meat juice was formed inside the film. Meanwhile, the *b** value rapidly decreased in all samples at the first day of storage. At the same day of storage, there was no significant difference observed among three treatments, except in the first and the fourth day which the control sample showed higher *b** value ($P < 0.05$). Any differences in *a** and *b** value was observed between PLA/SP and PLA/SP + Ped samples indicated that Ped in PLA/SP + Ped did not affect these values.

The apparent change in redness of pork samples was reported as redness index (*a**/*b**). Table 14 shows that redness index of all samples significantly increased during storage period. This can be explained by the reduction of *b** value while *a** value was slightly constant. Hui *et al*. (2001) explained that the thickness of oxymyoglobin layer, which contributed to redness of meat, increased with the storage time. This is because the respiratory activity of meat is reduced during chilled storage and oxygen is not consumed so rapidly. When this meat is fully bloom after exposure to the air, the oxymyoglobin layer is therefore thicker. However, after 14 days of storage, redness index of pork samples packaged with PLA/SP and PLA/SP + Ped was significantly lower than that of control ($P < 0.05$). In agreement with several studies, the packaging affected the changes in color of the packaged meat sample (Boakye and Mittal, 1996; Hui *et al*., 2001; Lee *et al*., 2003). The condensed water vapor inside the package extracted the water-soluble meat pigment led to a pale surface color (Lee *et al*., 2003). Packaging with low oxygen permeable allows not enough oxygen to enter the packaging to bloom the meat to any degree. This condition favors metmyoglobin formation led to brown coloration of meat (Hui *et al*., 2001). Moreover, hydrogen sulphide (H_2S) produced by microorganisms could react with myoglobin to form sulphmyoglobin which alter pork color to green (Hui et al., 2001). Meanwhile, the unpackaged one was exposed to the air with high oxygen content which caused the changes of myoglobin to oxymyoglobin consequently made the meat become more redness. Color appearance is the most important sensory attribute of fresh meat for retail sale. When purchasing meat, consumers judge its quality chiefly by means of the color of its surface; they prefer the bright red oxymyoglobin (Djenane *et al*., 2001).

Table 13. Changes in biogenic amines content in pork meat which were not packaged (control) and packaged with PLA/SP and PLA/SP + Ped during storage time at 4 °C

Parameter	Treatments	Storage time (days)				
		0	1	4	7	14
Putrescine (mg/kg)	Control	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	13.33 ± 2.78 bAB
	PLA/SP	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	17.67 ± 1.80 bB
	PLA/SP + Ped	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	11.56 ± 2.58 bA
Cadaverine (mg/kg)	Control	0.00 ± 0.00 aA	0.00 ± 0.00 aA	1.04 ± 0.09 aA	3.96 ± 0.24 cB	25.94 ± 1.63 dB
	PLA/SP	0.00 ± 0.00 aA	0.00 ± 0.00 aA	3.11 ± 0.69 bB	7.57 ± 0.94 cC	34.17 ± 0.32 dC
	PLA/SP + Ped	0.00 ± 0.00 aA	0.00 ± 0.00 aA	1.36 ± 0.04 bA	2.70 ± 0.05 bA	13.15 ± 0.47 cA
Tyramine (mg/kg)	Control	0.00 ± 0.00 aA	1.06 ± 0.82 aA	6.13 ± 0.33 bA	15.85 ± 0.96 dB	23.20 ± 0.85 eB
	PLA/SP	0.00 ± 0.00 aA	3.03 ± 0.16 bB	8.33 ± 0.28 cC	14.99 ± 0.71 dB	32.59 ± 0.49 eC
	PLA/SP + Ped	0.00 ± 0.00 aA	0.32 ± 0.09 bA	7.67 ± 0.17 cB	11.32 ± 0.18 cA	15.79 ± 0.21 dA
Spermine (mg/kg)	Control	6.30 ± 0.17 bcA	6.40 ± 0.21 cAB	6.94 ± 0.14 dA	5.91 ± 0.21 bA	5.15 ± 0.44 aA
	PLA/SP	6.59 ± 0.28 aA	6.05 ± 0.11 aA	6.68 ± 0.70 aA	5.80 ± 0.26 aA	5.74 ± 0.85 aA
	PLA/SP + Ped	4.19 ± 3.63 aA	6.58 ± 0.35 aB	6.74 ± 0.38 aA	5.89 ± 0.05 aA	5.75 ± 0.89 aA
BAI (mg/kg)	Control	0.00 ± 0.00 aA	1.06 ± 0.82 aA	7.17 ± 0.41 bA	19.81 ± 1.19 cB	62.47 ± 5.17 dB
	PLA/SP	0.00 ± 0.00 aA	3.03 ± 0.16 bB	11.44 ± 0.96 cC	22.56 ± 1.39 dC	84.43 ± 1.61 eC
	PLA/SP + Ped	0.00 ± 0.00 aA	0.32 ± 0.09 aA	9.04 ± 0.19 bB	14.02 ± 0.16 cA	40.50 ± 2.65 dA
Meat quality	Control	good	good	acceptable	acceptable	spoiled
	PLA/SP	good	good	acceptable	low	spoiled
	PLA/SP + Ped	good	good	acceptable	acceptable	low

Data are means ± SD of triplicate determinations. Different lower case letters in the same row indicate significant difference ($p < 0.05$). Different upper case letters in the same column within the same parameter indicate significant difference at ($p < 0.05$).

Table 14. Changes in color of pork meat which were not packaged (control) and packaged with PLA/SP and PLA/SP + Ped during storage time at 4 °C

Parameter	Treatments	Storage time (days)				
		0	1	4	7	14
*L**	Control	46.00 ± 4.42 aA	46.11 ± 1.48 aA	46.37 ± 1.47 aA	47.53 ± 1.41 aA	47.17 ± 3.01 aA
	PLA	45.86 ± 3.88 aA	45.56 ± 5.09 aA	46.29 ± 4.55 aA	45.93 ± 2.01 aA	48.89 ± 1.28 aA
	PLA + pediocin	43.80 ± 3.19 aA	46.40 ± 3.28 aA	46.31 ± 2.75 aA	45.38 ± 5.78 aA	47.08 ± 1.07 aA
*a**	Control	7.14 ± 1.03 abA	9.04 ± 0.77 cB	7.78 ± 0.60 bB	6.72 ± 1.26 aA	7.39 ± 0.99 abB
	PLA	6.67 ± 0.40 bA	7.36 ± 0.91 bA	6.73 ± 0.52 bA	6.88 ± 0.94 bA	5.69 ± 0.78 aA
	PLA + pediocin	6.91 ± 0.79 aA	6.90 ± 0.74 aA	6.67 ± 0.42 aA	6.16 ± 1.38 aA	6.23 ± 0.85 aA
*b**	Control	4.17 ± 0.47 cA	4.26 ± 0.26 cB	2.68 ± 0.53 bB	1.68 ± 0.60 aA	1.21 ± 0.71 aA
	PLA	4.13 ± 0.54 cA	2.65 ± 1.97 bA	2.17 ± 0.38 abA	1.86 ± 0.71 abA	1.51 ± 0.59 aA
	PLA + pediocin	4.34 ± 0.54 dA	2.50 ± 1.04 cA	2.14 ± 0.50 bcA	1.58 ± 0.74 abA	1.29 ± 0.37 aA
Δ*E*	Control	-	2.31	2.10	3.44	3.62
	PLA	-	1.67	2.31	2.46	4.72
	PLA + pediocin	-	2.09	2.36	2.75	3.54
Redness index	Control	1.73 ± 0.31 aA	2.12 ±0.11 abA	2.99 ± 0.49 abA	4.25 ± 1.18 bA	8.48 ± 4.93 cB
	PLA	1.64 ± 0.20 aA	5.15 ± 4.37 bB	3.23 ± 0.84 abA	4.03 ± 1.07 bA	4.31 ± 1.65 bA
	PLA + pediocin	1.61 ± 0.22 aA	3.42 ±1.98 abAB	3.28 ± 0.82 abA	5.39 ± 3.76 bcA	5.14 ± 1.30 cA

Data are means ± SD of five determinations. Different lower case letters in the same row indicate significant difference ($p < 0.05$). Different upper case letters in the same column within the same parameter indicate significant difference at ($p < 0.05$)

The total color difference between the samples at day 0 and each measurement days was recorded as ΔE value. The increase of ΔE implies that the greater change in the absolute value of color space coordinates as compared with that of the day 0 has occurred. The ΔE value of all samples increased significantly ($P < 0.05$). Boakye and Mittal (1996) and Lee *et al*. (2003) reported that the total color difference of chilled beef and pork meat increased linearly with an increase in ageing. After 7 days of storage, ΔE of all treatments reached to above 2.3 which corresponded to a "just noticeable difference" or JND (Sharma, 1997). This implied that the changing of pork color was observed by naked eye for all of pork samples, both unpackaged and packaged one, after 7 days of storage.

4.4.4 pH changes

The pH of pork samples packaged with PLA/SP and PLA/SP + Ped did not significantly change ($p > 0.05$) maintaining the level of 5.7-5.8 until the end of storage (Figure 14). However, after 14 days of storage, the pH value of unpackaged sample significantly increased and was higher when compared with the packaged ones ($P < 0.05$). The increase in pH value during storage might be due to the accumulation of the microbial metabolites exceptionally ammonia from amino acid metabolism of bacterial spoilage in pork meat (Hui *et al*., 2001; Lee *et al*., 2003; Biswas *et al*., 2012; Qin *et al*., 2013).

4.4.5 Sensory evaluation

Changes in the sensory characteristic of pork samples during storage at 4 ± 2 °C in retail chilled refrigerator were compared between the control group and those packaged with PLA/SP and PLA/SP + Ped (Figure 15). The evaluation of the color attributes showed a tendency of declining as the storage period extended and was lower than 3.0 in all samples at day 14. According to Lee *et al*. (2003), the color score lower than 3.0 was implied that the products lost its market value. The color score of PLA/SP and PLA/SP + Ped samples was not different throughout the storage period ($p > 0.05$) and still higher than 3.0 until day 7, indicating that the consumers

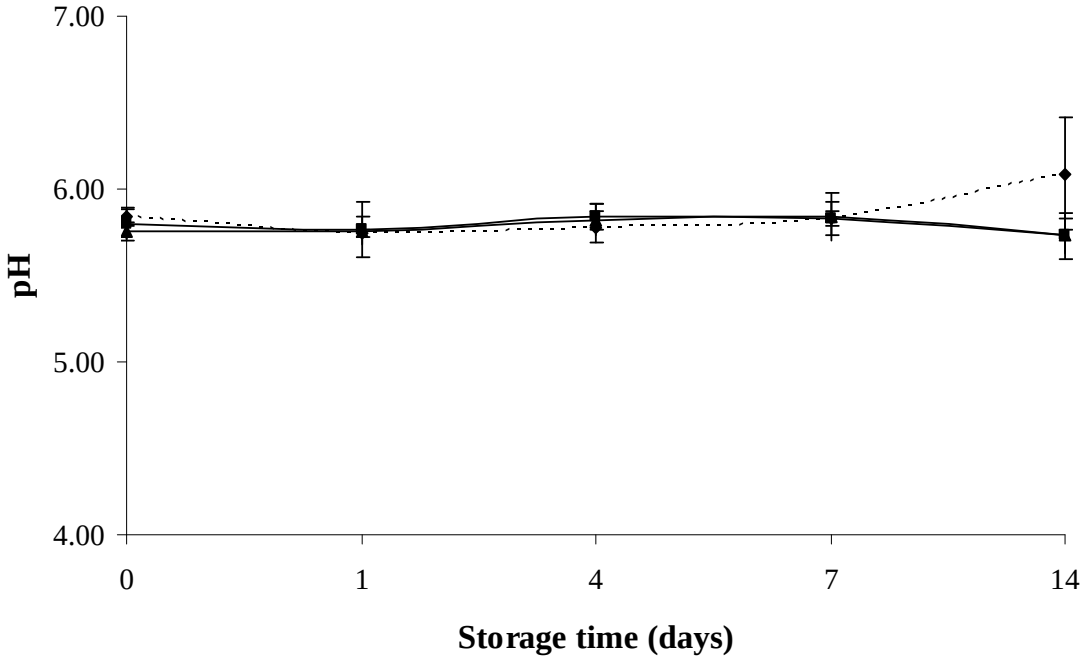

Figure 14. Changes in pH of pork meats during chilled storage (♦: control, unpackaged, ■: PLA/SP, ▲: PLA/SP + Ped). Bars represent the standard deviation of five determinations.

accepted the color of these samples until day 7 of storage. In contrast, the control sample obtained color score of 2.9 after day 4, indicating that the discoloration apparently occurred. This result was in agreement with ΔE value obtained from instrumental evaluation.

The odor (Figure 15b) and appearance (Figure 15c) scores of pork samples unpackaged and packaged with PLA/SP declined to be below 3.0 after day 7, while the score of PLA/SP + Ped sample remained over 3.0 until day 7, maintaining the market value. It was noticed that the off-odor of pork meat samples was detected when the total aerobic bacteria reached 7 log CFU/cm^2. This relationship was supported by Tewari *et al*. (1999) and Lee *et al*. (2003).

Among three samples, PLA/SP + Ped obtained the highest score of overall acceptability throughout the storage time, whilst the control group obtained the lowest score. Control and PLA/SP samples had declined below 3.0 after 4 and 7

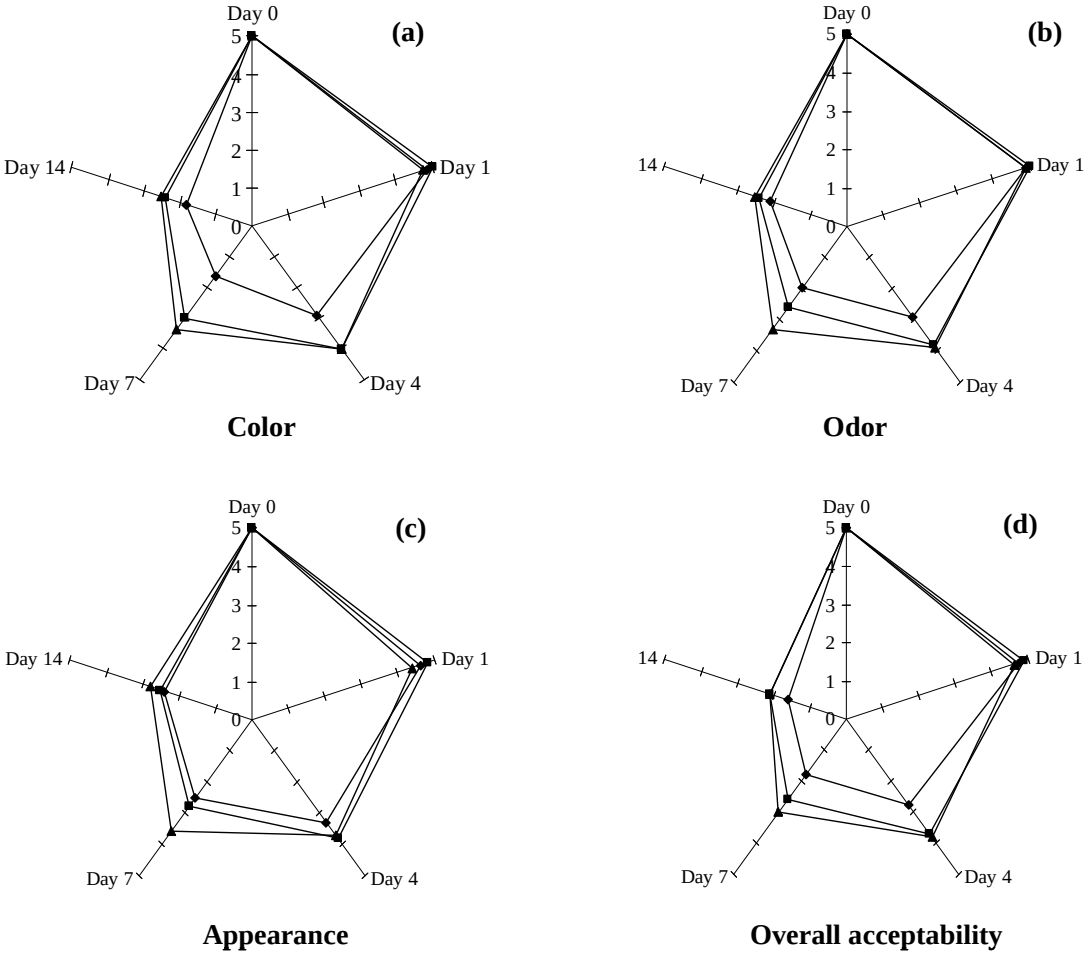

Figure 15. Sensory characteristics of pork meat unpackaged (♦) and packaged with PLA/SP (■) and PLA/SP + Ped (▲). Data points are means of fifteen determinations.

days of storage, respectively, whereas PLA/SP + Ped did in day 14. This can be concluded that the pork meat packaged with PLA/SP + Ped was higher score for all sensory attributes and was more attractive when compared with the others. These results appeared to be in good agreement with those of microbiological analysis, biogenic amine content and color characteristic, which refers to the fact that discoloration, off-odor, appearance and overall acceptability of chilled pork are related to microbial growth, biogenic amine content and color changes.

4.5 Conclusion

Poly(lactic acid)/sawdust particle biocomposite film incorporated with pediocin (PLA/SP + Ped) significantly affected the quality and shelf life of fresh pork meat stored at 4 ± 2 °C. Based on sensory characteristic and biogenic amine index of packaged pork, PLA/SP + Ped could extend shelf life of packaged pork by maintaining consumer acceptance of the products up to 7 days during storage in retail chilled condition, which was at least 3 days longer than those observed for the control unpackaged and packaged with PLA/SP film. From the microbiological point of view, however, one important drawback of the application of PLA/SP + Ped in food preservation is the limited inhibitory effect of pediocin on *Listeria* but not other pathogenic and spoilage bacteria.

CHAPTER 5

DETECTION AND CHARACTERIZATION OF PUTATIVELY NOVEL ANTIMICROBIAL PEPTIDES PRODUCED BY *WEISSELLA HELLENICA* BCC 7293

5.1 Abstract

Weissella hellenica BCC 7239, isolated from Thai fermented pork sausage called Nham, produced two putatively novel bacteriocins, 7293A and 7293B. Both bacteriocins had broad antimicrobial spectra and exceptionally inhibited several important Gram-negative food-borne bacteria (*Pseudomonas aeruginosa, Aeromonas hydrophila, Salmonella* Typhimurium and *Escherichia coli*). The highest amount of bacteriocin was produced in MRS and APT media at 30 °C without agitation. Bacteriocin 7293A showed relatively higher antimicrobial activity than bacteriocin 7293B. However, pH and thermal stability of bacteriocin 7293A was lower. These bacteriocins were of proteinaceous nature, in which the complete inactivation of their antimicrobial activity after treatment by proteolytic enzymes, including trypsin, α-chymotrypsin, pepsin and protease K was observed, whilst lipase and α-amylase exhibited no effect. Antimicrobial activity of both peptides was also not inactivated by organic solvents (ethanol, isopropanol, acetone, acetronitrile) and surfactants (Tween 20, Tween 80 and Triton X-100). Bacteriocin 7293A and B exhibited bactericidal effect against both Gram-positive and Gram-negative indicators without cell-lysis. According to ESI/MS analysis, the molecular masses of bacteriocin 7293A and B were determined to be 6249.302 and 6489.716 Da, respectively. Because their molecular masses were not similar to those of other known bacteriocins, both bacteriocin 7293A and B could be putatively novel bacteriocins.

5.2 Introduction

One of the most important problems always found in food industry is the contamination of pathogenic bacteria (Marie *et al*., 2012). Among the techniques used to control the microbial contamination in food, the application of natural antimicrobial agents has received wide attention. The demand for natural, chemical preservative-free, minimally processed, and healthy products with microbial safety is increasing (Deegan*et al*., 2006; Papagianni and Anastasiadou, 2009). Bacteriocins or antimicrobial peptides produced by lactic acid bacteria (LAB) are members of natural antimicrobial agents which have received great attention (Cleveland *et al*., 2001; Cotter *et al*., 2005; Zacharof and Lovitt, 2012). Although many bacteriocins from LAB, such as nisin and pediocin, have been approved and widely used in food products (Zacharof and Lovitt, 2012), the inability to inhibit Gram-negative pathogens limits their applications (Cotter *et al*., 2005; Deegan *et al*., 2006; Gillor*et al*., 2008). Some bacteriocins from LAB could exhibit antimicrobial activity against Gram-negative bacteria when unpurified form was used (De Kwaadsteniet *et al*., 2005; Lin *et al*., 2008; Gong *et al*., 2010; Ravi *et al*., 2011; Bendjeddou *et al*., 2012; Marie *et al*., 2012; Jena *et al*., 2013;) or they were used together with chelating agent such as EDTA (Cutter and Siragusa, 1995; Lappe *et al*., 2009; Martin-Visscher *et al*., 2010). Till date, only few purified LAB bacteriocins active against Gram-negative bacteria have been reported. Purified enterocin AS-48 exhibited antimicrobial activity against *Escherichia coli*(Gálvez *et al*., 1989), whilst purified enterocin E-760 was active against many strains of *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica*, *Yersinia pseudotubercolosis*, *Citrobacter ifreundii*, *Klebsiella pneumonia*, *Shigella dysenteriae* and *Campylobacter jejuni* (Line *et al*., 2008). However, these bacteriocins might be subjected to crtical scrutiny on the safety especially since the producer strains were isolated from non-food origins and had no safe history of use in food.

Amongst the bacteria belonging to *Weissella* genus, a food origins LAB, only few strains could produce bacteriocin (Papathanasopoulos *et al*., 1997; Srionnual *et al*., 2007; Pal and Ramana, 2010; Masuda *et al*., 2011; Papagianni and Papamichael, 2011: Leong *et al*., 2013). This study reported bacteriocin from *Weissella* genus which is active against Gram-negative bacteria including *P*.

aeruginosa ATCC 27853, *A. hydrophila* B1 AhB1, *S.* Typhimurium DMST 0562 and *E. coli* ATCC 25922. In this study, the optimal culture medium which maximized bacteriocin production was investigated. Purification and characterization of these bacteriocins were performed. Effects of hydrolytic enzymes, pH, temperature, chemicals and organic solvents on their antimicrobial activity were also evaluated.

5.3 Materials and Methods

5.3.1 Bacterial strains and culture conditions

Strain BCC 7293, a bacteriocin-producing strain, was isolated on a deMan Rogosa and Sharpe (MRS) agar (Difco^{TM}, USA) plates containing 0.5% $CaCO_3$ from Thai traditional fermented pork and was identified by 16S rDNA gene sequencing. Gram staining and catalase test were performed as a preliminary step in the screening of LAB (Sawa *et al.*, 2009).

Strain BCC 7293 was stored at -80°C in MRS medium with 15% glycerol and propagated in MRS broth at 30°C for 18 h before use. Indicator strains (Table 17) for the determination of antimicrobial activities were propagated at appropriate temperatures (30 or 37 °C) for 18-24 h before use. LAB strains were grown in MRS medium, and the other Gram-positive and Gram-negative indicator strains were grown in Tryptic Soy Broth (TSB, Merck, Germany) supplemented with 0.6% yeast extract (Merck, Germany).

5.3.2 Determination of bacteriocin activity

Bacteriocin activity assay was performed using the spot-on-lawn method as previously described (Zendo *et al*. (2005). *Lactobacillus sakei* subsp. *sakei* JCM 1157^{T} was used as an indicator strain unless otherwise mentioned. The titer of antimicrobial activity in the culture supernatant in production experiments was expressed as activity units (AU) per millilitre, in which the reciprocal of the highest dilution at which growth inhibition was still detectable. The minimum inhibitory concentration (MIC) of the bacteriocin for the various indicator strains were determined with purified bacteriocin solutions using the spot-on-lawn method

described above. The MIC was defined as the minimum bacteriocin concentration that yielded a clear zone of growth inhibition in the indicator lawn.

5.3.3 Bacteriocin production in different culture media

To compare the effectiveness of different culture media on the production of the bacteriocin produced by *Weissella hellenica* BCC 7293, TGE (1% tryptone, 1% yeast extract, 1% glucose, 0.2% Tween 80, 0.03 mM $MnSO_4$ and 0.02 mM $MgSO_4$), WYG (1.5% whey protein isolate, 1.5% yeast extract and 1.0% glucose), CYG (1.5% casein sodium salt, 1.5% yeast extract and 0.5% glucose), TSB, MRS and APT broths were compared. The media were sterilized by autoclaving at 121 °C for 15 min. A 1% (v/v) inoculum of *W. hellenica* BCC 7293 was added to 50 ml of culture medium in 125 ml Erlenmeyer flask. The samples were incubated at 30 °C without shaking. After 18 h of incubation, the samples were taken and antimicrobial activity against *Lb. sakei* ssp. *sakei* JCM 1157^T was determined using the spot-on-lawn method.

5.3.4 Time course of bacteriocin production

The overnight culture of *W. hellenica* BCC 7293 was added to 50 ml of MRS broth in 125 ml Erlenmeyer flask at the final concentration of 1% (v/v) and incubated at 30 °C without shaking. The samples were taken at different time intervals to determine the viable counts of the producing strain. The changes in pH were measured using a pH meter (Mettler-Teledo, Swizerland), and the antimicrobial activity (AU/ml) was determined using the critical dilution spot-on-lawn method as described above using *Lb. sakei* JCM 1157^T as an indicator strain.

5.3.5 Purification of bacteriocin from *W. hellenica* BCC 7293

Bacteriocin purification was carried out by a three-step procedure using the supernatant of a 1-L culture of *W. hellenica* BCC 7293 grown for 18 h in MRS broth at 30 °C. The culture was adjusted to pH 3.0 with 1 M HCl in order to recover bacteriocin adsorbed on the cells, and then the cells were removed by centrifugation at 8,000 × g for 15 min at 4 °C. The cell-free supernatant was mixed

with 20 g of Amberlite XAD-16 (Sigma-Aldrich, St Louis, MO, USA), synthetic hydrophobic resin previously activated with 50% (v/v) isopropanol. The mixture was gently shaken at 4 °C for 24 h and transferred to a column (25 mm internal diameter, 300 mm length). After the mixture was washed with 200 ml of MilliQ water and 200 ml of 50% ethanol, bacteriocins were eluted with 100 ml of 70% isopropanol containing 0.1% trifluoroacetic acid (TFA). The active eluted solution was placed in a rotary evaporator to remove isopropanol. The resulting solution was then diluted with 50 mM sodium phosphate buffer (pH 5.7) to 100 ml and loaded onto a SP-Sepharose Fast Flow cation-exchange column (15 mm internal diameter, 100 mm length; GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM sodium phosphate buffer (pH 5.7). The column was washed serially with 100 ml of the same phosphate buffer. Bacteriocin was eluted with 50 ml of 50 mM sodium phosphate buffer containing 1 M NaCl (pH 5.7). For further purification, the active eluted solution was applied to a reverse-phase column (3-ml RESOURCE RPC column; Amersham biosciences, Uppsala, Sweden) incorporated in the LC-2000 Plus HPLC system (Jasco, Tokyo, Japan) and eluted with a gradient of MilliQ water-acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min as follows: 0 to 0.1 min, 5 to 30% acetonitrile; 0.1 to 10 min, 30% acetonitrile; 10 to 35 min, 30 to 80% acetonitrile; 35 to 40 min, 80 to 100% acetonitrile and 40 to 50 min, 100% acetonitrile. Active fractions were placed in a Speed-Vac Concentrator (Savants, Farmingdale, NY, USA) to thoroughly remove the acetonitrile. Active fractions containing bacteriocin were pooled and subjected to MIC determination and further characterization. The antimicrobial activity of each fraction in the purification steps was determined as described earlier. The protein concentration (mg/ml) of each fraction was determined according to the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

5.3.6 Molecular mass determination

Molecular mass of purified bacteriocin 7293A and B were determined by electrospray ionization time of flight mass spectrometry (ESI-TOF MS) with a JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan).

5.3.7 Mode of action of bacteriocin 7293

The bacteriocidal effect of bacteriocin 7293A and B was assayed using the modified method described by Hu *et al*. (2008) based on the killing manner toward the indicator strain, *Weissella paramesenteroides* JCM 9890^T and *S.* Typhimurium DMST 0562. Purified bacteriocin 7293A and B were added in the middle of the logarithmic phase of the growth curve (4 h of incubation) of indicator strains to final concentration of 5-fold of MICs. The effect on the optical density at 600 nm and the bacterial viability were then evaluated. Purified nisin at the concentration of 5-fold of MIC was used as a positive control for *W. paramesenteroides* JCM 9890^T.

5.3.8 Effect of enzymes, chemicals, pH and temperature on activity of bacteriocins

Purified bacteriocin 7293A (28.8 µM) and 7293B (37.0 µM) were used in the following experiments. The sensitivity of the bacteriocins to hydrolytic enzymes (Sigma-Aldrich, St. Louis, MO), including trypsin (pH 7.5), α-chymotrypsin (pH 7.5), pepsin (pH 3.0), proteinase K (pH 7.5), lipase (pH 7.5) and α-amylase (pH 7.5), was examined by incubating the purified bacteriocin in the presence of 1.0 mg/ml of each enzyme at 37 °C for 3 h (Castro *et al*., 2011). The samples were then heated at 100 °C for 10 min to inactivate the enzymes. The purified bacteriocin without enzyme and 1.0 mg/ml enzyme solution without purified bacteriocin were used as controls.

Effect of chemicals, including organic solvents and surfactants, on the antimicrobial activity of bacteriocin was also investigated. Various organic solvents such as acetone, acetonitrile, ethanol and isopropanol were added to purified bacteriocin solution at 1:1 ratio. Untreated purified bacteriocin solution and organic solvent with equal volume of sterile distilled water were used as controls. All samples were thoroughly mixed and kept at room temperature for 5 h (Karaoğlu*et al*., 2003) before antimicrobial test.

Effect of chemicals on antimicrobial activity of bacteriocin 7293A and B was investigated by incorporating non-ionic (Triton X-100, Tween 20, Tween 80),

anionic (sodium dodecyl sulphate) surfactant, EDTA and urea. All agents were added to purified bacteriocin to yield the final concentration of 1% (w/v). Untreated purified bacteriocin and chemicals at this concentration in sterile distilled water were used as controls. All samples were incubated at 30 °C for 5 h (Pinto *et al*., 2009) before antimicrobial test.

To examine the heat and pH stability of bacteriocin 7293, the purified bacteriocin solution was adjusted to pH values between 3.0 and 11.0 using 1 M HCl or 1 M NaOH. The the preparations were incubated at 25 °C (24 h), 80 °C (30 min), 100 °C (30 min) and 121 °C (15 min). The bacteriocin activity of the pH 3.0 sample without heat treatment was defined as 100% (Sawa*et al*., 2009).

The residual antimicrobial activities of controls and treated samples were determined by critical dilution spot-on-lawn technique using *Lb. sakei* subsp. *sakei* JCM 1157^{T} as an indicator strain.

5.4 Results and Discussion

5.4.1 Identification of strain BCC 7293

The isolate BCC 7293 was identified as *Weissella hellenica* with 99.58% identity to *Weissella hellenica* NCFB 2973^{T} accession no. S67831 (Collins *et al*., 1993) based on sequence analysis of the 16S rDNA intergenic spacer (Table 15).

5.4.2 Bacteriocin production in different culture media

The maximum amount of bacteriocin produced by *W. hellenica* BCC 7293 was obtained in MRS and APT media at 30 °C. Lower amount of bacteriocin was produced in CYG and TSB, whilst, it could not be produced in TGE and WYG (Figure 16).

The type or composition of culture medium, especially nitrogen and carbon sources, always has an influence on bacteriocin production (Cheigh*et al*., 2002). In the case of *W. hellenica* BCC 7293, the bacteriocin production was maximized when the producer strain was cultured in MRS and APT media at 30 °C for 18 h (Figure 16). As reported by several studies, MRS was an optimized culture

Table 15. Similarity of 16s rDNA of strain BCC 7293 compared with closely related species

Rank	Name	Strain	Accession	Pairwise Similarity (%)
1	*Weissella hellenica*	NCFB 2973(T)	S67831	99.58
2	*Weissella paramesenteroides*	ATCC 33313(T)	ACKU01000017	98.37
3	*Weissella thailandensis*	FS61-1(T)	AB023838	97.49
4	*Weissella confusa*	JCM 1093(T)	AB023241	96.45
5	*Weissella cibaria*	KACC 11862(T)	AEKT01000037	96.05
6	*Weissella oryzae*	SG25(T)	AB690345	95.69
7	*Weissella viridescens*	NRIC 1536(T)	AB023236	95.1
8	*Weissella minor*	NRIC 1625(T)	AB022920	95.1
9	*Weissella koreensis*	KCTC 3621(T)	AKGG01000017	94.77
10	*Weissella soli*	DSM 14420(T)	AY028260	94.76
11	*Weissella ceti*	1999-1A-09	FN813251	94.61

medium for bacteriocin production by LAB since it was rich in organic nitrogen and carbon sources and minerals, which enhanced the growth of the producer and bacteriocin biosynthesis (Biswas*et al*., 1991; Daba*et al*., 1993; Cheigh*et al*., 2002). The production of bacteriocin by the other species of *Weissella* was also performed in MRS (Papathanasopoulos*et al*., 1997; Srionnual*et al*., 2007; Pringsulaka*et al*., 2012). APT broth was used to cultivate *W. hellenica* QU 13 for the production of weissellicin Y and M (Masuda *et al*., 2011). Although the cultivation was performed in the same culture medium, *W. hellenica* BCC 7293 produced bacteriocins with different antimicrobial spectra and molecular masses, compared with those produced by *W. hellenica* QU 13. This indicated that even the same species, different strains could produce different bacteriocins.

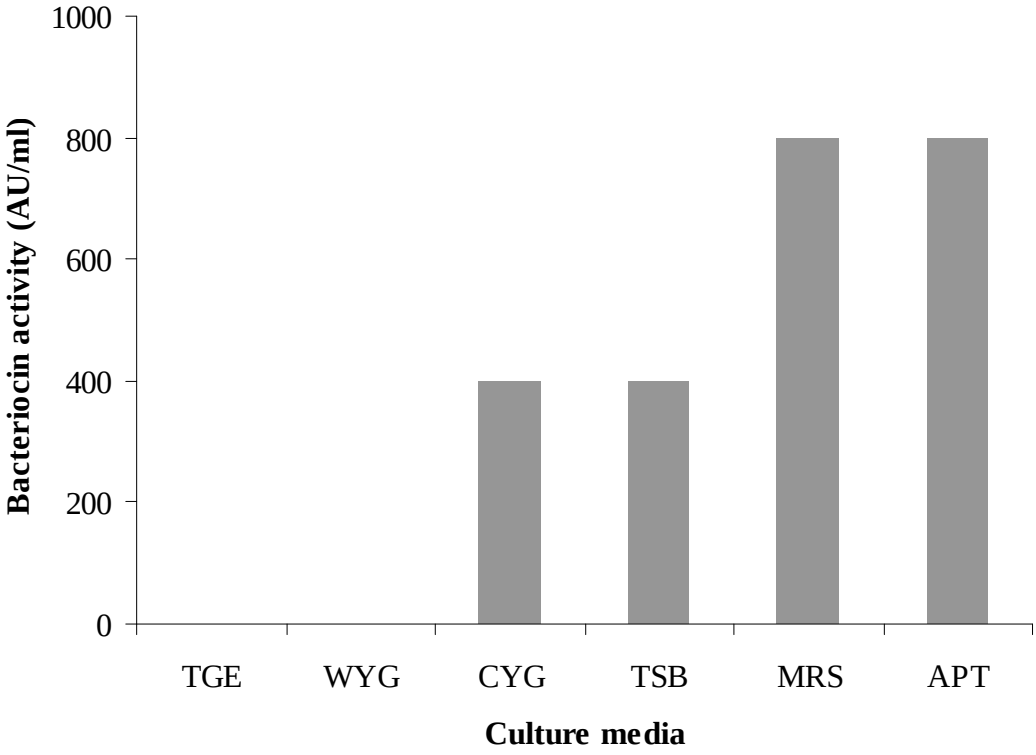

Figure 16. Bacteriocin production by *W. hellenica* BCC 7293 in different culture media (TGE: Tryptone Glucose Yeast Extract containing 1% tryptone, 1% yeast extract, 1% glucose, 0.2% Twen80, 0.03 mM $MnSO_4$ and 0.02 mM $MgSO_4$; WYG: Whey Protein Yeast Extract Glucose containing 1.5% whey protein isolate, 1.5% yeast extract and 1.0% glucose; CYG: Casein Yeast Extract Glucose containing 1.5% casein sodium salt, 1.5% yeast extract and 0.5% glucose). Data are means of duplicate determinations from two independent experiments.

5.4.3 Production of bacteriocin from *W. hellenica* BCC 7293

Time course of bacteriocin production by *W. hellenica* BCC 7293 in MRS broth was investigated. *W. hellenica* BCC 7293 produced bacteriocin during early logarithmic growth phase and the maximum antimicrobial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T of 800 AU/ml was detected at 12 h of growth.

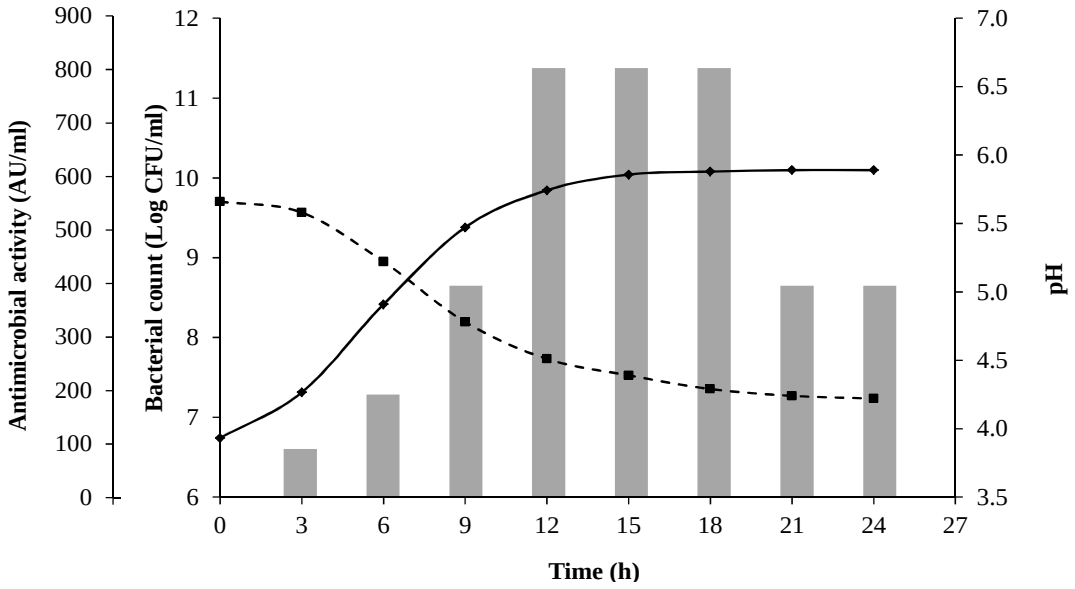

Figure 17. Growth kinetics and bacteriocin biosynthesis of *W. hellenica*BCC 7293 cultured in MRS broth at 30 °C. Cellular growth (♦), bacteriocin production (bar) and pH changes (■). Data points and bars are means of duplicate determinations from two independent experiments.

No changes in antimicrobial activity were found during 12-18 h at the stationary phase. After that, the antimicrobial activity decreased (Figure 17). As this antimicrobial substance was produced only during the exponential phase, it can be considered to show primary metabolite kinetics, like other bacteriocins (Srionnual *et al*., 2007; Papagianni and Papamichael, 2011; Pringsulaka *et al*., 2012). The reduction in the antimicrobial activity may be attributed to proteolytic degradation by non-specific protease, adsorption on producer cell (Xie *et al*., 2009) or aggregation of bacteriocin (Kingcha *et al*., 2012).

5.4.4 Purification of bacteriocin from *W. hellenica* BCC 7293

Since the maximum amount of bacteriocin was produced after 18 h, the culture supernatant was harvested at 18 h of incubation and then was used for bacteriocin purification. A three-step purification procedure, which included hydrophobic interaction, cation-exchange chromatography and reversed-phase HPLC was performed. The total antimicrobial activity, yield and the purification fold of the

bacteriocin, along with the purification procedure, are summarized in Table 16. Forty percent of the activity in the culture supernatant was recovered by hydrophobic interaction chromatography (Amberlite XAD-16). After being subjected to cation-exchange chromatography (SP-sepharose), the bacteriocin activity was recovered in 1.0 M NaCl fraction. This fraction was applied to reversed-phase HPLC. Two peaks with antimicrobial activity, termed as A and B, were obtained (Figure 18) and designated as bacteriocin 7293A and B, respectively. Finally, approximately 25.6 and 12.8% of the total activity of the culture supernatant were obtained after these purification steps. Bacteriocin 7293A and B did not have any synergistic activity (data not shown).

5.4.5 Molecular mass determination

ESI-TOF MS analysis showed that purified bacteriocin 7293A and B had molecular masses of 6249.302 and 6489.716 Da, respectively (Figure 19). These molecular masses were different from those of any known bacteriocins. Therefore, we concluded that these bacteriocins were putatively novel.

5.4.6 Antimicrobial spectra

The inhibitory spectra of bacteriocin 7293A and B are shown in Table 17. Bacteriocin 7293A and B exhibited the same antimicrobial spectra. However, the antimicrobial activity of bacteriocin 7293A was stronger than those of bacteriocin 7293B. Bacteriocin 7293A and B had broad antimicrobial spectra against not only Gram-positive but also Gram-negative bacteria including *A. hydrophila, E. coli, P. aeruginosa*and *S.* Typhimurium. Amongst indicator strains used in this study, bacteria belonging to genus *Weissella* were the most sensitive strains to bacteriocin 7293A and B. This confirmed the general characteristic of bacteriocin which is active mostly against the bacteria closely related to the bacteriocin producer (Srionnual*et al*., 2007). Furthermore, both bacteriocins 7293A and B did not inhibit the growth of the other bacteriocin-producers including *Enterococcus faecium* BCC 49239, *E. faecium* JCM 5804^{T}, *L. garvieae* BCC 43578 (Tosukhowong*et al*., 2011), *L. lactis*s sp. *lactis* NCDO 497 and *Pediococcus pentosaceus* BCC 3772 (Kingcha *et al*., 2012).

Table 16. Purification of bacteriocin 7293A and B produced by *W. hellenica* BCC 7293

Purification step	Volume (ml)	Total activity (AU)[a]	Yield (%)	Total protein (mg)[b]	Specific activity (AU/mg)	Purification (fold)
Supernatant	1000	800000	100	20450	39	1
Amberlite	100	320000	40	1238	258	7
SP-sepharose	45	288000	36	244	1179	30
RP-HPLC						
Bacteriocin 7293A	1	204800	25.6	0.18	1137778	29084
Bacteriocin 7293B	1	102400	12.8	0.24	426667	10907

[a]Antimicrobial activity [in arbitrary units (AU)] was assayed by the spot-on-lawn method using *Lb. sakei*s sp. *sakei* JCM 1157^T as an indicator strain.

[b]The protein concentration (in mg/ml) was determined by the Lowry method (Lowry *et al*., 1951).

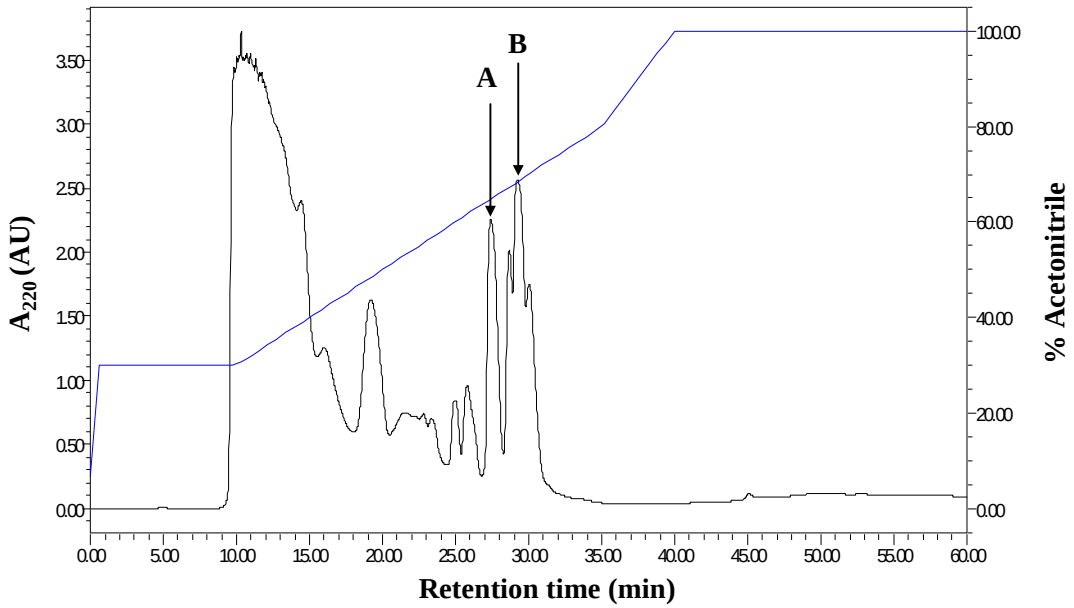

Figure 18. Reversed phase-HPLC of bacteriocin from *W. hellenica* BCC 7293. The bacteriocin activity was detected in the fraction containing the peaks as indicated by A and B.

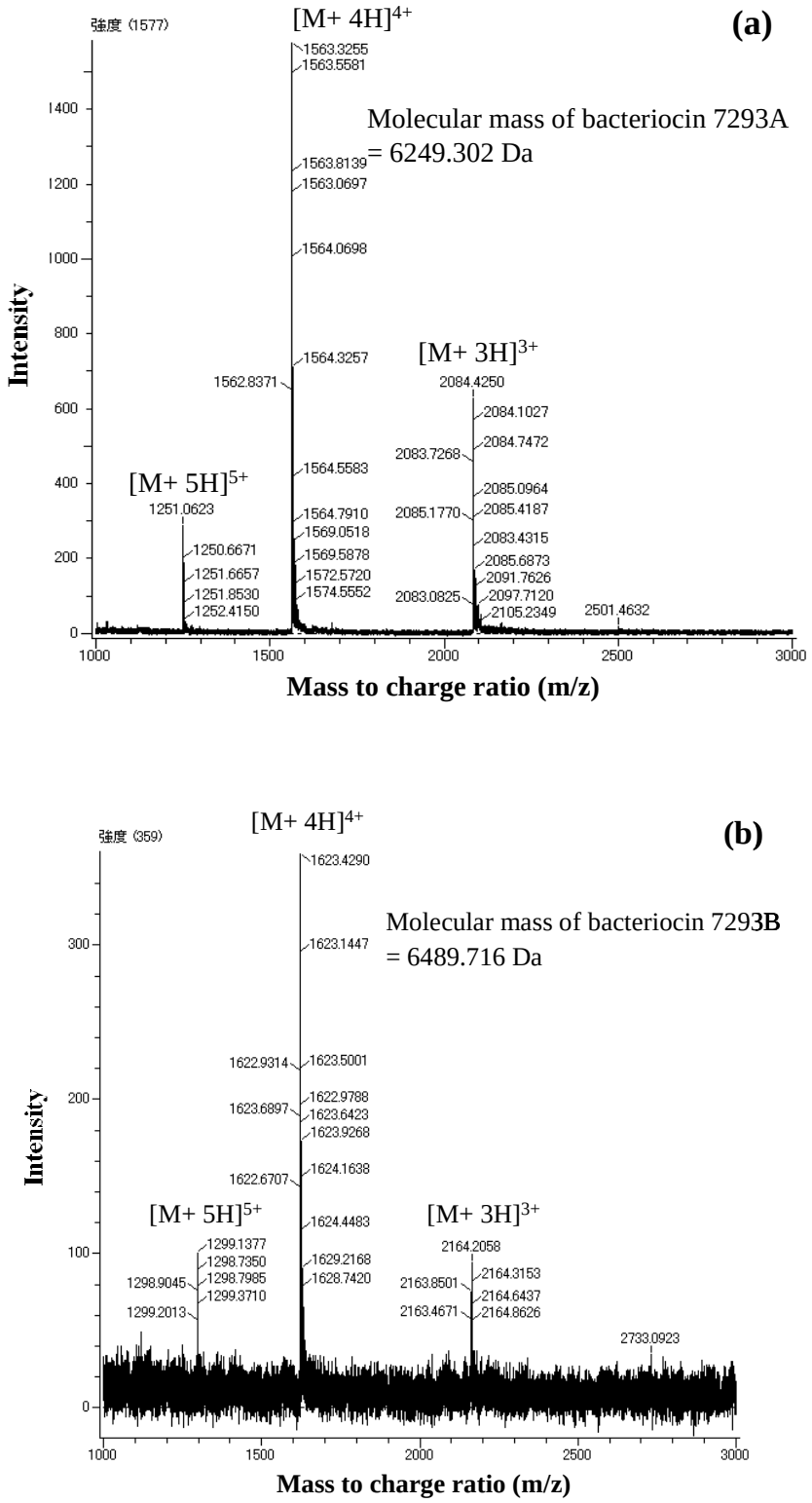

Figure 19. ESI-TOF/MS spectra of purified bacteriocins 7293A (a) and 7293B (b).

This characteristic would be an advantage of the application of bacteriocin 7293 in food system both in the form of purified bacteriocin and starter culture since the beneficial bacteria would not be killed by these bacteriocins.

Although bacteriocins from LAB have been frequently reported, those from *Weissella* genus have been rarely investigated (Papathanasopoulos *et al*., 1997; Srionnual, Yanagida *et al*., 2007; Pal and Ramana, 2010; Masuda *et al*., 2011; Papagianni and Papamichael, 2011; Leong *et al*., 2013; Chen *et al*., 2014). To our knowledge, this study is the first report of bacteriocin from *Weissella* genus, exhibiting the antimicrobial activity against at least 4 species of food-borne pathogenic Gram-negative bacteria including *A. hydrophila*, *E. coli*, *P. aeruginosa* and *S.* Typhimurium. Purified bacteriocin 7293A and B could inhibit Gram-negative bacteria without the use of chelating agents such as EDTA which weaken cell wall integrity (Cintas *et al*., 2001). With the trend toward foods with chemical-free preservatives, especially for health-concerned consumers (Deegan*et al*., 2006; Papagianni and Anastasiadou, 2009), bacteriocin 7293A and B could be used as an effective natural food preservative.

Table 17. Antimicrobial spectra of bacteriocin 7293A and B

Indicator strains	MIC (µM)	
	Bacteriocin 7293A	Bacteriocin 7293B
Gram-positive bacteria		
Bacilllus cereus DMST 5040	N.A.	N.A.
Bacillus cereus B34	N.A.	N.A.
Bacillus cereus B9	N.A.	N.A.
Bacillus circulans JCM 2504^T	0.007	0.0181
Bacillus coagulans JCM 2257^T	0.0035	0.009
Bacillus subtilis ATCC 6633	0.9001	2.3113
Bacillus subtilis ssp. *subtilis* JCM 1465^T	0.0281	0.1445
Enterococcus faecalis JCM 5803^T	1.8002	4.6227
Enterococcus faecium BCC 49239	N.A.	N.A.
Enterococcus faecium JCM 5804^T	N.A.	N.A.
Kocuria rhizophila NBRC 12708	1.8002	2.3113
Lactobacillus plantarum ATCC 14917^T	N.A.	N.A.
Lactobacillus sakei ssp. *sakei* JCM 1157^T	0.0141	0.0361
Lactococcus garvieae BCC 43578	N.A.	N.A.
Lactococcus lactis ssp. *lactis* ATCC 19435^T	0.0141	0.0361
Lactococcus lactis ssp. *lactis* NCDO 497	N.A.	N.A.
Leuconostoc mesenteroides ssp. *mes.* JCM 6124^T	0.0281	0.1445
Listeria inocua ATCC 33090^T	0.0563	0.1445
Listeria monocytogenes ATCC 19115	0.0281	0.0722
Micrococcus luteus MIII	N.A.	N.A.
Mucococcus luteus ATCC 9341	N.A.	N.A.
Pediococcus dextrinicus JCM 5887^T	N.A.	N.A.
Pediococcus pentosaceus BCC 3772	N.A.	N.A.
Pediococcus pentosaceus JCM 5885	0.0141	0.0361
Staphylococcus aureus ATCC 23235	3.6004	9.2454
Staphylococccus aureus ATCC 25923	0.0563	0.1445

Table 17. Antimicrobial spectra of bacteriocin 7293A and B (cont.)

Indicator strains	MIC (μM)	
	Bacteriocin 7293A	Bacteriocin 7293B
Gram-positive bacteria		
Staphylococcus aureus ATCC 6538	N.A.	N.A.
Staphylococcus aureus Cowan 1	N.A.	N.A.
Methicillin-resistant *Staphylococcus aureus* (MRSA)	N.A.	N.A.
Staphylococcus xylosus BCC 3710	0.4501	1.1557
Weissella confuse JCM 1093^T	0.0281	0.0722
Weissella cibaria JCM 12495^T	0.225	0.5778
Weissella paramesenteroides JCM 9890^T	0.0004	0.0011
Weissella hellenica JCM 10103^T	0.0035	0.009
Gram-negative bacteria		
Aeromonas hydrophila B1 AhB1	0.1125	0.2889
Escherichia coli ATCC 25922	0.225	0.5778
Escherichia coli O157:H7	N.A.	N.A.
Pseudomonas aeruginosa ATCC 27853	0.0563	0.1445
Salmonella Typhimurium DMST 0562	0.225	0.5778
Vibrio parahaemolyticus Vp1	N.A.	N.A.
Vibrio algenolyticus Va	N.A.	N.A.
Vibrio harveyi Vh1	N.A.	N.A.
Vibrio sp. Brood 4.27	N.A.	N.A.

Data are means of duplicate determinations from two independent experiments. N.A., no activity detected at the highest concentration of applied bacteriocin 7293A (28.8 μM) and bacteriocin 7293B (37.0 μM)

ATCC, American Type Culture Collection, Rockville, MD; BCC, Biotech Culture Collection, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand; DMST, Culture Collection for Medical Microorganism Department of Medical Sciences Thailand; JCM, Japan Collection of Microorganisms, Saitama,

Japan; NBRC, NITE Biological Resource Center, Chiba, Japan; NCDO, National Collection of Dairy Organisms (Reading, United Kingdom)

5.4.7 Mode of action of bacteriocin 7293

Bacteriocin 7293A and B exhibited the same mode of action, bactericidal effect without cell lysis, against both *W. paramesenteroides* and *S.* Typhimurium since the addition of bacteriocin 7293A and B to the indicators could reduce the bacterial population without any decreasing or increasing of cell density. In contrast, the addition of nisin, a bacteriolytic bacteriocin significantly reduced viable cell count and cell density (Figure 20 and 21).

Bacteriocin 7293A and B plausibly acted on the cytoplasmic membrane of target cells by forming hydrophilic pores which caused an efflux of important cellular metabolites and subsequent cell death as suggested by Papagianni and Papamichael (2011). However, the reduction of viable count of *S.* Typhimurium (Figure 21) was slower than that of *W. paramesenteroides* (Figure 20). This result might imply the absence of an outer membrane layer in Gram-positive bacteria (De La Fuente-Salcido *et al*., 2012). Therefore, the presence of an outer lipopolysaccharide membrane of Gram-negative bacteria could retard bactericidal effect of bacteriocin 7293A and B.

5.4.8 Effect of enzymes, chemicals, pH and temperature on activity of bacteriocins

The antimicrobial activity of bacteriocin 7293A and B were affected by hydrolytic enzymes, chemicals, pH and temperature (Table 18 and Figure 22). Both two bacteriocins were completely inactivated by proteolytic enzymes, which included trypsin, α-chymotrypsin, pepsin and proteinase K. While α-amylase and lipase did not affect the bacteriocin activity.

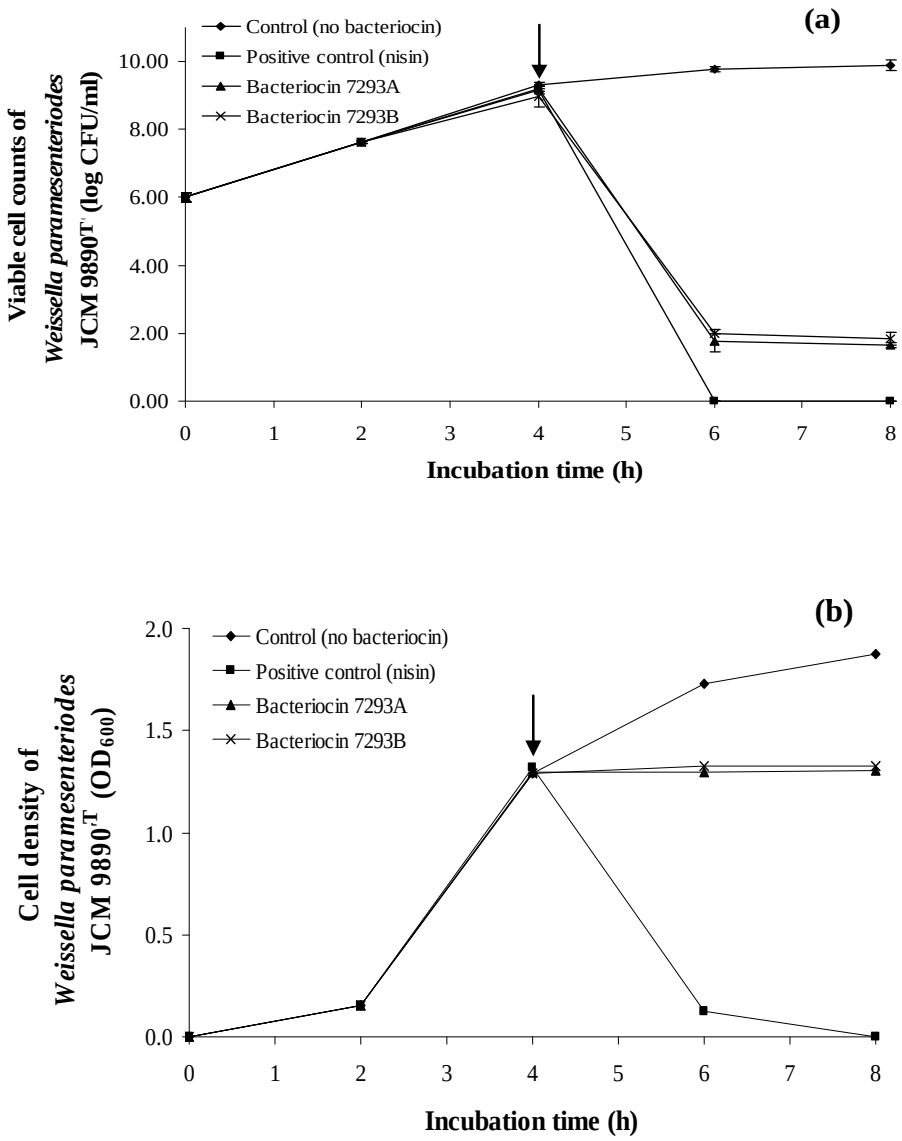

Figure 20. Effect of bacteriocin 7293A and 7293B on viable cell counts (a) and cell density (b) of *W. paramesenteroides* JCM 9890^{T}. Bacteriocin 7293A and B were added to the suspension of the indicator cells after 4 h of incubation to final concentration of 5-fold of MICs. Cell density in optical density was measured at 600 nm. Purified nisin at the concentration of 5-fold of MIC was used as positive control for *W. paramesenteroides* JCM 9890^{T}. Bars represent the standard deviation of duplicate determinations from two independent experiments.

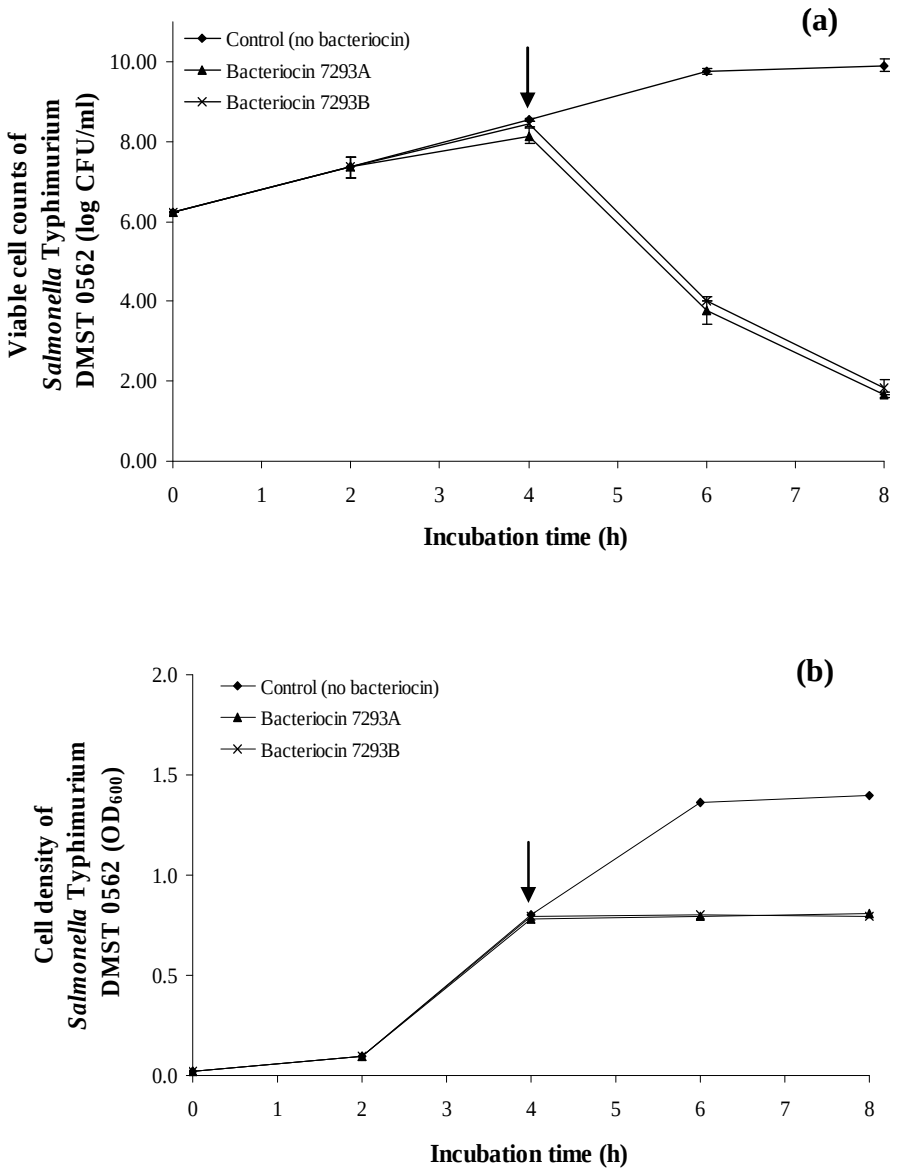

Figure 21. Effect of bacteriocin 7293A and 7293B on viable cell counts (a) and cell density (b) of *S.* Typhimurium DMST 0562. Bacteriocin 7293A and B were added to the suspension of the indicator cells after 4 h of incubation to final concentration of 5-fold of MICs. Cell density in optical density was measured at 600 nm. Bars represent the standard deviation of duplicate determinations from two independent experiments.

The result confirmed that these antimicrobial substances were proteinaceous in nature and not containing lipid or carbohydrate groups in its molecule (Riley and Chavan, 2007). The absence of lipid moieties in the bacteriocin molecules was confirmed by the stability of bacteriocin activity in the presence of organic solvents (Osmanağaoğlu, 2007). As shown in Table 18, treatment of bacteriocins with organic solvents including acetone, acetonitrile, ethanol and isopropanol did not cause any losses in bacteriocin activity. The complete inactivation of bacteriocin activity by proteolytic enzymes also ensured the safety for the consumer since these bacteriocins could be completely deactivated by enzymes in human gastrointestinal tract and then were not active against beneficial bacteria in human intestine.

The antimicrobial activity of bacteriocin 7293A and B was not affected by 1% (w/v) Tween 20, Tween 80 and Triton X-100. Only the exposition of the purified bacteriocin to 1% urea resulted in the reduction of the bacteriocin activity. The increased activity of bacteriocin 7293A and B in combination with EDTA and SDS was observed. EDTA and SDS acted as destabilizer which alter the permeability of the cell membrane of sensitive bacteria and subsequently increased the susceptibility to bacteriocins (Shafa and Salton, 1960; Alakomi *et al*., 2000). The sensitivity of bacteriocin 7293A and B to chemicals were similar to the other bacteriocins (De Kwaasdsteniet *et al*., 2005; Albano *et al*., 2007; Pinto *et al*., 2009; Jiang *et al*., 2012). However, the sensitivity to surfactants and urea seems to be bacteriocin-dependent. Activity of bacteriocin ST5Ha produced by *E. faecium* ST5Ha was not affected by the presence of urea (Todorov *et al*., 2010).

The effects of pH and heat treatment on antimicrobial activity of bacteriocin 7293A and B were investigated (Figure 22). The activity of both bacteriocins decreased upon the exposure to elevated temperatures and high pH. Under heat treatment at 121 °C for 15 min, the activity of bacteriocin 7293A was completely inactivated (Figure 22a). In contrast, bacteriocin 7293B showed higher stability against high temperatures and pH when compared with bacteriocin 7293A (Figure 22b). Even at 121 °C and pH 3, it still retained 50% of its initial activity. Moreover, bacteriocin 7293B retained 100% activity of its activity after exposure to pH 3 at 25 °C (24 h), 80 °C (30 min) and 100 °C (30 min).

Table 18. Effect of enzymes and chemicals on antimicrobial activity of bacteriocin 7293A and 7293B

Treatment	Relative activity (%) after treatment[a]	
	Bacteriocin 7293A	Bacteriocin 7293B
Untreated*	100	100
Enzymes		
Trypsin	0	0
Alpha-Chymotrypsin	0	0
Pepsin	0	0
Protease-K	0	0
Lipase	100	100
Alpha-Amylase	100	100
Organic solvents		
Acetone	100	100
Acetonitrile	100	100
Ethanol	100	100
Isopropanol	100	100
Chemicals		
Tween20	100	100
Tween80	100	100
EDTA	200	200
SDS	1600	1600
Triton-X-100	100	100
Urea	25	25

[a]The activity of an untreated sample was defined as 100%.

Data are means of duplicate determinations from two independent experiments.

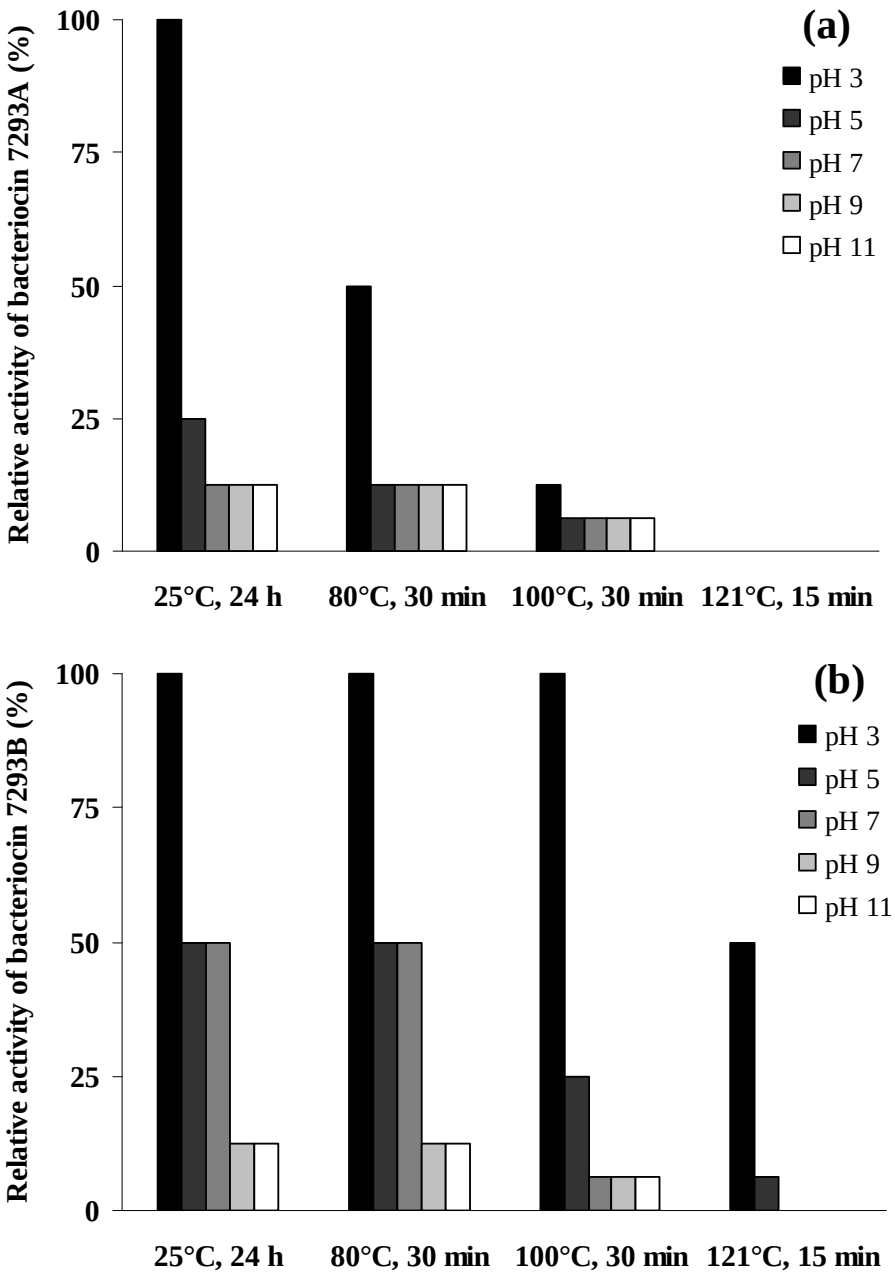

Figure 22. Effect of pHs and temperatures on the activities of purified bacteriocin 7293A (a) and 7293B (b). Purified bacteriocins adjusted to a pH range of 3.0-11.0 were incubated at 25 °C for 24 h, 80 °C for 30 min, 100°C for 30 min and 121 °C for 15 min. The activity of the samples at pH 3.0 without any treatment was considered to be 100%. Bars are means of duplicate determinations from two independent experiments.

As a common feature of bacteriocins, bacteriocin 7293A and B were stable in the wide range of pH and temperature (Papagianni, 2003; Papagianni and Papamichael, 2011). However, bacteriocin 7293A and B seemed to be more stable in low pH. In food application point of view, low-pH food was suitable for these bacteriocins. Since bacteriocin 7293B was more stable in high temperature than bacteriocin 7293A, it could be applied even in food packaging which high temperature is always used to produce antimicrobial packaging.

Further work is in progress to study the structure of bacteriocin 7293A and B. Until now, no signals were obtained when bacteriocin 7293A and B were subjected to N-terminal amino acid sequencing by automated Edman degradation. This suggested that the N terminus of bacteriocin 7293A and B was blocked. Various fragmentation procedures, such as BNPS-skatole, cyanogen bromide treatment, endoprotease V8 digestion and acid hydrolysis, were performed to obtain a partial amino acid sequence of these bacteriocins. However, any treatments could not provide appropriated fragments of bacteriocin 7293A and B (data not shown).

5.5 Conclusion

Weissella hellenica BCC 7293, isolated from Thai fermented pork sausage called Nham, produced two bacteriocins designated as bacteriocin 7293A and B. Their unique molecular masses and antimicrobial spectra against both Gram-positive and Gram-negative food-borne pathogens suggested that bacteriocin 7293A and B could be putatively novel bacteriocins. The sensitivity to proteolytic enzymes and stability in organic solvents, pH and temperature of both bacteriocins suggested the potential use of bacteriocin 7293A and B in food and food packaging applications.

CHAPTER 6

APPLICATION OF PUTATIVELY NOVEL BACTERIOCIN PRODUCED BY *WEISSELLA HELLENICA* BCC 7293 IN PLA/SP BIOCOMPOSITE FILM FOR FOOD PACKAGING

6.1 Abstract

The objective of this study was to develop an antimicrobial biodegradable food packaging by using Bacteriocin 7293 (Bac7293), a putatively novel antimicrobial peptide produced by *Weissella hellenica* BCC 7293. Bac7293 was loaded into poly(lactic acid)/sawdust particle biocomposite film (PLA/SP) using diffusion coating technique. The maximum amount of Bac7293 incorporated into the pre-heated PLA/SP was achieved at 19.54 ± 2.87 $\mu g/cm^2$ after 30 min of diffusion coating, whilst PLA film without sawdust particle could not absorb Bac7293. According to the JIS Z 2801:2000 standard testing method, the produced PLA/SP impregnated with Bac7293 (PLA/SP + Bac7293) effectively inhibited both Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa, Aeromonas hydrophila, Escherichia coli* and *Salmonella* Typhimurium). Antimicrobial activity of PLA/SP + Bac7293 film remained unchanged even after storage at 25 °C for 12 months. In a food model study, PLA/SP + Bac7293 film effectively inhibited the growths of *P. aeruginosa*, *A. hydrophila*, *L. monocytogenes*, *E. coli*, *S.* Typhimurium and *S. aureus* artificially inoculated on fresh Pangasius fillet during storage under refrigeration. A reduction of 2 to 5 log CFU/cm^2 of all tested bacteria on fish fillet was observed. The overall migration of PLA/SP + Bac7293 film to food simulants was maximized in isooctane (3.67 ± 0.47 mg/dm^2) which was much lower than the overall migration limit regulated by the commission of the European communities.

6.2 Introduction

The growth of global consumption of fish as an animal protein has doubled since the 1970s. In particular, the consumption of freshwater fish has grown massively in recent decades, primarily in East Asia (Delgado *et al*., 2003). Pangasius fish (*Pangasius hypophthalmus*) is one of the most famous freshwater fish consumed not only in Asia but also in the European Union (EU), USA and Canada (Tong Thi *et al*., 2013). Pangasius fish products in the forms of skinned and boneless fillets are the most popular forms being sold in supermarkets in over 80 countries (Orban *et al*., 2008; Tong Thi *et al*., 2013).

Despite the high economic importance of this fishery product, the food safety issue according to microbiological contamination still remains as evidence from the notifications and rejection of the contaminated products by EU (Little *et al*., 2012). The microbiological contamination of pangasius fish consists of a diverse mixture of Gram-positive and Gram-negative bacteria including *Listeria*, *Staphylococcus*, *Aeromonas*, *Escherichia*, *Pseudomonas* and *Salmonella* (ICMSF, 2005; Sarter *et al*., 2007; Little *et al*., 2012; Noseda *et al*., 2012; Tong Thi *et al*., 2013). The contaminations of *Listeria* and *Salmonella* in pangasius fish fillets were predominantly detected and alerted by EU Rapid Alert System for Food and Feeds (RASFF) during 2005 and 2010 (Little *et al*., 2012; Noseda *et al*., 2013).

Several techniques have been introduced to control the microbial contamination in fish fillet such as treating live fish with antibiotic (Sarter *et al*., 2007) and pesticides (Little *et al*., 2012) during aquaculture, washing fish fillets with chlorinated water (Tong Thi *et al*., 2013), treating fish fillets with tannic acid and modified atmosphere packaging (MAP) (Maqsood and Benjakul, 2010).

Due to the trend toward the natural products without antibiotics or chemical preservatives, bacteriocins, the antimicrobial peptides produced by lactic acid bacteria, have been used to control the growth of spoilage and pathogenic microorganisms in foods (Deegan *et al*., 2006; Zacharof and Lovitt, 2012). The desirable properties of bacteriocins that make them suitable for food preservation are (i) are generally recognized as safe (GRAS) substances, (ii) are inactive and nontoxic on eukaryotic cells and the consumers, (iii) become inactivated by digestive protease,

having little influence on the consumer's gut microbiota, (iv) usually active in wide range of pH and temperature and (v) have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria (Helander *et al*., 1997; Cleveland *et al*., 2001; Gálvez *et al*., 2007; Hwanhle *et al*., 2014). During the last decades, innovative bioactive films enriched with bacteriocins have been developed and received a consideration attention for control microbial contamination in foods (Coma *et al*., 2008; Jin *et al*., 2009; Cao-Hoang *et al*., 2010; Theinsathid *et al*., 2012). The important role of packaging system in this sense is maintaining the concentration of bacteriocins above the active concentration and continuously releasing bacteriocins to kill the target microorganisms contaminated on foods (Appendini and Hotchkiss, 2002).

Recently, Woraprayote *et al*. (2013) have successfully developed the technique to incorporate hydrophilic antimicrobial peptide, pediocin, into hydrophobic PLA film to provide high efficiency antimicrobial biodegradable packaging. The film played significant role to carry and release bacteriocin into food matrix to reduce the population of target microorganism up to 99% during chilled storage. However, the antimicrobial activity of this packaging was limited against only *Listeria monocytogenes*, thus it was not sufficient to control the microbial contamination in pangasius fish fillet which consists of both Gram-positive and Gram-negative bacteria. Our previous study demonstrated the wide spectrum of inhibition of Bacteriocin 7293, a new found antimicrobial peptide produced by *Weissella hellenica* BCC 7293, against all of the above mentioned bacteria contaminated in pangasius fish fillets. Therefore, the application of Bacteriocin 7293 with PLA/SP could be a promising means to control the growth of target microorganisms in pangasius fish fillets.

To our knowledge, no study has reported the use of antimicrobial biodegradable film containing bacteriocin to control the growth of pathogenic and spoilage microorganisms in pangasius fish fillets. Thus, the objective of present work was to develop an antimicrobial biodegradable packaging impregnated with Bacteriocin 7293 to control the growth of *Aeromonas*, *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Listeria* and *Salmonella* on pangasius fish fillets during refrigerated storage.

6.3 Materials and Methods

6.3.1 Bacterial strains and culture conditions

Weissella hellenica BCC 7293 was isolated from Nham, a traditional fermented pork sausage in Thailand. *W. hellenica* BCC 7293 was maintained as frozen stock held in -80 ºC in De Man, Rogosa and Sharpe (MRS) culture broth containing 15% (w/v) glycerol. Prior to use, culture was streaked on MRS agar and grown at 30 ºC for 18 h. A single colony obtained from the plate was grown twice in MRS broth and incubated at 30 ºC for 18 h without shaking. Bacterial strains that were used as indicators for the antimicrobial assay were propagated in culture broth for 18-24 h at the temperature (30 ºC or 37 ºC) recommended by culture collection.

6.3.2 Determination of antimicrobial activity

Antimicrobial activity assay was performed using the spot-on-lawn method as previously described by Zendo *et al*. (2005). The antimicrobial activity of bacteriocin preparation was expressed as activity unit (AU) per ml, in which the reciprocal of the highest two-fold serial dilution at which growth inhibition was still detectable.

6.3.3 Preparation of partially purified Bacteriocin 7293

W. hellenica BCC 7293 was grown in CYG broth at 30 ºC for 18 h. Antimicrobial substance was recovered from cell surface by adjusting the pH of culture broth to 2.0. Cells were removed by centrifugation at 10,000 x g for 15 min at 4 ºC using a Hi-speed centrifuge (Beckman: Avanti J-E). The cell-free supernatant was partially purified by hydrophobic interaction chromatography using amberlite XAD-16 polymeric resin (Sigma, USA). In brief, a 25 g of the resin was activated in 50% (v/v) isopropanol at 4 °C for 24 h. Thereafter, isopropanol was completely removed from the resin by washing with one volume of deionized water. The activated resin was added into a 1,000 ml of the culture supernatant with gently mixed and kept at 4 °C for 24 h. The resin was loaded into the Econo fast flow column 2.5 × 30 cm (Bio-rad, USA) and washed with 100 ml of deionized water, followed by 100

ml of 50% (v/v) ethanol in deionized water. The active fraction was eluted with 100 ml of 70% (v/v) isopropanol in deionized water containing 0.1% (v/v) trifluoro acetic acid (TFA). The eluent was then evaporated to get rid of isopropanol using rotary evaporator (40 °C for 30 min) and then freeze-dried at -60 ºC, 0.1 mBar and kept at 4 ºC prior to use. This preparation was designated as Bac7293.

6.3.4 Elaboration of antimicrobial PLA/SP film coated with Bac7293 (PLA/SP + Bac7293)

PLA/SP biocomposite film was fabricated using a blown film extrusion as described by Woraprayote *et al*. (2013). Firstly, 5% (w/w) sawdust particles (SP) in the range of 100 – 300 µm was mixed with PLA resin (Nature work®) and subjected to a twin screw extruder (SHJ-36 twin screw extruder product line, Nanjing Chengke Machinery, China). Then, the composite film was fabricated via blown film technique. The thickness of the composite film was adjusted to 400-500 micrometer by controlling the take up speed.

Incorporation of Bac7293 into PLA/SP biocomposite film was performed by diffusion coating method according to Woraprayote *et al*. (2013). Briefly, the film was pre-heated at 90 °C for 2 h in air-circulating oven. The pre-heated film was removed from the oven and immediately placed into 0.2% (w/v) Bac7293 solution. The film surface area to Bac7293 solution ratio was 8 cm^2: 5 ml. The coating was performed in static condition for 30 min at 25 °C. Then, the films were removed from the Bac7293 solution and washed three times with 5 ml of deionized water by shaking in the solution for 1 min for each time in order to eliminate the un-adsorbed Bac7293. The washed films were dried under laminar flow for 60 min and stored at 4 ± 2 °C in a refrigerator prior to bacterial inhibition tests.

To quantitatively determine the Bac7293 adsorption on composite film during diffusion coating, the amount of Bac7293 retained in the solution was analyzed as the total protein content according to the method of Lowry *et al*. (1951). Bac7293 adsorption on the film was calculated by the following equation:

$$\text{Bac7293 adsorbed } (\mu g/cm^2) = (P1 - P2)/A$$

where *P1* and *P2* represent the total protein (µg) of pediocin solution before and after adsorption, respectively and *A* represents the total surface area (cm^2) of the films used in the experiment. Absorption of Bac7293 onto PLA/SP film (µg/cm^2) was plotted as a function of soaking time (min) (Figure 23).

6.3.5 Evaluation of antimicrobial efficiency of PLA/SP + Bac7293

The quantitative evaluation of the antimicrobial activity of PLA/SP + Bac7293 was evaluated as their efficacy after 24 h under JIS Z 2801:2000 testing conditions (Anonymous, 2000). A PLA/SP film without Bac7293 coating was used as the negative control. Approximately 10^5 to 10^6 cells of either *Aeromonas hydrophila* B1, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19115, *Pediococcus pentosaceus* JCM 5885, *Pseudomonas aeroginosa* ATCC 27853, *Salmonella* Typhimurium DMST 0562 or *Staphylococcus aureus* ATCC 25923 was inoculated onto each respective test PLA/SP or PLA/SP + Bac7293 film. The inoculated specimens were incubated at 37 °C at a relatively high humidity (approximately 90% RH) for 24 h. The bacterial cells were harvested from the film surface using 10 ml of SCDLP (soybean-casein digest broth with lecithin and polysorbate) broth and serial dilutions plated onto TSB agar plate for enumeration of the viable cells of indicator strains after culturing at 37 °C for 24 h. The value of antimicrobial activity was expressed as the difference in the logarithmic value of viable cell counts between antimicrobial products and untreated products (negative control) after incubation of bacteria.

6.3.6 Stability of antimicrobial activity of PLA/SP + Bac7293

The PLA/SP + Bac7293 films were sterilized under UV light for 15 min each side and incubated at 4 °C and 25 °C. After 3, 6, 9 and 12 months of incubation, the tested films were removed and the antimicrobial activity was tested according to JIS Z 2801:2000 standard testing method using *W. paramesenteroides* JCM 9890^T as the indicator strain. The residual antimicrobial activity of the film samples was reported as a percentage of antimicrobial activity of the samples after storage compared with that of time zero.

6.3.7 Challenge test of inhibition of pathogenic microorganisms in chilled pangasius fillet

The antimicrobial effectiveness of the PLA/SP + Bac7293 films was evaluated using raw pangasius fish fillet. Sections (5 × 5 cm^2) of pangasius fillet were UV-sterilized under a biosafety hood for 15 min. Sterilized fish fillet sections were then inoculated with 250 µl of the cocktail inoculum of *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19115, *Salmonella* Typhimurium DMST 0562, *Staphylococcus aureus* ATCC 25923, *Aeromonas hydrophila* B1 and *Pseudomonas aeruginosa* ATCC 27853 to obtained the final bacterial density of each indicator on fish surface of 10^5 CFU/cm^2. The inoculated fish samples were then packed in direct contact with the antimicrobial PLA/SP + Bac7293, placed on the tray and stored at 4 ± 2 °C. Samples packaged with uncoated film and unpackaged were used as negative controls. After 0, 1, 3, 5 and 7 days of chilled storage, selective viable counts of indicator strains were performed. For the analysis, the samples were placed in sterilized plastic bags, 10 ml of sterile 0.1% (w/v) peptone solution were added, and then the samples were homogenized for 1 min in Stomacher Lab Blender (Seward; UK). Determination of *A. hydrophila* and *P. aeruginosa* count was carried out on GSP agar (Pseudomonas Aeromonas Selective Agar Base, Merck, Germany) after incubation at 37 °C for 18-24 h. *A. hydrophila* and *P. aerugiosa* was recognized as yellow and red-violet colony on this culture medium, respectively. *L. monocytogenes* was enumerated on PALCAM agar plates supplemented with PALCAM antimicrobic supplement (BD Difco™, Becton, Dickinson and Company, NJ, USA) after incubation at 37 °C for 24-48 h. On PALCAM agar, the grey-green colony with black precipitate was recognized as *L. monocytogenes*. *E. coli* was enumerated on Eosin Methylene Blue Agar Modified (Holt-Harris & Teague) (EMB, BD, BBL™, Becton, Dickinson and Company, NJ, USA). *E. coli* was recognized as blue-black colony with green metallic sheen on this medium after incubation at 37 °C for 18-24 h. *S.* Typhimurium was enumerated as red colony with black centers on XLD agar (BD, Difco™, Becton, Dickinson and Company, NJ, USA) after incubation at 37 °C for 18-24 h. Staphylococci count was determined using Baird-Parker agar supplemented with Egg-yolk Tellurite Emulsion 20% (Merck, Darmstadt, Germany) as a medium after

incubation at 30 °C for 24-48 h. Staphylococci was recognized as black colony surrounded by clear zone on this medium. The experiments were performed in triplicate and the results were expressed as CFU/cm^2.

6.3.8 Migration of PLA/SP + Bac7293 into packaged fish fillet

The overall migration as the total mass of compounds transferred from the packaging material to the food simulants was evaluated according to Directive 97/48/EC (The Commission of the European Communities, 1997). In this study, 4 simulants were used including water, 3% acetic acid (w/v) in aqueous solution as a stimulant of aqueous food with pH of lower than 4.5, 10% ethanol (v/v) in aqueous solution as a stimulant of aqueous foodstuff with pH of higher than 4.5 and isooctane as a fatty food stimulant.

Total immersion migration tests were performed as described by Tovar *et al*. (2005). A 12 cm^2 piece of PLA/SP + Bac7293 was placed in glass tube containing 20 ml of the simulant (area-to-volume ratio 6 dm^2/L). The migration tested tubes were then incubated at 40 °C for 10 days in an incubator. After exposure, film samples were removed and the simulants were evaporated by rotary evaporation. The solid residue remained in the tube was then gravimetrically determined. Six independent replicates were analyzed for each stimulant. The overall migration value was expressed as mg of the migrated film materials/dm^2 of food contact surface.

6.3.9 Statistical analysis

Antimicrobial experiments were conducted in triplicate, with two observations per film treatment for each replicate. The overall migration testing was performed for six replications. Data value was expressed as the mean ± SD. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 17.0 software. Duncan’s Multiple Range Test (DMRT) was used for comparison of mean values at a significant level of 0.05.

6.4 Results and Discussion

6.4.1 Antimicrobial activity of Bac7293

After 18 h of incubation in CYG medium at 30 °C, the culture supernatant was subjected to partial purification with hydrophobic interaction chromatography of Amberlite XAD-16. The obtained partially purified bacteriocin 7293 was designated as Bac7293. Bac7293 was then freeze-dried to produce Bac7293 powder. Based on this production procedure, 2.81 g of Bac7293 powder was obtained from 1 l of culture supernatant. The antimicrobial activity (AU/mg) of Bac7293 powder against the target microorganisms was shown in Table 19. Bac7293 exhibited antimicrobial activity not only against Gram-positive bacteria but also against Gram-negative bacteria. To our knowledge, only few studies have discovered bacteriocins from lactic acid bacteria of food origin with antimicrobial activity against Gram-negative bacteria (Gong *et al*., 1210; Ravi *et al*., 2011; Marie *et al*., 2012; Chen *et al*., 2014). In addition, Bac7293 was more effective against Gram-positive than Gram-negative indicators except *P. aeruginosa*. This result was in agreement with general characteristic of bacteriocins of Gram-positive bacteria which exhibit high antimicrobial activity against Gram-positive bacteria or closely related bacteria (Klaenhammer, 1993; Jack *et al*., 1995). In addition, many bacteriocins produced by Gram-positive bacteria can kill Gram-negative species that are likely to have the same ecological niche (Jack *et al*., 1995).

6.4.2 Protein adsorbed and antimicrobial efficiency of PLA/SP + Bac7293

The adsorption of Bac7293 was monitored by the total protein adsorbed onto PLA and PLA/SP films throughout the diffusion coating. After coating, PLA/SP film adsorbed high amount of Bac7293, while no protein was adsorbed on PLA film. The result indicated that the sawdust particle and coating technique used in this study effectively embedded Bac7293 into PLA film which was in agreement with the result of our previous study (Woraprayote *et al*., 2013).

Table 19. Antimicrobial activity of Bac7293

Indicator strains	Antimicrobial activity (AU/mg)
Gram-positive bacteria	
Listeria monocytogenes ATCC 19115	4326
Staphylococcus aureus ATCC 25923	1082
Gram-negative bacteria	
Aeromonas hydrophila B1	270
Escherichia coli ATCC 25922	68
Pseudomonas aeruginosa ATCC 27853	1082
Salmonella Typhimurium DMST 0562	68

Figure 23 shows that the maximum amount of Bac7293 incorporated into the PLA/SP film was achieved at 19.54 ± 2.87 µg/cm^2 after 30 min of diffusion coating. The adsorption capacity of this system was higher than those of other reports. Nakanishi *et al*. (2001) reviewed that the amount of protein adsorbed is on the order of several mg/m^2 or ng/cm^2, varying with the kind of protein, type of surface and adsorption conditions. Lactocin 705 maximally adsorbed on multilayer-LLDPE film at 0.07 ± 0.02 µg/cm^2 (Massani *et al.*, 2013). The amount of nisin adsorbed on hydrophilic and hydrophobic PE film was recorded at 4.83 ± 0.3 µg/cm^2 and 1.8 ± 0.1 µg/cm^2, respectively (Karam *et al*., 2013), whilst pediocin PA-1/AcH could be adsorbed by PLA/SP film with maximum amount of 11.63 ± 3.07 µg protein/cm^2 (Woraprayote *et al*., 2013).

The adsorption kinetic of Bac7293 to the PLA/SP at 25 °C was shown in Figure 23. Bac7293 adsorption exhibited a steep initial slope (in the first 10 min of diffusion coating) followed by the attainment of a plateau after 30 min of diffusion suggested that a time of 30 min was needed to reach equilibrium. Therefore, the time of 30 min was selected as a time for Bac7293 coating on PLA/SP film at 25 °C. This result was similar to Woraprayote *et al*. (2013), suggesting that the affinity of Bac7293 and pediocin for PLA/SP was similar. The longer times (on the order of hour) were necessary to adsorb other bacteriocins to other hydrophobic and hydrophilic surfaces (Guerra *et al*., 2005; Ibarguren *et al*., 2010; Massani *et al*., 2013).

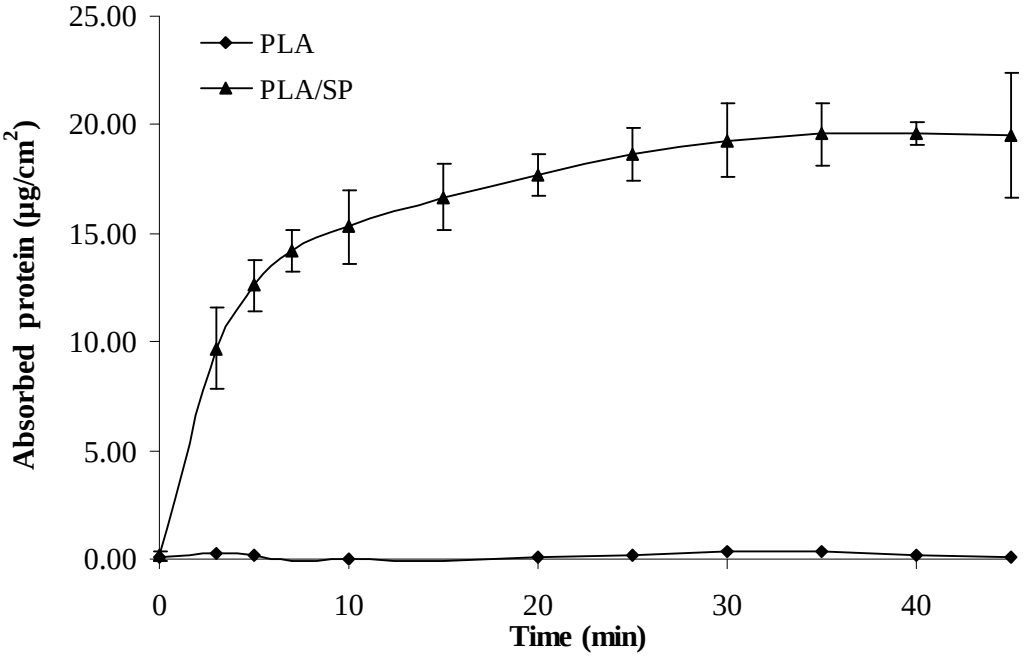

Figure 23. Absorption kinetics of Bac7293 in PLA film. Bars represent the standard deviation of duplicate determinations from three independent experiments.

To ensure that the adsorbed Bac7293 could desorb from the film to exhibit antimicrobial activity against the sensitive microorganisms, the determination of the antimicrobial activity of PLA/SP + Bac7293 was performed by determining the reduction in the number of viable bacteria (as log CFU/cm^2) over the exposure time according to the JIS Z 2801:2000 testing method. In this study, after 24 h of exposure to the film, the viable counts of each indicator strains were in the range of 3.03 to 4.78 log CFU/cm^2 reduction compared to the control PLA/SP film (PLA/SP film without Bac7293) (Table 20). The value of antimicrobial activity obtained by this standard testing method shall not be less than 2.0 for the antimicrobial efficacy of antimicrobial products (Anonymous, 2000). Based on this standard value, PLA/SP + Bac7293 could be designated as high efficiency antimicrobial product. The result are similar to that for PLA/SP + Ped reported by Woraprayote *et al*. (2013), where the 3 log reduction of *L. monocytogenes* population in model food system was observed after contacted with the film. PLA/SP + Bac7293 was more active against Gram-positive indicators

Table 20. Antimicrobial efficiency of PLA/SP + Bac7293

Indicator strains	Antimicrobial activity (log reduction)
Gram-positive bacteria	
Listeria monocytogenes ATCC 19115	4.78
Staphylococcus aureus ATCC 25923	4.56
Gram-negative bacteria	
Aeromonas hydrophila B1	3.12
Escherichia coli ATCC 25922	3.03
Pseudomonas aeruginosa ATCC 27853	4.10
Salmonella Typhimurium DMST 0562	3.03

than Gram-negative indicators which was in agreement with the antimicrobial property of Bac7293 powder.

6.4.3 Stability of antimicrobial activity of PLA/SP + Bac7293

The stability of antimicrobial activity of Bac7293 powder and Bac7293 adsorbed on PLA/SP in real storage conditions (refrigerated temperature at 4 °C and room temperature at 25 °C) was investigated. Antimicrobial activity of Bac7293 powder decreased with an increasing time and completely lost after 12 month of storage at 25 °C whereas no changes in its activity were observed at 4 °C (Table 21). The result indicated that absorption of Bac7293 onto PLA/SP improved the stability of Bac7293, thus protecting its antimicrobial activity. This result was similar to those of pectin-Nisaphlin® and pectin-nisin complexes which the complexes played an important role to improve the thermal stability of bacteriocin (Liu *et al*., 2009). Other previous studies also showed that bacteriocins retain their activity when applied to various surfaces (Daeschel and McGuire, 1992; Ming *et al*., 1997; Scannell *et al*., 2000). However, a detailed mechanism remains to be investigated.

The decrease in antimicrobial activity of Bac7293 in partially purified Bac7293 powder may be associated to the interaction with proteolytic enzymes, proteins, fat and sugar remaining in the powder. Whilst PLA/SP film selectively

Table 21. Stability of antimicrobial activity of partially purified Bac7293 and PLA/SP + Bac7293

Samples		Residual activity (%)
Bac7293 powder	4 °C, 3 months	100
	4 °C, 6 months	100
	4 °C, 12 months	100
	25 °C, 3 months	50
	25 °C, 6 months	25
	25 °C, 12 months	0
PLA/SP + Bac7293	4 °C, 3 months	100
	4 °C, 6 months	100
	4 °C, 12 months	100
	25 °C, 3 months	100
	25 °C, 6 months	100
	25 °C, 12 months	100

adsorbed Bac7293, only small amount of contaminants allowed to react with Bac7392 was not enough to cause the reduction of antimicrobial activity.

6.4.4 Challenge test of microbial inhibition in chilled pangasius fillet

The antimicrobial effectiveness of PLA/SP + Bac7293 against targets microorganisms artificially inoculated on pangasius fish fillets is shown in Figure 24. PLA/SP + Bac7293 film effectively inhibited the growth of all tested microorganisms including *L. monocytogenes, S. aureus, A. hydrophila, P. aeruginosa, E. coli* and S. Typhimurium. A reduction of 4 to 6 log cycles of all tested bacteria on fish fillet was observed.

Packaging with PLA/SP + Bac7293 reduced the *Listeria* population in pangasius fish fillets when compared with the control and PLA/SP packaged samples throughout the storage time (Figure 24a). The 2-log reduction of *Listeria* count was observed in PLA/SP + Bac7293 sample after 5 days of chilled storage.

The growth of *Staphylococcus* in control unpackaged fish fillets was slightly increased from 5.2 log CFU/cm^2 to 7.4 log CFU/cm^2 during chilled storage.

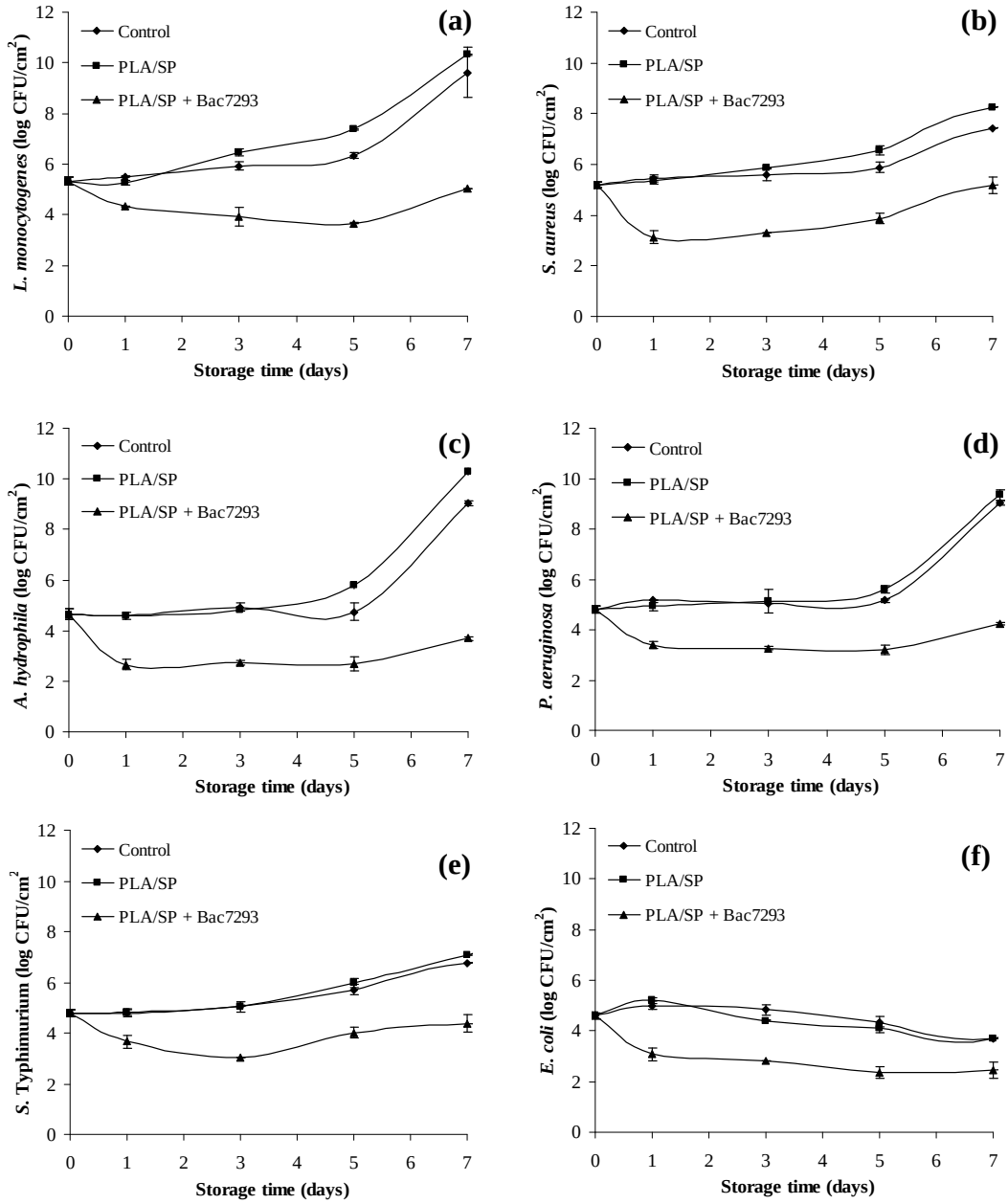

Figure 24. Challenge test of inhibition of pathogenic microorganisms in chilled pangasius fillet. Bars represent the standard deviation of duplicate determinations from three independent experiments.

Packaging with PLA/SP + Bac7293 showed the reduction of *Staphylococcus* by about 2 to 3 log CFU/cm^2, compared with the control (Figure 24b).

The growth of *A. hydrophila* and *P. aeruginosa*, a Gram-negative pathogenic and spoilage bacteria always found in fresh water fish including pangasius fish (Hänninen *et al*., 1997; Bhowmik *et al*., 2009), were also inhibited by PLA/SP + Bac7293 throughout the storage period. PLA/SP + Bac7293 reduced 2.0 and 1.4 log cycle of *A. hydrophila* and *P. aeruginosa*, respectively, after 1 day of chilled storage (Figure 24c and 24d).

The viable count of *S.* Typhimurium was also inhibited by PLA/SP + Bac7293 (Figure 24e). In control and PLA/SP packaged samples, *S.* Typhimurium counts slightly increase initially from 4.7 to 7.0 log CFU/cm^2 throughout the chilled-storage period. The growth reduction of up to 2.7 log CFU/cm^2 was observed in PLA/SP + Bac7293 packaged sample compared with the others.

During the first three days of chilled storage, no change in the *E. coli* counts were observed in control and PLA/SP packaged samples (~4.5 log CFU/cm^2) and the counts naturally decreased at the end of storage as shown in Figure 24f. The counts of *E. coli* were significantly reduced (~2 log CFU/cm^2) by packaging with PLA/SP + Bac7293 at first day of storage.

These results reconfirmed the antimicrobial efficacy of PLA/SP + Bac7293 against indicator strains as previously observed in *vitro* and demonstrated the potential used of PLA/SP + Bac7293 to control the growth of *L. monocytogenes, S. aureus, A. hydrophila, P. aeruginosa, E. coli* and S. Typhimurium on pangasius fish fillet in retail chilled storage condition.

Other studies reported a similar reduction behavior in the populations of the target microorganism when food preparations were packaged with the high efficiency antimicrobial food packaging containing bacteriocins. Generally, the high efficiency antimicrobial packaging could reduce the growth of target bacteria by at least 2 log cycles within 1 to 3 days of contacting (Ming *et al*., 1997; Scannell *et al*., 2000; Dawson *et al*., 2002; Lee *et al*., 2003; Mauriello *et al*., 2004; Guerra *et al*., 2005; Woraprayote *et al*., 2013).

It was noted that the antimicrobial activity of PLA/SP + Bac7293 was decreased when applied to pangasius fish fillets (Figure 24). The alteration of bacteriocin bioavailability in food systems may be caused by the portioning of

bacteriocins into polar or non-polar food components and the interaction with food components such as phospholipids, glutathione S-transferase and other endogenous food enzymes, (Cleveland *et al*., 2001; Chollet *et al*., 2008; Sant'Anna *et al*., 2011; Malheiros *et al*., 2012).

6.4.5 Migration of PLA/SP + Bac7293

In order to quantify the total release of possible components of PLA/SP and PLA/SP + Bac7293 film to food, the overall migration of packaging materials was evaluated according to the Directive 97/48/EC. Table 22 depicts the overall migration value of PLA/SP + Bac7293 into all food simulants was higher than those of PLA/SP ($p < 0.05$). The additional mass presented in the migrant of PLA/SP + Bac7293 might be the contribution of Bac7293 migrated from PLA/SP film during tested. This assumption was supported by the presence of protein content in water, one of tested food simulant, after film exposure (data not shown).

Among four food simulants, the maximum migration of PLA/SP + Bac7293 film (3.67 ± 0.47 mg/dm^2) was observed after exposure in isooctane, a fatty food simulant. This result may be explained by the fact that isooctane could absorbed in the film structure, leading to the swelling of the polymer matrix, thereby enlarging the gaps between the molecules and enhancing the migration of small particles including PLA degradation products (Fortunati *et al*., 2012) and bacteriocin. However, this value was far below the overall migration limit (OML: 10 mg/dm^2) regulated by European directive 2002/72/EC. None of the studied PLA films exceeded the overall migration limit of 60 mg/kg or 10 mg/dm^2 (Fortunati *et al*., 2012; Mattioli *et al*., 2013). This result suggested that PLA/SP + Bac7293 is in compliance with the overall migration limit and could be used safely as a food contact materials for low acid, high acid and fatty foods.

Table 22. Overall migration of PLA/SP and PLA/SP + Bac7293 films into food simulants

Simulant	Overall migration (mg/dm^2)	
	PLA/SP	PLA/SP + Bac7293
Water	1.18 ± 0.68 a	3.00 ± 0.48 b
3% acetic acid	1.56 ± 1.34 a	3.44 ± 0.38 b
10% ethanol	1.33 ± 0.47 a	3.17 ± 0.24 b
Isooctane	1.52 ± 0.20 a	3.67 ± 0.47 b

Data were expressed as mean ± SD of six independent experiments.

Different lower case letters in the same row indicate significant difference ($p < 0.05$).

6.5 Conclusion

Bacteriocin 7293 could also be impregnated into PLA/SP film to produced high antimicrobial efficiency packaging, similar to pediocin. PLA/SP played the role to maintain the antimicrobial activity of Bac7293 for at least 12 months at 25 °C. The produced PLA/SP + Bac7293 effectively inhibited the growth of both Gram-positive and Gram-negative bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Escherichia coli* and *Salmonella* Typhimurium) both in *vitro* and food models during chilled storage. The overall migration of PLA/SP + Bac7293 was much lower than the overall migration limit regulated by the commission of the European communities. This suggested high potential application of PLA/SP + Bac7293 as a good antimicrobial packaging for pangasius fish fillets.

CHAPTER 7

SUMMARY AND FUTURE WORKS

7.1 Summary

The use of bioactive films enriched with antimicrobial peptides from lactic acid bacteria as an antimicrobial food packaging have been received a consideration attention. Biodegradable materials, especially poly(lactic acid) (PLA), have been more emphasized due to the trend toward active and green packaging. However, direct incorporation of antimicrobial peptide to PLA film was limited by the hydrophobic characteristics of PLA. In this study, the emerging technology to incorporate antimicrobial peptide from lactic acid bacteria to PLA film has successfully been developed. Pediocin PA-1/AcH from *Pediococcus pentosaceus* BCC 3772 was impregnated into poly(lactic acid)/sawdust particle biocomposite film (PLA/SP) using diffusion coating technique to produce food packaging with high antimicrobial efficiency. The optimization of pediocin production was performed in order to improve the productivity and reduce the browning index of pediocin preparation, thus providing the desirable quantity and quality of pediocin preparation for use in food packaging. The challenge test of inhibition and effect of PLA/SP + Ped on microbiological property of packaged pork indicated that the antimicrobial activity of the film was limited only on *Listeria monocytogenes*. In order to broaden the antimicrobial spectrum of the packaging, putatively novel antimicrobial peptide, Bacteriocin 7293 from *Weissella hellenica* BCC 7293, which active against both Gram-positive and Gram-negative food-borne pathogenic and spoilage bacteria was discovered and introduced to PLA/SP film. The PLA/SP film with Bacteriocin 7293 effectively inhibited several important bacteria reported to contaminate on Pangasius fish fillet. This validated the incorporating technique and suggested the feasibility of the use of PLA/SP enriched with antimicrobial peptide from lactic acid bacteria as a high efficiency antimicrobial biodegradable packaging for a variety of foods. The following are the detailed summary.

1. Culture medium and incubation condition for the production of pediocin PA-1/AcH by *Pediococcus pentosaceus* BCC 3772 were optimized.

According to the analysis of Plackett-Burman and central composite design (CCD) under response surface methodology (RSM), the optimal medium for pediocin production contained 1.5% (w/v) of sodium caseinate, 1.5% (w/v) of yeast extract and 0.5% (w/v) of glucose. The production of pediocin in the optimal medium with optimal pH of 6.5 and optimal incubation temperature of 30 °C not only improved pediocin productivity but also the color of pediocin preparation, thus increasing the possibility of its use in food packaging.

2. A method for incorporating hydrophilic antimicrobial peptide into hydrophobic film was developed using pediocin PA-1/AcH and poly(lactic acid) (PLA) as a model. Natural sawdust particle (SP) played an important role in embedding pediocin into the PLA film. Pre-conditioning of the poly(lactic acid)/sawdust particle (PLA/SP) biocomposite film by dry-heat treatment not only improved anti-listeria activity but also increased tensile strength with no effect on film color, transparency and solubility in water. PLA/SP biocomposite film coated with pediocin can be used as a good anti-listeria biodegradable packaging for pork and other high-moisture foods.

3. Poly(lactic acid)/sawdust particle biocomposite film incorporated with pediocin (PLA/SP + Ped) significantly affected the quality and shelf life of fresh pork meat stored at 4 ± 2 °C. Based on sensory characteristic and biogenic amine index of packaged pork, PLA/SP + Ped film could the extension the shelf life of packaged pork by maintaining consumer acceptance of the products up to 7 days during storage in retail chilled condition, which is at least 3 days longer than those observed for the control unpackaged and packaged with PLA/SP film. However, from the microbiological property point of view, the antimicrobial activity of PLA/SP + Ped was limited only on *Listeria*, an important food borne pathogen always founded in pork meat and product.

4. Two putatively novel antimicrobial peptides were discovered. *Weissella hellenica* BCC 7293, isolated from Thai fermented pork sausage called Nham, produced two bacteriocins designated as bacteriocin 7293A and B. Their unique molecular masses and antimicrobial spectra against both Gram-positive and Gram-negative food-born pathogens suggested that bacteriocin 7293A and B could be novel bacteriocins. The sensitivity to proteolytic enzymes and stability in organic

solvents, pH and temperature of both bacteriocins suggested the potential use of bacteriocin 7293A and B in food and food packaging applications.

5. Bacteriocin 7293 could be impregnated into PLA/SP film to produce antimicrobial packaging by the technique developed in this study. PLA/SP played the role to maintain the antimicrobial activity of Bac7293 for at least 12 months at 25 °C. The produced PLA/SP + Bac7293 effectively inhibited the growth of both Gram-positive and Gram-negative bacteria (*Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Escherichia coli* and *Salmonella* Typhimurium) both in *vitro* and in food model, pangasius fish fillets, during chilled storage. The overall migration of PLA/SP + Bac7293 was much lower than the overall migration limit regulated by the commission of the European communities. This suggested high potential application of PLA/SP + Bac7293 as a good antimicrobial packaging for pangasius fillets.

7.2 Future works

1. The elongation at break and optical property of PLA/SP + Ped film should be improved to produce more flexible film with transparent property.

2. The complete amino acid sequences and structures of bacteriocin 7293A and B should be further studied in order to better understand the physicochemical properties of these two bacteriocins.

3. To ensure the use of PLA/SP + Bac7293 as a packaging for pangasius fish fillets, the effect of PLA/SP + Bac7293 on quality and shelf life of packaged fish fillets should be investigated.

4. The combination of several antimicrobial peptides should be applied to PLA/SP film to produce the antimicrobial film with broader antimicrobial spectrum.

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