การผลิตแบคเทอริโอซินจาก *Lactococcus lactis* TISTR 1401 และการประยุกต์ใช้ในผลิตภัณฑ์ลูกชิ้นหมู

นายสาโรช โกษารักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2549

PRODUCTION OF BACTERIOCINS

BY *LACTOCOCCUS LACTIS* TISTR 1401 AND

APPLICATION IN PORK MEATBALLS

Saroj Gosaarak

A thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Food Technology

Suranaree University of Technology

Academic Year 2006

PRODUCTION OF BACTERIOCINS BY

***Lactococcus lactis* TISTR 1401 AND**

APPLICATION IN PORK MEATBALLS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

_____

(Asst. Prof. Dr. Piyawan Gasaluck)

Chairperson

_____

(Assoc. Prof. Dr. Kanok-Orn Intrapichet)

Member (Thesis Advisor)

_____

(Asst. Prof. Dr.Suphitchaya Chanthachum)

Member

_____ _____

(Assoc. Prof. Dr. Saowanee Rattanaphani) (Asst. Prof. Dr. Suwayd Ningsanond)

Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

สาโรช โกษารักษ์ : การผลิตแบคเทอริโอซินจาก *Lactococcus lactis* TISTR 1401 และการประยุกต์ใช้ในผลิตภัณฑ์ลูกชิ้นหมู (PRODUCTION BACTERIOCINS BY *LACTOCOCCUS LACTIS* TISTR 1401 AND APPLICATION IN PORK MEATBALLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กนกอร อินทราพิเชฐ, 80 หน้า

จุดประสงค์การทดลองเพื่อคัดเลือกเชื้อแบคทีเรียแลคติกที่สามารถผลิตสารแบคทีริโอซินและเพื่อประยุกต์ใช้สารแบคทีริโอซินที่ยังไม่ผ่านการทำให้บริสุทธิ์ที่ผลิตจากเชื้อแบคทีเรียที่คัดเลือกได้เพื่อยืดอายุการเก็บผลิตภัณฑ์ลูกชิ้นหมู ทำการคัดเลือกแบคทีเรียแลคติกจำนวน 8 สายพันธุ์ ที่ได้จากศูนย์จุลินทรีย์ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ประกอบด้วยสายพันธุ์ *Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteroides* TISTR 053, *Lb. acidophilus* TISTR 450, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* supsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 และ *Lactococcus lactis* TISTR 1401 และจากมหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จำนวน 1 สายพันธุ์ คือ *Lb. casei* spp. *rhamnosus* SN11 เพื่อทดสอบความสามารถในการผลิตแบคเทอริโอซิน พบว่า *Lc. lactis* TISTR 1401 มีค่ากิจกรรมการยับยั้งต่อแบคทีเรียทดสอบ (*Bacillus* sp., TISTR 908, *B. cereus* TISTR 687, *B. subtilis* TISTR 008 และ *Staphylococcus aureus* TISTR 118) สูงที่สุดเมื่อเทียบกับแบคเทอริโอซินที่ผลิตได้จากแบคทีเรียแลคติกสายพันธุ์อื่นๆที่ทำการศึกษา แบคเทอริโอซินที่ผลิตจาก *Lc. lactis* TISTR 1401 ในอาหารเลี้ยง MRS broth มีค่าความสามารถในการยับยั้งเชื้อจุลินทรีย์ทดสอบ *Bacillus* sp. TISTR 908 เท่ากับ 3,200 AU/ml นอกจากนั้นการปรับปรุงการผลิตแบคเทอริโอซินจาก *Lactococcus lactis* TISTR 1401 โดยควบคุมค่าความเป็นกรด-ด่าง ของอาหารเลี้ยงเชื้อเหลว MRS ให้คงที่ที่ 6.5 สามารถเพิ่มจำนวนเซลล์ของแบคทีเรียและค่ากิจกรรมการยับยั้งของแบคเทอโอซินได้สูงสุด ที่ 12,800 AU/ml ที่ระยะเวลาการเจริญที่ 8 ชั่วโมง เมื่อเปรียบเทียบกับการผลิตโดยไม่มีการควบคุมค่าความเป็นกรด-ด่าง ซึ่งมีค่ากิจกรรมการยับยั้งเท่ากับ 3,200 AU/ml ที่ระยะเวลาการเจริญที่ 10 ชั่วโมง แบคเทอริโอซินที่ผลิตได้มีเสถียรที่ระดับความเป็นกรด-ด่าง ที่ 2 ถึง 8 และสามารถทนความร้อนได้ถึง 80 องศาเซลเซียส เป็นเวลา 20 นาที โดยไม่สูญเสียค่ากิจกรรมการยับยั้ง อย่างไรก็ตามกิจกรรมการยับยั้งของแบคเทอริโอซินจะสูญเสียทั้งหมดด้วยเอนไซม์โปรติเนส

การประยุกต์ใช้สารละลายแบคเทอริโอซินไม่บริสุทธิ์ที่ผลิตโดยการควบคุมค่าความเป็นกรด-ด่างที่ 6.5 เพื่อการยืดอายุการเก็บของผลิตภัณฑ์ลูกชิ้นหมูโดยการจุ่มเคลือบ แล้วบรรจุในถุงพลาสติกผนึกแบบปกติ และแบบสุญญากาศ เก็บในอุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 12 วัน

จากผลการวิเคราะห์ปริมาณจุลินทรีย์ในตัวอย่างลูกชิ้นหมูระหว่างการเก็บ พบว่า การจุ่มเคลือบลูกชิ้นหมูลงในสารละลายแบคเทอริโอซินความเข้มข้นเต็มที่ สามารถลดปริมาณจุลินทรีย์ทั้งหมดในตัวอย่างลูกชิ้นได้ 1.96 log cfu/g สำหรับการบรรจุแบบปกติ และ 1.42 log cfu/g สำหรับการบรรจุแบบสุญญากาศ เมื่อเทียบกับตัวอย่างที่ไม่ได้เคลือบด้วยแบคเทอริโอซิน และตัวอย่างที่จุ่มเคลือบด้วยสารละลายแบคเทอริโอซินที่เจือจางเข้มข้นลงครึ่งหนึ่ง พบว่าจำนวนจุลินทรีย์ทั้งหมดในตัวอย่างลูกชิ้นจุ่มเคลือบด้วยสารละลายแบคทีริโอซินที่เจือจางลงครึ่งหนึ่งไม่แตกต่างกับตัวอย่างควบคุมอย่างชัดเจน ($P > 0.05$) ตรวจพบเชื้อแบคทีเรียแลคติกในตัวอย่างได้เฉพาะในวันที่ 12 ทั้งในตัวอย่างควบคุมและตัวอย่างที่มีการเคลือบด้วยสารละลายแบคเทอริโอซิน จุลินทรีย์กลุ่ม *Enterobacteriaceae* และ *Pseudomonas* sp. ในตัวอย่างลูกชิ้นหมูที่บรรจุแบบสุญญากาศมีจำนวนลดลงเล็กน้อยเมื่อมีการเคลือบด้วยสารละลายแบคเทอริโอซิน อย่างไรก็ตามพบว่า สำหรับตัวอย่างบรรจุแบบปกตินั้นไม่พบการลดลงของแบคทีเรียทั้งสองชนิดนี้ นอกจากนี้ยังไม่พบการเจริญของเชื้อ *Brochothrix thermosphacta* ตลอดระยะเวลาการเก็บ

คุณภาพทางเคมี กายภาพ และทางประสาทสัมผัสของลูกชิ้นเคลือบด้วยสารแบคทีริโอซิน พบว่าระดับความเป็นกรด-ด่าง และค่าปริมาณกรดทั้งหมด (ร้อยละ) ไม่มีการเปลี่ยนแปลง โดยคงที่ที่ 6.5 และ ร้อยละ 0.3 ตามลำดับ การจุ่มลูกชิ้นในสารละลายแบคเทอริโอซินไม่ส่งผลต่อสีของลูกชิ้นหมู โดยที่ค่า L และ a ไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญตลอดระยะเวลาการเก็บ ยกเว้นค่า b ของตัวอย่างที่มีการจุ่มในสารละลายแบคเทอริโอซินความเข้มข้นเต็มที่จะมีค่า b สูงกว่าอย่างมีนัยสำคัญ ($P < 0.05$) การประเมินคุณภาพทางประสาทสัมผัสของตัวอย่างลูกชิ้นด้วยวิธีวิเคราะห์คุณภาพเชิงพรรณา (QDA) พบว่า การใช้แบคเทอริโอซินไม่ทำให้เกิดความแตกต่างอย่างมีนัยสำคัญ ($P > 0.05$) ของคุณลักษณะการเยิ้มน้ำ การเกิดเมือก กลิ่นเน่าเสีย กลิ่นการหืน ลักษณะปรากฏโดยรวม และการยอมรับโดยรวมของตัวอย่าง อย่างไรก็ตาม ตัวอย่างที่เคลือบด้วยแบคเทอริโอซินส่งผลให้การยอมรับของผู้ทดสอบลดลงในด้านกลิ่นผิดปกติ นอกจากนี้ การใช้แบคเทอริโอซินในการทดลองนี้ส่งผลให้สีของลูกชิ้นหมูเข้มขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) โดยเฉพาะอย่างยิ่งในตัวอย่างที่บรรจุแบบปกติ สำหรับการบรรจุลูกชิ้นแบบสุญญากาศทำให้การยอมรับโดยรวมของตัวอย่างลูกชิ้นที่เคลือบด้วยแบคเทอริโอซินลดลงเมื่อเทียบกับการบรรจุแบบปกติ

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา _____

SAROJ GOSAARAK : PRODUCTION OF BACTERIOCINS BY *LACTOCOCCUS LACTIS* TISTR 1401 AND APPLICATION IN PORK MEATBALLS. THESIS ADVISOR : ASSOC. PROF. KANOK-ORN INTARAPICHET, Ph.D. 80 PP.

BACTERIOCIN/LACTIC ACID BACTERIA/*LACTOCOCCUS LACTIS*

The objectives of the study were to select bacteriocin producing lactic acid bacteria and to apply crude bacteriocins produced by selected bacteria to extend shelf life of pork meatball products. Eight strains of lactic acid bacteria (LAB) obtained from Thailand Institute of Scientific and Technological Research (TISTR): *Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteroides* TISTR 053, *Lb. acidophilus* TISTR 450, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* supsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 and *Lactococcus lactis* TISTR 1401, and *Lactobacillus casei* spp. *rhamnosus* SN11 obtained from Prince of Songkla University (PSU) were used for selection of their ability to produce bacteriocins. From the bacteriocin ability test, results indicated that strain of *Lc. lactis* TISTR 1401 exhibited the maximum inhibition activity against indicator bacteria (*Bacillus* sp. TISTR 908, *B. cereus* TISTR 687, *B. subtilis* TISTR 008 and *Staphylococcus aureus* TISTR 118) compared with other LAB strains. Bacteriocins produced by *Lc. lactis* TISTR 1401 in MRS broth showed the inhibition activity of 3,200 AU/ml against the indicator bacteria, *Bacillus* sp. TISTR 908. Production of bacteriocins with constantly controlled pH at 6.5 during fermentation gave higher cell population and bacteriocin activity compared with control

fermentation without pH controlling. The inhibition activity reached 12,800 AU/mL at 8 h of microbial growth and 3,200 AU/mL at 10 h of microbial growth for controlled-pH and uncontrolled pH fermentation, respectively. Bacteriocin supernatant was stable at a pH range of 2 to 8 and heat stable at 80 ^{o}C for 20 min without loss of its activity. However, the bacteriocin supernatant was completely lost its activity when proteinase enzyme was added.

Studies on application of bacteriocin supernatant produced by *Lc. lactis* TISR 1401 by controlling pH at 6.5 for extended shelf life of pork meatball were performed. The pork meatballs were dipped and coated with crude bacteriocin supernatant (CBS), stored at 4 ^{o}C for 12 days in aerobically packed and vacuum packed. The CBS concentration at full strength and half strength were used, compared with control treatment. Total bacterial counts of the full strength CBS coated meatballs were 1.96 and 1.42 log cfu/g for aerobically and vacuum packed, respectively, lower than those treated with half strength and controlled samples. It was found that there was no significant difference between total bacterial counts of meatballs coated with half strength CBS and controlled samples. In case of LAB population, it was detected only at day 12. Slight decrease of *Enterobacteriaceae* and *Pseudomonas* sp. was observed in the meatballs treated with full strength CBS and packed in vacuum condition. However, deceasing of these bacteria was not observed in aerobically packed samples. In addition, *Brochothrix thermosphacta* was not observed throughout the storage period.

For chemical and physical qualities of coated pork meatballs during storage, pH and total titratable acidity were constant at 6.5 and 0.3 %, respectively. The dipping of pork meatballs in CBS did not affect color and no significant changes of L

and a values were observed throughout the storage period. However, an increase in b value (yellowness) was found for the sample treated with CBS ($P < 0.05$).

For sensory quality, the experiment was evaluated by quantitative descriptive analysis (QDA). The acceptance of panelists for color and abnormal odor decreased for the samples treated with CBS. In addition, the use of CBS gave darker color of the pork meatballs ($P < 0.05$). In case of aerobically packed condition, the significant differences were not observed for the following attributes: water purge, slime, spoiled odor, oxidized odor, overall appearance and overall acceptance. For vacuum packed condition, the significant differences of slime, spoiled odor and oxidized odor were not observed. However, the overall acceptance of panelists was lower compared with those of aerobically packed and controlled treatment.

School of Food Technology Student's Signature _____

Academic Year 2006 Advisor's Signature _____

ACKNOWLEDGEMENTS

During my graduate studies I have been fortunate enough to receive help and encouragement from several people. I would like to recognize and sincerely thank them who have made my studies at the Suranaree University of Technology a pleasant one and who help make a completion of this degree possible:

Assoc. Prof. Dr. Kanok-Orn Intarapichet, my major advisor, for her guidance, assistance, professional and personal advice, and her genuine interest in my education.

Asst. Prof. Dr. Piyawan Gasaluck and Asst. Prof. Dr. Suphitchaya Chanthachum, for their guidance and knowledge.

Appreciation would also due to the entire member of food technology and biotechnology laboratories whose immense help and friendships have made to my work in laboratory enjoyable and memorable. This study was partially supported by Suranaree University of Technology.

Most importantly, I would like to thank my family for giving me the opportunity to pursue my graduate studies and for their love and support.

Saroj Gosaarak

CONTENTS

Page

ABSTRACT (IN THAI)…….……...……...…..I

ABSTRACT (IN ENGLISH)……...…….……...III

ACKNOWLEDGEMENT…….....VI

CONTENTS……..…….....VII

LIST OF TABLES……...……..XII

LIST OF FIGURES…….……..XIV

LIST OF ABBREVIATIONS……XV

CHAPTER

I INTRODUCTION

1.1 Introduction……...…..……..1

1.2 Research objectives…….…….....3

1.3 Research hypothesis……..……...…….3

1.4 Expected results……...…..……3

1.5 References……...……..……4

II LITERATURE REVIEWS

2.1 Bacteriocins…….….6

2.1.1 Bacteriocins and classification……..……..…….……..6

2.1.2 Mode of action…….……..……...…..7

2.1.3 Nisin…….……..…...……..8

CONTENTS (Continued)

Page

2.2 Bacteriocin-producing lactic acid bacteria……...9

2.2.1 Species of *Lactococcus lactis*…….……...…..……..10

2.2.2 Strain of *Lactococcus lactis* TISTR 1401……...……..11

2.3 Bacteriocins Production……..……..…….11

2.4 Bacteriocins and meat products……..……14

2.4.1 Application of bacteriocins in meat products…….....…..14

2.4.2 Microbial spoilage of meat products……..……...16

2.4.3 Production of pork meatballs in Thailand……..……...16

2.5 References……..……...…….18

III SELECTION, PRODUCTION, CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *LACTOCOCCUS LACTIS* TISTR 1401

3.1 Abstract…….……...……..22

3.2 Introduction……23

3.3 Materials and methods……...25

3.3.1 Bacterial strains and cultures…….……..…….....25

3.3.2 Selection of bacteriocin-producing strain……..……26

3.3.3 Optimum temperature of *Latococcus lactis* TISTR 1401…….…26

3.3.4 Controlled-pH fermentation……...……..……26

3.3.5 Preparation of crude bacteriocin supernatant (CBS)…….....27

CONTENTS (Continued)

Page

3.3.6 Bacteriocin assay and activity determination……..…..27

3.3.7 Inhibitory spectrum of bacteriocins produced

by *Lactoccus lactis* TISTR 1401…….…….…….28

3.3.8 Effect of heat treatment……...……..29

3.3.9 pH and enzyme sensitivity……..…...……...29

3.4 Results and discussion……...……..30

3.4.1 Selection of bacteriocin-producing strain……..……30

3.4.2 Optimum temperature for *Lactococcus lactis* TISTR 1401……..32

3.4.3 Controlled-pH fermentation……..……...33

3.4.4 Inhibitory spectrum of bacteriocins produced

by *Lactococcus lactis* TISTR 1401…….…….….37

3.4.5 Effect of heat treatment……..…….……..39

3.4.6 pH and enzyme sensitivity…….....……...41

3.5 Conclusions.……...43

3.6 References……...……...44

IV APPLICATIONSOF BACTERIOCINS PRODUCED BY *LACTOCOCCUS LACTIS* TISTR 1401 IN PRODUCTION OF PORK MEATBALLS

4.1 Abstract…….…….49

4.2 Introduction……50

CONTENT (Continued)

Page

4.3 Materials and methods……...53

4.3.1 Bacterial strains and culture conditions…….....……53

4.3.2 Preparation of bacteriocin

of *Lactococcus lactis* TISTR 1401……..……..……..…..53

4.3.3 Bacteriocin assay and activity determination……..…...…...54

4.3.4 Pork meatball manufacture……..……..……55

4.3.5 Trials…….....…….56

4.3.6 Enumeration of microbial population……...…….57

4.3.7 pH and titratable acidity of pork meatballs.……...…..…57

4.3.8 Color determination…….……..……58

4.3.9 Sensory evaluation……...…….58

4.3.10 Statistical analyses…….....……..58

4.4 Results and discussion.....59

4.4.1 Enumeration of microbial population……...…….59

4.4.1.1 Microbial population of pork meatballs in

aerobically packed condition…….……59

4.4.1.2 Microbial population of pork meatballs in

vacuum packed condition……..…….…...……61

4.4.2 pH and titratable acidity of pork meatballs.……..65

4.4.3 Color characteristics of pork meatballs……...….....66

4.4.4 Sensory evaluation of pork meatballs……..…….69

4.5 Conclusions……72

CONTENT (Continued)

Page

4.6 References…….....……...73

V SUMMARY……...……..76

APPENDIX……..…….……..….79

CURRICULUM VITAE……..……..80

LIST OF TABLES

Table **Page**

2.1 Bacteriocins characterized from lactic acid bacteria….....7

3.1 Antibacterial activity of crude bacteriocin supernatant (CBS)
produced by nine strains of LAB against 4 strains of
Gram-positive indicator bacteria……..…….…….30

3.2. Inhibitory spectrum of bacteriocins produced
by *Lactococcus lactis* TISTR 1401……..……..38

3.3 Effect of heat treatment to the bacteriocin activity of CBS
produced by *Lactocossus lactis* TISTR 1401…...…….....…….40

3.4 Effect of pH and enzyme treatments to bacteriocin activity of CBS
produced by *Lactoccus lactis* TISTR 1401 against indicator bacteria…….……..42

4.1 Microbial population (log cfu/g) of pork meatballs during storage
at 4^{o}C in aerobically packed condition…….……...……..….63

4.2 Microbial population (log cfu/g) of pork meatballs during storage
at 4^{o}C in vacuum packed condition…….……...64

4.3 pH and titrable acidity (%) of pork meatballs during storage at 4 ^{o}C in
aerobically packed condition……...……...66

4.4 pH and titrable acidity (%) of pork meatball during storage
at 4 ^{o}C in vacuum packaged condition……...…….……..66

4.5 Color values (L, a, b Hunter color system) of pork meatballs
during storage at 4 ^{o}C in aerobically packed condition……...….……..68

LIST OF TABLES (Continued)

Table **Page**

4.6 Color values (L, a, b Hunter color system) of pork meatballs during storage at 4 ^{o}C in vacuum packed condition…….……..68

LIST OF FIGURES

Figures **Page**

2.1 The Structure of nisin……..……...……...9

2.2 Pork meatball production in pilot-plant scale……..……...……17

2.3 Drying process of pork meatballs…….……..……17

3.1 Growth of *Lactococcus lactis* TISTR 1401 in MRS broth at 30, 35, 37 and 40 ^{o}C in shaking incubator…….…….33

3.2. Growth of *Lactococcus lactis* TISTR 1401 (log cfu/ml) in MRS broth at 37 ^{o}C in pH controlled and uncontrolled condition……34

3.3. Growth of *Lactococcus lactis* TISTR 1401 (Absorbance) in MRS broth at 37 ^{o}C in pH controlled and uncontrolled condition…….……...……34

3.4. Bacteriocins activity of *Lactoccus lactis* TISTR 1401 during growth…..…...…..35

3.5 The inhibition activity of *Lactococcus lactis* TISTR 1401 in MRS broth determined by agar well diffusion assay……...…….36

4.1 Pork meatball manufacturing in Food processing laboratory, SUT…….……..55

4.2 Pork meatballs with control treatment in aerobically and vacuum packed……56

4.3 Sensory characteristics of pork meatballs during storage at 4 ^{o}C in aerobically packed condition……..…….……...69

4.4 Sensory characteristics of pork meatballs during storage at 4 ^{o}C in vacuum packed condition……..……...……..71

LIST OF ABBREVIATIONS

aa	Amino acid
AU	Arbitrary unit
^{o}C	Degree Celsius
CBS	Crude Bacteriocin Supernatant
cfu	Colony forming unit
Da	Dalton
DMRT	Duncan’s New Multiple’s Range Test
e.g.	For example
et al.	et alia (and others)
Fig.	Figure
g	Gram
h	Hour
HCl	Hydrochloric
x g	Gravitational acceleration
L	Liter
LAB	Lactic acid bacteria
M	Molar
min	Minute
mg	Milligram
mL	Milliliter

LIST OF ABBREVIATIONS (Continued)

mm	Millimeter
NaOH	Sodium Hydroxide
pp.	Page
rpm	Revolution per minute
SUT	Suranaree University of Technology
TISTR	Thailand Institute of Scientific and Technological Research
v/v	Volume : volume
w/v	Weight : volume
w/w	Weight : weight
%	Percent
μm	Micrometer

CHAPTER I

INTRODUCTION

1.1 Introduction

Lactic acid bacteria produce a variety of antimicrobial compounds, including bacteriocins. Bacteriocins are proteinaceous compounds, exhibit the inhibition activity against many Gram-positive bacteria including *Listeria monocytogenes*, *Staphylococcus aureus, Bacillus cereus* and lactic acid bacteria, and they are active mainly against closely related bacterial species (Mataragas , Metaxopoulos, Galiotou, and Drosinos, 2003; Deegan, Cotter, Hill and Ross, 2006). Bacteriocin-producing bacteria has been studied for recent year, especially nisin, bacteriocin produced by *Lactococcus lactis,* has been approved as GRAS (Generally recognized as Safe).

Eight strains of lactic acid bacteria obtained from Thailand Institute of Scientific and Technological Research (TISTR): *Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteroides* TISTR 053, *Lb. acidophilus* TISTR 450, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* supsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 and *Lc. lactis* TISTR 1401 and *Lactobacillus casei* spp. *rhamnosus* SN11 obtained from Department of Food Technology, Prince of Songkla University (PSU) were used for selection of their ability to produce bacteriocins. The most suitable strain was selected for bacteriocin production and also characterization.

Production of bacteriocin is influenced by many factors and the most important are pH, temperature and medium composition. Controlled pH constantly during fermentation has exhibited higher bacteriocin activity and cell population, results to the increased bacteriocin activity corresponded with the increased cell numbers (Yang and Ray, 1994; Morgan, Galvin, Kelly, Ross, and Hill, 1999; Morgan, Galvin, Ross, and Hill, 2001). Most of bacteriocins are produced during the active growth phase but often there is a sharp decrease in activity at the end of the log phase due to protein degradation, protein aggregation, etc. Maximum bacteriocin activity can be harvested by collecting it immediately when activity peaks (Morgan et al., 1999; Onda, Yanagisa, Tsuji, Shinohara, and Yokotsuka, 2003). Preparation of bacteriocins for applied in food system can be also performed by spray-dried (Morgan et al., 2001; Silva, Carvalho, Teixeira, and Gibbs, 2002) instead partial purification which high cost and production time. The inhibition activity of bacteriocin supernatant should be considered to performed in foods before enlarge the production for spray-dried.

Pork meatball products are economically important meat products with a high consumption in Thailand. Concern about the high level of chemical preservative compounds added to meatballs has led to investigation of the use of bacteriocin in meatballs. Even bacteriocin (especially nisin) is extremely heat stable peptide (Holzapfel, Geisen, and Schillinger, 1995) and its activity remain at temperature during meatball production, but the absent of bacteriocin activity due to its binding to meat particles is normally occurred (O'keeffe and Hill, 1999). However, there are no investigation s of the use of bacteriocins on the meatball preservation.

During cooking process (heat at 80^{o}C for 10-15 min) of pork meatballs, most of spoilage microorganisms are eliminated, but some bacterial spores and heat resistant bacteria are exist. In addition, if the sanitation system of meatball factory is poor, re-contamination of pathogens on the surface of meatballs can be a major problem for the product quality. However, dipping or surface treatment of bacteriocin should be an alternative way of application of bacteriocins in meat products (Palumbo and Williums, 1993; Göğüş, Bozoglu, and Yurdugul, 2004).

1.2 Research objectives

The objectives of this research were:

(1) To select the most suitable bacteriocin-producer from lactic acid bacteria obtained from stock culture of Thailand Institute of Scientific and Technological Research (TISTR).

(2) To produce of crude bacteriocins with high inhibition activity against selected indicator bacteria and perform some major physiochemical characteristics.

(3) To apply crude bacteriocins in pork meatballs for extending shelf life.

1.3 Research hypothesis

Bacteriocins produced from selected bacteriocin-producing strain had ability to inhibit growth of closely related bacterial species including Gram-positive bacteria, spoilage bacteria and pathogens. The application of bacteriocins by dipping or surface treatment could inhibit the growth of spoilage bacteria in pork meatballs during storage at 4 ^{o}C.

1.4 Expected results

The satisfied results of extended shelf life of pork meatballs, when crude bacteriocin supernatant was applied, would lead to the production of bacteriocins in large scale with low-cost culture medium.

1.5 References

Deegan, L.H., Cotter. P.D., Hill, C., and Ross, P. (2006). Bacteriocin: Biological tools for bio-preservation and shelf-life extension. **Int. Dairy J.** 16 (9): 1058-1071.

Göğüş, U., Bozoglu, F., and Yurdugul, S. (2004). The effects of nisin, oil-wax coating and yogurt on the quality of refrigerated chicken meat. **Food Control.** 15 (7): 537-542.

Holzapfel, W.H., Geisen, R., and Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. **Int. J. Food Microbiol.** 24 (3): 343-362.

Mataragas, M., Metaxopoulos, J., Galiotou, M., and Drosinos, E.H. (2003). Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. **Meat Sci.** 64 (3): 265-271.

Morgan, S.M., Galvin, M., Kelly, J., Ross, R.P., and Hill, C. (1999). Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. **J. Food Prot.** 62 (9): 1011-1016.

Morgan, S.M., Galvin, M., Ross, R.P., and Hill, C. (2001). Evaluation of a spray-

dried Lacticin 3147 powder for the control of *Listeria monocytogenes* and *Bacillus cereus* in a range of food systems. **Lett. Appl. Microbiol.** 33 (5): 387-391.

O'keeffe, T. and Hill, C.(1999). Bacteriocins/Potential in food preservation**. Encyclopedia of food Microbiology** (vol.1) (pp 183-191).New York: Academic press.

Onda, T., Yanagisa, F., Tsuji, M., Shinohara, T., and Yokotsuka, K. (2003). Production and purification of a bacteriocin peptide produced by *Lactococcus* sp. Strain GM005, isolated from *Miso*-paste. **Int. J. Food. Microbiol.** 87 (1-2): 153-159.

Palumbo, S.A., and Williams, A.C. (1993). Control of *Listeria monocytogenes* on the surface of frankfurter by acid treatments. **Food Microbiol.** 11 (4): 293-300.

Silva, J., Carvalho, A.S., Teixeira, P., and Gibbs, P.A. (2002). Bacteriocin production by spray-dried lactic acid bacteria. **Lett. Appl. Microbiol.** 34 (2): 77-81.

Yang, R., and Ray, B. (1994). Factors influencing production of bacteriocins by lactic acid bacteria. **Food Microbiol.** 11 (4): 281-291.

CHAPTER II

LITERATURE REVIEWS

2.1 Bacteriocins

2.1.1 Bacteriocins and classification

Bacteriocins are ribisomally synthesized, extracellularly released bioactive peptide or peptide complexes which have a bactericidal or bacteriostatic effect on other species. In all cases, the producer cell exhibits specific immunity to the action of its own bacteriocins. Bacteriocin-producing strains can be readily identified in a deferred antagonism assay, in which colonies of the putative producer are overlaid with a bacterial lawn of a sensitive strain. After further incubation, zone of inhibition are visible in the sensitive lawn. The term 'bacteriocins' was originally coined in 1953 specifically to define protein antibiotics of the colicin type (produced by *Escherichia coli*), but is now accepted to include peptide inhibitors from any genus. They are generally considered to act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipid bilayers (Daw and Falkiner, 1996; Deegan, Cotter, Hill, and Ross, 2006). Bacteriocins have been classified into three major groups as show in Table 2.1. Class I encompasses the small, post-translationally modified, board host range lantibiotics, of which nisin is the best-known example. Class II includes the small, heat-stable unmodified peptides, while class III contains larger, heat-labile molecules.

Table 2.1 Bacteriocins characterized from lactic acid bacteria

Bacteriocin	**Producer**	**Inhibitory spectrum[a]**	**Size (aa)**
Class I: Lantibiotics			
Nisin (A and Z)	*Lactococcus lactis*	Broad	34
Lacticin 481	*Lactococcus lactis*	Broad	27
Lactocin S	*Lactococcus sake*	Broad	37
Class II: Non-lantibiotic, small, heat-stable			
Lactococcin A	*Lactococcus lactis*	Narrow	54
Lactocin F	*Lactobacillus johnsonii*	Narrow	57, 48
Mesenterocin 52B	*Leuconostoc mesenteroides*	Narrow	32
Pediocin-like bacteriocin:			
Sakacin A	*Lactobacillus sake*	Medium	41
Pediocin AcH/PA-1	*Pediococcus acidilactici*	Medium	44
Leucocin A-UAL-187	*Leuconostoc gelidum*	Medium	37
Enterocin 1146/A	*Enterococcus faecium*	Medium	47
Class III: Large, heat-labile			
Helveticin J	*Lactobacillus helveticus*	Narrow	333

[a]Narrow spectrum indicates bacteriocins that only affect the producer genus; medium spectrum indicates bacteriocin that affect the producer genus and members of one or two other genera, aa = number of amino acid

Source: O'keeffe and Hill, 1999.

2.1.2 Mode of action

Bacteriocins of lactic acid bacteria can work via different mechanisms to exert an antimicrobial effect. The cell envelope is generally the target. Nisin has a board spectrum of inhibition activity against Gram-positives and also inhibits the out growth of *Bacillus* spp. and *Clostridium* spp. spores. In contrast, lactococcin A has narrow

spectrum, being active only against other lactococcal strains. The initial electrostatic attraction between the target cell membranes and the bacteriocin peptide is thought to be the driving force for subsequent event. Nisin forms pores that disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death. Other lantibiotics also form pores include Lacticin 3147, Pep5, subtilin and edidermin (McAuliffe, Ross, and Hill, 2001). However, it has long been recognized that nisin also interferes with cell wall biosynthesis. It has now been established that this phenomenon is mediated by the ability of nisin bind lipid II which is peptidoglycan precursor, thus inhibiting cell wall biosynthesis. Such binding is also intrinsic to the ability of nisin to form pores. In case of class II bacteriocins, they predominantly act by forming pores, causing dissipation of the cell membranes, depletion of intracellular ATP and leakage of amino acids and ions (Deegan et al., 2006).

2.1.3 Nisin

Nisin is a natural, toxicological safe and antibacterial food preservative, produced by *Lactococcus lactis*. Nisin exhibits antimicrobial activity toward a wide range of Gram-positive vegetative bacteria, and is particularly effective against bacterial spores. Nisin belongs to a group of bacteriocins collectively know as lantibiotics, ribosomally synthesized, relatively small polycyclic polypeptide, consisting 34 amino acids and has a molecular mass of 3354 (Delves-Broughton, 1990). Lantibiotics are named because they contain, beside protein amino acids, the unusual amino acids lanthionine and/or β-methyllanthionine, both of which form interchain ether bridges (Helzapfel, Geisen, and Schillinger, 1995; Guinane, Cotter,

Hill, and Ross, 2005). Nisin also contains another two unusual amino acids, dehydroalanine and dehydrobutyrine. Lanthionine is known to introduce a high level of hydrophobicity, and a high proportion of basic amino acids which give nisin a net positive charge. Nisin can form dimmers or even oligomers, which possibly arise through a reaction between the dehydroamino acids and amino groups of two or more nisin molecules. In aqueous solution, nisin is most soluble at pH 2. Increase of pH affects solubility and stability of nisin (Davies and Delves-Broughton, 1999; Parente and Ricciardi, 1999). The structure of nisin is shown in Figure 2.1.

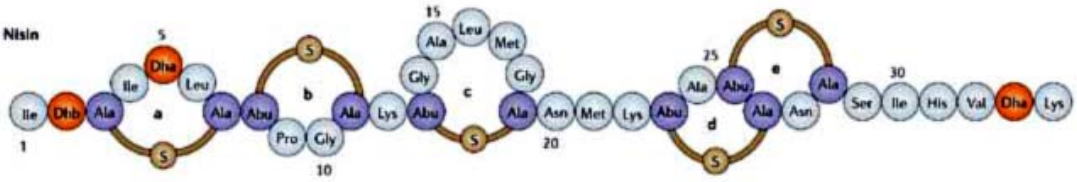


Figure 2.1 The Structure of nisin: Aba: aminobutyric; Dha: dehydroalanine; Dhb: dehydrobutyrine (β-methyldehydroalanine); Ala-S-Ala: lanthionine; Aba-S-Ala: β-methyllanthionine.

Source: modified from O'keeffe and Hill (1999)

2.2 Bacteriocin-producing lactic acid bacteria

Studies of bacteriocins produced by lactic acid bacteria have been investigated in various species include *Lactococcus* spp., *Lactobacillus* spp., *Pediococcus* spp., etc. Many authors have reported that many strains of LAB are bacteriocin-producing

lactic acid bacteria. Bacteriocin-producing LAB isolated from fermenting olive brines, *Lb. pentosus* B96 has shown to have bacteriocin activity against indicator bacteria, *Weissella paramesenteroides* (Delgado, Brito, Peres, Noé-Arroyo, and Garrido-Fernández, 2005). Lacticin 3147 bacteriocin produced by *Lc. lactis* 3147 has been shown to have bacteriocin activity against foodborne pathogens include *Listeria monocytogenes* (Morgan, Galvin, Kelly, Ross, and Hill, 1999; Guinane et al., 2005). Strain of *Lc. lactis* subsp. *lactis*, isolated from Kimchi, are also produced nisin-z (Park, Itoh, Kikuchi, Niwa, and Fujisawa, 2003) as same as strain of *Lc. lactis* IO-1 (Ishizaki and Ueda, 1995; Sonomoto, Chinachoti, Endo, and Ishizaki, 2000). *Lb. sake* isolated from meat and meat products has been shown to have bacteriocin activity against various type of Gram-positive bacteria (Schillinger and Lücke, 1989).

2.2.1 Species of *Lactococcus lactis*

Lc. lactis is the most important organism in the manufacture of fermented dairy products such as sour milk, cream, butter, fresh cheeses and many varieties of semi-hard cheeses. *Lc. lactis* is a non-sporulating, non-motile and typically 0.5-1.5 μm in length. Lactococci are homofermentative microaerophillic Gram-positive bacteria which grow at a temperature of 10 ^{o}C, but not at 45 ^{o}C, and produce L (+) lactic acid from glucose. They are characterized by ovoid cells of lactococci themselves extend into a chain, which makes them difficult to differentiate from lactobacilli. Certain strains of the species *Lc. lactis* produce a multitude of different antimicrobial compounds, including bacteriocins. These compounds occur as final products of the metabolism process (Wijtzes, Bruggeman, Nout, and Zwietering, 1997).

2.2.2 Strain of *Lactococcus lactis* TISTR 1401

Lc. lactis TISTR 1401 was obtained from Thailand Institute of Scientific and Technological Research (TISTR). This strain is deposit to *Lc. lactis* IO-1 or *Lc. lactis* JCM 7638, grow at optimum temperature of 37 ^{o}C in MRS broth according to culture description document of TISTR. In addition, *Lc. lacti*s TISTR 1401 is a highly potent strain producing L-lactate from xylose as well as glucose, and also reported to produce nisin-z (Matsusaki, Chinachoti, Sonomoto, and Ishizaki, 1998; Sonomoto et al., 2000; Park et al., 2003).

2.3 Bacteriocin production

Study of bacteriocin production, MRS broth is usually used as medium and may be supplemented with other substance to increase bacteriocin activity. Since the cost of culture medium is expensive, then production of bacteriocin is limit for study only in small-scale. Nieto-Lozano, Reguera-Useros, Peláez-Martínez, and Torre, (2005) reported the production of bacteriocins produced by *Pediococcus acidilactici* in MRS broth, and its bacteriocin has shown the inhibition activity against *Listeria monocytogenes* and *Clostridium perfringens*. Most bacteriocins are produced during the active growth phase but often there is a sharp decrease in activity at the end of the log phase due to protein degradation, adsorption to cell surface, protein aggregation, complex formation, etc. (Paynter, Brown, and Hayasaka, 1997; O'keeffe and Hill, 1999). Maximum amounts of bacteriocins can be harvested by collecting immediately at the activity peak (Parente and Ricciardi, 1999; O'keeffe and Hill, 1999; Mataragas, Metaxopoulos, Galiotou, and Drosinos, 2002). Bacteriocins could be removed from the fermentation by either batchwise or continuously by adsorbants. Bacteriocin

production seems to be stimulated by stress factors such as low temperature and competing microorganisms. A lower specific growth rate may lower acid production thus making less successful competition and necessitating other factors such as bacteriocin production for competition. Production may be controlled by a two-component regulatory mechanism through signal transduction, a cell-cell communication system known as quorum sensing. Molecular techniques may allow one strain to produce a number of bacteriocins, thus increasing the spectrum of bacteriocins sensitive to that strain. Protein engineering may increase activity, stability and host range. It is possible to make nisin synthetically and the sequence has been altered to examine the specific role to different amino acids. However, such engineering must overcome regulatory hurdles. An ideal protocol for bacteriocin production would be one which is application for large scale purification having low production and recovery costs, leading to a bacteriocin yield greater than 50% and purity greater than 90% (O'Keeffe and Hill, 1999).

There are many factors that influence production of bacteriocins by lactic acid bacteria. The important factors are pH, temperature and medium composition (Yang and Ray, 1994; Mataragas et al., 2002). Onda et al. (2003) reported that fermentation by constantly controlled pH gave the higher bacteriocin titres. Production of bacteriocins produced by *Lactococcus* sp. GM005 isolated from Miso-paste, in GYP2 medium, controlled pH of 6.0, gave the maximum activity of bacteriocins of 1,280 AU/ml. Delgado et al. (2005) performed the optimization of bacteriocin production by *Lb. pentosus* B96 in MRS broth and reported that bacteriocin activity was influenced by temperature and NaCl concentration.

Many studies have shown that the highest bacteriocin titres usually are obtained at pH and temperature values lower than the optimum ones for growth (Parente and Ricciardi, 1999). Mataragas et al. (2002) reported the bacteriocins produced by *Leuconostoc* in 1.5 liter of MRS broth. The optimum pH and temperature for growth were 6.0-6.5 and 30^{o}C and for bacteriocin production were 5.5 and 25^{o}C. Nelson, Rua, and Pastrana (2001) reported that bacteriocin production from *Lc. lactis* subsp. *lactis* was greatly inhibited by the nitrogen, buffer, and to a lesser extent, sugar concentration in the medium, nevertheless, the used phosphorous source produced a light stimulatory effect on bacteriocin synthesis

Economically reliable processes have to be developed for bacteriocin production. Optimizing production and enhancing stability and activity are necessary for such an economic breakthrough. In addition, bacteriocin-producing starter cultures may prevent the growth of spoilage and pathogenic microorganisms more efficiently in food and feed products if the production in situ and stability of active bacteriocin are increased. Fermentation should be based on cheap substrates, and a suitable and low-cost downstream processing strategy devised to produce bacteriocins for directly use as food biopreservative, or biomass as starter culture for in situ bacteriocin production. Morgan et al. (1999) demonstrated a large scale of lacticin 3147 production while using reconstituted demineralized whey powder as media, while bacteriocin activity was corresponded with cell number, both of cell number and bacteriocin activity reached maximum when the pH of fermentation was constantly held at 6.5 at 30^{o}C. Yang and Ray (1993) showed that the production of bacteriocins include nisin, pediocin AcH, Leucoccin Lcm1 and sakacin A in simple medium can be increased by growing the cells at optimum pH and supplemented with nutrients

specific for a species.. In the case of *Lc. lactis* IO-1 (deposit to *Lc. lactis* TISTR-1401), optimal pH may also be affected by the culture medium. Nisin Z production with strain IO-1 was optimal at pH 6.0 in xylose media and at pH 5.5 in glucose media.

For purification of bacteriocins, the cationic and hydrophobic nature of bacteriocins is used for their recovery from complex fermentation broth which contains high levels of peptide (10-30 g/l compared to a bacteriocin concentration of 10-100 mg/l) (Parente and Ricciardi, 1999). Laboratory purification protocols usually include an ammonium sulphate precipitation step, followed by various combinations of ion-exchange and hydrophobic interaction chromatography with a final RP-HPLC (Reverse Phase-High Performance Liquid Chromatography) purification step.

2.4 Bacteriocins and meat products

2.4.1 Application of bacteriocins in meat products

Most of applications of bacteriocins in food are usually investigated in dairy products, canned foods and wine. Concerning about the toxicological safety of nitrite used in cured meat has led to investigation into the use of nisin to allow a reduction in nitrite level. Therefore, there are many research works on applications of bacteriocins in meat products. Bacteriocins produced by LAB associated with meat and meat fermentations such as *Pediococcus*, *Leuconostoc*, *Carnobacterium* and *Lactobacillus* spp. are likely to have much greater potential as meat preservatives (Abee, Krockel, and Hill, 1995).

Various types of meat products has been studied. Fish sausage treated with 50 ppm of nisin was acceptable after storage at ambient temperature for 20-22 days

compared with the control, which were acceptable only for 2 days (Raju, Shamasundar, and Udupa, 2003). Infant milk formulation added with Lacticin 3147-enriched whey powder has been shown the inhibition activity against *Listeria monocytogenes* Scott A, resulted in greater than a 99% kill of *L. monocytogenes* within 3 h at 30 ^{o}C. Lacticin 3147 has also shown the inhibition activity against *Listeria* and *Bacillus* in natural yogurt, cottage cheese and soup, resulted with 99.9% and 85 % reduction of *Listeria* in yogurt and cottage cheese, respectively. In addition, 80 % reduction of *Bacillus* in soup was observed (Morgan, Galvin, Ross, and Hill, 2001). Bacteriocin of entercin AS-48 exhibited the inhibition activity against *Listeria monocytogenes* and *Staphylococcus aureus* in model sausages (Ananou et al., 2005). Inhibition effect of bacteriocin produced by *Pediococcus acidilactici*, against *Listeria monocytogenes* and *Clostridium perfringens* on Spanish raw meat surface was observed by Neito-Lozano et al. (2005). The inhibition effect of the combination of yogurt and nisin against *Salmonella* in refrigerated chicken meat was found for mesophilic aerobic bacteria and *Salmonell*a with 2.1 and 1.97 log reductions (Göğüş, Bozoglu, and Yurdugul, 2004). Alexander and Richard (2000) demonstrated the inhibition effect of nisin against many spoilage species include *Brochothrix thermosphacta*, *Escherichia coli* O157:H7, *Lb. sakei*, *Lb. curvatus*, *Leuconostoc mesenteroides*, *Listeria monocytogenes*, *Salmonella thyphimurium*, *Serratia grimesii* and *Shewanalla putrefaciens* in ham and bologna.

However, uneconomically high levels are required to achieve good control of spoilage bacteria in meat products (O'keeffe and Hill, 1999). Morgan et al. (2001) reported the application of Lacticin 3147 powder in food system required the concentration 10% of product weight which was not appropriate due to un-economic.

The complexity of food matrix could interfere the bacteriocin activity and direct adding to food products should be considered.

2.4.2 Microbial spoilage of meat products

The predominant bacteria associated with spoilage of refrigerated beef and pork, are *Brochothrix thermosphacta*, *Carnobacterium* spp., Enterobacteriaceae, *Lactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp. and *Shewanella putrefaciens*. The main defects in meat are off-odors and off-flavors, however, discoloration and gas production also occur. Bacteria associated with the spoilage of refrigerated meat products causing defects such as sour, off-flavors, discoloration, gas production, slime production and decrease in pH, consist of *Brochothrix thermosphacta*, *Carnobacterium* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Weissella* spp. (Borch, Kant-Muermans, and Blixt., 1996).

2.4.3 Production of pork meatballs in Thailand

Pork meatballs are normally prepared from pork, salt, phosphate, tapioca flour, pepper, sugar and ice. Formulations of pork meatballs could be differ. Minced pork and other ingredients are mixed and homogenized in chopper machine, formed into meatballs by hand or automatically forming machine in 60-65 ^{o}C water (Figure 2.2), then cooked in 80 ^{o}C water for 10-20 min, cooled in cold water, dried at room temperature or under cold air and then packed in plastic bags (Figure 2.3). Most of commercial pork meatballs in Thailand are aerobically packed in plastic bags, only some premier brands are vacuum packed.

(A) (B)

Figure 2.2 Pork meatball production in pilot-plant scale: (A), pork meatball batter in former machine; (B), pork meatballs after forming in 60 ^{o}C water

Figure 2.3 Drying process of pork meatballs

2.5 References

Abee, T., Krockel, L., and Hill, C. (1995). Bacteriocin: modes of action and potentials in food preservation and control of food poisoning. **Int. J. Food Microbiol.** 28: 169-185.

Alexander, O.G. and Richard, A.H. (2000). Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. **Food Res. Int.** 33: 83-90.

Ananou, S.,et al. (2005). Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. **Int. J. Food Microbiol.** 103: 179-190.

Borch, E., Kant-Muermans, M., and Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. **Food Microbiol.** 33: 103-120.

Daw, M.A., and Falkiner, F.R. (1996). Bacteriocin: Nature, function and structure. **Micron.** 27 (6): 467-479.

Deegan, L.H., Cotter. P.D., Hill, C., and Ross, P. (2006). Bacteriocin: Biological tools for bio-preservation and shelf-life extension. **Int. Dairy J.** 16 (9): 1058-1071.

Delgado, A., Brito, D., Peres, C., Noé-Arroyo, F., and Garrido-Fernández, A. (2005). Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. **Food Microbiol.** 22 (6): 521-528.

Delves-Broughton, J. (1990). Nisin and its uses as a food preservative. **Food Technol.** 44: 100-117.

Devies, E.A. and Delves-Broughton, J. (1999). Bacteriocins/nisin**. Encyclopedia of food Microbiology** (vol.1) (pp 191-198). New york: Academic press.

Guinane, C.M., Cotter, P.D., Hill, C., and Ross, R.P. (2005) Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. **J. Appl. Microbiol.** 98: 1316-1325.

Göğüş, U., Bozoglu, F., and Yurdugul, S. (2004). The effects of nisin, oil-wax coating and yogurt on the quality of refrigerated chicken meat. **Food Control.** 5: 537-542.

Holzapfel, W.H., Geisen, R., and Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. **Int. J. Food Microbiol.** 24: 343-362.

Ishizaki, A., and Ueda T.(1995). Growth kinetics and product inhibition of *Lactococcus lactis* IO-1 culture in xylose medium. **J. Ferment. Bioeng.** 80 (3): 287-290.

Mataragas, M., Metaxopoulos, J., Galiotou, M., and Drosinos, E.H. (2003). Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. **Meat Sci.** 64 (3): 265-271.

Matsusaki H., Chinachoti, N., Sonomoto, K., and Ishizaki, A. (1998) Purification, identification, and effective production of a peptide antibiotic produced by *Lactococcus lactic* OI-1 (JCM 7638). **Annals of the New York Academy of Sciences.** 864 (1): 422–427.

McAuliffe, O., Ross, R.P., and Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. **FEMS Microbiol. Rev.** 25 (3): 285-308.

Morgan, S.M., Galvin, M., Kelly, J., Ross, R.P., and Hill, C. (1999). Development of

a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. **J. Food Prot.** 62 (9): 1011-1016.

Morgan, S.M., Galvin, M., Ross, R.P., and Hill, C. (2001). Evaluation of a spray-dried Lacticin 3147 powder for the control of *Listeria monocytogenes* and *Bacillus cereus* in a range of food systems. **Lett. Appl. Microbiol.** 33 (5): 387-391.

Nelson, P.G., Rua, M.L., and Pastrana, L. (2001). Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. **Int. J. Food Microbiol.** 70: 267-281.

O'keeffe, T. and Hill, C.(1999). Bacteriocins/Potential in food preservation**. Encyclopedia of food Microbiology** (vol.1) (pp 183-191).New york: Academic press.

Parente, E. and Ricciardi, A. (1999). Production, recovery and purification of bacteriocins from lactic acid bacteria. **Appl. Microbiol. Biotechnol.** 52 (5): 628-638.

Park, S., Itoh, K., Kikuchi, E., Niwa, H., and Fujisawa, T. (2003). Identification and characteristics of nisin Z-producing *Lactococcus lactis* subsp. *lactis* isolated from Kimchi. **Current microbiology.** 46: 385-388.

Paynter, M.J.B., Brown, K.A., and Hayasaka, S.S.(1997). Factors affecting the production of an antimicrobial agent, plantaricin F, by *Lactobacillus lantarum* BF001. **Lett. Appl. Microbiol.** 24: 159-165.

Raju, C.V., Shamasundar, B.A., and Udupa, S.K.(2003). The use of nisin as preservative in fish sausage stored at ambient (28±2^{o}C) and refrigerated (6±2^{o}C). **Int. J. Food Sci. Tech.** 38: 171-185.

Schillinger, U. and Lücke F. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. **Appl. Envi. Microbiol.** 55 (8): 1901-1906.

Sonomoto, K., Chinachoti, N., Endo, N., Ishizaki, A. (2000). Biosynthetic production of nisin Z by immobilized Lactococcus lactis IO-1. **J. Mol. Catal. B: Enzymatic.** 10: 325-334.

Wijtzes, T., Bruggeman, M.R., Nout, M.J.R., and Zwietering, M.H. (1997). A computerised system for the identification of lactic acid bacteria. **Int. J. Food Microbiol.** 38: 65–70.

Yang, R., and Ray, B. (1994). Factors influencing production of bacteriocins by lactic acid bacteria. **Food Microbiol.** 11 (4): 281-291.

CHAPTER III

SELECTION, PRODUCTION AND CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *LACTOCOCCUS LACTIS* TISTR 1401

3.1 Abstract

Bacteriocin supernatant produced by *Lactococcus lactis* TISTR 1401 was showed the most antagonistic activity against Gram-positive indicator bacteria compared with other 8 strains of lactic acid bacteria obtained from Thailand Institute of Scientific and Technological Research (TISTR) and Prince of Songkla University (PSU) included *Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteroides* TISTR 053, *Lb. acidophilus* TISTR 450, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* supsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 and *Lb. casei* spp. *rhamnosus* SN11. The optimum growth temperature of *Lc. lactis* TISTR 1401 was investigated in order to make use of the culture for bacteriocin production by constantly controlled pH during fermentation. The temperatures of fermentation were set at 30, 35, 37, and 40 ^{o}C. The pHs of the fermented media were constantly controlled at 6.0, 6.5 and 7.0 compared with that of uncontrolled media. The optimum growth temperature was observed at 37 ^{o}C with the maximum cell count obtained within 8 h of fermentation. The highest activity of crude bacteriocins was obtained from controlled pH fermentation at 37 ^{o}C with the

maximum activity of 12,800 AU/ml compared to only 3,200 AU/ml from the uncontrolled pH experiment. Bacteriocins produced by *Lc. lactis* TISTR 1401 showed the inhibition activity against many type of Gram-positive bacteria but none with Gram-negative. In addition, the crude bacteriocins obtained were heat stable, proteinaceous as well as stable in neutral to acid conditions.

Keywords: nisin, bacteriocins, *Lactococcus lactis*

3.2 Introduction

The elimination of food spoilage and pathogenic organisms has become the focus of many researchers. In recent years, consumer demands for fresh, minimally processed safe food, in addition to concern over the use of chemical preservatives in foods, the application of bio-preservatives has prompted substantial interest (Deegan, Cotter, Hill, and Ross, 2006). Bacteriocins produced by lactic acid bacteria (LAB) are seen as alternatives to traditional preservatives for ensuring food safety, and potential applications in foods have been readily identified (Moreno, Lerayer, Baldini, and Leitão, 2000; Park, Itoh, Kikuchi, Niwa, and Fujisawa, 2003; Nieto-Lozano, Reguera-Useros, Peláez-Martínez, and Torre, 2005).

Bacteriocins are ribosomally synthesized antimicrobial compounds produced by many different bacterial species including many members of lactic acid bacteria (Schillinger and Lücke, 1989; Avonts, Uytven, and Vuyst, 2004; Aslim, Yuksekdag, Sariyaka, and Beyatli, 2005; Ekinci and Barefoot, 2006). Some bacteriocins produced by LAB, such as Nisin, inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes* and many other Gram-positive spoilage microorganisms (Schillinger and Lücke, 1989; Parente and

Ricciardi, 1999). Nisin is the only bacteriocin considered to be GRAS (generally recognized as safe) by US FDA (Food and Drug Administration) (O'Sullivan, Ross and Hill, 2002; Schneider et al., 2006). For this reason, bacteriocins have attracted considerable interest for use as natural food preservatives in recent years. Bacteriocin production is influenced by many factors and the most important are pH, temperature and medium composition (Mataragas, Metaxopoulos, Galiotou, and Drosinos, 2002; Todorov and Dick, 2005). Bacteriocin production is associated with bacterial growth, bacteriocin production rates can be improved in continuous fermentations whereas high growth rates can be maintained. In addition, controlling of pH improves the growth of LAB, resulting in improved bacteriocin production (Parente and Ricciardi, 1999). Growth at optimal temperature usually results in optimal bacteriocin production (Yang and Ray, 1993). Morgan, Galvin, Kelly, Ross, and Hill (1999) reported that when pH of growth media was maintained at 6.5, production of Lacticin 3147 produced by *Lactococcus lactis* subsp. *lactis* DPC3147 in 10% skim milk powder solution significantly increased. In addition, temperature stress or growth at sub-optimal temperature may result in an increase of specific bacteriocin production rate. However, optimum cell growth does not always give high bacteriocin production (Matsusaki, Chinachoti, Sonomoto, and Ishizaki, 1998; Mataragas et al., 2002) whereas bacterial strains, culture media and type of desire bacteriocin are also necessary to consider.

The aim of this work was to optimize bacteriocin production by *Lc. lactis* TISTR 1401 in controlled pH media, as a function of temperature condition during the fermentation.

3.3 Materials and methods

3.3.1 Bacterial strains and cultures

The bacteriocin producer *Lactococcus lactis* TISTR 1401 was obtained from Microbiological Resources Center, TISTR (Thailand Institute of Scientific and Technological Research). The Gram-positive indicator bacteria included *Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteriodes* TISTR 053, *Lactobacillus acidophilus* TISTR 450, *Lactobacillus brevis* supsp. *brevis* TISTR 860, *Lactobacillus delbruckii* subsp. *bulgaricus* TISTR 892, *Lactobacillus sake* TISTR 911, *Lactococcus lactis* TISTR 1401, *Staphylococcus aureus* TISTR 118, *Bacillus subtilis* TISTR 008, *Bacillus cereus* TISTR 687, *Bacillus* sp. TISTR 908 and a Gram-negative bacteria, *Escherichia coli* TISTR 780. The others strains of Gram-negative bacteria of *Pseudomonas aerogenosa* and *Enterococcus aerogenosa* were obtained from Suranaree University of Technology stock culture. The lactic acid bacteria isolated from Plasomfuk (Thai traditional fermented fish), *Lactobacillus casei* spp. *rhamnosus* SN11 was obtained from Department of Food Technology, Prince of Songkla University (PSU). Handling of the LAB was done according to the recommendation documented by TISTR. All indicator bacteria cultures were stored at -40 ^{o}C in 10% (w/v) skim milk powder and re-activated by transferring 1 ml of stock culture to 5 ml TSB (Typtic soy broth, Hi-media, Mumbai, India) or MRS broth (Hi-media, Mumbai, India) for lactic acid bacteria. The culture was overnight incubated at room temperature, in a shaker (Unimax 100, Heidolph, Schwabach, Germany) at 50 rpm, then transferred to 25 ml TSB and incubated in a shaking incubator (Orbital Incubator S-150, Stuart Sciencetific, UK) at each optimum temperature.

3.3.2 Selection of bacteriocin-producing strain

Overnight cultures of 9 strains of LAB were 1 % (v/v) inoculated to 25 ml MRS broth, incubated at each optimum temperature (according to a recommendation from TISTR), in a shaking incubator (Orbital Incubator S-150, Stuart Sciencetific, UK) at 100 rpm for 12-16 h to reached stationary phase (Schillinger and Lücke, 1989). The cultures were prepared for crude bacteriocin supernatant (CBS) by centrifugation at 12,000 x g for 20 min at 4 ^{o}C with a Sorvall® RC SC-plus centrifuge (Thermo Sciencetific, Waltham, USA) for determination of bacteriocin activity by agar well diffusion assay. Four strains of *Bacillus* sp. TISTR 908, *B. subtilis* TISTR 008, *B.cereus* TISTR 687, *S. aureus* TISTR 118 were used as indicator bacteria. The inhibition activity was reported as diameter (mm) of a clear inhibition zone.

3.3.3 Optimum temperature of *Lactococcus lactis* TISTR 1401

The *Lc. lactis* TISTR 1401 culture of 1 % (v/v) was inoculated in 100 ml MRS broth and shaked at 100 rpm in a shaking incubator (Orbital Incubator S-150, Stuart Sciencetific, UK) at different temperatures of 30, 35, 37 and 40 ^{o}C. Samples were taken every hour for the first 12 h and then at 14, 16, 20 and 24 h for enumeration of LAB in MRS agar, incubated at 37 ^{o}C for 24-36 h (Cai, Ng, and Farber, 1997).

3.3.4 Controlled-pH fermentation

The bacteriocinogenic *Lc. Lactis* TISTR 1401 strain was grown in MRS broth, supplemented with 2 % (w/v) yeast extract (Hi-media, Mumbai, India), 0.2 % (w/v) glucose (Ajax Finechem, Australia) and 0.2 % (w/v) meat extract (Merck, Darmstadt, Germany) according to Nieto-Lozano et al. (2005), at 37 ^{o}C, micro-aerobically

incubated in a stainless bioreactor with slow agitation (100 rpm) at 37 ^{o}C for 24 h. The pH of fermentation was maintained constantly at 6.0, 6.5 and 7.0 by addition of 1 M NaOH via a 718 STAT Titrino (Metrohm, Ireland) according to Morgan et al. (1999). Uncontrolled-pH fermentation was also investigated. Samples were taken with a sterile syringe every hour for the first 12 h and then were taken at 14, 16, 20 and 24 h. Total viable count was performed on MRS agar at the appropriate dilution as duplicates. The experiment was done in 2 replications.

3.3.5 Preparation of crude bacteriocin supernatant (CBS)

The fermentate was centrifugation at 12,000 x g for 20 min at 4 ^{o}C with a Sorvall® RC SC-plus centrifuge (Thermo Sciencetific, Waltham, USA). The supernatant was collected and neutralized to pH 6.5 with 1 M NaOH, in order to exclude a potential inhibitory effect caused by a decrease of pH (Ogunbanwo, Sanni, and Onilude, 2003). To exclude the inhibitory effect of possible production of hydrogen peroxide, the supernatant was treated with catalase (Sigma Chemical Co., St. Louis, MO, USA) by dissolving in a phosphate buffer at pH 7.0, to the final concentration of catalase (Sigma, St. Louis, USA) at 1 mg/ml, and then incubated for 30 min at room temperature (Nieto-Lozano et al., 2005). The supernatant was filtered through a 0.22-μm pore-size cellulose acetate syringe filter (PALL, New York, USA) and stored at 4 ^{o}C before use.

3.3.6 Bacteriocin assay and activity determination.

Bacteriocin activity was determined by the agar well diffusion method, slightly modified from Schillinger and Lücke (1989). Overnight culture of *Bacillus*

sp. TISTR 908 of 1 % (v/v) was inoculated into 15 ml of warm seeded agar (Tryptic soy soft agar, contained 0.75 % (w/v) agar). A seeded agar was overlaid on NA (Nutrient agar, Merck, Darmstadt, Germany). Wells of approximately 6 mm were bored in the seeded agar. A 40 µl volume of a two-fold serial dilution of a prepared CBS was dispensed into each well as duplicate. The plates were kept at 4 ^{o}C for 2 h for pre-diffusion, then incubated at 37 ^{o}C for 24 h (Buncic, Avery, Rocourt, and Dimitrijevic, 2001). Calculation of bacteriocin activity was performed as the inverse of the last dilution that gave a clear zone of inhibition, arbitrary units (AU) were expressed per ml (1/dilution X 25).

3.3.7 Inhibitory spectrum of bacteriocins produced by *Lactococcus lactis* TISTR 1401

The agar well diffusion assay was utilized to detect the inhibition of the sensitive strains. The bacteriocin was produced by *Lc. lactis* TISTR 1401 in MRS broth supplemented with 2 % (w/v) yeast extract, 0.2 % (w/v) glucose and meat extract, controlled pH constantly at 6.5 by addition of 1 M NaOH via a 718 STAT Titrino (Metrohm, Ireland). Harvest of bacteriocins was conducted when the bacteriocin activity reached maximum according to previous studied. Fermented media was centrifuged at 12,000 x g for 20 min at 4 ^{o}C. The supernatant was neutralized to pH 6.5 with 1 M NaOH and filtered through a 0.22 µm pore-size cellulose acetate syringe filter (PALL, New York, USA). Determination of bacteriocin activity was performed as described above.

3.3.8 Effect of heat treatment

Aliquots 5 ml of sterile CBS were heated at 70, 80, 90 and 100 ^{o}C for 10, 15 and 20 min in a thermostatically controlled water bath (GP-400, Neslab instruments, Newington, USA) and at 121 ^{o}C for 20 min in an autoclave (Sanyo Vertical Labo Autoclave, NB Scientific, Edison, NJ) (Suma, Misra, and Varadaraj, 1998). The treated samples were tested for antibacterial activity against the indicator bacteria *Bacillus* sp. TISTR 908. Untreated culture concentrated served as a positive control.

3.3.9 pH and enzyme sensitivity

Aliquots 50 ml of sterile CBS were adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 0.1 M HCl and 0.1 M NaOH, incubated for 4 h at room temperature (Ogunbanwo et al., 2003) and then assayed for bacteriocin activity against indicator bacteria *Bacillus* sp. TISTR 908. Effect of enzymes on the bacteriocin activity was determined by treating sterile CBS pH 6.5 with enzyme solutions. Each of catalase (Sigma, St. Louis, USA) and proteinase (Sigma, St. Louis, USA) was added to the pH 7.0 bacteriocin solution to the final enzyme concentration of 1 mg/ml, and then incubated for 2 h at 37 ^{o}C (Chin, Shim, Kim, Yang, and Yoon, 2001; Yamato, Ozaki, and Ota, 2003). The remaining activity was measured using the well diffusion assay method as described above.

3.4 Results and discussion

3.4.1 Selection of bacteriocin-producing strain

To compare bacteriocin production among the LAB strains, the nine stains were assayed for their production using the agar well diffusion assay method. The CBS produced by *Lc. lactis* TISTR 1401 showed stronger inhibition activity than others (Table 3.1). This strain showed the most potent antibacterial activity against *Bacillus* sp. TISTR 908, *B. subtilis* TISTR 008, *B. cereus* TISTR 687 and *S. aureus* TISTR 118 with the inhibition zone of 22.0, 17.5, 16.4 and 11.3 mm, respectively. The strain of *Lc. lactis* TISTR 1401 is deposited to *Lc. lactis* IO-1 (JCM 7638), a highly potent strain producing L-lactate from xylose. The peptide of strain OI-1 was deduced to be nisin Z (3.34 kDa molecular weight) (Matsusaki et al., 1998; Park et al., 2003).

Table 3.1 Antibacterial activity of crude bacteriocin supernatant (CBS) produced by nine strains of LAB against 4 strains of Gram-positive indicator bacteria.

Strain of LAB	Strains	Inhibition zone (mm) of indicator bacteria			
		Bacillus sp. TISTR 908	*B. cereus* TISTR 687	*B. subtilis* TISTR 008	*S. aureus* TISTR 118
Lb. plantarum	TISTR 050	7.1 (+)	6.9 (+)	6.6 (+)	-
Pediococcus acidilactici	TISTR 051	-	-	-	-
Leu. mesenteriodes	TISTR 053	-	-	-	-
Lb. acidophilus	TISTR 450	7.9 (+)	7.1 (+)	-	-
Lb. brevis supsp.*brevis*	TISTR 860	-	-	-	-
Lb. delbruckii subsp. *bulgaricus*	TISTR 892	-	-	-	-
Lb. sake	TISTR 911	-	-	-	-
Lb. casei spp. *rhamnosus*	SN 11	-	-	-	-
Lc. lactis	TISTR1401	22.0 (+++)	16.4 (++)	17.5 (++)	11.3 (++)

Symbol: -, No inhibition zone: +, small inhibition zone (<10 mm): ++, intermediate inhibition zone (11-20 mm): +++, large inhibition zone (>21 mm).

Bacteriocin supernatant produced by *L. plantarum* TISTR 050 did not inhibit growth of *S. aureus* TISTR 118 but showed slight inhibition activity against *Bacillus* sp. TISTR 908, *B. subtilis* TISTR 008 and *B. cereus* TISTR 687 with the inhibition zone 7.1, 6.6 and 6.9 mm, respectively. The cell-free supernatant bacteriocins produced by strain of *L. acidophilus* TISTR 450 also showed a small inhibition zone against *Bacillus* sp. TISTR 908 (7.9 mm) and *B. cereus* TISTR 687 (7.1 mm). When the pH of cell-free supernatant was adjusted to 6.5 and catalase was added, there was no reduction of inhibition (data not shown). This indicated that the inhibitory activity was not due to hydrogen peroxide or acid production but due to an antimicrobial compound excreted into the culture media (Schillinger et al., 1989). Moreover, when 3% (w/v) hydrogen peroxide was loaded into agar well seeded with 10^6 cfu of indicator bacteria, only strain of *Bacillus* sp. TISTR 908 that was not sensitive to hydrogen peroxide (data not shown). The strain *Bacillus* sp. TISTR 908 also showed the highest sensitivity to bacteriocin supernatant produced by *Lc. lactis* TISTR 1401. Bacteriocins produced by strains of *P. acidilactici* TISTR 051, *Leu. mesenteriodes* TISTR 053, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* subsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 and *Lb. casei* spp. *rhamnosus* SN11 did not show the inhibition activity against the 4 strains of indicator bacteria as shown in Table 3.1.

Bacteriocin production is influenced by many factors included strain of LAB, growth temperature, pH and medium composition (Mataragas et al., 2002). Number of non bacteriocin-producing LAB observed in this study indicated that they might not produce bacteriocin in this growth condition. The test indicator bacteria might not be suitable, or it did not produce any bacteriocin-like substances. The indicator bacteria in this study were normally used in bacteriocin activity assay especially in the

study of screening and inhibitory spectrum of bacteriocin-producing bacteria (Schillinger and Lücke, 1989; Moreno et al., 2000).

These results demonstrated that the most suitable bacteriocin-producer strain was *Lc. lactis TISTR* 1401 because of its outstanding inhibition activity. Strain of *Bacillus* sp. TISTR 908 was considered as major indicator bacteria for further study.

3.4.2 Optimum temperature for *Lactococcus lactis* TISTR 1401

The optimum temperature of *Lc. lactis* TISTR 1401 in MRS broth could be considered at 37 ^{o}C (Figure 3.1). The highest viable cell count of *Lc. lactis* TISTR 1401 at 37 ^{o}C reached 8.54 log cfu/ml in 8 h, whereas incubation at 30, 35 and 40 ^{o}C, the highest viable cell counts reached 8.26 log cfu/ml in 11 h, 8.35 (9 h) and 8.10 log cfu/ml in 14 h, respectively. It was obvious that the growth of bacteriocin producer strain *Lc. lactis* TISTR 1401 at 37 ^{o}C was better than at other temperatures. The optimum temperature at 37 ^{o}C was agreed with the recommendation of the TISTR, Matsusaki et al. (1998) and previous studies using an automatically turbidity measurement (Bioscreen C) (data not shown).

Bacteriocins are primary metabolite excrete while their activities might be correlated with population of cells (Parente and Ricciardi, 1999; Morgan et al., 1999). The hypothesis of this study was to demonstrate that the optimum temperature for cell growth should be the optimum for bacteriocin production as well. Therefore, the bacteriocin production was conducted at 37 ^{o}C in future study.

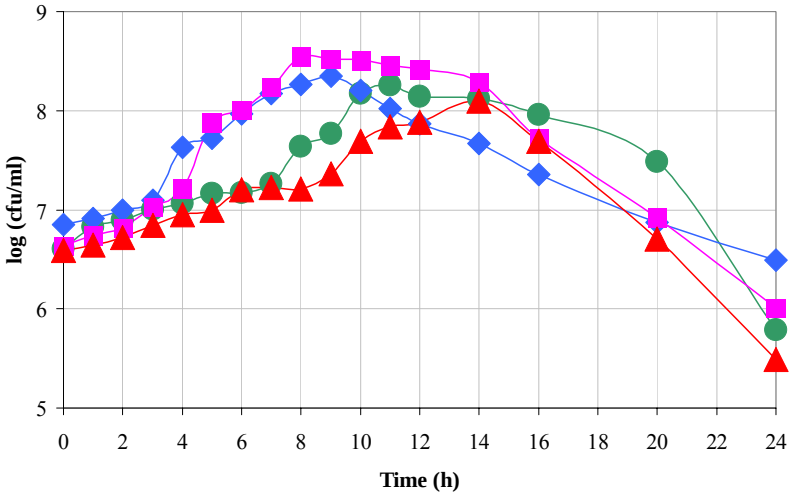


Figure 3.1 Growth of *Lactococcus lactis* TISTR 1401 in MRS broth at 30, 35, 37 and 40 ^{o}C in shaking incubator. ●, at 30 ^{o}C; ◆, at 35 ^{o}C; ■, at 37 ^{o}C ; ▲, 40 ^{o}C.

3.4.3 Controlled-pH fermentation

When the pH of fermentation of *Lc. lactis* TISTR 1401 was constantly held at 6.0, 6.5, 7.0 and uncontrolled, at 37 ^{o}C then the viable cell counts of LAB and absorbance at 600 nm were determined. Viable cell counts of *Lc. lactis* TISTR 1401 and absorbance of fermented media during 24-h period are shown in Figure 3.2 and 3.3. The maximum number of cell count was obtained during 8-10 h for fermentation at pH 6.0 (9.08 log cfu/ml) and 7.0 (9.66 log cfu/ml) and during 6-7 h at pH 6.5 (10.57 log cfu/ml) while the maximum growth (7.75 log cfu/ml) in uncontrolled pH media was prolonged till 14 h. It was obvious that the cell densities in the fermented media were in agreement with the growth of the bacterial culture. The maximum of absorbance (in term of absorbance reading at 600 nm) and bacterial growth were observed at similar time of fermentation with the maximum reading at 2.526, 2.684

and 2.434 for pH 6.0, 6.5 and 7.0, respectively. However, the maximum reading of the uncontrolled media was observed in 24 h with only 1.297.

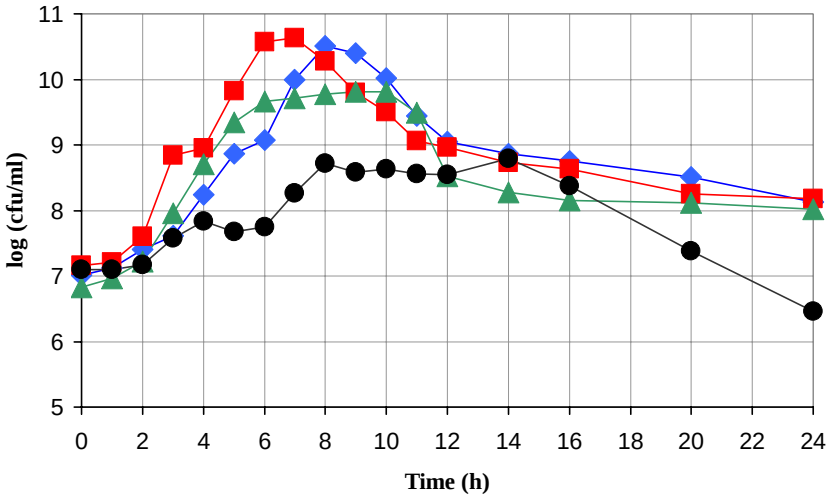


Figure 3.2 Growth of *Lactococcus lactis* TISTR 1401 (log cfu/ml) in MRS broth at 37 °C in pH controlled and uncontrolled condition. ●, with no pH control imposed; ◆, constant pH of 6.0; ■, constant pH of 6.5; ▲, constant pH of 7.0.

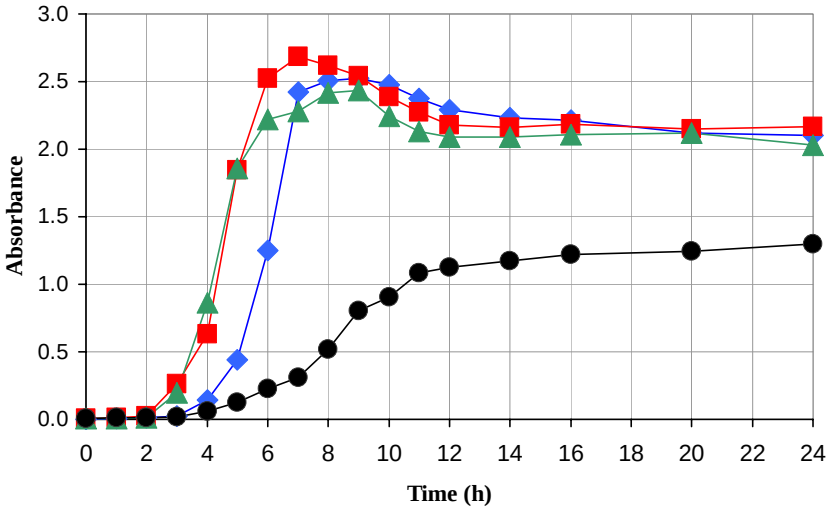


Figure 3.3. Growth of *Lactococcus lactis* TISTR 1401 (Absorbance) in MRS broth at 37 °C in pH controlled and uncontrolled condition. ●, no pH control imposed; ◆, constant pH of 6.0; ■, constant pH of 6.5; ▲, constant pH of 7.0: Absorbance measured at 600 nm.

Bacteriocin production of *Lc. Lactis* TISTR 1401 in controlled pH fermentation revealed that bacteriocin production could be maximized by constant maintaining the pH of growth media at 6.5 (Figure 3.4). Bacteriocin activity reached the maximum of 12,800 AU/ml (Arbitrary unit per ml) in 6 h when the growth media was constantly held at pH 6.5 while the uncontrolled pH media produced the activity only 3,200 AU/ml (Figure 3.5 B) in 10 h. However, the growth media constantly held at pH 6.0 and 7.0 also produced bacteriocin activity up to 12,800 AU/ml (Figure 3.5 A) but at the later time of fermentation, 10 and 7 h, respectively. Most of bacteriocins are produced during the active growth phase and maximum bacteriocin amount can be harvested by collecting it immediately when activity reach the highest peaks (Morgan et al., 1999; O'Keeffe and Hill, 1999; Onda, Yanagisa, Tsuji, Shinohara, and Yokotsuka, 2003).

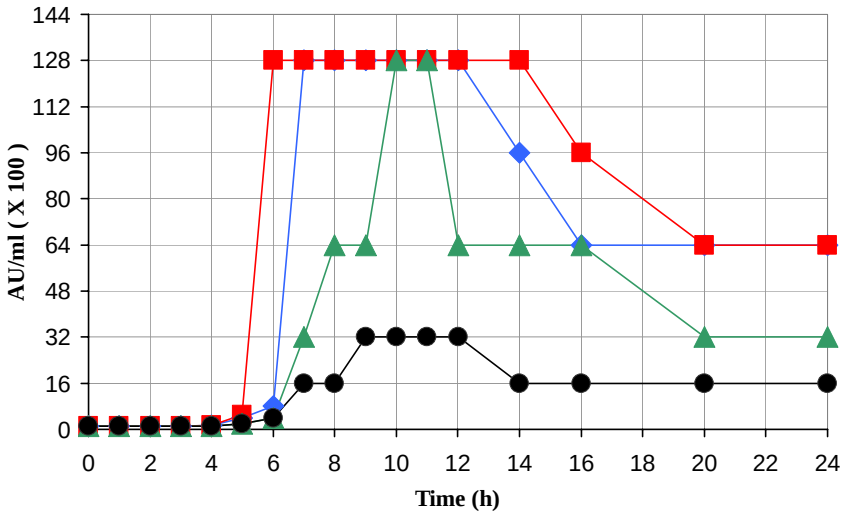
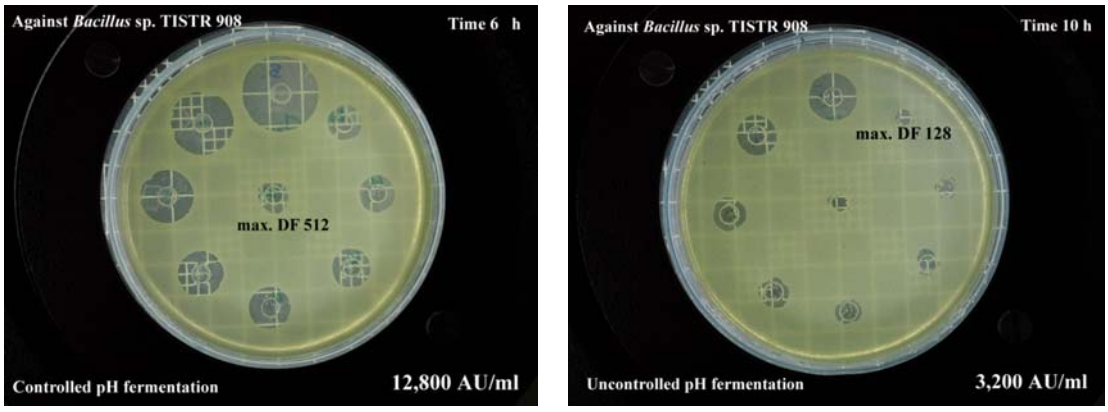


Figure 3.4. Bacteriocins activity of *Lactococcus lactis* TISTR 1401 during growth. ●, AU/ml with no pH control imposed; ◆, constant pH of 6.0; ■, constant pH of 6.5; ▲, constant pH of 7.0.

The effect of pH on bacteriocin production has been well documented, control of pH during growth results in higher bacteriocin titers (Yang and Ray, 1993; Bogovič-Matijašić and Rogelj, 1998; Morgan et al., 1999; Morgan, Galvin, Ross, and Hill, 2001). Bacteriocin activity produced by *Lc. lactis* TISTR 1401 increased dramatically at pH 6.5 and reached the maximum at 12,800 AU/ml in a shorter time than at pH 6.0 and 7.0. The results of control pH of bacteriocin production at 6.5 were in agreement with Morgan et al. (1999). Moreover, bacteriocin produced at pH 6.5 could be suitable for incorporation to a wide range of foods especially in meat products. The results shown in Figure 3.2, 3.3 and 3.4, high concentrations of bacteriocins were produced during the active growth or log phase to the initial stationary phase according to the studied of Morgan et al. (1999), Yang and Ray (1993), Mataragas et al. (2002) and Onda et al. (2003).



(A) **(B)**

Figure 3.5 The inhibition activity of *Lactococcus lactis* TISTR 1401 in MRS broth determined by agar well diffusion assay: (A), inhibition activity at 6 h, controlled pH at 6.5; (B), inhibition activity at 10 h, uncontrolled pH.

Therefore, fermentation of bacteriocin-producing bacteria at controlled pH of 6.5 was considered as more suitable than at pH 6.0 and 7.0. In addition, when fermentation performed at pH 6.5, the fermented media had the maximum bacteriocin activity last for 8 h while at pH 6.0, 7.0 and uncontrolled pH, the activity last only 5, 1 and 3 h, respectively. Decreasing of bacteriocin activities was observed at 11, 14, 12 and 12 h for the fermented media controlled pH at 6.0, 6.5, 7.0 and uncontrolled pH, respectively (Figure 3.4). Decrease in bacteriocin activity was observed during the late of fermentation for both controlled and uncontrolled pH media. O' Keeffe and Hill (1999) described this phenomena as due to the protein degradation, adsorption to cell surface, protein aggregation and complex formation.

3.4.4 Inhibitory spectrum of bacteriocins produced by *Lactococcus lactis* TISTR 1401

The antibacterial activity of the bacteriocin supernatant produced by *Lc. lactis* TISTR 1401 against 16 indicator bacteria is shown in Table 3.2. The inhibition activity was positively observed of 8 from 13 strains of Gram-positive indicator bacteria. However, all of three Gram-negative indicator bacteria, *E. coli*, *Pseudomonas aerogenosa*, *Enterococcus aerogenosa* were not sensitive to bacteriocin supernatant. The most sensitive bacteria was *Bacillus* sp. TISTR 908, with 23.6 mm of inhibition zone. From earlier research works, some of *Lactococcu*s strains including *Lc. lactis* JCM 7638 showed inhibitory activity against following bacteria: *L. monocytogenes* (Cai et al., 1997; Morgan et al., 1999; Con and Gökalp, 2000; Moreno et al., 2000), *B. cereus* (Morgan et al., 1999), *S. aureus* (Morgan et al., 1999; Con and Gökalp, 2000), *Clostridium* spp. (Park et al., 2003), *E. coli* (Con and Gökalp,

Table 3.2. Inhibitory spectrum of bacteriocins produced by *Lactococcus lactis* TISTR 1401

Indicator bacteria	Strain	Origin	Average zone (mm)	Inhibition activity
Gram-positive bacteria				
Lactobacillus plantarum	050	TISTR	12.3	++
Pediococcus acidilactici	051	TISTR	8.3	+
Leuconostoc mesenteriodes	053	TISTR	19.9	++
Lactobacillus acidophilus	450	TISTR	-	-
Lactobacillus brevis supsp. *brevis*	860	TISTR	-	-
Lactobacillus delbruckii subsp. *bulgaricus*	892	TISTR	-	-
Lactobacillus sake	911	TISTR	13.4	++
Lactobacillus casei spp. *rhamnosus*	SN11	PSU	-	-
Lactococcus lactis	1401	TISTR	-	-
Staphylococcus aureus	118	TISTR	10.0	+
Bacillus subtilis	008	TISTR	10.5	++
Bacillus cereus	687	TISTR	16.0	++
Bacillus sp.	908	TISTR	23.6	+++
Gram-negative bacteria				
Escherichia coli	780	TISTR	-	-
Pseudomonas aerogenosa	-	SUT	-	-
Enterococcus aerogenosa	-	SUT	-	-

Symbol: -, No inhibition zone: +, small inhibition zone (7-10 mm): ++, intermediate inhibition zone (10-20 mm): +++, large inhibition zone (20-30 mm).

2000), *Weisella spp*. (Park et al., 2003). However, some of the LAB isolates did not show antimicrobial activity on agar well diffusion but its antimicrobial was shown on agar spot test, as reported by Schillinger and Lücke (1989) and Con and Gökalp (2000).

Selection of indicator bacteria for the study of bacteriocin activity is dependent on the target organisms of food product. There are many groups of indicator bacteria has been used such as *L. innocua* MP 2418 (Schneider et al., 2006), *L. monocytogenes* Scott A and *S. aureus* DPC 10 (Morgan et al., 1999), *Lb. acidophilus* A201 7 (Yamato, Ozaki, and Ota, 2003), *Weisella paramesenteroides* (Delgado, Brito, Peres, Noé-Arroyo, and Garrido-Fernández, 2005), *Lc. lactis* subsp. *lactis* ATCC 19435 (Zendo, Koga, Shigeri, Nakayama, and Sonomoto, 2006), *Lb. delbrueckii* subsp. *lactis* ATCC 4797 (Ekinci and Barefoot, 2006), *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 (Avonts et al., 2004), *Lb. plantarum* NCDO 955 (Park et al., 2003), *Lb. helveticus* ATCC 15009 (Bogovič-Matijašić and Rogelj, 1998**)** and *B. cereus* (Morgan et al., 2001), etc. In this study, strain of *Bacillus* sp. TISTR 908 was used as indicator bacteria. Species of *Bacillus* was considered as the contaminant species of ingredients for meat products, especially in pork meatball production because its spores which normally found in wheat and tapioca flour (Berghofer, Hocking, Miskelly, and Jansson, 2003).

3.4.5 Effect of heat treatment

The effect of heat treatment on bacteriocin activity was determined using *Bacillus* sp. TISTR 908 as indicator bacteria. The inhibitory substance produced by *Lc. lactis* TISTR 1401 was considered to be nisin Z, molecular weight about of 4 kDa

and heat stable (Park et al., 2003). CBS produced by *Lc. lactis* TISTR 1401 was considered to be heat stable, as the inhibition activity (3,200 AU/ml) remained constant after heating at 80 ^{o}C for 20 min, but a half decreased in activity was observed when heating at 90 ^{o}C from 10 to 20 min (Table 3.3). Heating at 100 ^{o}C for 20 min, the activity decreased down to 800 AU/ml, therefore, when CBS was autoclaved (at 121 ^{o}C for 20 min) its activity remained only 100 AU/ml. This result indicated that crude bacteriocin produced by *Lc. Lactis* TISTR 1401 was heat stable at the temperature under 100 ^{o}C. Bacteriocins obtained from this study could be used in food production especially in meat products that processes under 90 ^{o}C.

Table 3.3 Effect of heat treatment to bacteriocin activity of CBS produced by *Lactococcus lactis* TISTR

Temperature (^{o}C)	**Time (min)**	**Inhibition activity (AU/ml)**
70	10	3,200
	15	3,200
	20	3,200
80	10	3,200
	15	3,200
	20	3,200
90	10	1,600
	15	1,600
	20	1,600
100	10	1,200
	15	1,200
	20	800
121	20	100
Control	-	3,200

3.4.6 pH and enzyme sensitivity

Bacteriocins differ greatly with regard to their sensitivity to inactivation by changes in pH and enzyme. Many are stable only in acid and neutral condition, and are even inactivated at pH 8.0 such as nisin. Nisin shows increase solubility in an acid environment and becomes less soluble as the pH increases. Nisaplin, the commercial preparation of nisin, contained approximately 2.5 % of nisin. Nisaplin solution is stable at high temperature in an autoclave up to 121 ^{o}C for 15 min in the pH range 3.0-3.5 (< 10 % activity loss) whereas pH values below and above this range cause a marked decrease in activity (Davies and Delves-Broughton, 1999). Decrease in solubility of nisin might directly affect the decrease in antibacterial activity. In this study, the effect of pH and enzymes on activity of bacteriocins was carried out using *Bacillus* sp. TISTR 908 as indicator bacteria. It was observed that bacteriocins produced by *Lc. lactis* TISTR 1401 were stable at pH below 7 while the activity remained constant at 6,400 AU/ml (Table 3.4). When the pH treatment was adjusted at 8, then a half reduced in activity was observed. Moreover, the antibacterial activity of bacteriocin supernatant was completely loss when pH treatment was at 9 or above. From this study, application of a neutral pH stable bacteriocins in low acid food especially non fermented meat products should be very interesting.

For enzyme treatment, the antibacterial activity was completely loss on treatment with proteinase. However, catalase had no reduction in the antibacterial activity compared with the untreated sample. These indicated that bacteriocin supernatant produced by *Lc. lactis* TISTR 1401 was a proteinaceous and also rules out the possible role of hydrogen peroxide in bringing about the inhibition on sensitive bacteria (Suma et al.,1998; Schneider et al., 2006). It was stable during frozen storage

and slightly decrease during stored at 4 ^{o}C for a month (data not shown), which was in agreement with Schillinger and Lücke (1989).

Table 3.4 Effect of pH and enzyme treatment to bacteriocin activity of CBS produced by *Lactococcus lactis* TISTR 1401.

pH / Enzyme Treatment	**Inhibition activity (AU/ml)**
Control	6,400
2	6,400
3	6,400
4	6,400
5	6,400
6	6,400
7	6,400
8	3,200
9	0
10	0
11	0
12	0
Catalase	6,400
Proteinase	0

3.5 Conclusions

From this study, LAB obtained from TISTR and PSU provided bacteriocin-producing ability. Strains of *P. acidilactici* TISTR 051, *Leu. mesenteroides* TISTR 053, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* subsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911 and *Lb. casei* spp. *rhamnosus* SN11 did not produce bacteriocins when group of *Bacillus* species and *S. aureus* TISTR 118 were used as indicator bacteria. Bacteriocin-producing LAB in this study were *Lb. plantarum* TISTR 050, *Lb. acidophilus* TISTR 450 and *Lc. lactis* TISTR 1401. It was obvious that strain of *Lc. lactis* TISTR 1401 was the most suitable strain for bacteriocin production.

Bacteriocins of *Lc. lactis* TISTR 1401 showed the inhibition activity against some of Gram-positive indicator bacteria, but none against Gram-negative bacteria. The optimum temperature for growth of *Lc. lactis* TISTR 1401 was 37 ^{o}C which was in agreement with the recommendation of TISTR. Fermentation of this bacterial culture in MRS broth supplemented with 2 % (w/v) yeast extract and 2 % (w/v) glucose and meat extract at constantly controlled pH of 6.5 proved to give the maximum cell growth with the highest bacteriocin activity. Antibacterial substance produced by *Lc. lactis* TISTR 1401 was a heat-stable, proteinaceous and stable in range from acid to neutral pH.

3.6 References

Aslim, B., Yuksekdag, Z.N., Sariyaka, E., and Beyatli, Y. (2005). Determination of bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products. **LWT.** 38 (6): 691-694.

Avonts, L., Uytven, E.V., and Vuyst, L.D. (2004). Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. **Int. Dairy J.** 14 (11): 947-955.

Berghofer, L.K., Hocking, A.D., Miskelly, D., and Jansson, E. (2003). Microbiology of wheat and flour milling in Australia. **Int. J. Food Microbiol.** 85: 137-149.

Bogovič-Matijašić, B., and Rogelj, I. (1998**).** Bacteriocin complex of *Lactobacillus acidophilus* LF221-production studies in MRS media at different pH values and effect against *Lactobacillus helveticus* ATCC 15009. **Process Biochem.** 33 (3): 345-352.

Buncic, S., Avery, S.M., Rocourt, J., Dimitrijevic, M. (2001). Can food-related environmental factors induce different behaviour in two key serovars, 4b and 1/2a, of Listeria monocytogenes?. **Int. J. Food Microbiol.** 65: 201-212.

Cai, Y., Ng, L.K., and Farber, J.M. (1997). Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean-sprouts. **J. Appl. Microbiol.** 83 (4): 499-507.

Chin, H.S., Shim, J.S., Kim, J.M., Yang, R., and Yoon, S. (2001). Detection and antibacterial activity of a bacteriocin produced by *Lactobacillus plantrum*. **Food Sci. Biotechnol.** 10 (4): 335-341.

Çon, A.H. and Gökalp, H.Y. (2000). Production of bacteriocin-like metabolites by lactic acid cultures isolated from sucuk samples. **Meat Sci.** 55 (1): 89-96.

Devies, E.A. and Delves-Broughton, J. (1999). Bacteriocins/nisin**. Encyclopedia of food Microbiology** (vol.1) (pp 191-198). New york: Academic press.

Delgado, A., Brito, D., Peres, C., Noé-Arroyo, F., and Garrido-Fernández, A. (2005). Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. **Food Microbiol.** 22 (6): 521-528.

Deegan, L.H., Cotter. P.D., Hill, C., and Ross, P. (2006). Bacteriocin: Biological tools for bio-preservation and shelf-life extension. **Int. Dairy J.** 16 (9): 1058-1071.

Ekinci, F.Y. and Barefoot, S.F. (2006). Fed-batch enhancement of jenseniin G, a bacteriocin produced by *Propionibacterium thoenii* (*jensenii*) P126. **Food Microbiol.** 23 (4): 325-330.

Enan, G., El-Essawy, A.A., Uyttendaele, M., and Debevere, J. (1996). Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. **Int. J. Food Microbiol.** 30 (3): 189-215.

Harlander, S.K. (1993). Regulatory aspects of bacteriocin use. **Bacteriocins of lactic acid bacteria.** Academic Press.11: 223-247.

Kelly, W.J., Asmundson, R.V., and Huang, C.M. (1996). Isolation and characterization of bacteriocin-producing lactic acid bacteria from ready-to-eat food products. **Int. J. Food Microbiol.** 33 (2-3): 209-218.

Mataragas, M., Metaxopoulos, J., Galiotou, M., and Drosinos, E.H. (2003). Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. **Meat Sci.** 64 (3): 265-271.

Matsusaki H., Chinachoti, N., Sonomoto, K., and Ishizaki, A. (1998) Purification, identification, and effective production of a peptide antibiotic produced by *Lactococcus lactic* OI-1 (JCM 7638). **Annals of the New York Academy of Sciences.** 864 (1): 422–427.

Mayrhofer, S., Paulsen, P., Smulders, F.J.M., and Hilbert, F. (2004). Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. **Int. J. Food Microbiol.** 97 (1): 23-29.

Moreno, I., Lerayer, A.L.S., Baldini, V.L.S., and Leitão, M.F. de F. (2000). Characterization of bacteriocin produced by *Lactococcus lactis* strains. **Brazilian J. Microbiol.** 31: 182-192.

Morgan, S.M., Galvin, M., Kelly, J., Ross, R.P., and Hill, C. (1999). Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. **J. Food Prot.** 62 (9): 1011-1016.

Morgan, S.M., Galvin, M., Ross, R.P., and Hill, C. (2001). Evaluation of a spray-dried Lacticin 3147 powder for the control of *Listeria monocytogenes* and *Bacillus cereus* in a range of food systems. **Lett. Appl. Microbiol.** 33 (5): 387-391.

Nieto-Lozano, J.C., Reguera-Useros, J.I., Peláez-Martínez, M.C., and Torre, A.H. (2006). Effect of a bacteriocin produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* on Spanish raw meat. **Meat Sci.** 72 (1): 57-61.

Ogunbanwo, S.T., Sanni, A.I., and Onilude, A.A. (2003). Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. **African J. Biotechnol.** 2 (8): 219-227.

O'keeffe, T. and Hill, C.(1999). Bacteriocins/Potential in food preservation**. Encyclopedia of food Microbiology** (vol.1) (pp 183-191). New york: Academic press.

Onda, T., Yanagisa, F., Tsuji, M., Shinohara, T., and Yokotsuka, K. (2003). Production and purification of a bacteriocin peptide produced by *Lactococcus* sp. Strain GM005, isolated from *Miso*-paste. **Int. J. Food. Microbiol.** 87 (1-2): 153-159.

O'Sullivan, L.O., Ross, R.P., and Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. **Biochemie.** 84: 593-604.

Parente, E. and Ricciardi, A. (1999). Production, recovery and purification of bacteriocins from lactic acid bacteria. **Appl. Microbiol. Biotechnol.** 52 (5): 628-638.

Park, S., Itoh, K., Kikuchi, E., Niwa, H., and Fujisawa, T. (2003). Identification and characteristics of nisin Z-producing *Lactococcus lactis* subsp. *lactis* isolatedfrom Kimchi. **Current microbiology.** 46: 385-388.

Paynter, M.J.B., Brown, K.A., and Hayasaka, S.S. (1997). Factors affecting the production of an antimicrobial agent, plantaricin F, *Lactobacillus plantarum* BF001. **Lett. Appl. Microbiol.** 24 (3): 159-165.

Rossland, E., Langsrud, T., Granum, P.E., and Sorhaug, T. (2005). Production of antimicrobial metabolites by strains of *Lactobacillus* or *Lactococcus* co-cultured with *Bacillus cereus* in milk. **Int. J. Food Microbiol.** 98 (2): 193-200.

Schillinger, U. and Lücke F. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. **Appl. Envi. Microbiol.** 55 (8): 1901-1906.

Schneider, R., Fernádez, F.J., Aguilar, M.B., Guerrero-Legarreta, I., Alpuche-Solis, A., and Ponce-Alquicira, E. (2006). Partial characterization of a class IIa pediocin produced by *Pediococcus parvutus* 133 strain isolated from meat (Mexican 'chorizo'). **Food Control.** 17 (11): 909-915.

Suma, K., Misra, M.C., and Varadaraj, M.C. (1998). Plantaricin LP84, a broad spectrum heat-stable bacteriocin of *Lactobacillus plantarum* NCIM 2084 produced in a simple glucose broth medium. **Int. J. Food Microbiol.** 40 (1-2): 17-25.

Tarelli, G.T., Carminati, D., and Giraffa, G. (1994). Production of bacteriocins active against *Listeria monocytogenes* and *Listeria* innocua from dairy *enterococci*. **Food Microbiol.** 11 (3): 243-252.

Todorov, S.D. and Dick, L.M.T. (2005). Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a strain isolated from Boza. **Food Technol. Biotechnol.** 43 (2): 165-173.

Yamato, M., Ozaki, K., and Ota, F. (2003). Partial purification and characterization of the bacteriocin produced by *Lactobacillus acidophilus* YIT 0154. **Microbiol. Res.** 158 (2): 169-172.

Yang, R. and Ray, B. (1994). Factors influencing production of bacteriocins by lactic acid bacteria. **Food Microbiol.** 11 (4): 281-291.

Zendo, T., Koga, S., Shigeri, Y., Nakayama, J., and Sonomoto, K. (2006). Lactococcin Q, a novel two-peptide bacteriocin produced by *Lactococcus lactis* QU 4. **Appl. Environ. Microbiol.** 72 (5): 3383-3389.

CHAPTER IV

APPLICATION OF BACTERIOCINS PRODUCED BY *LACTOCOCCUS LACTIS* TISTR 1401 FOR STORAGE EXTENSION OF PORK MEATBALLS

4.1 Abstract

The objective of this study was to extend the shelf life of pork meatballs by using crude bacteriocins as a preservative. Crude bacteriocins produced by *Lactococcus lactis* TISTR 1401 with constantly controlled pH at 6.5 during fermentation was applied to preserve pork meatballs by coat dipping in pork meatballs surface in comparison with untreated pork meatballs. The treated meatballs were coated with full strength and half strength of the crude bacteriocin supernatant (CBS), aerobically packed and vacuum packed, stored in a cold room at 4 ^{o}C for 12 days. At day 12, the full strength CBS treatment showed the highest inhibition activity against total aerobic bacteria with microbial counts of 1.96 and 1.42 log cfu/g lower than that of control treatment, and 1.71 and 1.30 log cfu/g lower than that of half strength treatment for both aerobically and vacuum packed, respectively. Lactic acid bacteria were detected only at day 12 while species of *Brochothrix thermosphacta* was not detected throughout the storage period. *Pseudomonas* and *Enterobacteriaceae* were found slightly decreased at day 12 in vacuum packed samples. However, this observation was not found in aerobically packed samples, treatment of bacteriocins

could not inhibit the growth of *Pseudomonas* and *Enterobacteriaceae*. The pH and total titratable acidity of the meatballs of all treatments did not change throughout storage period, pH and titratable acidity were almost constant at 6.5 and 0.3%, respectively. Color measurement with L, a, b Hunter color system, No significant differences were found for the L and a values in all treatments during storage. However, the pork meatballs coated with CBS gave higher b values ($P < 0.05$) than did control samples, indicating that bacteriocins gave more yellowness to the meatballs. By sensory evaluation, treatments with CBS did not affect water purge, spoiled odor, compared with control treatment. However, the CBS gave strong abnormal odor of the culture media used for bacteriocin production. No significant differences were found for overall acceptance among the treatments, with slightly more acceptance for control samples over the CBS treated ones.

Keywords: bacteriocins, *Lactococcus lactis*, meatballs

4.2 Introduction

Bacteriocins produced by *Lactococcus* species, especially nisin, are widely used in European countries since the lactic acid bacteria have generally been regarded as safe (GRAS status). Bacteriocins from LAB have attracted particular attention in recent years in an attempt to develop their potential commercial application. Nisin is the only commercially produced bacteriocin from *Lactococcus lactis* for using in over 48 countries which approved by Food and Drug Administration (FDA). Nisin has been shown to be effective in a number of food systems, inhibiting the growth of a wide range of Gram-positive bacteria, including many important foodborne pathogens (Deegan, Cotter, Hill, and Ross, 2006). Concern about the toxicological safety of

chemical preservative in food products, nisin has been used to allow a reduction in chemical preservative level such as sodium benzoate or potassium sorbate.

During the last decade, numerous papers have been published on the used of biopreservation including examination from different lactic acid bacteria having ability to produce bacteriocins. Some of the bacteriocins were able to inhibit the growth of *L. monocytogenes* in meat and meat products (Holzapfel, Geisen, and Schillinger, 1995; Hugas, 1998). Nisin has been found for many applications in the food industries. However, nisin is not widely used in meat products, uneconomically high levels are required to achieve good control of *Clostridium botulinum*, perhaps as a consequence of nisin binding to meat particles, uneven distribution, poorly solubility in meat systems, or possibly interference in activity by meat phospholipids (O'keeffe and Hill, 1999). A major problem on the applications of bacteriocins in meat products is the binding of bacteriocin peptide with meat proteins during heat process, whereas, applied of bacteriocin solution on surfaces of meat products after heat process should be considered as the alternative way of application.

Pork meatball products are economically important meat products with a high consumption in Thailand. Commercially, pork meatballs are mostly aerobically packed in plastic bags. However, those sold in supermarket (especially for premium customer) are normally packed in vacuum condition. In general, the meatballs either aerobically packed or vacuum packed have a shelf life labelled for 15 days at 0-5 ^{o}C. Since in the process of making meatballs, the batter is heated to the temperatures of about 80 – 90 ^{o}C only, most vegetative cells are killed. Recontamination and growth of microorganisms could take place in cooling and packing steps. In general, product handling of meat products after cooking could cause recontamination of about 0.5-2

log cfu/g of total bacteria, mainly lactic acid bacteria (LAB) (Samelis, Kakouri, and Ramentzis, 2000).

To control microbial survival and out growth of microorganisms in foods, food preservation procedures are used. Tapioca and wheat flour are usually used in pork meatball production, to increase water holding capacity and also increase production yield. Since these flours are normally contaminate with large number of *Bacillus* spp. (Berghofer, Hocking, Miskelly, and Jansson, 2003), after heating process, heat stable spores of *Bacillus* spp. still remain in food product. However, *Bacillus sp.* has been shown to be the most sensitivity to the bacteriocins produced by *Lactococcus lactis* TISTR 1401 according to the results of previous study in Chapter III. Other bacteria species that could be responsible to the spoilage of pork meatballs include LAB, *Pseudomonas, Enterobacteriaceae* and *Brochothrix thermosphacta*. Species of *Pseudomonas* were identified as the strictly aerobic spoilage microbial in meat and meat products (Viana, Gomide, and Vanetti, 2005). The *Enterobacteriaceae* are a large family of the human intestinal microflora including many of the more familiar pathogens, such as *Salmonella* and *Escherichia coli*. Species of *Brochothrix thermosphacta* is also found to be a numerical significant component of the microflora of meat and meat products stored under vacuum packed (Samelis et al., 2000), this bacterium changes from a major contaminant to a minor part of the final population after storage (Cayré, Garro, and Vignolo, 2005).

In this study, the growth of total aerobic bacteria, *Enterobacteriaceae*, *Pseudomonas*, LAB and *Brochothrix thermosphacta* on pork meatballs in aerobically packed and vacuum packed conditions, stored at 4 ^{o}C were monitored in order to evaluate the efficacy of crude bacteriocins produced by *Lactococcus lactis* TISTR

1401. The pork meatballs were treated with crude bacteriocins in different concentrations and stored for 12 days.

4.3 Materials and methods

4.3.1 Bacterial strains and culture conditions

The bacteriocin producer *Lc. lactis* TISTR 1401 and indicator bacteria *Bacillus* sp. TISTR 908 was obtained from Microbiological Resource Center, Thailand Institute of Scientific and Technological Research (TISTR). *Lc. lactis* TISTR 1401 culture was stored at -40 ^{o}C in 10 % (w/v) skim milk powder and re-activated by transferring 1 ml of stock culture to 5 ml MRS broth (Hi-media, Mumbai, India). The culture was incubated overnight at room temperature, shaked 50 rpm on shaker (Unimax 100, Heidolph, Schwabach, Germany), then transferred to 25 ml MRS broth and incubated in a shaking incubator (Orbital Incubator S-150, Stuart Sciencetific, UK) at 37 ^{o}C. In addition, indicator bacteria *Bacillus* sp. TISTR 908 was re-activated in Typtic soy broth, TSB (Hi-media, Mumbai, India).

4.3.2 Preparation of bacteriocin of *Lactococcus lactis* TISTR 1401

Bacteriocin producer strain *Lc. lactis* TISTR 1401 was re-activated overnight, then 1 % (v/v) of the culture was inoculated in 2 liters of MRS broth supplemented with 2 % (w/v) yeast extract (Hi-media, Mumbai, India), 0.2 % (w/v) glucose (Ajax Finechem, Australia) and 0.2 % (w/v) meat extract (Merck, Darmstadt, Germany) according to Nieto-Lozano, Reguera-Useros, Peláez-Martínez, and Torre. (2005). The fermentation was performed in a stainless bioreactor, micro-aerobically, slow agitation at 100 rpm, at 37 ^{o}C. The pH of fermentation was constantly maintained at

6.5 by addition of 1 M NaOH via a 718 STAT Titrino (Metrohm, Ireland) according to Morgan, Galvin, Kelly, Ross, and Hill (1999). Harvesting of bacteriocin was performed when the cell population reached the maximum during initial-stationary phase by monitoring the absorbance at 600 nm. The fermented broth was centrifuged at 12,000 x g at 4 ^{o}C for 20 min (Sorvall® RC SC-plus centrifuge, Thermo Sciencetific, Waltham, USA), The supernatant was neutralized pH to 6.5 with 1 M NaOH. Catalase from bovine liver (Sigma Chemical Co., St. Louis, MO, USA) was added at 1 mg/ml final concentration, incubated for 30 min at room temperature. To eliminate the residual organisms, the supernatant containing bacteriocins was heated at 80 ^{o}C for 20 min in an autoclave (LABO Autoclave, Sanyo, NB Scientific, Edison, N.J.), cooled in ice immediately. Crude bacteriocin supernatant (CBS) was stored at 4 ^{o}C until use.

4.3.3 Bacteriocin assay and activity determination

Bacteriocin activity was determined by the agar well diffusion method according to Mogan et al. (1999), with slightly modified. The culture of *Bacillus* sp. TISTR 908, 1 % (v/v), was inoculated into 15 ml of molten seeded agar (Tryptic soy soft agar, containing 0.75 % (w/v) agar). A seeded agar was overlaid on NA (Nutrient agar, Merck, Darmstadt, Germany). Wells of approximately 6 mm were bored in the seeded agar. A 40 µL volume of a two-fold serial dilution of a prepared CBS was dispensed into each well as duplicate. The plates were kept at 4 ^{o}C for 2 h for pre-diffusion, then incubated at 37 ^{o}C for 24 h (Buncic, Avery, Rocourt, and Dimitrijevic, 2001; Suma, Misra, and Varadaraj, 1998). Calculation of bacteriocin activity was

performed as the inverse of the last dilution that gave a clear zone of inhibition, were expressed as arbitrary unit per millilitre (AU/ml).

4.3.4 Pork meatball manufacture

Fresh pork was obtained from a local market in Nakhon Ratchasima Province. The meatball batter ingredients containing 10 kg lean pork, 2 % salt, 0.4 % sodium phosphate, 15 % ice and 4 % tapioca starch, were mixed, finely chopped in a chopper machine, formed into meatballs in 60 ^{o}C water for 5 min then in 80 ^{o}C water for 20 min (Figure 4.1 A) and dried at room temperature for 10 min (Figure 4.1 B). The meatballs were dipped in defined CBS solution and then cooled in cold room for 10 min.

(A) **(B)**

Figure 4.1 Pork meatball manufacturing in Food processing laboratory, SUT: (A), heating of pork meatballs; (B), drying of pork meatballs at room temperature.

4.3.5 Trials

To investigate the effectiveness of the CBS produced by *Lc. lactis* TISTR 1401 in controlling microbial growth of pork meatballs during storage, trials of aerobically and vacuum packed of coated meatballs were performed compared with the control samples. Half strength of the CBS was prepared by adding the original CBS obtained with one fold of sterile phosphate buffer pH 6.5. The meatball samples were separately packed in polyethylene bag, approximately 30 g/packs as shown in Figure 4.2, stored at 4 ^{o}C during storage time (12 days). Samples were taken for analysis every 3 days.

(A) **(B)**

Figure 4.2 Pork meatballs with control treatment in aerobically (A) and vacuum packed (B).

4.3.6 Enumeration of microbial population

The meatballs were sampled at three day intervals for microbial enumeration for total aerobic plate count, lactic acid bacteria, *Pseudomonas*, *Brochothrix thermosphacta* and *Enterobacter*. The sample of 25 g was homogenized (1:10) with dilution medium (0.1 % peptone water) (Hi-media, Mumbai, India) in a stomacher (Stomacher Lab-blender model 400, Seward, London, England) for 1 min. The serial dilutions of 10-fold solutions were made and plated on the selective media: PCA agar (Hi-media, Mumbai, India) for total aerobic plate count (TAPC) and incubated at 25 ^{o}C for 24-48 h (Nel, Lues, Buys, and Venter, 2004); MRS agar (Hi-media, Mumbai, India) for lactic acid bacteria and incubated at 35 ^{o}C for 24-48 h (Viana et al., 2005); *Pseudomonas* isolation agar (Hi-media, Mumbai, India) for *Pseudomonas* and incubated at 20 ^{o}C for 48 h; STAA (Streptomycin sulphate thallous acetate actidione) (Oxoid, Sydney, Australia) agar for *Brochothrix thermosphacta* and incubated at 25 ^{o}C for 48 h (Cayre et al., 2005); and VRBG (Violet red bile agar with glucose) (Merck, Darmstadt, Germany) agar for Enterobacteriaceae and incubated at 37 ^{o}C for 48 h.

4.3.7 pH and titratable acidity of pork meatballs

The pH of pork meatballs was determined with a pH electrode connected to a 718 STAT Titrino. A pork meatball sample of 10 g was mixed with 10 ml of distilled water prior to pH measurement (Benkerroum, Daoudi, and Kamal, 2003). Titrable acidity of pork meatballs was measured by titration, with NaOH 0.1 M, of a filtrate from a mixture of 10 g of samples with 50 ml of distilled water via a 718 STAT

Titrino at end point pH 7.0 (Castano, García Fontán, Fresno, Tornadijo, and Carballo, 2002).

4.3.8 Color determination

Color determination of pork meatball samples was performed immediately in triplicates per sample by hand color meter (CR-300, Minota Chroma Meter, Minolta, Japan). Color values were presented in terms of L, a, b Hunter Color system.

4.3.9 Sensory evaluation

Sensory evaluation of pork meatballs was performed using the quantitative descriptive analysis (QDA) method with unstructured line score of 10 cm, from less intensity to high intensity (Stone and Sidel, 1985) by 6 trained panelists. Sensory attributes consisted of appearance, color, amount of purge, slime, abnormal, oxidized and spoiled odor and overall acceptance.

4.3.10 Statistical analyses

Statistical analyses were performed using the SAS for windows (SAS Institute Inc., North Carolina, USA, 1999). Each trial was repeated twice and each determination was done in duplicate for microbial analysis and triplicate for chemical analysis. Analysis of variance (P = 0.05) and student *t*-test were performed for comparison of means. Duncan's multiple range test (DMRT) was also performed.

4.4 Results and discussion

4.4.1 Enumeration of microbial population

4.4.1.1 Microbial population of pork meatball in aerobically packed condition.

Bacteria population by total aerobic plate counts (TAPC) of meatball batter were 6.18, 4.98, 4.73, 5.52 and 3.59 log cfu/g for total viable count, *Enterobacteriaceae*, *Pseudomonas* spp., lactic acid bacteria and *Brochothrix thermosphacta*, respectively. The initial bacterial population might be dependent on quality of raw materials, process temperature, ingredients used, type of contaminant bacteria, e.g. (Davies and Board, 1998). The microbial TAPC of meatballs stored at 4 ^{o}C in aerobically packed condition are shown in Table 4.1. TAPC is widely used to determination of bacterial contamination in food (Nel et al., 2004). At day 0, there were no bacteria presented in the samples of both of control and full strength CBS treated, whereas the microbial counts of 4.09 log cfu/g observed in the sample treated with half strength CBS. In addition, the TAPCs of control and half strength CBS treated samples stored from day 3 to day 12 were relatively similar ($P > 0.05$) in numbers ranged from 3.41 to 6.25 log cfu/g. There were no TAPCs observed in the samples treated with full strength CBS in day 0 to day 6 and was only 3.51 log cfu/g observed in day 9. Significant differences were found among treatments in day 12 that the counts of full strength CBS treated was about 2 log cycles lower ($P < 0.05$) than those of control and half strength CBS treated ones, with the counts of 4.30, 6.26 and 6.01 log cfu/g, respectively.

Species of *Enterobacteriaceae* are used as an indicator microorganism for possible faecal contamination in food (Alberle et al., 2001). It was appeared that these species were absent in both control and full strength CBS samples. However, small

numbers were observed in samples treated with half strength CBS. It was suggested that contamination occurred during meatball preparation, but not due to the lack of inhibition ability of the CBS. However, even though no significant differences ($P > 0.05$) were found for *Enterobacteriaceae* counts at all time of storage; at day 12, lower count of about 1 log cycle was observed in sample treated with full strength CBS. The numbers of counts of this bacterial group were 5.28, 5.06 and 4.22 log cfu/g for control, half strength and full strength CBS treated, respectively.

Pseudomonas spp. represents the largest group of bacteria usually present in fresh foods and is also expected to occur in meat. Researches have shown that *Pseudomonas* spp. predominates in fresh meat stored aerobically at chilled temperature, indicating *Pseudomonas* spp. to be psychrophilic (Nel et al., 2004). Pseudomonas was not observed in control samples but observation was made for the sample treated with half strength CBS starting from day 3 to day 12 from 3.83 to 5.02 cfu/g, respectively. For the sample treated with full strength CBS, observation was made in day 12 of 4.23 cfu/g. Most of LAB are responsible for spoilage of meat products such as slime produced by *Leuconostoc* sp. The presence of LAB is shown in Table 4.1. The LAB was absent between day 0 to day 9 however, similar numbers ($P > 0.05$) of the LAB was observed in day 12 for all treatments. Most of bacteriocins produced by LAB are normally extremely active against related species especially groups of Gram-positive bacteria including those in the group of LAB itself. Species of *B. thermosphacta* was not found in the samples of all treatments throughout 12 days of storage. *B. thermosphacta* is normally eliminated at 70 ^{o}C for 5 minute. Therefore, it was possible that heat during cooking the meatballs could be able to kill

this organism. However, Cutter and Siragusa (1998) had demonstrated the significant inhibition activity of nisin against *B. thermosphacta*.

4.4.1.2 Microbial population of pork meatball in vacuum packed condition.

Microbial counts of pork meatballs are shown in Table 4.2. At day 0, there were no microbial counts observed in control and full strength CBS treated samples. However, quite high numbers of 4.88 log cfu/g of microorganisms were observed in the samples treated with half strength of CBS. This could be suggested that contamination occurred during meatball preparation. After 12 day storage in vacuum packed, the microbial counts of control and half strength CBS treated were similar in numbers ($P > 0.05$), 6.16 and 6.04 log cfu/g, respectively and higher when compared with that of full strength CBS treated sample ($P > 0.05$) which was 4.74 log cfu/g.

There were no *Enterobacteriaceae* growth in pork meatballs treated with full strength CBS throughout 12 days of storage while sample treated with half strength CBS contained 3.43 log cfu/g at the beginning of storage. The microbial growth was suppressed till day 6 to day 12 whereas the microbial count were about log 4 cfu/g to log 5 cfu/g with no significant differences. Undetectable of *Enterobacteriaceae* in full strength treatment might be possible because of the effect of bacteriocins at the sufficient concentration. Although, bacteriocins from LAB, nisin, is only active against Gram-positive bacteria but it also inhibits against some groups of Gram-negative bacteria especially in weak cells (Davies and Delves-Broughton, 1999). The growth of *Pseudomonas* spp. was undetectable in full strength CBS treated as similar to that of *Enterobacteriaceae*. For control treatment, growth of *Pseudomonas* spp. was detected after day 6 at 3.86 log cfu/g and then increased in day 12 to 6.36 log

cfu/g. Numbers of this microorganism were found in half strength treated samples from day 0 to day 12 in the range between 4 to 6 log cfu/g. There was no significant difference between control and half strength treatments. However, no growth of *Pseudomonas* spp. was observed during storage.

Growth of the LAB in CBS treated samples were not detected till day 12 which were 3.66 and 3.74 log cfu/g for half strength and full strength BCS treated, respectively. It was observed from day 9 to day 12 for the control samples with the counts of 5.50 and 4.32 log cfu/g, respectively. Species of *B. thermosphacta* was not detected throughout the storage period according to the elimination by heat.

According to results in this study, treatment of bacteriocins at full strength concentration was capable to inhibite growth of aerobic bacteria which determined by aerobic plate counts in both aerobically and vacuum packed conditions. The absence of *B. thermosphacta*, *Pseudomonas* and lactic acid bacteria indicated the high effective cooking process and good sanitation. Half strength treatment gave no satisfied results due to low of inhibition activity. According to the microbial standard count of 1×10^5 cfu/g for meat products, it could be mentioned that full strength CBS treatment of pork meatballs could extend the product shelf life for at least 6 days longer compared with the untreated one.

Table 4.1 Microbial population (log cfu/g) of pork meatballs during storage at 4^{o}C in aerobically packed condition

Storage Time (days)	Aerobic count			*Enterobacteriaceae*			*Pseudomonas sp.*			Lactic acid bacteria			*Brochothrix thermosphacta*		
	C	**H**	**F**	**C**	**H**	**F**	**C**	**H**	**F**	**C**	**H**	**F**	**C**	**H**	**F**
0	-	4.09	-	-	3.53	-	-	-	-	-	-	-	-	-	-
3	3.41	3.51	-	-	-	-	-	-	-	-	-	-	-	-	-
6	4.21	4.78	-	-	4.62	-	-	3.83	-	-	-	-	-	-	-
9	4.80	4.49	3.57	4.87	4.90	-	-	4.82	-	-	-	-	-	-	-
12	6.26[a]	6.01[a]	4.30[b]	5.28	5.06	4.22	-	5.02	4.23	4.72	4.43	4.25	-	-	-

[a] Mean within a row for each treatment (C, H and F) lacking a common letter (a through c) differ significantly ($P < 0.05$).

C: control treatment, H: half strength treatment, F: full strength treatment

- : ND, not detected or less than minimum level of sensitivity of enumeration method.

Table 4.2 Microbial population (log cfu/g) of pork meatballs during storage at 4^{o}C in vacuum packed condition

Storage Time (days)	Aerobic count			*Enterobacteriaceae*			*Pseudomonas*			Lactic acid bacteria			*Brochothrix thermosphacta*		
	C	H	F	C	H	F	C	H	F	C	H	F	C	H	F
0	-	4.88	-	-	3.43	-	-	3.90	-	-	-	-	-	-	-
3	4.35	4.15	-	-	-	-	-	3.89	-	-	-	-	-	-	-
6	5.59	4.25	3.55	4.03	3.87	-	3.86	4.40	-	-	-	-	-	-	-
9	6.72	6.03	3.71	5.76	4.70	-	6.35	6.00	-	5.50	-	-	-	-	-
12	6.16[a]	6.04[a]	4.74[b]	5.45	5.34	-	6.36	4.44	-	4.32	3.66	3.74	-	-	-

[a] Mean within a row for each treatment (C, H and F) lacking a common letter (a through c) differ significantly (P <0.05).

C: control treatment, H: half strength treatment, F: full strength treatment

- : ND, not detected or less than minimum level of sensitivity of enumeration method.

4.4.2 pH and titratable acidity of pork meatballs

Titratable acidity and pH changes of pork meatballs during storage at 25 ^{o}C is shown in Table 4.3 for aerobically packed condition and in Table 4.4 for vacuum packed condition. In this study, pH and titrable acidity exhibited no significant changes ($P > 0.05$) during storage with pH ranged between 6.52 and 6.55 and total titratable acidity ranged between 0.33 to 0.39 % as lactic acid. The pH of pork meatball batter in this study was 6.50 (data not shown) and then constant after processing. At high pH (> 6.0) meat is spoiled more rapidly than at normal pH meat (5.4-5.8) (Borch, Kant-Muermans, and Blixt, 1996), in addition, bacteria able to grow well at high pH including *B. thermosphacta*, *Enterobacteriaceae* and *Lactobacillus* sp.

The pH of cooked meat products may decrease from pH 6.0-6.5 to pH 5.0-5.3 during storage due to the activity of lactic acid bacteria (Dykes, Cloete, and von Holly, 1991; Borch et al., 1996). From results of this study, pH and total titratable acidity were almost constant at 6.5 during storage periods which correlated to the absence of growth of lactic acid bacteria in pork meatball samples. The significant changes of pH and titratable acidity might be observed if the samples were stored longer.

Table 4.3 pH and titrable acidity (%) of pork meatballs during storage at 4 ^{o}C in aerobically packed condition

Storage Time (day)	pH			Titratable acidity (%)		
	C	H	F	C	H	F
0	6.54	6.55	6.53	0.39	0.33	0.37
3	6.54	6.53	6.54	0.36	0.35	0.36
6	6.54	6.53	6.54	0.35	0.38	0.37
9	6.52	6.52	6.54	0.35	0.36	0.35
12	6.54	6.53	6.53	0.36	0.35	0.37

C: control treatment, H: half strength treatment, F: full strength treatment.

Table 4.4 pH and titrable acidity (%) of pork meatballs during storage at 4 ^{o}C in vacuum packed condition

Storage Time (day)	pH			Titratable acidity (%)		
	C	H	F	C	H	F
0	6.54	6.55	6.53	0.35	0.34	0.37
3	6.54	6.53	6.54	0.37	0.36	0.35
6	6.54	6.53	6.54	0.34	0.36	0.37
9	6.52	6.52	6.54	0.35	0.37	0.35
12	6.54	6.53	6.53	0.37	0.37	0.35

C: control treatment, H: half strength treatment, F: full strength treatment.

4.4.3 Color characteristics of pork meatballs

Color characteristics of pork meatballs in aerobically packed during storage measured in terms of L, a, b Hunter color values are shown in Table 4.5. There were no significant differences among treatments for L and a values of the samples under

aerobically packed condition throughout storage period, with L values ranging from 75.75 to 77.59 and a values ranging from 1.86 to 2.88.

Yellowness of sample was represented with b value. In this study, significant differences were found at day 3. More intensity of yellowness found in samples treated with CBS in both levels of concentrations because of color of MRS broth used for CBS production. Even though there were not statistically different ($P > 0.05$), slightly higher b values were observed for the samples treated with full strength CBS. The b values of half and full strength CBS treated samples were ranging from 8.24 to 8.42 and 8.60 to 9.34, respectively.

For vacuum packed condition (Table 4.6), significant differences were not observed for L and a values throughout storage time with the values ranged from 75.03 to 75.97 and 3.05 to 4.12, respectively. Similar to the aerobically packed samples, the b values of the samples treated with CBS were slightly higher than those of control due to the color of MRS broth used for CBS preparation. Again, the full strength of CBS provided darker color to the meatballs than did the half strength. The b values of meatballs ranged from 7.09 to 7.54, 7.91 to 8.53, and 8.86 to 9.24 for control, half strength and full strength CBS treated samples, respectively.

Table 4.5 Color values (L, a, b Hunter color system) of pork meatballs during storage at 4 ^{o}C in aerobically packed condition

Storage Time (days)	L-value			a-value			b-value		
	C	H	F	C	H	F	C	H	F
0	76.30	75.87	75.75	2.77	3.07	3.05	7.77	8.42	9.34
3	76.77	75.98	75.79	2.82	2.88	2.88	6.93^{b}	8.27^{ab}	8.72^{a}
6	77.35	76.16	76.69	2.57	2.57	2.71	7.25	8.24	8.98
9	77.25	76.01	75.95	1.98	2.53	2.60	7.48	8.39	8.60
12	77.59	76.68	76.75	1.86	2.73	2.58	7.51	8.42	8.74

[a] Mean within a row for each treatment (C, H and F) lacking a common letter (a through c) differ significantly ($P < 0.05$). C: control treatment, H: half strength treatment, F: full strength treatment.

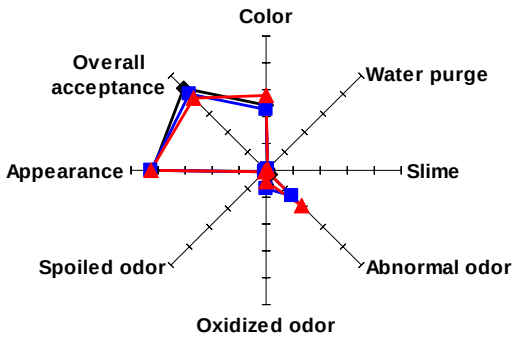
Table 4.6 Color values (L, a, b Hunter color system) of pork meatball during storage at 4 ^{o}C in vacuum packed condition

Storage Time (days)	L-value			a-value			b-value		
	C	H	F	C	H	F	C	H	F
0	76.40	75.25	74.22	3.05	3.28	3.49	7.53	8.53	9.24
3	75.97	75.90	74.78	3.68	3.40	3.56	7.09^{b}	8.40^{ab}	8.92^{a}
6	76.29	75.34	75.03	3.59	3.66	3.44	7.15	8.32	9.03
9	75.78	75.13	74.86	3.74	3.96	3.64	7.42	8.37	8.86
12	76.49	75.19	75.11	3.76	4.12	3.66	7.54^{b}	7.91^{b}	9.03^{a}

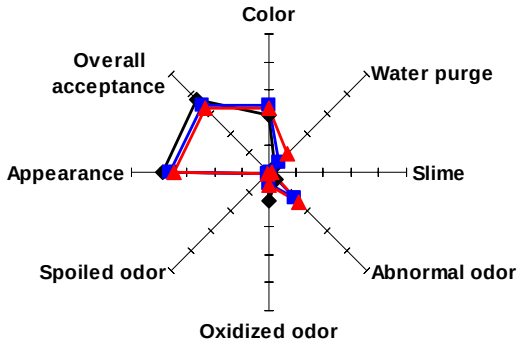
[a] Mean within a row for each treatment (C, H and F) lacking a common letter (a through c) differ significantly ($P < 0.05$). C: control treatment, H: half strength treatment, F: full strength treatment.

4.4.4 Sensory characteristics of pork meatballs

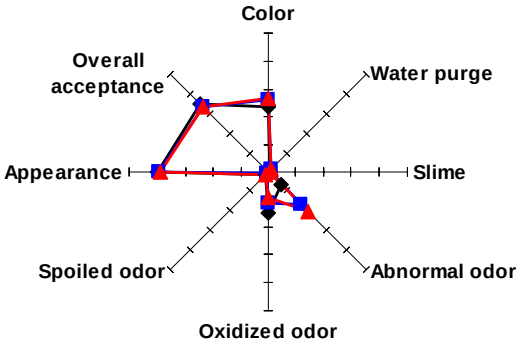
Decision of customer to choose the meatballs is mostly based on the appearance including color, purge of water, etc. Keeping color attractiveness is of primary importance since color is the first quality attribute consumers use to evaluate meat quality and therefore plays a major role in influencing purchase decisions. At the first day of storage (day 0), after being packed for 3-4 h and stored at 4 ^{o}C, the pork meatballs were sampled for sensory evaluation and throughout the storage period. Sensory characteristics of all the samples treated differently with and without CBS are presented in Figure 4.3 for aerobically packed and Figure 4.4 for vacuum packed condition. For aerobically packed condition, the appearance, water purge, slime, spoiled odor and oxidized odor of the meatballs among all treatments were not significantly differences. However, slightly darker color of the full strength CBS treated samples was observed over those treated with the half strength and control samples due to the color of the bacterial culture media. The abnormal odor due to the culture media also reported for the CBS treated samples. Oxidized odor of all samples was not found to be significantly different at each time of sampling, increasing of odor intensity was observed along the time of storage. Borch et al. (1996) reported that strong oxidized odor indicating the low quality or nearly expired products. At the end of storage period, the control meatballs had the most acceptance score, this could be due to less culture odor and lighter in color.



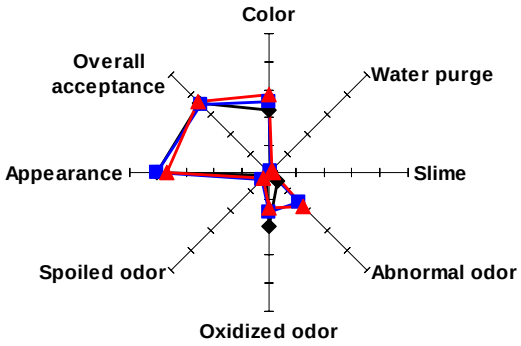
(A) Day 0



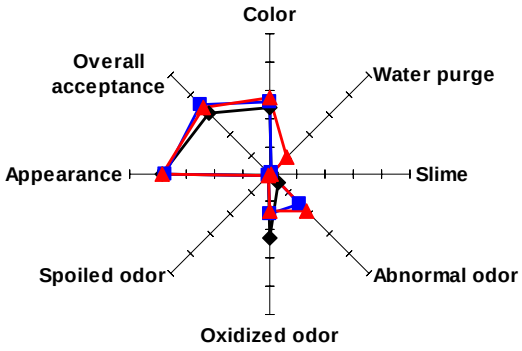
(B) Day 3



(C) Day 6



(D) Day 9



(E) Day 12

Figure 4.3 Sensory characteristics of pork meatballs during storage at 4 ^{o}C in aerobically packed condition: Control treatment (◆), Half strength treatment (■), Full strength treatment (▲). (A), Day 0; (B), Day 3; (C), Day 6; (D), Day 9; (E), Day 12.

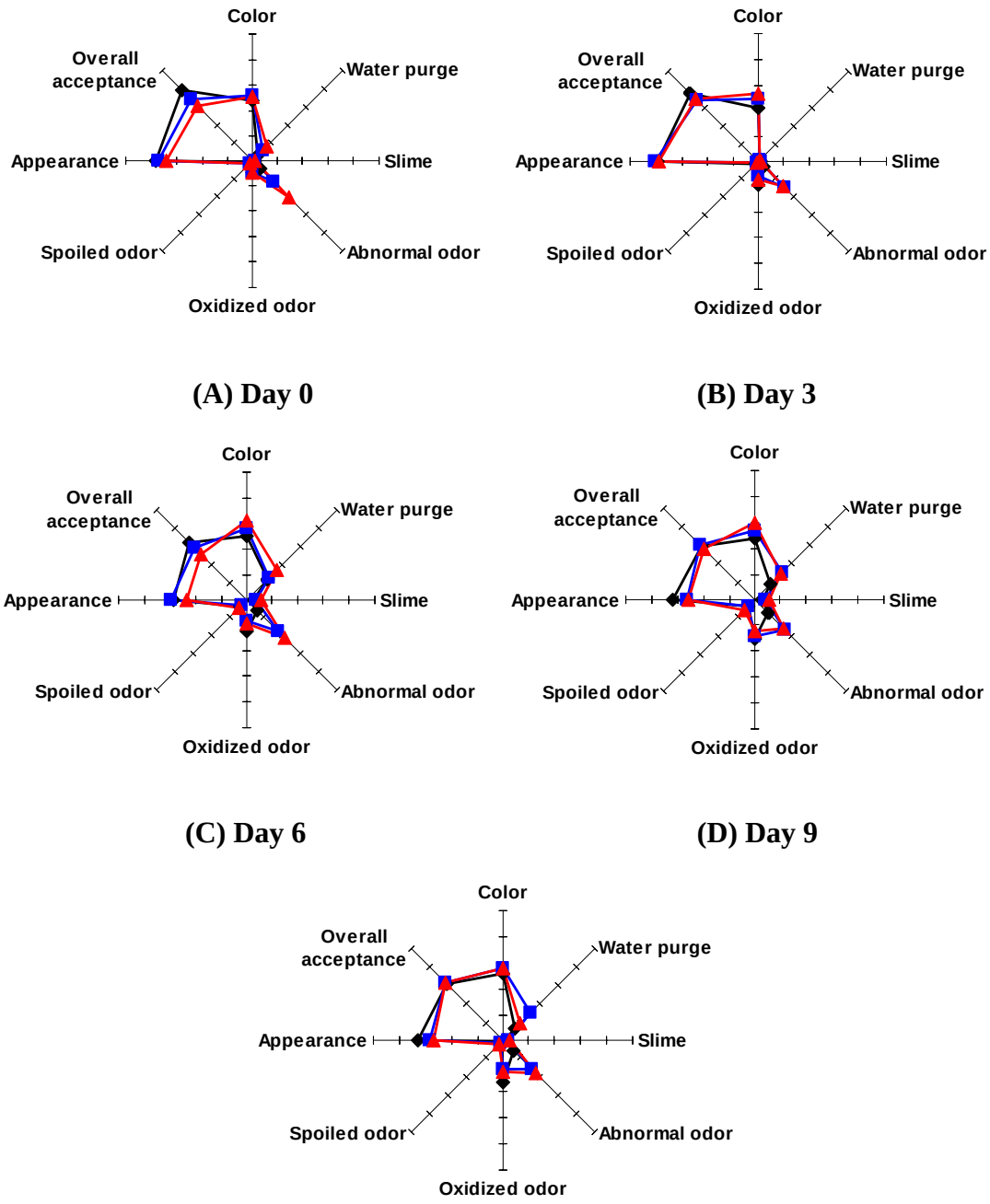


Figure 4.4 Sensory characteristics of pork meatballs during storage at 4 ^{o}C in vacuum packed condition: Control treatment (◆), Half strength treatment (■), Full strength treatment (▲). (A), Day 0: (B), Day 3; (C), Day 6; (D), Day 9; (E), Day 12.

In vacuum packed condition (Figure 4.4), water purge of the CBS treated samples, full strength CBS were reported higher than those of control samples. In general, high water purge indicates higher risk of microbial growth in foods (Hugas, 1998). But in this case, high water purge could be due to the liquid residue left on meatball surface from coating with CBS. Stronger odor of culture media was also reported in the samples treated with CBS, the full strength CBS treated samples obtained the higher score. Significant differences ($P > 0.05$) were not found for slime, oxidized and spoiled odor for all treatments. However, due to the darker color and stronger culture media odor, the pork meatballs treated with CBS were less accepted ($P < 0.05$) than the control meatballs. Therefore, elimination of color and odor from the culture media for bacteriocin production is still needed for further study.

4.5 Conclusions

Crude bacteriocins produced by *Lactococcus lactis* TISTR 1401 with controlled pH at 6.5 was successfully applied to extend the shelf life of pork meatballs. By surface application on the meatballs, stored aerobically packed and vacuum packed condition and kept at 4 ^{o}C, the shelf life of meatballs treated with full strength crude bacteriocin supernatant (CBS) could be extended for 6 days longer than the control meatballs, according to the standard microbial count of 1 x 10^5 cfu/g for meat products. pH and total titratable acidity of the treated meatballs were not significantly different ($P > 0.05$) from control meatballs throughout storage period. Color, measured by L, a, and b values and sensory panelists, of the CBS treated meatballs were darker than those of control meatballs. Darker color and strong odor of the culture media of the CBS caused the panelists to less accept the products

compared with the control one. Therefore, elimination of media color and odor is needed for further study

4.6 References

Benkerroum, N., Daoudi, A., and Kamal, M.(2003). Behaviour of *Listeria monocytogenes* in raw sausages (merguez) in presence of a bacteriocin-producing lactococcal strain as a protective culture. **Meat Sci.** 63: 479-484.

Berghofer, L.K., Hocking, A.D., Miskelly, D., and Jansson, E. (2003). Microbiology of wheat and flour milling in Australia. **Int. J. Food Microbiol.** 85: 137-149.

Borch, E., Kant-Muermans, M., and Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. **Food Microbiol.** 33: 103-120.

Buncic, S., Avery, S.M., Rocourt, J., and Dimitrijevic, M. (2001). Can food-related environmental factors induce different behaviour in two key serovars, 4b and 1/2a, of *Listeria monocytogenes*? **Int. J. Food Microbiol.** 65: 201-212.

Castano, A., García Fontán, M.C., Fresno, J.M., Tornadijo, M.E., and Carballo, J. (2002). Survival of *Enterobacteriaceae* during processing of Chorizo de cebolla, a Spanish fermented sausage. **Food Control.** 13: 107-115.

Cayré, M.E., Garro, O., and Vignolo, G. (2005). Effect of storage temperature and gas permeability of packing film on the growth of lactic acid bacteria and *Brochothrix thermosphacta* in cooked meat emulsions. **Food Microbiol.** 22: 505-512.

Cutter, C.N., and Siragusa, G.R.(1998). Incorporation of nisin into a meat binding system to inhibit bacteria on beef surfaces. **Lett. Appl. Microbiol.** 27: 19-23.

Davies, A. and Board, R. (1998). **The microbiology of meat and poultry.** London. Academic Press.

Deegan, L.H., Cotter, P.D., Hill, C., and Ross, P. (2006). Bacteriocin: Biological tools for bio-preservation and shelf-life extension. **Int. Dairy J.** 16 (9):1058-1071.

Dykes, G.A.,Cloete, T.E., and von Holly, A. (1991). Quantification of microbial populations associated with the manufacture of vacuum-packaged, smoked Vienna sausages. **Int. J. Food. Microbiol.** 13: 239-248.

Holzapfel, W.H., Geisen, R., Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. **Int. J. Food Microbiol.** 24: 343-362.

Hugas, M.(1998). Bacteriogenic lactic acid bacteria for the biopreservation of meat and meat products. **Meat Sci.** 98: 139-150.

Morgan, S.M., Galvin, M., Kelly, J., Ross, R.P., and Hill, C. (1999). Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. **J. Food Prot.** 62 (9): 1011-1016.

Nel, S., Lues, J.F.R., Buys, E.M., and Venter, P. (2004).Bacterial populations associated with meat from the deboning room of a high throughput red meat abattoir. **Meat Sci**. 66: 667-674.

Nieto-Lozano, J.C., Reguera-Useros, J.I., Peláez-Martínez, M.C., and Torre, A.H. (2006). Effect of a bacteriocin produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* on Spanish raw meat. **Meat Sci.** 72(1): 57-61.

O'keeffe, T. and Hill, C.(1999). Bacteriocins/Potential in food preservation**.** **Encyclopedia of food Microbiology** (vol.1) (pp 183-191). New York: Academic press.

Samelism L.K., Kakouri, A., and Ramentzis, J. (2000). Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4°C. **Food Microbiol.** 17 (3): 329-340.

Schillinger, U. and Lücke F. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. **Appl. Envi. Microbiol.** 55 (8): 1901-1906.

Stone, H., and Sidel, J.L.(1985). **Sensory evaluation practices.** London. Academic Press.

Suma, K., Misra, M.C., and Varadaraj, M.C.(1998). Plantaricin LP84, a broad spectrum heat-stable bacteriocin of *Lactobacillus plantarum* NCIM 2084 produced in a simple glucose broth medium. **Int. J. Food Microbiol.** 40: 17-25.

Viana, E.S., Gomide, L.A.M., and Vanetti, M.C.D. (2005). Effect of modified atmospheres on microbiological, color and sensory properties of refrigerated pork. **Meat Sci.** 71: 696-705.

CHAPTER V

SUMMARY

Out of nine culture strains of lactic acid bacteria (*Lactobacillus plantarum* TISTR 050, *Pediococcus acidilactici* TISTR 051, *Leuconostoc mesenteroides* TISTR 053, *Lb. acidophilus* TISTR 450, *Lb. brevis* supsp. *brevis* TISTR 860, *Lb. delbruckii* supsp. *bulgaricus* TISTR 892, *Lb. sake* TISTR 911, *Lactococcus lactis* TISTR 1401 and *Lb. casei* spp. *rhamnosus* SN11) used for screening of bacteriocins production, *Lc. lactis* TISTR 1401 was found to be the most effective bacteria producing bacteriocins with the maximum inhibition activity against indicator bacteria used (*Bacillus sp,* TISTR 908, *B. cereus* TISTR 687, *B. subtilis* TISTR 008 and *Staphylococcus aureus* TISTR 118). Based on the inhibition activity against *Bacillus sp.* TISTR 908, the *Lc. lactis* TISRT 1401 was selected for further crude bacteriocin production and applied for extending the shelf life of pork meatballs stored in aerobically packed and vacuum packed condition, and kept in a cold room at 4 ^{o}C for 12 days.

Crude bacteriocins produced by *Lc. lactis* TISTR 1401 showed the inhibition activity against Gram-positive bacteria including group of LAB, group of *Bacillus* and *Staphylococcus aureus*. The bacteriocins obtained were proved to be heat-stable at 80 ^{o}C for 20 min without loss of activity, stable at pH range 2 to 8 and inactivated by proteinase. Increase of inhibition activity of bacteriocins produced by *Lc. lactis* TISTR 1401 could be performed by controlled pH at 6.5 during fermentation while

bacteriocin activity was related to their cell density. Bacteriocins activity against *Bacillus* sp. TISTR 908 reached the maximum of 12,800 AU/ml at only 6 h of fermentation period.

Full strength of crude bacteriocin supernatant (CBS) was successfully applied by coat dipping on the pork meatball surface for extending the shelf life. At day 12 of meatball storage, the total microbial counts were slightly lower than the limit standard count of 5 x 10^5 cfu/g set for meat products, 4.30 and 4.74 log cfu/g for aerobically packed and vacuum packed condition, respectively, and 6.26 and 6.16 log cfu/g, respectively for control samples. It could be suggested that the full strength CBS treated meatballs had at least 6 days longer shelf life compared with control meatballs with slightly different in color and sensory characteristics. However, less acceptance was found for the treated meatballs due to the color and odor of bacterial culture media on the meatball surface which darker and stronger compared with the control samples. Therefore, elimination of dark color and strong odor is needed for further study.

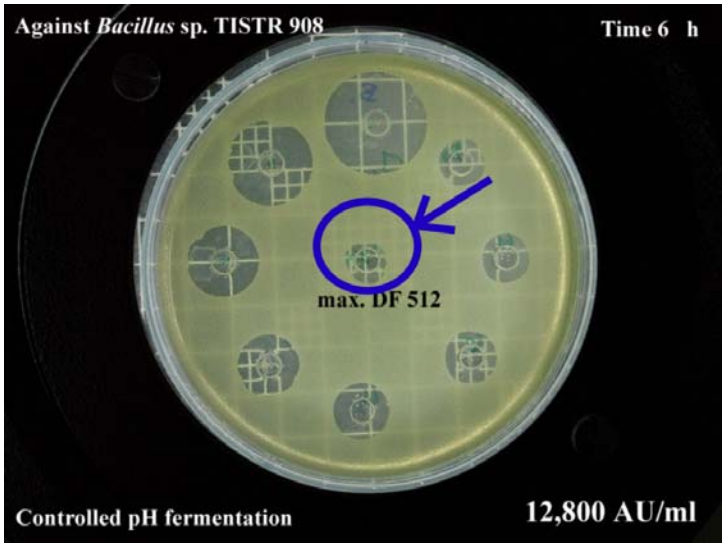
APPENDIX

CALCULATION OF ARBITRARY UNIT

APPENDIX

CALCULATION OF ARBITRARY UNIT

Various concentrations of crude bacteriocin supernatant (CBS) were loaded with 40 µl into each well. The maximum of inhibition zone was observed and used for calculation of arbitrary per ml.



Example:

Arbitrary Unit per ml = (maximum of dilution factor X 1000) / volume of CBS (µl)

= (512 X 1000) / 40

= 12,800 AU/ml

CURRICULUM VITAE

NAME: Mister Saroj Gosaarak **DATE OF BIRTH:** December 20, 1979

EDUCATION AND EXPERIENCE:

1997-2001	B.Sc. (Food Technology), Suranaree University of Technology.
2001-2002	Asst. production manager, Tia Ngee Hiang (chaosua) co., ltd.
2003-2004	Graduate Student Exchange Programe, Laboratoire de Génie Chimique, ENSIACET-INP, Toulouse, France
2003-2007	RA&TA, Suranaree University of Technology, Thailand.
2002-2007	M.Sc. (Food Technology), Suranaree University of Technology.

RESEARCH PRESENTATION EXPERIENCE: (Poster Presentation)

Gosaarak, S., and Intarapichet, K. (2005). **Selection of bacteriocin-producing thermo tolerance lactic acid bacteria for application in Thai traditional meat products.** The 7th Agro-Industrial Conference, Bitech, Bangna, Thailand. 22-24 June, 2005.

Gosaarak, S., and Intarapichet, K. (2005). **Controlled pH fermentation of *Lactobacillus lactis* TISTR 1401 for crude bacteriocin production.** The 7th Agro-Industrial Conference, Bitech Bangna, Bangkok, Thailand. 22-24 June, 2005.

Taillandier, P., Tataridis, P., Gosaarak, S., Mathieu, F., and Strehaiano,P. (2004). **Mixed cultures between wine microorganisms using a membrane bioreactor.** International Congress on Biotechnology in Food Industry, CLERMONT-FERRAND (France). 11-13 July, 2004.