ISOLATION, SELECTION AND IDENTIFICATION OF PROTEINASE-PRODUCING BACTERIA FROM FISH SAUCE FERMENTATION TO BE USED AS STARTER CULTURES

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การแยก คัดเลือก และ ระบุสายพันธุ์แบคทีเรียที่ผลิตเอนไซม์โปรติเนส จากน้ำปลาเพื่อใช้เป็นกล้าเชื้อ

นางสาวศิริวรรณ ณะวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOLATION, SELECTION AND IDENTIFICATION OF PROTEINASE-PRODUCING BACTERIA FROM FISH SAUCE FERMENTATION TO BE USED AS STARTER CULTURES

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

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ศิริวรรณ ณะวงษ์ : การแยก คัดเลือก และระบุชนิดแบคทีเรียที่ผลิตเอนไซม์โปรติเนสจากกระบวนการหมักน้ำปลาเพื่อใช้เป็นกล้าเชื้อ (ISOLATION, SELECTION AND IDENTIFICATION OF PROTEINASE-PRODUCING BACTERIA FROM FISH SAUCE FERMENTATION TO BE USED AS STARTER CULTURE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 162 หน้า.

วัตถุประสงค์ของการศึกษานี้เพื่อคัดแยกและระบุชนิดของแบคทีเรียที่สร้างโปรติเนส จากตัวอย่างน้ำปลาที่หมักในระยะเวลาต่างๆ และศึกษาผลของอุณหภูมิ ค่าความเป็นกรด-ด่าง และความเข้มข้นของเกลือโซเดียมคลอไรด์ที่เหมาะสมต่อการเจริญ และผลิตโปรติเนสของแบคทีเรียที่คัดเลือกได้คัดแยกแบคทีเรียที่สร้างโปรติเนสจากตัวอย่างน้ำปลาที่หมักในช่วง 1-12 เดือน น้ำคาวปลา และเกลือสมุทร จำนวนทั้งสิ้น 50 ตัวอย่าง จากแบคทีเรียที่คัดแยกได้ทั้งสิ้น 308 ไอโซเลท พบว่า 27 ไอโซเลท สามารถย่อยโปรตีนจากปลากะตักได้ดี เมื่อระบุชนิดของแบคทีเรียที่คัดเลือกได้โดยอาศัยลักษณะทางสัณฐานวิทยาและสรีรวิทยา พบว่าเป็นแบคทีเรียแกรมบวกรูปร่างเซลล์เป็นท่อนที่สร้างสปอร์ (spores) แบคทีเรียแกรมลบรูปร่างเซลล์เป็นท่อน และแบคทีเรียแกรมบวกรูปร่างเซลล์กลม จากผลการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene พบว่าแบคทีเรียดังกล่าวข้างต้นจัดอยู่ในสกุล *Virgibacillus*, *Halomonas*, *Bacillus*, *Brevibacterium*, *Corynebacterium* และ *Staphylococcus* แบคทีเรียที่สร้างโปรติเนสซึ่งย่อยโปรตีนปลาได้ดีที่สุด 3 ไอโซเลท คือ SK33, SK37 และ SK1-1-5 โดย SK33 และ SK37 มีความเหมือนของลำดับนิวคลีโอไทด์กับ *Vigibacillus halodenitrificans* DSM 10037 96% และ 95% ตามลำดับ ส่วน SK1-1-5 มีความเหมือนกับ *Staphylococcus saprophyticus* ATCC 15305 95% ดังนั้น SK33 และ SK37 จัดอยู่ในสกุล *Virgibacillus* และ SK1-1-5 จัดอยู่ในสกุล *Staphylococcus* จากการวิเคราะห์ผลด้วย Phylogenetic tree เมื่อประเมินผลร่วมกับลักษณะทางสัณฐานวิทยาและสรีรวิทยา และความเหมือนของลำดับนิวคลีโอไทด์ของ 16S rRNA gene พบว่า *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 และ *Staphylococcus* sp. SK1-1-5 มีแนวโน้มที่จะเป็นแบคทีเรียชนิดใหม่

สภาวะที่เหมาะสมต่อการเจริญและสร้างโปรติเนสของแบคทีเรียที่คัดเลือกแต่ละไอโซเลทแตกต่างกัน *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 และ *Staphylococcus* sp. SK1-1-5 เจริญได้ดีที่ระดับเกลือโซเดียมคลอไรด์เข้มข้น 18, 20 และ 15% ตามลำดับ ในอาหาร fish broth ที่มีความเป็นกรด-ด่างเริ่มต้นเท่ากับ 7.0 ทุกไอโซเลทเจริญได้ดีที่สุดที่อุณหภูมิ 35 องศาเซลเซียส *Virgibacillus* sp. SK33 สร้างโปรติเนสได้ดีที่สุดเมื่อเจริญในอาหาร fish broth ที่มีระดับเกลือโซเดียมคลอไรด์เข้มข้น 25% ที่ 40 องศาเซลเซียส ทั้ง *Virgibacillus* sp. SK37 และ *Staphylococcus*

sp. SK1-1-5 สร้างโปรติเนสได้ดีในอาหารที่มีระดับเกลือโซเดียมคลอไรด์เข้มข้น 5% ที่ 35 องศาเซลเซียส สารสกัดจากยีสต์ และกลูโคสไม่จำเป็นต่อการเจริญและผลิตโปรติเนสของแบคทีเรียทั้งสามไอโซเลท และเมื่อทดลองเตรียมกล้าเชื้อเพื่อเร่งกระบวนการหมักน้ำปลาจากปลากะตัก พบว่าเมื่อเติมกล้าเชื้อลงในวัตถุดิบคือปลากะตักที่ผ่านการย่อยด้วยเอนไซม์อัลคาเลสในระดับ 0.25% (65 องศาเซลเซียส นาน 2 ชั่วโมง) และเอนไซม์เฟโวไซม์ในระดับ 0.5% (50 องศาเซลเซียส นาน 4 ชั่วโมง) และมีเกลือสมุทรที่ระดับความเข้มข้น 25% หลังจากหมักเป็นเวลา 120 วัน ปริมาณกรดอะมิโนแอลฟาของปลากะตักหมักที่เติม *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 และ *Staphylococcus* sp. SK1-1-5 มีค่าสูงกว่าตัวอย่างควบคุม (วัตถุดิบที่ไม่เติมกล้าเชื้อ) ผลที่ได้จากการศึกษานี้แสดงถึงแนวโน้มในการใช้ประโยชน์แบคทีเรียทั้ง 3 ไอโซเลท ในกระบวนการหมักน้ำปลา

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

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SIRIWAN NAWONG : ISOLATION, SELECTION AND IDENTIFICATION OF PROTEINASE-PRODUCING BACTERIA FROM FISH SAUCE FERMENTATION TO BE USED AS STARTER CULTURES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D. 162 PP.

PROTEINASE-PRODUCING BACTERIA/FISH SAUCE FERMENTATION/ /PROTEINASE PRODUCTION/STARTER CULTURE

The objectives of this study were to isolate and identify proteinase-producing bacteria from various periods of fish sauce fermentation. In addition, the effects of temperature, pH, and NaCl concentration on bacterial growth and proteinase production of selected strains were investigated. Proteinase-producing bacteria were isolated from 50 samples of fish sauce fermented for 1-12 months, fish juice, and solar salt samples. A total of 308 bacterial isolates were collected, and 27 of them could hydrolyze anchovy proteins. Bacterial identification was performed using morphological and physiological characteristics. These bacterial isolates were Gram-positive and spore-forming rod, Gram-negative rod, and Gram-positive cocci. Based on 16S rRNA gene sequences, they were identified as *Virgibacillus*, *Halomonas*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, and *Staphylococcus*. Three selected isolates producing the highest proteinases were SK33, SK37 and SK1-1-5. SK33 and SK37 showed sequences homology to *Vigibacillus halodenitrificans* DSM 10037 96% and 95% similarity, respectively. SK1-1-5 showed 95% similarity to *Staphylococcus saprophyticus* ATCC 15305. Thus, SK33 and SK37 were closely related to the genus *Virgibacillus*. SK1-1-5 was identified as the genus *Staphylococcus*. On the basis of the

phylogenetic analyses and the combination of morphological, physiological characteristics and 16S rRNA gene sequences, three isolates namely *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were likely to be new species.

Optimum conditions for growth and proteinase production of each selected isolate were different. The optimum NaCl concentration for growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were 18, 20 and 15%, respectively, in fish broth at the initial pH 7. All isolates grew very well at 35^{o}C. *Virgibacillus* sp. SK33 optimally produced proteinase in the fish broth containing 25% NaCl at 40^{o}C. Both *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 showed their optimum temperature for proteinase production in fish broth containing 5% NaCl at 35^{o}C. Yeast extract and glucose were not needed for bacterial growth and proteinase production of three selected strains. The three selected isolates were also tested for their ability to accelerate protein hydrolysis of anchovy. Three starter cultures were added into anchovy hydrolysate prepared from 0.25% Alcalase® (65^{o}C for 2 h) and 0.5% Flavourzyme® (50^{o}C for 4 h) containing 25% solar salt. After incubation for 120 days, ∝-amino content of samples inoculated by *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5 were higher than the control (without starter culture). These studies demonstrated the potential of three selected strains to be used as starter culture for fish sauce fermentation.

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Siriwan Nawong

CONTENTS

Page

ABSTRACT (Thai)....……..……..……....I

ABSTRACT (English)…….…….…….....…...III

ACKNOWLEDGEMENTS...……..…….…….....…..V

CONTENTS.…….....VII

LIST OF TABLES..……..……....X

LIST OF FIGURES..……...…..XIII

LIST OF ABBREVIATIONS..……...…...XVII

CHAPTER

I INTRODUCTION

1.1 Introduction…….……1

1.2 Research objectives ……..…..…3

1.3 Research hypothesis……3

1.4 Scope of the study…….…..4

1.5 Expected results…….....4

1.6 References…….....5

II LITERATURE REVIEWS

2.1 Fish sauce fermentation……..….…..10

2.2 Halophilic bacteria……..…11

2.2.1 Habitats of halophilic bacteria…….....…11

2.2.2 Physiology of halophilic bacteria……...…….....…13

CONTENTS (Continued)

Page

2.3 Microorganisms in fish sauce fermentation…….....15

2.4 Characteristic of proteinase-producing bacteria…….....18

2.4.1 *Staphylococcus* sp…….....…18

2.4.2 *Virgibacillus* sp……...……..23

2.5 Identification of halotolerant/halophilic bacteria……...…..….27

2.6 Roles of the starter culture in food fermentations…….…....31

2.7 References…….…….…...34

III CHARACTERIZATION AND IDENTIFICATION OF PROTEINASE PRODUCING PRODUCING BACTERIA ISOLATED FROM THAI FISH SAUCE FERMENTATION

3.1 Abstract……...……..48

3.2 Introduction…….…….....49

3.3 Materials and methods…….....51

3.3.1 Collection of fish sauce samples…….……...…..…51

3.3.2 Isolation of bacteria from fish sauce samples……...…….....51

3.3.3 Screening and selection of proteinase-producing bacteria……..…....52

3.3.4 Determination of proteolytic activity…….…….…....52

3.3.5 Determination of biogenic amines…….…….…....53

3.3.6 Identification of proteinase-producing bacteria…….…….....54

3.4 Results and discussion……..60

3.4.1 Isolation of proteinase-producing bacteria from fish sauce samples……...…....60

CONTENTS (Continued)

Page

3.4.2 Screening and selection of proteinase-producing bacteria……65

3.4.3 Identification of proteinase-producing bacteria……65

3.5 Conclusions……...….92

3.6 References……..…….....93

IV OPTIMIZATION OF GROWTH AND PROTEINASE PRODUCTION CONDITIONS OF SELECTED BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION

4.1 Abstract……...…...…...102

4.2 Introduction……...…....103

4.3 Materials and methods……..…105

4.3.1 effect of temperature, NaCl concentration and pH……...…….....105

4.3.2 Effect of nitrogen and carbon source……..….....106

4.3.3 Effect of aeration……...….....106

4.3.4 Effect of inoculum size……...…..…..107

4.3.5 Application of starter cultures for fish sauce fermentation……..…...108

4.3.6 Statistical analyses…….....109

4.4 Results and discussion…….…..…108

4.4.1 temperature, NaCl concentration and pH…….....108

4.4.2 Nitrogen and carbon source…….……...117

4.4.4 Effect of aeration…….....…122

4.4.5 Effect of inoculum size…….....123

CONTENTS (Continued)

Page

4.4.6 Application of starter cultures for fish sauce fermentation….....125

4.5 Conclusions……...……...….129

4.5 Conclusions……...……...….129

4.6 References……...……...…...130

V SUMMARY..……...…….141

APPENDICES

Appendix A……..……...……...143

Appendix B……..……...……..154

Appendix C…….……...……...….159

CURRICULUM VITAE……...……...161

LIST OF TABLES

Table **Page**

2.1 Differential characteristics of genera *Micrococcus* and *Staphylococcus*.....19

2.2 Characteristics for differentiating species in the genus *Staphylococcus*.....22

2.3 Characteristics for differentiating species in the genus *Virgibacillus*……..…...26

3.1 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene……..……..…….59

3.2 Bacterial counts (CFU/g, ml) of various samples collected from four different fish sauce plants……...……..……..61

3.3 Proteinase-producing bacteria obtained from fish sauce samples fermented at various time……..…….66

3.4 TCA-soluble oligiopeptide content and total viable counts of the selected isolates in fish broth containing 25% NaCl, pH 7 and incubated at 35^{o}C for 5 days with initial cell counts of about 5 Log CFU/ml……..…..67

3.5 Biogenic amine content of isolates obtained from various sources of fish sauce fermentation process……..……67

3.6 Comparison of phenotypic characteristics of the selected Gram-positive rod and other genera…….....71

LIST OF TABLES (Continued)

Table		Page
3.7	Comparison of phenotypic characteristics of the selected Gram-positive coccus and other species	75
3.8	16S rRNA sequence similarity and relation of Gram-positive rods	84
3.9	16S rRNA sequence similarity and relation of Gram-positive cocci	84
3.10	16S rRNA gene sequence similarity of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and related species	86
3.11	16S rRNA gene sequence similarity of *Staphylococcus* sp. SK1-1-5, *Staphylococcus* sp. SKM20-7-9 and related species	87
3.12	Similarity of 16S rRNA gene sequence of three proteinase-producing bacteria isolates compared with other bacteria from nucleotide sequence database (NCBI)	91
4.1	Bacterial counts (Log CFU/ml) of all strains cultured in fish broth (pH 7) containing various glucose concentrations and incubated at 35^{o}C with initial cell counts of about 5 Log CFU/ml	121
4.2	Growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 cultured in fish broth (pH 7) incubated at 35^{o}C, at various shaking speeds	122

LIST OF FIGURES

Figure **Page**

3.1 Colonies of proteinase-producing bacteria from fish sauce on skim milk salt agar containing 25% NaCl, isolation step (a-b), pure culture step (c) and proteolytic test (d)……...…….....62

3.2 Scanning electron micrographs of selected isolates on JCM 168 medium and incubated at 35^{o}C for 5 days: SK33 (a), SK37 (b) and SK1-1-5 (c) (Bar=1 µm)……...……68

3.3 Gram stain of the selected isolates on JCM 168 medium at their optimum NaCl concentration of each isolate and incubated at 35^{o}C for 5 days , SK33 (18%NaCl) (a), SK37 (20% NaCl) (b) and SK1-1-5 (15% NaCl) (c) (Bar=1 µm, Magnification: x 1000)……..…….…..69

3.4 Endospores of SK33 (a) and SK37 (b) on JCM 168 medium at their optimum NaCl concentration and incubated at 35^{o}C for 3 weeks, Bar=1µm……..…70

3.5 Scanning electron micrographs of *Virgibacillus* sp. SK33 in fish broth containing 0 (a), 0.5 (b), 3 (c), 5 (d), 10 (e), 15 (f), 18 (g), 20 (h), 25 (i) and 30% NaCl (j)…….....…….....80

LIST OF FIGURES (Continued)

Figure		Page
3.6	Scanning electron micrographs of *Virgibacillus* sp. SK37 in fish broth containing 0 (a), 0.5 (b), 3 (c), 5 (d), 10 (e), 15 (f), 18 (g), 20 (h), 25 (i) and 30% NaCl (j)…….…….……..….81	
3.7	Scanning electron micrographs of *Staphylococcus* sp. SK1-1-5 in fish broth containing 0 (a), 0.5 (b), 3 (c), 5 (d), 10 (e), 15 (f), 18 (g), 20 (h), 25 (i) and 30% NaCl (j)……..… 82	
3.8	Gel electrophoresis of PCR products obtained from the amplification of 16S rRNA gene using primers POmod/ PC3mod r (a) and P3mod f/PC5 r (b)…….…….……..… 83	
3.9	Phylogenetic tree of proteinase-producing bacteria Gram-positive rod isolated from fish sauce fermentation, based on 16S rRNA gene sequence data…….……..…88	
3.10	Phylogenetic tree of proteinase-producing bacteria Gram-positive cocci isolated from fish sauce fermentation, based on 16S rRNA gene sequence data……..…….....89	
4.1	Bacterial counts (Log CFU/ml) of *Virgibacillus* sp. SK33 (a), *Virgibacillus* sp. SK37 (b) and *Staphylococcus* sp. SK1-1-5 (c) in fish broth containing 5% and 25%NaCl, and pH 7, and incubated at various temperatures……...…….…..110	

LIST OF FIGURES (Continued)

Figure		Page
4.2	α-Amino peptides produced by the selected strains in 5% NaCl (a) and 25% NaCl (b) in fish broth incubated at various temperatures…….……..…112	
4.3	Bacterial counts (Log CFU/ml) *Virgibacillus* sp. SK33 (+18% NaCl) (a), *Virgibacillus* sp. SK37 (+20% NaCl) (b), and *Staphylococcus* sp. SK1-1-5 (+15% NaCl)(c) (initial pH 7) incubated at 35^{o}C……..…….……..……....114	
4.4	Bacterial counts (Log CFU/ml) and α-amino content (mM) of *Virgibacillus* sp. SK33 (+18% NaCl) (a), *Virgibacillus* sp. SK37 (+20% NaCl) (b) and *Staphylococcus* sp. SK1-1-5 (+15% NaCl) (c) in fish broth at various initial pHs , 35^{o}C……...116	
4.5	Bacterial counts (Log CFU/ml) of *Virgibacillus* sp. SK33 (+18% NaCl) (a), *Virgibacillus* sp. SK37 (+20% NaCl) (b) and *Staphylococcus* sp. SK1-1-5 (+15% NaCl) (c) in fish broth containing various ratios of fish extract and incubated at 35^{o}C initial pH 7…….……..…….….118	
4.6	α-Amino peptides of media containing anchovy extract at varied proportions and inoculated with *Virgibacillus* sp. SK33 (+18% NaCl) (a) and *Virgibacillus* sp. SK37 (+20% NaCl) (b)……..…120	

LIST OF FIGURES (Continued)

Figure		Page
4.7	Effect of inoculum size on growth (Log CFU/ml) of *Staphylococcus* sp. SK1-1-5 (a) and *Virgibacillus* sp. SK33 (b) on JCM 168 medium containing 25%NaCl, incubated at 35^{o}C and initial pH 7…….……..……...123	
4.8	α-Amino peptides produced by *Staphylococcus* sp. SK1-1-5 (a) and *Virgibacillus* sp. SK33 (b) at various inoculum levels…….…..…..124	
4.9	Growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 during fish sauce fermentation incubated at 35^{o}C, pH 7……...……126	
4.10	Changes of pH during fermentation of samples inoculated by 3 selected strains. SK33=*Virgibacillus* sp. SK33, SK37=*Virgibacillus* sp. SK37, SK1-1-5=*Staphylococcus* sp. SK1-1-5…….…….127	
4.11	α-Amino content of fish sauce inoculated with 3 selected strains. Abbreviations were the same as Figure 4.10…….....129	

LIST OF ABBREVIATIONS

ANOVA	Analysis of varience
BLAST	Basic local alignment search tool
bp	Base pair
°C	Degree Celsius
CFU	Colony forming unit
cm	Centrimeter
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
et al.	et alia (and others)
(m, µ) g	(milli, micro) Gram
GC	Gas chromatography
h	Hour
HPLC	High performance liquid chromatograph
(m, µ) l	(milli, micro) Liter

LIST OF ABBREVIATIONS (Continued)

(m, µ) M	(milli, micro) Molarity
min	Minute
(m, µ) mol	(milli, micro) Mole
N	Normality
%	Percentage
PCR	Polymerase chain reaction
rpm	Round per minute
s	Second
SAS	Statistical analysis system
sp.	Species
supsp.	Supspecies
%v/v	Percentage volume by volume
%w/v	Percentage weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce is a condiment produced mainly in Southeast Asian countries, particularly in Thailand. Presently, the members of fish sauce consumers are increasing not only in Southeast Asia, but also in several continents owing mainly to the increasing Asian population abroad and the popularity of Asian cuisines. Thailand is the largest fish sauce producer in Southeast Asia. There are approximately 390 fish sauce factories in Thailand, which utilize about 64,000 metric tons of fish annually (Bovornreungroj, 2005). However, the traditional fermentation time of fish sauce production is considered too long for industrial scale production and expansion. It normally takes approximately 8-12 months from salting to maturity. Therefore, if the fermentation period could be shortened, the production cost will be reduced and at the same time the conversion of insoluble protein to soluble protein will be increased. These would be commercially advantage for the industry.

In the past, many investigators attempted to accelerate fish sauce fermentation using a variety of methods. These included the addition of acid and alkaline (Gildberg and SHi, 1984; Thongthai and Suntinanalert, 1991), raising temperature (Lopetcharat and Park, 2002), the reduction of salt concentration and/or the addition of enzymes (Beddows and Ardeshir; 1979, Gildberg and Shi; 1994). However, these methods has some limitations. The use of chemicals, such as hydrochloric acid in acid hydrolysis

method is a harsh condition and causes adverse sensory characteristics in the finished product. Raising the temperature results in high energy expenditure. Therefore, it is considered impractical for the industry. The addition of commercial enzyme is not convenient and costly. Direct inoculation of proteinase-producing microorganisms could be an alternative.

Many microorganisms were found during fish sauce fermentation by many investigators. They were identified as the species of the following genera; *Micrococcus*, *Tetragenococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Lactobacillus*, *Coryneform*, *Pseudomonas*, *Proteus* (Saisithi, Kasemsarn, Liston and Dollar, 1966; Thongthai and Suntinanalert, 1991; Saisithi, 1994; Thongsanit, Tanasupawat, Keeratipibul and Jatikavanich, 2002; Fukami, Funatsu, Kawasaki and Watabe, 2004). Other halophilic bacteria, including *Halobacterium*, *Halococcus* and *Halobacillus* were also reported (Thongthai and Suntinanalert, 1991; Chaiyanan, et al., 1999). The proteinase producing bacteria found in fish sauce fermentation are *Halobacterium*, *Filobacillus* and *Halobacillus* (Chaiyanan et al., 1999; Kanlayakrit, Bovornreungroj, Oka and Goto, 2004; Hiraga et al., 2005; Namwong et al., 2006). The important role of proteinase-producing bacteria was to hydrolyze protein during fish sauce fermentation process in the presence of high salt concentration. *Halobacillus* sp. SR5-3 produced serine proteinase which showed the highest activity at 20% NaCl incubated in modified JCM 168 medium containing 10% NaCl. This strain grew well at 30^{o} C and 15% NaCl in the modified JCM 168 medium (Namwong et al., 2006). *Filobacillus* sp. RF2-5 also produced serine proteinase and was highly stable activity at 25% NaCl (Hiraga et al., 2005). The optimum NaCl concentration for growth and proteinase-production of *Filobacillus* sp. RF2-5 was at 10%. However,

the starter culture, which was prepared from proteinase-producing bacteria isolated from fish sauce fermentation process, has not yet been reported and successfully applied to accelerate fish sauce fermentation.

This research was aimed at isolating halophilic bacteria from fish sauce fermentation at different periods and selecting halophilic and/or halotolerant proteinase-producing bacteria for starter culture development. The selected strains were identified, based on morphological and biochemical characteristics, and 16S rRNA sequences. The starter cultures prepared from the selected strains were evaluated for their ability to accelerate protein hydrolysis of anchovy.

1.2 Research objectives

The objectives of this research were:

1) To isolate and identify proteinase-producing bacteria from various periods of fish sauce fermentation

2) To select proteinase-producing bacteria based on their proteolytic activity and biogenic amines formation for starter culutre development.

3) To investigate the effect of temperature, pH, and salt concentration on bacterial growth, cell morphology and proteinase production of the selected strains.

1.3 Research hypothesis

A number of bacterial species found in fish sauce play crucial roles during fish sauce fermentation. Some strains could efficiently produce proteinase at high salt concentration, and could be developed as a starter culture to reduce fish sauce fermentation time.

1.4 Scope of the study

Proteinase-producing halotolerant and halophilic bacteria were screened and isolated from fish sauce fermented for 1, 3, 5, 7, 9 and 12 months. These bacteria should not produce biogenic amines, especially histamine. The identification of halobacteria was carried out according to Grant, Kamekura, McGenity and Ventosa (2001) and Holt, Krieg, Sneath, Staley and Williams (1994). Phenotypic and genomic analyses were achieved using biochemical tests and/or the gene sequence analysis ampified by polymerase chain reaction (PCR) technique (Stan-Lotter et al., 2002). Factors affecting growth and proteinase production of the selected strains were studied, which included temperature, pH, and salt concentration. Starter culture from selected strains were prepared and applied to hydrolyze anchovy proteins.

1.5 Expected results

Results obtained from this study led to more understandings of bacteria producing proteinase during fish sauce fermentation. Proteinase-producing bacteria were identified to their species using molecular technique and were characterized to understand how various factors (temperature, pH and NaCl concentration) affected their proteinase production and growth. The knowledge obtained was critical for starter culture development and would ultimately reduce fermentation time and improve fish sauce production.

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CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce fermentation

Fish sauce is a fermented fish product resulted from hydrolysis of fish protein at high salt concentration (Chaveesuk, Smith and Simpson, 1993). The product is widely consumed in Southeast Asian countries (Fukami et al., 2004), and gained popularity worldwide. Fish sauce is called by different names, such as Patis in the Phillippines, Shotturu in Japan, Budu in Malaysia, Nam-pla in Thailand, Noucnam in Vietnam, Ketjapikan or Bakasang in Indonesia, Yuilu in China, and Ngapi in Burma (Fukami, 2002). Both marine and freshwater fish can be used as a raw material for fish sauce production (Heu, Kim, Cho, Godber and Pyeum, 1997). Fish and sea salt are mixed at a weight ratio of approximately 2:1 for marine fish and 3:1 for freshwater fish (Adamst, Cooke and Rattagool, 1985). Traditionally, whole fish is dumped on a concrete floor, and the excess liquid is allowed to drain off. Fish are thoroughly mixed with salt. The mixture is then placed to the tank. Another layer of salt is spreaded on top. After one week, the fish may float and rise to the top of the fermentation tank. It is necessary to keep the fish immersed at all times, otherwise they will become rancid. While the salting progresses, hydrolysis of fish tissues by fish gut enzymes provides the necessary nutrients for bacterial fermentation to begin. Time needed for the full flavor of fish sauce to develop varies from 8 to 18 months at 30-35^{o}C (Owen and Mendoza, 1985; Lopetcharat et al., 2001; Fukami et al., 2002). The finished product is

a clear dark-brown color, with pH 5.5-5.8 and 20.5-27.4% salt (Saisithi, 1994; Lopetcharat, et al., 2001), and has a distinct aroma and flavor. The flavor and aroma determine its quality and these characteristics develop progressively as the fermentation process advances (Saisithi et al., 1966; Eungruttanagorn, 2000; Lopetcharat et al., 2001).

Salting is used to prevent spoilage bacteria during fermentation by reducing water activity (a_w). The maximum amount of salt (NaCl) to prevent the growth of spoilage microorganisms is 15% (Lopetcharat et al., 2001; Bovornreungroj, 2005). Amount of salt used for fish sauce production is usually between 20 and 30% (Saisithi, 1994; Lopetcharat et al., 2001; Bovornreungroj, 2005). Most of spoilage bacteria originally present in fish will die off quickly after contacting with high salt concentration, leaving only the halotolerant rods to grow slowly in the medium. Proteinases responsible for fermentation come partly from fish digestive system and partly from bacteria naturally presented in the fish and salt. Fermentation process starts with the fish gut enzymes which gradually break down proteins into oligopeptides and amino acids. These products are, in turn, used as substrates for proteolytic and lactic acid bacteria. Microorganisms, responsible for the fermentation, therefore, are either halophilies or halotolerants (Saisithi, 1994). Fish tissue consists mainly of protein and lipid. Degradation pathways of the lipid component during the fermentation is not fully understood. When fish protein is hydrolyzed by both endogenous and exogenous proteinases, several amino acids and small peptides are accumulated (Owens and Mendoza, 1985; Saisithi, 1994; Lopetcharat et al., 2001; Yongsawatdigul, Choi and Udomporn, 2004). Amino acids found in fish sauce are taurine, aspartic acid, threonine, serine, glutaminc acid, glycine, alanine, valine, methionine, isoleucine,

leucine, tyrosine, phenylalanine, histidine, lysine, arginine and citrulline (Saisithi, 1994; Eungruttanagorn, 2000).

Yongsawatdigul et al. (2004) found that the amount of amino acids during fermentation of fish sauce increased gradually from 4,560.9 to 7,208.3 mg/100 ml at the end of 13 months of fermentation. Other compounds found in fish sauce are volatile acids. The amount of total volatile acids increased as fermentation time increased and reached the maximum at approximately 9 months. The volatile acids found in fish sauce, such as formic, acetic, propionic and iso-butyric acid, are belived to be by-products of lactic acid fermentation (Saisithi, 1966; Beddows, Ardeshir and Daud, 1980 ; Saisithi, 1994; Fukami et al., 2002; Mcfeeters, 2004). These compounds gave the unique odor to fish sauce.

One problem of fish sauce product is the presence of histamine and other biogenic amines. Some proteolytic bacteria can produce biogenic amines, including histamine, tyramine, cadaverine, putrescine, spermidine and spermine (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez and Mora-Ventura, 1999; Yongsawatdigul et al., 2004). The toxicological level of histamine depends on the ability of detoxification systems of the intestinal tract and the presence of other amines, such as putrescine and cadaverine (Rodriguez-Jerez et al., 1994). A limit of 20 mg histamine/ 100 g of fish sauce was suggested by the Canadian Food Inspection Agency (CFIA, 2003). According to Food and Drug Administration (FDA), the level of histamine 5 mg/100 g in any fish can cause poisoning (Lopetcharat, et al., 2001). Certain species of *Staphylococcus capitis, S. epidermidis,* and *Tetragenococcus muriaticus* found in salted anchovies and fish sauce have been reported to produce histamine about 40 mg/ 100 ml or more than 100 µg/ 100 ml in the presence of 3 to 10% NaCl at 30^{o}C

(Hernandez-Herrero et al., 1999; Kimura, Konagaya and Fujii, 2001). Ideally, it is strongly recommended to select bacteria which are unable to produce biogenic amines.

2.2 Halophilic bacteria

Halophilic bacteria are salt-loving organisms that inhabit in hypersaline environment. Halophiles have the capacity to balance the osmotic pressure of the environments and resist the denaturing effect of salts (Grant et al., 2001).

Kushner (1993) classified microorganisms based on their requirement for NaCl to five groups, which are non-halophiles (<0.2 M, ~1% salt), slight halophiles (0.2-0.5 M, ~1-3% salt), moderate halophiles (0.5-2.5, ~3-15% salt), borderline extreme halophiles (1.5-4.0 M, ~9-23% salt) and extreme halophiles (2.5-5.2 M, 15-32% salt). Halotolerant organisms can grow both in high salinity and in the absence of salt.

Many halophiles and halotolerant microoganisms can grow over a wide range of salt concentrations with requirement or tolerance for salt. Moreover, growth of these microoganisms depends on environmental and nutritional factors. Halophiles are distributed all over the world in hypersaline environments, many in natural hypersaline brines, in arid, coastal and even deep-sea locations, as well as in artificial salterns used to mine salt from the sea (Turper and Galinski, 1986). In addition, halophilic bacteria can be found in fermented fish and fish products, such as fish sauce, fermented fish pastes and salted fish.

2.2.1 Habitats of halophilic bacteria

Halophilic bacteria can thrive in hypersaline environments. Life is represented in both athalassohaline and thalassohaline conditions. Thalassohaline

environments contain sodium and chloride ions as the predominant ions. While athalassohaline environments are potassium, magnesium and sodium. One characteristic shared by athalassohaline and thalassohaline environments is pH which usually near neutral to slightly alkaline (Litchfield and Gillevet, 2002)

Dead Sea is the athalassohaline ecosystem. There are reports on the characterization of members of archaea and bacteria (Oren, Gurevich, Gemmell and Teske, 1995). Several halophilic bacteria are found as follows: *Halobacterum* spp., *Halococcus* spp., *Halobaculum gomorrense*, *Haloarcula marismortui*, *Haloarcula vallismortis*, *Haloferax volcanii*, *Flavobacterium*, *Pseudomonas*, *Chromobacterium*, *Halomonas* and *Bacillus*, particularly B. *marismortui*. (Chookietwattana, 2003). A novel species of the genus *Halorubrum* was isolated in the Atacama Saltern.

Many microorganisms were found on thalassohaline systems. A new genus belonging to the Archaea domain, *Halogeometricum*, was isolated and characterized by Montalvo-rodriguez et al. (1998). Several new species belonging to the genera *Haloterrigena*, *Haloferax* and *Haloarcula* have been also reported (Montalvo-rodriguez et al., 2000; Asker and Ohta, 2002; Ihara, Watanabe and Tamura, 1997). Representatives from the bacteria domain can be found in this environment. The genus *Thermohalobacter* was isolated from a saltern (Cayol et al., 2000). A new genus of halophilic bacteria, *Salinibacter*, was isolated and characterized (Anton et al., 2002) from a similar environment.

Several halophiles are often found on salted food, such as salted fish, meat and other food products. Villar, Ruiz-Holgado and Sanchez (1985) found that *Pediococcus halophilus* was a dominant bacterium at the end of the curing process of anchovies. While *Halomonas salina* was isolated from fully cured wet and dry

bachalao (dried salted codfish) that contains about 19% salt (Vihelmsson, Hafsteinwsson and Kristjansson 1996). *Pseudomonas beijerinckii* and *Halomonas halodenitrificans* were isolated from salted beans preserved in brine and meat curing brines, respectively. Moreover, *Halobacterium* sp., *Halococcus* sp., *Halobacillus* sp. SR5-3, *Filobacillus* sp. RF2-5, *Lentibacillus salicampi*, *Lentibacillus halophilus* sp. nov., and *L. juripiscarius* sp. nov were isolated from Thai fish sauce (Thongthai and Suntinanalert, 1991; Hiraga et al., 2005; Namwong et al., 2005, 2006;Tanasupawat et al., 2006).

2.2.2 Physiology of halophilic bacteria

Halophilic bacteria used amino acids as energy source and required a number of growth factors mainly vitamins. Electron transport chains containing cytochromes a, b and c were presented in halophilic bacteria and energy was conserved during aerobic growth via a proton motive force (Ventosa, Nieto and Oren 1998). Some strains of halophilic bacteria could grow in anaerobic condition.

Halophilic bacteria could withstand the osmotic forces that accompanied to their life in a high solute environment by accumulating organic compounds intracellulary that referred as compatible solutes (Margesin and Schinner, 2001). These compounds counteracted the tendency of the cell to become dehydrated under conditions of high osmotic strength by placing the cell in positive water balance with its surroundings and can be accumulated compatible solutes at high concentrations without interfering with cellular metabolism (Moral, Severin, Romos-Cormenzana, Truper and Galinski, 1994). Halophilic bacteria thrive in high osmolarity environment and excrete compatible solutes from their cytoplasm or uptake from the medium to achieve osmotic balance (Robert, Le Marrec, Blanco and Jebbar, 2000). In the case of

Halobactium, cells pumped large amounts of K^+ from the environment into the cell. The concentration of K^+ inside the cell was considerably greater than that the concentration of Na^+ outside the cell. Therefore, *Halobacterium* used an inorganic ion as a compatible solute and remained in positive water balance (Moral et al., 1994).

Cell wall of halophilic bacteria was stabilized by sodium ions. In low sodium environment, the cell wall of extremely halophilic bacteria was broken down. Extremely halophilic bacteria was similar to gram negative bacteria but cell wall was quite different. Sodium ion (Na^+) bound to outer surface of cell wall that was absolutely essential for maintaining cellular integrity. When insufficient Na^+ was presented, the cell wall was broken apart and the cell was lysed. Although extremely halophilic bacteria did not contain peptidogycan in the cell wall, the cell wall was composed of glycoprotein. The negative charges contributed by the carboxyl groups of amino acids in the cell wall. Glycoprotein was shielded by Na^+. When Na^+ was diluted, the negatively charged regions of the proteins actively repel each other and cell lysed (Margesin and Schinner, 2001). Cytoplasmic protein of halophilic bacteria contained very low levels of hydrophobic amino acids which probably represented an evolutionary adaptation to highly ionic cytoplasm of halophilic. The ribosomes of halophilic bacteria also required high K^+ levels for stability while ribosomes of nonhalophiles required no K^+. It appeared that the halophilic bacteria was highly adapted, both internally and externally, to life in highly ionic environment (Ventosa et al., 1998). Cellular component of *Halobacterium salinarium* exposing to the external environment required high Na^+ for stability while internal components required K^+ (Matheson, Sprott, McDonald and Tessier, 1976). Lipid membrane of halophilic bacteria had a low H^+ and Na^+ permeability at high salt concentration. Vossenberg,

Driessen, Grant and Koninge (1999) found pH and the salt concentration influenced on the proton and sodium ion permeability in lipid membrane of the halophiles. The proton permeability in lipid membrane of halophlies was independent of the salt concentration and was essentially constant between pH 7-9. Thus, the membranes of halophiles were stable over a wide range of salt concentrations and pH values were slightly alkaline and were well adapted to halophilic conditions.

2.3 Microorganisms in fish sauce fermentation

Fish sauce fermentation process contains very high concentration of salt (25-30%). Most of the microorganisms found in fish sauce are classified as halotolerant, slight halophiles, moderate halophiles and extreme halophiles. These are microflora from fish, solar salt and fermentation tank (Lopetcharat et al., 2001) and have been identified as the species of *Micrococcus, Bacillus, Achromobacter, Flavobacterium, Proteus, Pseudomonas, Staphylocococcus, Steptococcus, Pediococcus, Halococcus, Coryneform, Sarcina, Pseudomonas, Halobacterium* and *Lactobacillus* (Thongthai and Suntinanalert, 1991; Saisithi, 1994; Chaiyanan et al., 1999; Sanni, Asiedu and Ayemot, 2002; Fukami et al., 2004).

Ijong and Ohta (1996) found that total viable numbers of bacteria increase in first 10 days of Bakasang (Indonesian fish sauce) fermentation and then decreased gradually. Microorganisms predominantly found during Bakasang fermentation were *Micrococcus*, *Streptococcus* and *Pediococcus*. Moreover, some studies found acid-producing bacteria in Thai fish sauce and identified as *Pediococcus halophilus, Staphylococcus saprophyticus, Micrococcus varians* and *Paracoccus halodenitrificans* (Beddows et al., 1980; Owen and Mendoza, 1985; Saisithi, 1994). Other halophilic

lactic acid bacteria which grew well on media at high salt concentration and alkaline pH were also isolated and identified as *Aerococcus haloveridans*, *Peptococcus anaerobius, Tetragenococcus halophilus* and *T. muriaticus* (Saisithi, 1994; Tanasupawat et al., 2002; Thongsanit et al., 2002)

Gasaluck, Yokoyama, Kimura and Sugahara (1996) found that only *Bacillus* was isolated from the ripened fish sauce products. These bacteria could originate from both fish and salt used in the fermentation (Sanni et al., 2002). Tran and Nagano (2000) confirmed that the principal species of microorganisms isolated from Vietnamese fish sauce and Laotian fish sauce were *Bacillus subtilis*, respectively. Noguchi et al. (2004) isolated *B. vietnamensis* sp. nov. which was moderately halotolerant from Vietnamese fish sauce. Moreover, other Genus of *Bacillus* have been isolated from fish sauce, such as *Lentibacillus salicampi*, *L. juripiscarius* sp. nov., *L. halophilus* sp. nov., *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3 (Namwong et al., 2005, 2006; Hirage et al., 2005; Tanasupawat et al., 2006).

Proteolytic bacteria played an important role during fish sauce fermentation. There are several groups of proteolytic bacteria in fish sauce fermentation, including halophilic bacteria, halotolerant and lactic acid bacteria. These bacteria hydrolyze fish protein to peptides and amino acids (Lopetcharat et al., 2001). Some amino acids can be used as substrates for lactic acid bacteria.

Proteinase-producing bacteria found in fish sauce are *Pseudomonas* sp. (Vermelho et al., 1996), *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Pediococcus* sp., coryneforms (Thongthai and Suntinanalert, 1991; Fukami et al., 2002, 2004; Noguchi et al., 2004), *Halobacillus thailandensis* sp. nov., (Chaiyanan et al., 1999), *Tetragenococcus halophililus* and *T. muriaticus* (Satomi,

Kimura, Mizoi, Sato and Fujii, 1997; Tanasupawat et al.; Thongsanit et al., 2002). Some proteolytic bacteria of the genus *Halobacterium* are widely distributed in brines of high salt concentration. Nevertheless, only a few proteolytic bacteria have been isolated from Thai fish sauce.

Thongthai and Suntinanalert (1991) found that 203 out of 241 tested strains isolated from Thai fish sauce were gelatinolytic and most of these strains also degraded casein. Optimum salt concentration for growth of halophilic bacteria was found to be 20%, and the optimum pH between 6.5-7.5. (Thongthai and Suntinanalert, 1991; Saisithi, 1994; Thongsanit et al., 2002; Kobayashi et al., 2004). The bacteria seemed to grow better on a medium enclosed in container at 75.5% relative humidity. However, detailed studies on the properties of the proteolytic enzymes produced by such strains are not been made. It is known that bacteria have their intracellular concentrations of NaCl and KCl, which are approximately the same as the NaCl concentration of the growth medium. Halophilic bacteria's enzymes are thus active at salt concentrations which inhibit or even denature many enzymes of non–halophilic organisms. This must reflected in marked differences in the composition and properties of enzyme molecules. *Halobacterium* strains produced extracellular proteinases which degraded gelatin and casein (Thongthai and Suntinanalert, 1991; Chaiyanan et al., 1999). Moreover, proteolytic enzymes of halophilic bacteria had a high pH optimum and required divalent cations and high concentration NaCl or KCl for activity and stability (Ventosa et al., 1998; Demirijian, Moris and Cassidy, 2001; Dube, Singh and Alam 2001).

Tetragenococcus was classified in group of lactic acid bacteria and produce proteolytic enzymes. The bacterium was found distributed in Thai fish sauce,

especially *T. halophilus* and *T. muriaticus* (Tanasupawat et al., 2002; Thongsanit et al., 2002; Kobayashi et al., 2004). It was found that most of strains hydrolyzed casein and did not grow in 0% NaCl. But all strains grew at pH 6.5 and 9.0 at 40^{o}C. Recently, Hiraga et al. (2005) and Namwong et al. (2006) purified and characterized a serine proteinase from *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3, respectively. These bacteria was moderately halophilic bacteria isolated from Thai fish sauce. The enzyme of the strain RF2-5 showed the highest activity in the presence of 10% NaCl at 60^{o}C and pH 10-11. While strain SR5-2 showed optimum activity at 50^{o}C and pH 9-10 in 20% NaCl. They were highly stable in the presence of 25% NaCl. There are several previous studies on proteinase from bacteria isolated from fish sauce. However, parameters affecting growth and proteinase production of proteinase-producing bacteria isolated from fish sauce have not been thoroughly investigated. More importantly, these proteinases have not yet been reported in producing fish sauce in a shorter time and in other food production.

2.4 Characteristic of proteinase-producing bacteria

2.4.1 *Staphylococcus* sp.

Staphylococcus is a group of gram-positive bacteria. It is commonly found in animal, human and environment. This genus is closely related to the genus of *Micrococcus* which is separated on the basis of morphological and biochemical characteristics as shown in Table 2.1 (Kloos and Schleifer, 2001).

Genus *Staphylococcus* is spherical with 0.5-1.5 μm diameter and nonmotile. It does not form endospores. Cells arrangement can be singly, pairs, tetrads, short chains, and irregular clusters (grape-like). Colonies are smooth, raised, circular, entire

and translucent. Colonies pigment is cream-yellow, yellow, yellow-orange or orange depending on species. Most species are facultative anaerobes which grow better under aerobic than anaerobic conditions. Catalase test is always positive.

Table 2.1 Differential characteristics of the genus *Micrococcus* and *Staphylococcus*

Characteristics	*Micrococcus*	*Staphylococcus*
Irregular clusters cell arrangement	+	+
Tetrads cell arrangement	+	-
Capsule	-	-
Motility	-	-
Growth on Furazolidone agar	+	-
Anaerobic fermentation of glucose	-	+
Oxidase tests	+	-
Glycine in peptidoglycan	-	+
Teichoic acid present in cell wall	-	+
G+C of DNA (% Mol)	65-75	30-39

(Kloos and Schleifer, 2001)

Cell wall contains peptidoglycan and teichoic acid. The diamino acid present in the peptidoglycan is L-lysine and the interpeptide bridge of the peptidoglycan is glycine. The cells require nutritionally rich media including amino acids, several B group vitamins (thiamine and nicotinic acid and nicotinamide) and fermentable carbohydrates. *Staphylococcus* can produce acid from a variety of carbohydrates depending on species. *Staphylococcus* grows well at pH 7 and most species grow at 10% NaCl and 18-40^{o}C.

There are several *Staphylococcus* strains that have been found in a wide variety of fermented foods. They included fish sauce (Fukami et al., 2004), soy sauce mash (Tanasupawat et al., 1992; Andreas et al., 1998), bu-du (Chihara et al., 2002) and fermented fish (Tanasupawat et al., 1992). The members of *Staphylococcus* are as follows:

Staphylococcus cohnii has been predominated in fermented sausage (Paramithiotis et al., 2006; Drosinos et al., 2007). It is spherical with 0.5-1.2 μm in

diameter, non motile and non-sporeforming. Energy is obtained from nitrogen source. Kloos and Schleifer (2001) found that *S. cohnii* grow well in medium containing $(NH_4)_2SO_4$ as a nitrogen source. Most strains of *S. cohnii* were capable of producing acid as shown in Table 2.2. *S. cohnii* do not reduce nitrate. However, some strains retain their ability to hydrolyze gelatin and casein. *S. cohnii* grew well in NaCl concentration up to 10% and pH 7. Optimal temperature for growth was between 15^{o}C and 45^{o}C and optimum growth occurred at 30-40^{o}C (Kloos and Wolfshohl, 1991).

Staphylococcus nepalensis was isolated from fish sauce mash (moromi) made from frigate mackerel in Japan and was able to improve fish sauce odor. It was sphere-shape (approximataely 1 μm diameter), non-motile and non-sporeforming (Fukami et al., 2004). Most strains do not require an organic nitrogen source, but usually require vitamins B for growth (Kloos and Wolfshohl, 1991). Acids may be produced from a wide variety of carbohydrates (Fukami et al., 2004) (Table 2.2). *S. nepalensis* reduces nitrate and hydrolyzes urea, arginine and otnithine. *S. nepalensis* requires 10% NaCl for growth (Kloos and Wolfshohl, 1991). Moreover, some strains can grow in media containing 18-22% NaCl (Fukami et al., 2004). Temperature range for growth was between 20 and 40^{o}C with the optimum growth at 30^{o}C. *S. nepalensis* did not grow at 15 and 45 ^{o}C (Spergser et al., 2003).

Staphylococcus carnosus was originally isolated from fermented sausages and used as starter culture of the fermented sausage and cured ham as it showed effects on flavor formation (Kloos and Wolfshohl, 1991; Hammes and Knauf, 1994). *S. carnosus* was isolated from fish fermentation and soy sauces in Asia (Tanasupawat et al., 1991). Cells of *S. carnosus* are 0.5-1.0 μm in diameter, non-motile and non-spore forming. *S. carnosus* requires glucose, yeast extract and peptone for growth. It

shows positive tests on catalase and hydrolysis of gelatin but negative for hemolysis. It can produce acid as shown in Table 2.2. Generally, it can grow at NaCl concentrations up to 15% but some strains grow at only 6-8% NaCl. *S. carnosus* grow well at a pH range of 6.8-9.0 but no growth is observed at pH 4.5. Temperature range for growth is 18-42^{o}C

Staphylococcus piscifermentans was a new species isolated from fermented fish in Thailand, such as ka-pi, pla-chom, pla-ra and bu-du (Tanasupawat et al., 1992). This species was closely related to *Staphylococcus carnosus* which separated by urea and esculin hydrolysis, β-glucosidase activity, phosphatase activity and acid production from melezitose and sucrose. The characteristic of *S. piscifermentans* was described by Tanasupawat et al. (1992). *S. piscifermentans* was the group of gram-positive cocci. It was spherical with 1μm in diameter and required glucose, yeast extract and peptone for growth. It hydrolyzed arginine, gelatin, urea, Tween 80, esculin and showed negative results on hemolysis. Acid was produced as shown in Table 2.2. *S. piscifermentans* grew well at relatively high even at pH 6.8-9.0. This strain grew slightly at pH 5.0 and could not grow at pH 4.5. The optimum growth temperature was 42-45^{o}C. *S. piscifermentans* grew in the medium containing 6-10% NaCl and some strains grew in the medium containing 15% NaCl.

Staphylococcus condimenti was originally found in soy sauce mash and described as the new species by Andreas et al. (1998). *S. condimenti* was clearly separated from *S. carnosus* and *S. piscifermentans* by their phenotypic and biochemical characteristics, and also DNA sequences. *S. condimenti* was gram positive cocci and spherical with 1μm in diameter. It required about 48 h to reach log phase. It hydrolyzed urea and arginine but not aesculin. It could produce acid as shown in

Table 2.2 Characteristics for differentiating species in the genus *Staphylococcus*

Characteristics	S. *cohnii*[a]	*S. nepalensis*[b]	*S. carnosus*[c]	*S. piscifermentans*[d]	*S. codimenti*[e]
Acid from:					
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Mannose	+	+	NA	-	+
Maltose	+	NA	NA	NA	NA
Lactose	NA	+	NA	NA	NA
Fucose	-	NA	-	-	NA
Sucrose	+	+	-	NA	NA
Trehalose	+	+	+	+	+
Mannitol	+	+	NA	NA	+
Xylitol		NA	-	-	NA
Sorbitol	-	NA	NA	NA	+
Inositol	-	NA	NA	NA	NA
Adonitol	-	NA	NA	NA	NA
Dulcitol	-	NA	NA	NA	NA
Arabitol	-	NA	NA	NA	NA
Erythrose	-	NA	NA	NA	NA
Tagatose	-	NA	NA	NA	NA
Sorbose	-	NA	NA	NA	NA
Melibiose	-	NA	-	NA	NA
Raffinose	-	-	NA	-	-
Xylose	NA	+	-	-	NA
Arabinose	NA	+	-	-	-
Cellobiose	-	-	-	-	-
Salicin	-	NA	NA	NA	NA
Galactose	NA	NA	NA	NA	+
Melezitose	-	NA	NA	NA	NA
Turanose	-	+	-	-	-
Ribose	-	NA	NA	NA	-
Rhamnose	-	NA	NA	NA	NA
Gentibiose	-	NA	NA	NA	NA
Glycerol	+	+	NA	NA	+

[a] Kloos and Schleifer, 2001, [b] Fukami et al., 2004, [c] Tanasupawat et al., 1992
[d] Andreas et al., 1998

Table 2.2. Growth was found at 15 and 42°C and it could grow in the medium containing 0.5-15% NaCl.

2.4.2 *Virgibacillus* sp.

The genus *Virgibacillus* was firstly proposed by Heyndrickx et al. (1998). It was originally assigned to the genus *Bacillus* based on phenotypic characterization, fatty acid profile and genotypic characterization. Heyndrickx et al. (1998) reported that *Bacillus pantothenticus* represented a phylogenetic group sufficiently different from other *Bacillus* species to warrant the status of a separate genus for which they had proposed the name *Virgibacillus*. This genus could be distinguished from members of *Bacillus* rRNA group aerobic endospore-forming bacteria, such as *Halobacillus*, *Paenibacillus*, *Brevibacillus* and *Aneurinbacillus*.

Members of the genus *Virgibacillus* were gram positive rods (0.3-0.7 x 2.0-6.0 μm). Cells arrangement occurred singly, pair, short or long chain. Colony was small, circular, low convex and slightly transparent to opaque. This genus was catalase-positive, motile and produced endospores. Spores were usually spherical to ellipsoidal and located at terminal or subterminal position. It usually hydrolyzed gelatin, aesculin and casein. Moreover, it could produce acid from a variety of carbohydrate, depending on species. Growth of genus *Virgibacillus* was noticed between 5°C and 50°C with the optimum temperature at about 28°C or 37°C. Growth of *Virgibacillus* was stimulated by 4-10% NaCl. The G + C in DNA of *Virgibacillus* was 36-43% mol, which was less than *Bacillus* (32-69% mol) (Heyrman et al., 2003). Cell wall peptidoglycan of *Virgibacillus* contained *meso*- diaminopimelic acid type.

Sneath (1998) described sporulation stage of *Virgibacillus*. Most species of genus *Virgibacillus* produced endospores but some strains did not. This was important

for a taxonomic point of view, especially in the identification of bacterial isolates where non-sporulating *Virgibacillus* strains may be misidentified as a member of other genera. Factors affecting endospore formation included the temperature of growth, pH of the medium, aeration, presence of minerals in the medium and the concentration of the carbon or nitrogen source. Moreover, the activation of endospore may also be achieved by aging at low temperature.

Members of the genus *Virgibacillus* comprise nine species including as *V. pantothenticus* (Heyndrickx et al., 1998), *V. proomii* (Heyndrickx et al., 1999), *V. salexigens*, *V. carmonensis*, *V. necropolis*, *V. marismortui* (Heyrman et al., 2003), *V. halodenitrificans* (Yoon et al., 2004), *V. dokdonensis* (Yoon, Kang, Lee, Lee, and Oh, 2005) and *V. koreensis* (Lee, Lim, Lee, Park and Kim, 2006). But, there were only 4 strains isolated from salt containing environment as follows:

Virgibacillus marismortui was the group of moderately halophilic bacteria isolated from the Dead Sea by Arahal et al. (1999). It was gram-positive rod (2.0-3.6 x 0.5-0.7 μm), motile and produced spore. Spores were oval shape and positioned at terminal or subterminal position. It was strictly aerobic and grew in the presence of 5-25% NaCl at pH 6.0-9.0 (optimum at pH 7.5). Optimum growth was found at 10% NaCl and did not grow in the absence of NaCl. It could grow at 15-50^{o}C with the optimum temperature at 37^{o}C. It was catalase and oxidase positive. It hydrolyzed casein and gelatin but did not hydrolyze starch and Tween 80. It produced acid as shown in Table 2.3. Growth factors of *V. marismortui* were citrate, sucrose, mannitol and butyrate as a carbon source, while alanine and arginine could serve as a nitrogen source. Cell wall contained peptidoglycan of the *meso*-diaminopimelic acid type. The G + C content was 39 - 42.8% mol.

Virgibacillus halodenitrificans was a moderately halophilic bacteria, isolated from marine solar saltern of the Yellow Sea in Korea by Yoon et al. (2004). It was a gram variable rod with the size of approximately 0.6-0.8 x 2.0-4.0 μm. It was motile and produced spore with ellipsoidal shape located at terminal or subterminal position. It could produce acid as shown in Table 2.3. Catalase and oxidase tests were positive. Casein and gelatin were usually hydrolyzed but starch and Tween 80 were not. pH range for growth was between 5.8 and 9.6, however the optimum pH was 7.4-7.5. It showed slightly growth at pH 5.0 and could not grow at pH 4.5. The growth temperature was 10-45^{o}C and the optimum temperature for growth was around 35-40^{o}C. It could grow at 0-23% NaCl with the optimum at 3-7% NaCl. Cell wall peptidoglycan contained *meso*-diaminopimelic acid type. The G + C content was 38-39% mol.

Virgibacillus dokdonensis was slightly halophilic bacteria isolated from East Sea in Korea by Yoon et al. (2005). It was gram variable rod (0.6-0.8 x 2.5-5.0 μm) and motile. Spore shape was ellipsoidal or spherical and in terminal or subterminal position. Acids produced by *V. dokdonensis* were shown in Table 2.3. It hydrolyzed casein, gelatin, aesculin, starch and Tween 80 but not hypoxanthine, xanthines and tyrosine. Growth occurred at 15-50^{o}C and optimum growth temperature was 37^{o}C. The optimum pH for growth was 7.0-8.0. It slightly grew at pH 5.5 and could not grow at pH 5.0. Growth occurred at 0-23% NaCl and with the optimum at 4-5% NaCl. Cell wall contained peptidoglycan of the *meso*-diaminopimelic
acid type. The G + C content was 36.7 mol%.

Virgibacillus koreensis was isolated from a salt field on the Yellow Sea in Korea by Lee et al. (2006). It was motile and produced spores. Spore shape was

ellipsoidal and located at terminal position. It produced acid as shown in Table 2.3. It hydrolyzed aesculin but not gelatin. The temperature range for growth was 10-45^{o}C

Table 2.3 Characteristics for differentiating species in the genus *Virgibacillus*

Characteristics	*V. marismortui*[a]	*V. halodenitrificans*[b]	*V. dokdonensis*[c]	*V. koreensis*[d]
Acid from:				
Glucose	+	+	+	+
Fructose	+	NA	+	+
Mannose		NA	NA	+
Maltose	+	+	+	+
Lactose	-	NA	+	NA
Fucose	NA	NA	NA	+
Sucrose	-	+	+	NA
Trehalose	-	NA	NA	NA
Mannitol	-	NA	NA	+
Sorbitol	NA	-	+	NA
Inositol	NA	-	+	NA
Adonitol	NA	-	NA	NA
Melibiose	NA	NA	NA	+
Raffinose	NA	-	-	NA
Xylose	-	-	-	+
Arabinose	-	-	-	+
Cellobiose	NA	-	+	+
Galactose	-	NA	NA	+
Melezitose	NA	-	-	NA
Turanose	NA	NA	NA	+
Ribose	NA	+	+	NA
Rhamnose	NA	NA	NA	+
Glycerol	+	NA	NA	+

[a] Arahal et al., 1999, [b] Yoon et al., 2004, [c] Yoon et al., 2005, [d] Lee et al., 2006

with optimum at 25 ^{o}C. pH range for growth was 5.5-9.0 and optimum pH was at 7.0. It required 5-10% NaCl for proper growth and it could grow in media containing more

than 20% NaCl. Cell wall peptidoglycan contained *meso*-diaminopimelic acid type. The G + C content was 41 % mol.

2.5 Identification of halotolerant/halophilic bacteria

Many organisms can be identified on the basis of morphological characteristics. However, this approach is not reliable for all groups of organisms, including bacteria which possess limited morphological differentiation (Entis et al., 2001). Many techniques for bacterial identification rely on results of biochemical tests and assimilation assays (Reva, Sorokulova and Smironov, 2001). Such physiological tests have been performed using traditional microbiological methods or commercially available kits such as API system. The API has been tested extensively with reported accuracy ranging from 90.2-93% (Entis et al., 2001). Thongthai and Suntinanalert (1991) isolated 241 bacterial isolates from Thai fish sauce. All bacteria were identified based on phenotypic and biochemical characteristics. Twenty percent of isolates were identified as *Halobacterium* sp. which grew strictly in the presence of 20-30% NaCl, while 5% of the isolates were identified as *Halococcus* sp. that grew in the presence of 15-30% NaCl. Other bacterial isolates were identified as either halotolerant or moderately halophilic which showed growth at 0-30% NaCl and grew well in the presence of less salt. These isolates included *Micrococcus, Staphylococcus, Streptococcus, Bacillus* and *Corynebacterium*.

Study of halophilic bacterial classification was performed, based on their phenotypic and biochemical characteristics. Their phenotypic characteristics still have limitation for species identification. However, other techniques including cellular fatty acid analysis, DNA/DNA hybridization and DNA sequence analysis techniques

are recently developed for bacterial identification. The molecular techniques have been developed based on homologies in between of DNA, RNA and protein molecules and lead to evolutionary relationships of microorganisms (Priest and Austin, 1993).

For the analysis of fatty acid composition for chemotaxonomic characterization of bacterial membrane function, lipid are the major components of the bacterial membrane maintaining, particularly phospholipids. Due to bacteria grow well in different environments, they show distinct forms and functions. Thus, their lipid composition should be specific for each individual bacterium. Phospholipids and their fatty acid composition are important indicators in bacterial chemotaxonomy. Brown and Leff (1996) studied the comparison of fatty acid methyl ester (FAME) analysis with the use of API system for identification of aquatic bacteria. Aquatic bacteria were isolated from Cuyahoga River in Ohino using modified Nutrient agar and MacConkey agar. They found 127 isolates from both modified Nutrient agar and MacConkey agar. All identification from API system found that approximately 62.2% of isolates could be identified, while 99.2% could be identified by FAME analysis. FAME analysis was more efficient for identification. These results confirmed those of Santos et al. (1993) who found that the API system could not accurately identify some strains in species levels. Bernard, Bellefeuille and Evan (1991) described API system is not reliable for some group of bacteria which possess limited biochemical differentiation, whereas cellular fatty acid composition was found to be sufficiently unique or discriminatory to be used as a highly regarded taxonomic criterion by itself. However, cellular fatty acid composition analysis had some limitations. Deviation of bacteria from the recommended growth medium, growth conditions (temperature or

time) or extract preparation procedure will alter the fatty acid profiles, resulting in poor identification (Farber et al., 2001).

There are several techniques which permit different levels of identification, such as strain, species, or genus. The study of the polymerase chain reaction (PCR) technique is more useful for classification purpose. It is possible to amplify a genera or a part of gene from a very limited number of cells for subsequent DNA sequencing (Axelsson, 2004). The PCR consists of repeating cycles of alternating high and low temperatures that bring about the amplification. Each cycle consists of three steps as denaturation, annealing and extension. Denaturation at high temperature causes the double stranded target DNA to separate into single strands. In the annealing step, the temperature is lowered, and the primers bind to their complementary sequences on opposite strands of the target DNA. The temperature is raised again and DNA polymerase adds nucleotide bases onto the ends of both primers (extension), building two new polynucleotide chains according to the sequences within the target region. Thus, the original target is copied. The newly made copies then serve as templates for additional rounds. The cycle of denaturation, annealing, and DNA synthesis is then repeated many times. Because the productions of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product (Entis et al., 2001)

Another technique is hybridization which is often used in molecular genetics. It is very well adapted for the identification of species and even strains. Hybridization technique is based on the ability of single-strand DNA chains to reassemble in double-strand chains. The process of hybridization as DNA probe binds to a complementary region of single-stranded DNA. DNA probe is usually 20-2000 nucleotides base

sequence of DNA that is unique to a particular microbial group. Probes can identify specific genes, such as toxin genes, regions of ribosomal RNA or DNA sequences that have unknown function but are common to particular microbial groups. Tanasupawat et al., (1992) used DNA hybridization, phenotypic and biochemical characteristics to detect and identify new *Staphylococcus* (*S. piscifermentans* sp. nov.) from fermented fish in Thailand.

Chaiyanan et al., (1999) isolated halophilic bacteria strain fs-1 from fish sauce. This strain was gram-positve rod which showed high proteolytic activity and was proved to be a moderate halophile growing in media containing 3-15% NaCl. The 16S rRNA gene sequence and DNA/DNA hybridization were used to identify a novel of *Halobacillus*. Based on 16S rRNA gene sequence of bacterial isolate strain fs-1, it showed the highest level of similarity with *Halobacillus litoralis* (99.2%) and *Halobacillus halophilus* (97.2%). The results were confirmed by DNA/DNA hybridization. Strain fs-1 showed low level of similarity between type strains of the *Halobacillus litolaris* (49.2%), *Halobacillus trueperi* (40.1%) and *Halobacillus halophilus* (33.0%). Strain fs-1 was subsequently identified a new species of *Halobacillus thailandensis* sp. nov. Based on this study, the combination between 2 methods of 16S rRNA gene sequence and DNA/DNA hybridization increased accuracy in the identification. Noguchi et al. (2004) identified a new *Bacillus* species using 16S rRNA gene sequence technique and confirmed the genus by DNA/DNA hybridization technique. Moreover, they used phylogenetic analysis of all bacteria which closely related the genus *Bacillus*. Thongsanit et al. (2002) isolated halophilic lactic acid bacteria from fish sauce based on phenotypic and chemotaxonomic characteristics, including DNA relatedness. Halophilic lactic acid bacteria were

identified as *Tetragenococcus halophilus*, *Tetragenococcus muriaticus* and *Aerococcus viridans*.

Therefore, the molecular technique especially, 16S rRNA gene sequence and DNA/DNA hybridization are useful for halophilic bacteria identification. However, the combination of phenotypic characterization, was also important and should be used in combination with the molecular technique for accuracy identification.

2.6 Roles of the starter culture in food fermentations

Food fermentations are spontaneous processes caused by microorganisms derived from the raw materials or the environment and has been used as a method to preserve perishable food products (Buckenhuskes, 1993; Hansen, 2002). With the discovery of microorganisms, it became possible to improve the products and the fermentation processes by using isolated microorganisms and well characterized cultures. Then starter cultures have been prepared for various food fermentation process. Starter cultures are living microorganisms whose their metabolism play effective role in the fermentation process (Hammes, 1990; Gilliland, 1994).

Proteinase-producing bacteria are widely used in the production of fermented food, such as cheese, fermented sausage, fermented soybean products and soy sauce (Fermandez-Garcia et al., 1999). Preparation of bacterial starter culture depended on several factors, including salt concentration, incubation temperature, pH of medium, and ability of bacteria to produce proteinase (Miralles, Flores and Perez-Matinez, 1996).

Fungal proteinase had been used for centuries for preparation of soy sauce and other soy products. Soy sauce is the traditional fermented condiment commonly consumed by the people in Taiwan, Japan and China. The manufacturing processes

for soy sauce consists of two stages, including koji prepararion and mash aging (Yokotsuka and Sasaki, 1998). Traditionally, the soy sauce koji is generally prepared by growing koji mold, such as *Aspergillus oryzae* or *A. sojae*, on the mixture of the cooked-defatted soybean and roasted wheat flour. The finished koji is then placed into sodium chloride solution (18%) and lactic acid bacteria and yeast fermentation during the mash-aging period. Rapid growth of molds and high activity of enzymes including proteinase, amylase, lipase etc. in koji are essential to the reduction of the processing time and improving the quality (Baens-Arcega et al., 1996).

Ling and Chou (1996) found that soy sauce koji of *Aspergillus oryzae* was essential to the reduction of the processing duration and improved quality of the soy sauce product. In addition, they studied the preparation of soy sauce koji with *A. oryzae* on extruded raw materials. The extruded substrates were composed of wheat flour and defatted soybean at a ratio of 1:1, then extruded through a co-rotating twin-screw extruder. Results showed that *A. oryzae* grew well on the extruded substrate and also showed a higher final content of the total nitrogen, formol nitrogen and higher enzyme activity (proteinase, lipase, α-amylase, α-galactosidase and glutaminase) than that prepared from the traditional substrate. So the koji prepared with extruded substrate accelerated the fermentation process and improved the production of soy sauce, such as flavor, color and textures. Extruded substrate was more gelatinized (72.9%) than the traditional (21.3%) and the extruded substrate was more susceptible to the activities of proteinase, and possessed more porous structure than the traditional substrate. These physical and chemical changes caused by the extrusion process may account for the difference in the behavior of traditional processes.

Kobayashi and Hayashi (1998) developed the soy yeast starter *Zygosaccharomyces rouxii* AM-105, which was added to the mash (*moromi*) in the fermentation process for the production of soy sauce. The soy yeast *Z. rouxii* is important for aroma development in the soy sauce. The yeast starter with 16% NaCl was effective for maintaining both the viability and fermentative activity of *Z. rouxii* cells and preventing bacterial contamination especially *Lactobacillus* sp.

Omafuvbe, Abiose and Shonukan (2002) isolated *Bacillus subtilis, B. pumilus* and *B. licheniformis,* from soy-*daddawa*, a traditional soy product of Nigeria. Total viable count pattern of fermented soybean inoculated with single and mixed starter cultures of *Bacillus* species were similar. Use of mixed cultures (*Bacillus subtilis*, *B. pumilus*, *B. licheniformis*) showed an increased final pH, whereas the use of single *B. subtilis* did not. In addition, a single culture, *B. subtilis*, resulted in the highest level of proteolytic activity and free amino acid content.

However, the role of the starter culutres in fermented meat products is believed to be very important. Miralles et al. (1996) isolated *Staphylococcus* strains from raw-cured fermented Spanish products (*saichichon*) as a potential meat starter culture. *Staphylococcus* strains were isolated and identified as *S. xylosus* and *S. epidermidis*. *S. xylosus* showed cell-bound proteinase and lipases activity which were important for protein hydrolysis and flavor development. Although, *S. epidermidis* showed high proteinase activity, but it was a pathogen and not suitable to be used as starter culture.

Cagno et al. (2003) was successful in using *Lactobacillus plantarum* DPC2741 as a starter culture in cheese ripening. *L. plantarum* improved flavor and texture of semi-hard cheeses and accelerated ripening process. Moreover, when the partially purified proteinase of *Lactobacillus helveticus* PR4 and *Lactococcus lactis* subsp. ST2

were added into a medium containing β-casein, the growth and proteolytic activity of *L. plantarum* DPC 2741 were effectively increased. This was because, these partially purified proteinase hydrolyzed β-casein, resulting in the higher peptide and amino acid supply. These peptides and amino acid were the nitrogen source for bacterial growth, such as Glu, Val and Ile and other amino acids essential for the growth of *L. plantarum* DPC 2741.

Recently, Uchida et al. (2005) studied the effects of soy sauce koji and lactic acid bacteria on the fermentation of fish sauce from freshwater silver carp (*Hypophthalmichthys molitrix*). The addition of soy sauce koji (*Aspergillus oryzaes*) resulted in a remarkable increase of soluble components such as organic acids and amino acids that contributed to taste. Whereas the combination of lacitic acid bacteria (*Tetragenococcus halophilus*) and *A. oryzaes* in koji were similar to the use of koji alone (*A. oryzaes*). So koji alone (without lactic acid bacteria) was prepared for use in fish sauce fermentation. However, proteolytic starter culture prepared from halophilic/halotolerant bacteria has not yet been studied in the fish sauce fermentation.

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CHAPTER III

CHARACTERIZATION AND IDENTIFICATION OF PROTEINASE-PRODUCING BACTERIA ISOLATED FROM THAI FISH SAUCE FERMENTATION

3.1 Abstract

Proteinase-producing bacteria were isolated from 50 samples of fish sauce fermented at 1-12 months, fish juice, and solar salt samples. skim milk salt agar (+25% NaCl), standard methods caseinate agar (+25% NaCl), and halobacterium agar (+25% NaCl) were used for the isolation. Total of 308 bacterial isolates were collected and 27 of them could hydrolyze anchovy proteins and did not produce histamine and other biogenic amines. Bacterial identification was also performed using morphological and biochemical characteristics and 16S rRNA gene sequences. Twenty-seven proteinase-producing bacterial isolates were Gram-positive spores forming rod, Gram-negative rod, and Gram-positive cocci which were identified as *Virgibacillus*, *Halomonas*, *Bacillus firmus*, *Brevibacteium*, *Corynebacterium*, and *Staphylococcus*. Three isolates namely *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 showed highest proteolytic activity towards anchovy proteins based on TCA-soluble oligopeptide. These three potential could be used to prepare starter culture for fish sauce fermentation.

Keywords: Fish sauce fermentation, Proteinase production, 16S rRNA gene sequences

3.2 Introduction

Fish sauce is a clear brown liquid produced from fermentation of fish at high salt content (Saisithi, 1994). It is widely consumed in most countries of Southeast Asia. Fish sauce is basically produced by mixing fish and salt at a ratio of 3:1. The mixture is allowed to ferment for a long time at about 12-18 months at ambient temperature. Long fermentation process is a major factor limiting the growth of fish sauce industry. Reducing fermentation time using microorganisms as a starter culture was an alternative.

Many microorganisms were found during fish sauce fermentation which were identified as *Micrococcus*, *Tetragenococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Lactobacillus*, *Coryneform*, *Pseudomonas*, *Proteus* (Saisithi et al., 1966; Thongthai et al., 1991; Saisithi, 1994; Thongsanit et al., 2002; Fukami et al., 2004). Other halophilic bacteria, including *Halobacterium*, *Halococcus* and *Halobacillus* were also reported (Thongthai et al., 1991; Chaiyanan et al., 1999). Proteinase-producing bacteria were found to be dominant in fish sauce fermentation (Noguchi et al., 2004, 2005) and had important role to hydrolyze protein during fermentation process. Chaiyanan et al. (1999) isolated moderately halophilic bacterium from Thai fish sauce and identified as *Halobacillus thailandensis* sp. nov. It showed high proteolytic activity and could grow in a medium containing 0.5 to 30% NaCl with the optimum NaCl concentration at 10%. *Bacillus vietnamensis* was isolated from Vietnamese fish sauce. It was moderately halotolerant bacteria which grew at 0-15% NaCl. Recently, *Halobacillus* sp. SR5-3 and *Filobacillus* sp. RF2-5 were also isolated from Thai fish sauce, nam-pla. These were moderately halophilic bacteria which optimally grew at 10% NaCl and produced serine proteinase (Namwong et al., 2005, 2006). However,

proteinase-producing bacteria in fish sauce fermentation have not been thoroughly reported. Proteinase-producing bacteria are widely used in the production of fermented food, such as fermented sausage, fermented soy bean products. Terlabie, Dawson and Amoa (2006) found that *Bacillus subtilis* 72BP30 isolated from soybean-dawadawa (traditional fermented food in Africa) reduced the processing time (within 72 h) and improved product quality. Omafuvbe et al. (2002) also found that *B. subtilis, B. pumilus* and *B. licheniformis* were essential to the reduction of the processing duration of soy-daddawa (traditional soy product of Nigeria). Miralles et al. (1996) believed that *Staphylococcus* strains isolated from raw cured fermented Spanish products (saichichon) was a potential meat starter culture. Thus, proteinase-producing bacteria were assumed to be important for fermentation process. Another characteristic of proteinase-producing bacteria that used as a starter culture should not produce biogenic amines. Due to biogenic amines, especially histamine, were important in vasoactive effects in human (Fernandez-Garcia et al., 1999; Lehane and Olley, 2000). Nevertheless, the use of proteinase-producing bacteria as a starter culture has not been reported for fish sauce fermentation.

Isolation and identification of proteinase-producing bacteria, that are dominant microflora of fermentation process, is an important step in the development of the new starter culture. However, identification of proteinase-producing bacteria, particularly halotolerant/ halophilic bacteria, is not always possible using only phenotypic methods or other biochemical/physiological traits (Bernard et al., 1991; Santos et al., 1993). Several molecular methods have recently been applied for more rapid and reliable identification of halotolerant/ halophilic bacteria, such as DNA/DNA hybridization and DNA sequence analysis techniques (Priest and Anstin, 1993).

Several studies suggested that 16S rDNA sequences and phylogenetic relationships could be used to identify halotolerant/ halophilic bacteria (Stan-Lotter et al., 2002; Amoozegar et al., 2003; Yoon et al., 2003; Yoon et al., 2004). Moreover, Stackebrandt et al. (2002) also suggested that DNA/DNA hybridization techniques could be used to confirm the species when 16S rDNA gene sequences similarity was more than 97%. The objective of this study was to isolate and identify proteinase-producing bacteria from various periods of Thai fish sauce fermentation.

3.3 Materials and methods

3.3.1 Collection of fish sauce samples

Samples of fish sauce fermented at various time (1, 3, 5, 7, 9, 12 months) were collected at 4 different factories in Rayong province. Samples were randomly taken at the depth of 30-50 cm from the opening of fermentation using a water sampling device with 10 cm diameter. Solar salt was also collected from each factory by sampling at the surface and 20-30 cm beneath the surface. Fish sauce samples and solar salt were kept in a steriled bottle at 4^{o}C for further examination within 24 h.

3.3.2 Isolation of bacteria from fish sauce samples

Proteinase-producing bacteria were enumerated and isolated using skim milk salt agar containing 25% NaCl (Prasad and Seenayya, 2000), standard methods caseinate agar containing 25% NaCl (Atlas and Parks, 1997) and halobacterium agar containing 25% NaCl at pH 7 (Altas and Parks, 1997; Thongthai and Suntinanalert, 1991; Thongthai et al., 1992). Total plate count was also performed using plate count agar (PCA) containing 20% NaCl. Twenty five milliliters of fish sauce samples were placed into the bottle containing either 25 or 100 ml of 0.85% NaCl to obtain the

dilution of 1:1 and 1:4, respectively, and mixed throughly. The standard spread plate count method was used for enumeration of proteinase-producing bacteria. Each selected dilution was analyzed in duplicate. The plates were incubated under aerobic condition at 35^{o}C for 7 days. Results were recorded as logarithm of colony forming unit (Log CFU) per milliliter or gram. Different colonies grown on each media were selected and purified for further investigation. Purified bacteria were maintained on Skim milk salt broth containing 20% NaCl and kept at -20^{o}C with the addition of skim milk to the final concentration of 5%. For cell propagation procedure, the stock cultures were taken from -20^{o}C, thawed at room temperature. Two hundred microliters of each culture were inoculated into 2 ml of skim milk salt broth containing 20% NaCl. After incubation at 35^{o}C for 5-7 days, the culture was streaked on Skim milk salt agar containing 20% NaCl and incubated under aerobic condition at 35^{o}C for 5-7 days. Then, a single colony was collected for further study.

3.3.3 Screening and selection of proteinase-producing bacteria

The purified isolates were subcultured on the same medium containing 20% NaCl at 35^{o}C for 5-7 days. Proteolytic activity was tested using skim milk salt agar containing 20% NaCl. A single colony of each strain was streaked on skim milk salt agar and incubated at 35^{o}C under aerobic condition for 5-7 days or until growth was observed. A positive reaction for the proteolytic test was indicated by clear zone around the colony and was collected for proteolytic activity measurement.

3.3.4 Determination of proteolytic activity

Isolated proteinase-producing bacteria were tested for proteolytic activity. One loopful of pure culture was transferred into 10 ml skim milk salt broth containing either 10 or 20% NaCl at pH 7 and incubated under aerobic condition at 35^{o}C for 3 or

5 days, respectively. TCA-soluble oligopeptides content was analyzed using tyrosine as a standard (Lowry, Rosenbrough, Farr and Randall, 1951). Hydrolysis of anchovy proteins by the selected strain was also tested in fish broth containing 20% NaCl for 5 days at 35^{o}C. α-Amino acid and protein content were analyzed using trinitrobenzene sulfonic acid (TNBS) (Field, 1979) and dye binding method (Bradford, 1976), respectively.

3.3.5 Determination of biogenic amines

Biogenic amine production of all isolates were tested using Moller broth added 20% NaCl and 0.4% of various amino acids, including L-lysine, L-histidine, L-ornithine and L-tyrosine (Rodriguez-Jerez et al., 1994). The broths were incubated at 35^{o}C for 5-7 days. Presumptive biogenic amine formation was indicated when color was changed from yellow to purple.

To confirm biogenic amine formation, Moller broth with purple color were centrifuged at 13,000 rpm for 15 min. Supernatant were collected to determine biogenic amines concentration using high performance liquid chromatography (HPLC) (Hewlett-Packard HP 1100, Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with Hypersil BDS C_{18}-collumn. The mixture of acetonitrile and 0.02 M acetic acid (1:9) was used as solvent A and the mixture of 0.02 M acetic acid, acetonitrile and methanol (1:4.5:4.5) was used as solvent B with the flow rate of 1 ml/min. Isocratic elution was initiated with 50% solvent B for 5 min, subsequently the gradient elution was started and ended at 90% solvent B in 35 min. The column was equilibrated with 50% solvent A and B for 10 min before the next injection. The column was kept at 28^{o}C. The sample volume injected was 10 μl. Dansylation was carried out and biogenic amines were detected using a diode array detector set at 254

and 550 nm as a reference (Eerola, Hinkkanen, Linfords and Hirivi, 1993).

3.3.6 Identification of proteinase-producing bacteria

Proteinase-producing bacterial isolates that did not produce biogenic amines were characterized using phenotypic, biochemical, physiological, and ribosomal RNA gene.

3.3.6.1 Characterization of bacterial isolates

All isolates were determined as described in the Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986; Grant et al., 2001; Kloos and Schleifer, 1986; Meyer, 1989; Palleroni, 1984; Schleifer, 1986; and Vreeland, 1984).

A Morphological characteristics

Gram stain reaction, cell morphology, and spore and motility were examined as described by Cappuccino and Sherman (1999).

B Biochemical characteristics

Biochemical reactions were conducted following the standard determinative bacteriology procedure (Beishier, 1991; Smibert and Krieg, 1994; Holt et al., 1994 and Grant et al., 2001) in conjunction with API-staph and API-50CHB/E kits (BIO-Merieux, Marcy-I ' Etoile, France).

Oxidation fermentation medium (O/F medium) containing 20% NaCl was tested for oxidative and fermentative metabolism of glucose. Each strain was deeply stabbed into two tubes of O/F medium and steriled mineral oil (2-3 ml) was added to one of the tube. The inoculated O/F medium was incubated at 35^{o}C for 5-7 days. Yellow medium indicated glucose fermentation.

Starch agar containing 20% NaCl was used for starch hydrolysis test at 35^{o}C for 5-7 days. The plate was flooded with iodine solution. A clear zone

around the colony indicated positive result.

Each isolate was stabbed into the gelatin medium containing 20% NaCl, and incubated at 35^{o}C for 5-7 days. Gelatin hydrolysis was indicated by liquefying of the medium after the tube was kept at 4^{o}C for 30-40 minutes.

Bacterial cells were transferred to the surface of a glass slide. One drop of 3% hydrogen peroxide was added. Rapid gas formation indicated a positive result.

The filter paper (Whatman no. 4) was placed into a petridish and wet with 0.5 ml of 1% tetramethyl-p-phenylenediamine dihydrochloride. The bacterial cells were streaked onto the reagent zone of the filter paper. The development of a deep blue color at the inoculation site within 5-10 seconds indicated a positive result of oxidase test.

C Physiological characteristics

1) pH, temperature and salt tolerance tests

Effect of chemical and physical factors on bacterial growth was also investigated. Selected strains were tested for growth at different NaCl concentrations (0, 0.5, 3, 5, 10, 15, 18, 20, 23, 25 and 30%), pHs (4, 5, 5.5, 6.0, 7.0, 7.5, 8.0, 9.0, 10.0, 11.0 and 12.0) and temperatures (10, 15, 20, 25, 30, 35, 40, 45, 50 and 55^{o}C) using trypticase soy broth (TSB). The inoculum size of 1 ml (approximate 10^{6} CFU/ml) was aseptically transferred to 9 ml of TSB. Each condition was analyzed in duplicate and incubated under aerobic condition at 35^{o}C for 1-7 days. Growth was assayed by enumeration using JCM 168 medium containing 25% NaCl.

2) Effect of NaCl concentration on cell morphology

The effect of NaCl on cell morphology was observed using

scanning electron microscopy (SEM). *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were cultured in Nutrient broth (NA) with addition of different NaCl concentrations (0, 0.5, 3, 5, 10, 15, 18, 20, 25 and 30%). At 0 and 0.5% NaCl, samples were incubated for 1 day, while samples containing 3-5, 10-15, 18, 20-25 and 30% NaCl were incubated for 2, 3, 4, 5 and 7 days, respectively. When the incubation time was attained, cells were pre-fixed with 2% glutaraldehyde for 24 h, and washed with cacodylate buffer and fixed in 2% osmium tetroxide for 2 h until the black color was formed on the cells and washed again with cacodylate buffer. The fixed cells were slowly dehydrated by washing with ethanol at a sequencial concentration of 30, 50, 70, 90, 95 and 100 %**.** The cell samples were dried using a critical point dryer (CPD, SAMDRI/PVT-3B, Tousimis Research Corporation, Rockville, MD, U.S.A.) and then placed on the stub. The fixed cells were coated with gold using Ion Sputtering (JFC 1100E, JEOL, JEOL Ltd., Tokyo, Japan) and examined using SEM (JFC-6400 Scanning Microscope, JEOL, JEOL Ltd., Tokyo, Japan).

D Chemical component of cell wall

Diaminopimelic acid (cell wall component) was analyzed using thin layer chromatography (Komagata and Suzuki, 1987). Dried cells (10 mg) of SK33 and SK37 were hydrolyzed with 6N HCl at 100^{o}C for 18 h. The hydrolyzed solution was filtered and evaporated. The 400 µl of distilled water was added into dried sample. The solution was loaded onto cellulose HPTLC plate no.5787 and developed with MeOH: H_2O: 6N HCl: Pyridine (80:26:4:10). The cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol for detection.

E Ribosomal RNA gene

Sequencing of 16S rRNA was used for genotypic characterization.

There were four major steps, including genomic DNA extraction, Polymerase Chain Reaction (PCR) amplification of 16S rRNA, sequencing of PCR amplicon, and 16S rRNA sequence analysis.

Genomic DNA of the selected isolate of proteinase-producing bacteria was extracted using Wizard Genomic DNA Purified kit (Promega, Promega corporation, Madison, WI, USA). One loopful of the selected isolate was inoculated into 9 ml of JCM containing 20% NaCl broth, and incubated at 35°C for 5 days. Bacterial cells at the late exponential phase were harvested by centrifugation at 12,000 rpm for 2 min at 4°C. The supernatant was discarded, and the cell pellet was washed once with 240 µl of 50 mM EDTA (pH8), then 30 µl of 10 mg/ml lysozyme was added. The mixture was incubated at 37^{o}C for 30 min, followed by addition of 300 µl of Nuclei Lysis Solution (Promega, Promega corporation, Madison, WI, USA). Subsequently, samples were incubated at 80^{o}C for 5 min and kept at room temperature until cool. The mixture was then added with 1.5 µl of RNase Solution (Promega, Promega corporation, Madison, WI, USA), mixed by inversion, and incubated at 37^{o}C for 30 min. One hundred microliter of Protein Precipitation Solution (Promega, Promega corporation, Madison, WI, USA) was added and mixed using vortex device at high speed for 20 sec. After incubation at 0^{o}C for 5 min, the mixtures were centrifuged at 12,000 rpm for 5 min at 4^{o}C. The supernatant was transferred into a new sterilized microcentrifuge tube. Then, 300 µl of isopropanol was added into supernatant and centrifuged at 12,000 rpm for 5 min at 4^{o}C. The supernatant was discarded. The DNA pellet was added with 600 µl of 70% ethanol. The mixture was centrifuged at 12,000 rpm for 2 min at 4^{o}C. After the supernatant was discarded then dried at 37^{o}C for 1 h. Fifty microliter of TE buffer (10 mM Tris-HCl, pH 8 and 1 mM

EDTA) was added, and kept overnight at 4°C to allow DNA to dissolve. The extracted DNA was detected using 0.8% agarose (Low EEO Agarose, BIO 101, Inc., La Jolla, CA, USA) gels electrophoresis in TBE buffer pH 8.3.

Polymerase Chain Reaction (PCR) was performed using the Thermal Cycler (Thermo electron corporation Px2 Thermal Cycler, Bioscience Technologies Division, Waltham, MA, USA) with a primary heating step for 2 minutes at 94^{o}C, followed by 30 cycles of denaturation for 60 seconds at 94^{o}C, annealing for 60 seconds at 53^{o}C, and extension for 1.30 minutes at 72^{o}C. Each 25 µl of reaction mixture contained 2 µl of genomic DNA, 14.25 µl of MilliQ water, 2.5 µl of 10x buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM each concentration) 1.0 µl of each primer (PO mod f, PC3 mod r, P3 mod f and PC5 r) (20.0 pmoles/µl), and 0.25 µl of *Taq* DNA polymerase. The PCR amplified products were examined by electrophoresis using 1% agarose gel containing ethidium bromide (0.5 µg/ml). The size of PCR products was compared with 1 Kb DNA ladder. The separated PCR products were observed under short wavelength UV light. Size of the PCR products was approximately 1,500 bp.

Direct sequence of 16S rRNA gene were performed using Primer (PO mod f/ PC3 mod r, P3 mod f/ PC5 r) and Terminator Ready Reaction kit version 2.0 (Perkin Elmer, Applied Biosystems, Inc., Foster, CA, USA) in combination with an automated sequencing system. The gene was amplified using Thermal Cycler. An estimated amount of 100 ng of DNA was used for each reaction together with 5 pmol of primer, 4 µl of ready reaction mix and DI water to attain a 10 µl final volume. The same primers were used as previous PCR amplification. Cycle-sequencing PCR

and DNA precipitation with ethanol and sodium acetate were done following the manufacturer's protocol (Applied Bio-Systems, Foster, CA, USA). The precipited DNA was dried, and dissolved in deionized water. Then, sequencing was performed

Table 3.1 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene.

Primer	Primer sequence (5' to 3')	Target region[a]	Reference
PO mod	AGAGTTTGATCMTGG	8-22	Wilson et al. (1990)
PC3 mod r	GGACTAHAGGGTATCTAAT	787-806	Wilson et al. (1990)
P3 mod f	ATTAGATACCCTDTAGTCC	787-806	Wilson et al. (1990)
PC5 r	TACCTTGTTACGACTT	1402-1507	Wilson et al. (1990); Lane (1991)

[a] *E. coli* numbering
D = A:C:T, M = C:A, H = A:C:T, R means the complement of rDNA sequence.

using ABI377 Autometed DNA sequencer (Perkin Elmer, Applied Biosystems, Inc., Foster, CA, USA).

Nucleotide sequence data obtained from DNA sequencing software of ABI377 Automated DNA Sequence was interpreted and converted to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram. The sequence was compared to local alignment search of the GenBank database using the BLAST (Basic Local Alignment Search Tool) version 2.2.9 program of the National Center for Biotechnological Information (NCBI). (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic tree was inferred using the Maximum Pasimony method with software MEGA version 3.1 (Kumar, Tamura and Nei, 2004). The stability relationships were evaluated by a boot strap analysis of 1000 replications.

3.4 Results and discussion

3.4.1 Isolation of proteinase-producing bacteria from fish sauce samples

Bacterial population of fish sauce samples fermented for 1-3 months from plant A-C, solar salt, and fish juice were approximately 10^2-10^3 CFU/g, ml (Table 3.2). Viable cell count of fish sauce fermented at 5-12 months were relatively low at 30 CFU/ml. Skim milk salt agar and standard methods caseinate agar were selective media for proteinase-producing bacteria, while halobacterium agar was selective media for halophilic bacteria. From Table 3.2, bacterial cell counts of all media were comparable. Most of bacteria found in this study might be halophilic bacteria. Samples from factory (D) showed bacterial counts of approximetely 10^2 CFU/g, ml, and less than 30 CFU/g, ml in 12 month-old fermented sample (Table 3.2). The number of microorganisms decreased continuously as the fermentation time prolonged. These results were similar to those of Thongthai and Suntinanalert (1991) who found total bacterial counts were lower at the later stage of fermentation. Lopetcharat and Park (2002) reported that an increase in the number of halophilic bacteria increased at day 10 of Pacific whiting fish sauce fermentation, and rapidly decreased to an undetectable level at day 20. Moreover, Dissaraphong, Benjakul, Visessanguan and Kishimura (2006) found that total viable counts of fish sauce made from tuna viscera decreased to less than 1 log CFU/ml after 4 months while halophilic bacteria and proteolytic bacteria counts decreased gradually during fermentation and were lower than 1 log CFU/ml after 4 and 5 months, respectively. High salt concentration in the fermentation inhibited bacterial growth due to plasmolysis of the bacterial cells. The bacteria may exhibit cell morphological changes in high saline condition as evident by swelling, elongation or shrinkage (Zahran, 1997). Lowering water activity (*A*w)

Table 3.2 Bacterial counts (CFU/g, ml) of various samples collected from four different plants

Fish sauce production plant	Samples	Bacterial counts (CFU/g, ml)			
		Skim milk salt agar[a]	Standard methods caseinate agar[a]	Halobacterium agar[a]	Plate count agar[a]
A	1st month	$1.34x10^3$	$4.47x10^2$	$4.75x10^2$	$2.24x10^3$
	3rd month	$7.91x10^2$	$9.30x10^2$	$7.80x10^1$	$3.63x10^3$
	5th month	$1.10x10^3$	$9.64x10^1$	$4.67x10^2$	$3.84x10^2$
	7th month	<30 (10)	<30 (5)	0	$7.9x10^1$
	9th month	<30 (2)	<30 (1)	0	<30 (4)
	12th month	<30 (19)	<30 (1)	0	<30 (13)
	Fish juice[d]	$8.97x10^2$	$5.77x10^2$	$1.88x10^3$	$1.77x10^3$
	Solar salt	$1.00x10^3$	$1.07x10^3$	$2.00x10^3$	$2.90x10^3$
B	1st month	$2.10x10^3$	$3.38x10^3$	$3.38x10^3$	$5.80x10^2$
	3rd month	$1.55x10^3$	$9.18x10^3$	$9.75x10^2$	$4.00x10^2$
	5th month	<30 (2)	<30 (1)	0	<30 (1)
	7th month	<30 (5)	0	0	<30 (1)
	9th month	0	0	0	0
	12th month	0	0	0	0
	Solar salt	<30 (25)	0	0	<30 (5)
C	1st month	$1.78x10^3$	$1.46x10^2$	$1.28x10^2$	$2.08x10^3$
	3rd month	$4.50x10^2$	$8.92x10^2$	0	$4.02x10^3$
	5th month	0	0	0	<30 (6)
	7th month	<30 (7)	0	0	<30 (22)
	9th month	0	0	0	<30 (23)
	12th month	<30 (12)	0	0	<30 (2)
	Solar salt	$7.60x10^2$	<30 (17)	<30 (6)	$1.85x10^2$
D	1st month	>300[b]	>300[c]	>300[b]	$2.35x10^2$
	3rd month	>300[c]	>300[b]	>300[c]	<30 (1)
	5th month	>300[c]	>300[b]	>300[c]	<30 (2)
	7th month	$2.50x10^2$	$3.10x10^2$	$2.50x10^2$	<30 (3)
	9th month	$9.70x10^2$	$2.55x10^2$	$3.10x10^2$	<30 (3)
	12th month	<30 (15)	<30 (7)	<30 (14)	<30 (1)
	Solar salt	$8.25x10^2$	<30 (0)	<30 (11)	<30 (0)

[a] Medium containing 25% NaCl
[b] Small orange colonies at 1:50 sample dilution
[c] Small orange and white colonies at 1:50 sample dilution
[d] Fish juice was exudates collected from the initial salting.

reduces available water for all metabolic activities causing a longer lag phase. Moreover, in a very high ionic environment, enzymes are easily denatured and inactivated. Thus, metabolism of cells cannot properly function and eventually stop at

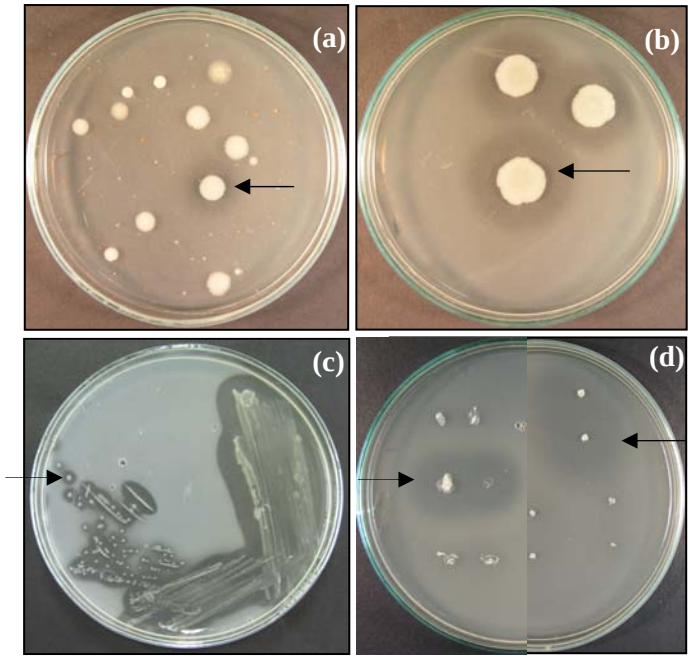

Figure 3.1 Colonies of proteinase-producing bacteria from fish sauce on skim milk salt agar containing 25% NaCl, isolation step (a-b), pure culture step (c) and proteolytic test (d).

high salt (Lopetcharat et al., 2001). It should be noted that bacterial counts of samples fermented for 7-9 months from factory D were higher than those collected from others. This could be due to variations in raw material and process among plants.

For samples collected from the factory D, viable cell counts on skim milk salt agar, Standard methods caseinate agar and halobacterium agar were higher than those obtained from plate count agar (PCA) (Table 3.2). Since, these media consisted of $MgSO_4$ $7H_2O$ and KCl, bacteria found in these samples were probably extremely or

moderately halophilic bacteria. These groups of bacteria required magnesium (Mg^{2+}), potassium (K^{+}) and calcium (Ca^{2+}) for their growth, while other do not strictly require these ions (Ventosa et al., 1998 and Grant et al., 2001).

3.4.2 Screening and selection of proteinase-producing bacteria

A total of 308 isolates was randomly selected from colonies grown on the selected media (Table 3.3). One hundred seventy-five out of 308 isolates were selected from the isolation step for screening proteolytic activity using Skim milk salt agar (Figure 3.1). In second isolation step, bacteria were recultivated using Skim milk salt broth for screening the proteinase-producing bacteria. Only 41 isolates were able to hydrolyze protein in Skim milk salt broth as measured by the TCA-soluble oligopeptides. Subsequently, forty-one isolates were cultivated in fish broth containing mainly fish tissue extract from anchovy, the main raw material used for fish sauce fermentation. This step was important to confirm proteolytic activity of the selected strains towards fish protein.

Nine out of 41 isolates showed proteolytic activity towards anchovy proteins, based on oligopeptide content measured by TNBS. Five out of 9 isolates (SK33, SK37, SK1-1-5, SKW19 and SKS20) exhibited the highest proteolytic activity (Table 3.4). It should be noted that all isolates grew well in fish broth with approximate cell count of 10^{6}-10^{7} CFU/ml when incubated at 35^{o}C for 5 days. Moreover, five isolates were found from the first month of fermentation and solar salt. Lopetcharat et al. (2001) found that protein degradation during fermentation occurred in the early-middle period (1-5 months) of the fish sauce fermentation process by endogenous enzymes (Lopetcharat et al., 2001). However, in this study, most of proteinase-producing bacteria were also found in the early stage of fermentation. It

should be implied that these bacterial had also important role in protein degradation during fermentation process. Thus, these isolates were selected for starter culture development.

All of isolated proteinase-producing bacteria were tested for biogenic amines production. Fourteen out of 308 isolates were positive in Moller broth. Histamine, tyramine and spermidine were the major biogenic amines produced by these 14 isolates. Putrescine, tryptamine and spermine were not produced by all isolates, Only 2 isolates were found to produce cadaverine of 0.18 and 1.85 ppm, respectively. All isolates produced high level of histamine (109.9-163.73 ppm) (Table 3.5). High levels of histamine in food result in vasoactive effects in human (Lehane and Olley, 2000). Biogenic amines are formed through the decarboxylation of specific free amino acids by exogenous decarboxylase released from microorganisms. Histamine-forming bacteria were likely to cause an increase in histamine **(**Lehane and Olley, 2000; Kimura et al., 2001; Tsai et al., 2005, 2006)**.** Histamine production varied depending on the strains. Thongsanit et al. (2002) isolated *Tetragenococcus halophilus* and *T. muriaticus* from Thai fish sauce which produced histamine ranging from 0.40 to 522.9 ppm. While high level of histamine (152.5-668.6 ppm) was produced by *T. muriaticus* isolated from traditional Japanese fermented fish sauce (Kimura et al., 2001). Tsai et al. (2006) isolated *Bacillus coagulans* and *B. megaterium* from fish sauce in Taiwan. These strains produced histamine more than 500 ppm. The fourteen isolated obtained from this study were found from solar salt and the early stage of fermentation (1 and 3 months). Therefore, it should be implied that these isolates were microflora from raw material, such as solar salt and anchovy. Yongsawatdigul et al. (2004) reported that the main source of biogenic amines was

associated with raw material, rather than the fermentation process.

Sodium chloride concentration at 5% could reduce growth and decarboxylase activity of biogenic amine forming bacteria (Tsai et al., 2005). So, these results revealed that biogenic amine forming-bacteria should be classified as halophilic bacteria rather than non-halophilic bacteria. However, these 14 isolates were not selected for further starter culture development because they were likely to increase biogenic amine content during fish sauce fermentation.

3.4.3 Identification of proteinase-producing bacteria

3.4.3.1 Characterization of bacterial isolates

Twenty seven isolates found at 1-9 months of fermentation and solar salt. These isolates were Gram-positive endospores forming rod, Gram-negative rod, and Gram-positive cocci. All isolates did not produce histamine in Moller broth and were tested for the following phenotypic characteristics as detailed in Table1 Appendix B. The variation of microorganism was likely due to microflora in the raw material (anchovy and solar salt) or in fish sauce fermentation tanks. Only 3 out of 27 isolates produced the highest proteinase activity, namely SK33, SK37 and SK1-1-5. There isolated for phenotypic characterization and were identified using API-system.

SK33 and SK37 were Gram-positive endospore-forming rod with the approximate size of 0.5-0.7 x 2.1-6.6 µm after cultivation on JCM 168 containing 18 and 20% NaCl, respectively, at 35^{o}C for 4 days (Figure 3.2 and 3.3). They were non motile. Terminal and subterminal ellipsoidal spores were observed (Figure 3.4). Colonies of SK33 and SK37 were circular, raised, white to cream color with 2-3 mm diameter. They grew at aerobic and anaerobic condition. In the presence of 0-30% NaCl, SK33 grew at 30-45^{o}C, pH 4-12, while SK37 grew at 20-45^{o}C, pH 4-1.

Table 3.3 Proteinase-producing bacteria obtained from fish sauce samples fermented at various times.

Fish sauce production plant	Samples	Number of isolate tested and selected from screening steps			
			Hydrolysis of		
		Total isolates selected	Skim milk salt agar	Skim milk broth	Fish broth
A	1st month	50	42	17	6
	3rd month	22	17	8	-
	5th month	10	4	-	-
	7th month	5	2	-	-
	9th month	2	-	-	-
	12th month	5	-	-	-
	Fish juice	56	38	8	1
	Solar salt	18	15	7	1
B	1st month	25	17	-	-
	3rd month	10	6	-	-
	5th month	2	-	-	-
	7th month	2	-	-	-
	9th month	-	-	-	-
	12th month	-	-	-	-
	Solar salt	10	9	-	-
C	1st month	15	8	-	-
	3rd month	10	6	-	-
	5th month	-	-	-	-
	7th month	5	1	1	1
	9th month	-	-	-	-
	12th month	5	1	-	-
	Solar salt	10	4	-	-
D	1st month	5	-	-	-
	3rd month	5	-	-	-
	5th month	5	-	-	-
	7th month	8	1	-	-
	9th month	8	-	-	-
	12th month	5	-	-	-
	Solar salt	10	4	-	-
	Total	308	175	41	9

Table 3.4 TCA-soluble oligiopeptide content and total viable counts of the selected isolates in fish broth containing 25% NaCl, pH 7 and incubated at 35°C for 5 days with initial cell count of about 5 Log CFU/ml.

Bacterial isolate code	TCA-soluble oligopeptide produced (mM)	Total viable counts (Log CFU/ml)
SK25	2.26±1.07	6.45
SK33	4.82±2.33	6.36
SK37	4.00±0.55	6.45
SK37-1	2.49±0.70	5.72
SK1-1-2	0.62±0.02	6.79
SK1-1-5	3.45±0.14	7.96
SKW19	4.85±0.33	6.11
SKS20	3.62±0.19	6.02
SKM20-7-9	2.52±0.22	7.00

Mean ± standard deviation

Table 3.5 Biogenic amine content of isolates obtained from various sources of fish sauce fermentation process

Bacterial isolate	**His[a] (ppm)**	**Put[b]**	**Cad[c]**	**Tpm[d]**	**Tym[e]**	**Spd[f]**	**Spm[g]**	**Source**
SKS-11	112.29±9.51	ND	ND	ND	39.99±0.7	10.81±0.19	ND	Solar salt
SKS-12	149.99±1.25	ND	ND	ND	37.9±2.62	12.51±0.50	ND	Solar salt
SKS-16	159.77±3.63	ND	0.18±0.24	ND	38.16±0.24	12.54±0.07	ND	Solar salt
SKS-23	163.75±11.81	ND	1.85±2.32	ND	36.59±0.64	11.46±0.19	ND	Solar salt
SK1-1-8	142.06±0.14	ND	ND	ND	38.92±2.16	11.54±0.19	ND	1st month
SK1-1-9	132.98±9.4	ND	ND	ND	32.78±0.52	10.68±0.35	ND	1st month
SK1-3-1	123.78±3.08	ND	ND	ND	32.14±0.43	9.70±0.12	ND	3rd month
SK1-3-2	129.03±2.18	ND	ND	ND	35.22±2.1	9.77±0.81	ND	3rd month
SK1-3-3	135.71±1.18	ND	ND	ND	31.07±3.11	9.20±0.70	ND	3rd month
SK1-3-4	122.92±21.42	ND	ND	ND	37.07±3.65	13.58±1.09	ND	3rd month
SK1-3-8	109.9±5.36	ND	ND	ND	39.31±1.15	13.66±0.86	ND	3rd month
SK1-3-9	134.74±3.37	ND	ND	ND	34.54±1.59	11.86±0.52	ND	3rd month
SK1-3-10	121.54±1.62	ND	ND	ND	33.56±1.4	11.97±11.97	ND	3rd month
SK1-3-14	115.64±9.83	ND	ND	ND	36.29±1.64	10.64±0.22	ND	3rd month

Mean ± standard deviation, ND = Not detected
[a] = Histamine, [b] = Putrescine, [c] = Cadaverine, [d] = Tryptamine, [e] = Tyramine, [f] = Spermidine, [g] = Spermine

They showed catalase and oxidase activities but no urease activity. They were not able to hydrolyze starch and Tween 80. They contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. They showed negative response to hemolysis.

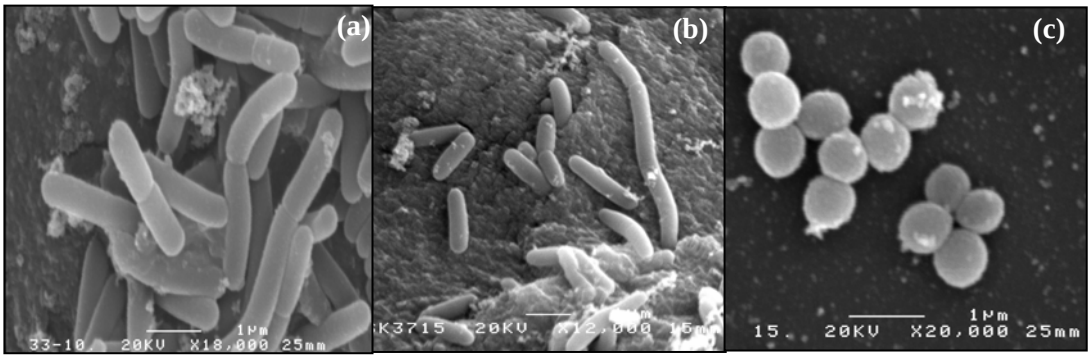

Figure 3.2 Scanning electron micrographs of selected isolates on JCM 168 medium and incubated at 35°C for 5 days: SK33 (a), SK37 (b), and SK1-1-5 (c) (Bar=1 μm)

SK1-1-5 were Gram-positive coccus and non-motile. Diameter of cell ranged from 0.5 to 1.1 μm after cultivation for 3 days at 35°C on JCM 168 containing 15% NaCl (Figure 3.2 and 3.3). Colonies on JCM 168 agar were circular, raised and white with pale yellow color. SK1-1-5 was catalase and urease positive, while negative response on oxidase and hemolysis. SK1-1-5 grew at 0-30% NaCl, 15-55°C and wide pH rang of 4-12. Moreover, growth was noticed under aerobic and anaerobic condition.

Phenotypic characteristics of three isolates (SK33, SK37 and SK1-1-5) compared with the reference are shown in Table 3.5 and 3.6. Phenotypic characteristics still had limitation for species identification. Isolates SK33

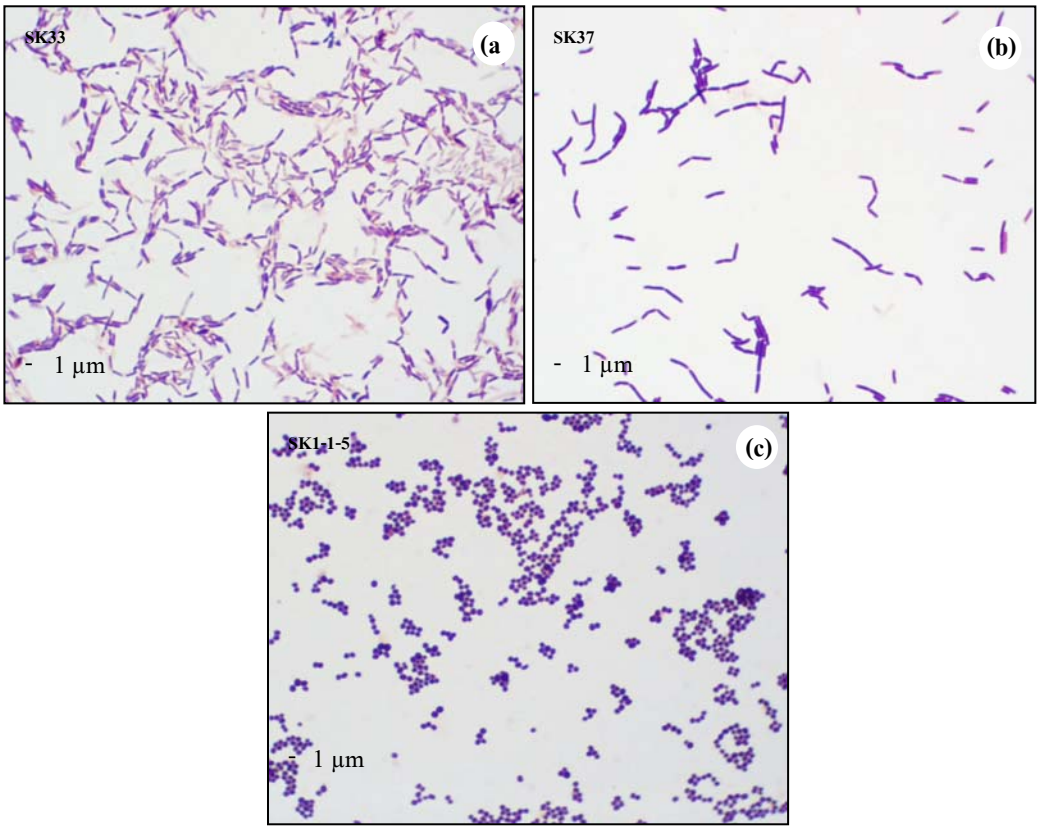

Figure 3.3 Gram stain of the selected isolates on JCM 168 medium at their optimum NaCl concentration of each isolate and incubated at 35^{o}C for 5 days , SK33 (18%NaCl) (a), SK37 (20% NaCl) (b) and SK1-1-5 (15% NaCl) (c) (Bar=1 µm, Magnification: x 1000)

identification. Isolates SK33 and SK37 were biochemically identified as *Brevibacillus brevis* with 98.9% and 99.3% similarity, respectively. However, SK33 and SK37 showed different phenotypic characteristic from *Brevibacillus brevis*. Shida et al. (1996) found that *Brevibacillus* sp. was motile and strictly aerobic. Moreover, Goto et al. (2004) also found that growth of *Brevibacillus* sp was typically inhibited at 5% NaCl, which was different from SK33 and SK37. SK1-1-5 showed similar biochemical characteristics to *Staphylococcus cohnii* subsp. *urealyticus* at 99.6%.

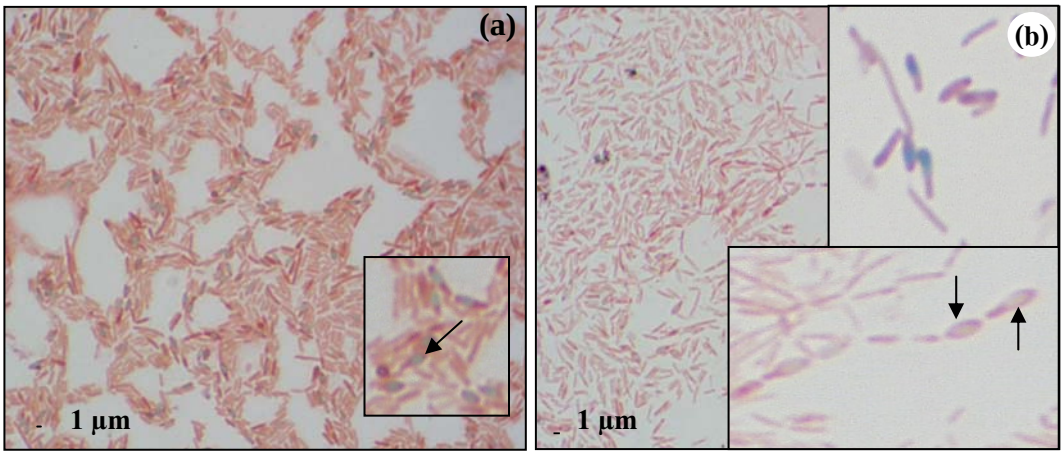

Figure 3.4 Endospores of SK33 (a) and SK37 (b) on JCM 168 medium at their optimum NaCl concentration and incubated at 35°C for 3 weeks, Bar=1µm (The arrow demonstrated shape and position of spore)

However, Kloos and Wolfshohl (1991) found that *S. cohnii* did not reduce nitrate and its growth was poor at 15%. However, our results indicated that SK1-1-5 grew well at 15% NaCl and could reduce nitrate. Thus, phenotypic and biochemical characteristics were not sufficient for identification these isolates.

Table 3.6 Comparison of phenotypic characteristics of the selected Gram-positive rods and other bacteria

Characteristics	Bacterial strain										
	SK33	SK37	*Bacillus halodenitrificans*	*Brevibacillus* sp.	*Bacillus marisflavi*	*Filobacillus* sp. RF2-5	*Bacillus aquimaris*	*Lentibacillus juripiscarins*	*Gracilibacillus halotolerans*	*Oceanobacillus iheyensis*	*Bacillus marimortui*
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Cell size (μm)	(0.5-0.7) x (2.1-4.1)	(0.6-0.7) x (3.0-6.6)	(0.6-0.8) x (2.0-4.0)	(0.7-0.9) x (3.0-5.0)	(0.6-0.8) x (1.5-3.5)	(0.5-0.8) x (2.0-4.0)	(0.5-0.7) x (1.2-3.5)	(0.4-0.5) x (1.5-6.0)	(0.4-0.6) x (2.0-5.0)	(0.6-0.8) x (1.5-2.6)	(0.6-0.8) x (1.5-2.6)
Gram	+, V	+, V	V	+, V	+, V	+, V	+, V	+, V	+, V	+, V	+, V
Aerobic growth	+	+	+	+	+	NA	+	+	+	NA	+
Anaerobic growth	+	+	+	d	-	NA	-	+	+	NA	-
Cell wall composition	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP
Spore shape[b]	E	E	E	E	E	ND	E	E	E	E	E
Spore position[c]	T	S, T	S, T	ND	C, S	T	C	T	T	S, T	S, T
Catalase test	+	+	+	+	+	+	+	+			+
Oxidase test	+	+	+	V	-	+	-	+	ND	ND	+
Motility	-	-	+	+	+	+	+	-	+	ND	+
Growth in pH	4-12	4-11	5.8-9.6	NA	4.5-8.0	NA	Inhibited at 4.5 or 9	5-9	5-10	6.5-10	6-9
Growth in NaCl (%)	0-30	0-30	0-23	Inhibited at 5% NaCl	0-16	0-25	2-18	3-30	0-20	0-21	5-25
Growth at temperature (°C)	30-45	20-45	10-45	30-48	10 and 47	45	10 and 44	10-45	6-50	15-42	15-50
Hydrolysis of:											
Starch	-	-	-	V	-	NA	+	-	NA	-	-
Lipase	-	-	-	NA	-	NA	+	+	+	+	-
Gelatin	+	+	+	+	NA	+	NA	+	NA	+	+

Table 3.6 (continued)

Characteristics	Bacterial strain[a]										
	SK33	SK37	*Bacillus halodenitrificans*	*Brevibacillus* sp.	*Bacillus marisflavi*	*Filobacillus* sp. RF2-5	*Bacillus aquimaris*	*Lentibacillus juripiscarins*	*Gracilibacillus halotolerans*	*Oceanobacillus iheyensis*	*Bacillus marimortui*
Acid from:											
Glycerol	-	-	NA	NA	+	NA	-	+	+	-	+
Erythritol	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Arabinose	-	-	NA	NA	-	NA	-	NA	+	NA	-
L-Arabinose	-	-	NA	NA	-	NA	-	NA	+	NA	-
D-Ribose	-	-	NA	NA	+	NA	+	+	NA	NA	-
D-Xylose	-	-	NA	NA	+	-	-	+	+	NA	-
L- Xylose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Galactose	-	-	+	NA	+w	-	-	-	+	NA	-
D-Glucose	-	-	+	NA	+	-	+	+	+	+	+
D-Fructose	-	-	+	NA	+	-	+	+	+	NA	+
D-Mannose	-	-	+	NA	+	-	-	-	+	+	+
L-Sorbose	-	-	NA	NA	-	NA	-	NA	NA	NA	
L-Rhamnose	-	-	-	NA	-	NA	-	-	+	NA	+
Dulcitol	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
Inositol	-	-	NA	NA		NA	-	-	NA	NA	-
D-Mannitol	-	-	+, V	NA	+	NA	-	-	NA	NA	-
D-Sorbitol	-	-	NA	NA	-	NA	-	-	-	NA	NA
Methyl-αD-mannopyranoside	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
Methyl-αD-glucopyranoside	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
N-Acetylglucosamine	-	-	NA	NA	-	NA	-	NA	+	NA	NA
Amygdaline	-	-	NA	NA	-	NA	-	-	NA	NA	NA
Arbutin	-	-	NA	NA	+	NA	-	NA	NA	NA	NA
Esculin	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
Salicin	-	+	NA	NA	+	NA	-	-	NA	NA	-

Table 3.6 (continued)

Characteristics	Bacterial strain[a]										
	SK33	SK37	*Bacillus halodenitrificans*	*Brevibacillus* sp.	*Bacillus marisflavi*	*Filobacillus* sp. RF2-5	*Bacillus aquimaris*	*Lentibacillus juripiscarins*	*Gracilibacillus halotolerans*	*Oceanobacillus iheyensis*	*Bacillus marimortui*
D-Celiobiose	-	+	NA	NA	+	NA	-	-	+	NA	NA
D-Maltose	+	-	NA	NA	+	NA	+	-	+	+	+
D-Lactose	-	-	NA	NA	-	NA	NA	NA	NA	NA	NA
D-Melibiose	-	-	-	NA	+	NA	-	-	+	NA	NA
D-Saccharose (Sucrose)	-	-	+	NA	+	NA	+	+	NA	NA	-
D-Trehalose	-	-	+	NA	+	NA	+	-	+	NA	-
Inulin	-	-	NA	NA	-	NA	-	-	+	NA	+
D-Melezitose	-	-	NA	NA	-	NA	-	-	+	NA	
D-Rafinose	-	-	NA	NA	+w	NA	-	-	+	NA	+
Amidon (Starch)	-	-	NA	NA	-	NA	+	NA	+	NA	-
Glycogen	-	-	NA	NA	-	NA	+	NA	+	NA	NA
Xylitol	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
Gentiobiose	-	-	NA	NA	+	NA	-	NA	NA	NA	NA
D-Turanose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Lyxose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Tagatose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Fucose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
L-Fucose	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
D-Arabitol	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
L-Arabitol	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
Potassium gluconate	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
Potassium 2-keto-gluconate	-	-	NA	NA	-	NA	-	NA	NA	NA	NA
Potassium 5-keto-gluconate	-	-	NA	NA	-	NA	+	NA	NA	NA	NA
β-galactosidase	-	-	NA	NA	-	NA	NA	NA	NA	NA	NA
Arginine dihydrolase	-	-	NA	NA		NA	NA	NA	-	NA	NA

Table 3.6 (continued)

Characteristics	Bacterial strain[a]										
	SK33	SK37	*Bacillus halodenitrificans*	*Brevibacillus* sp.	*Bacillus marisflavi*	*Filobacillus* sp. RF2-5	*Bacillus aquimaris*	*Lentibacillus juripiscarins*	*Gracilibacillus halotolerans*	*Oceanobacillus iheyensis*	*Bacillus marimortui*
Lysine decarboxylase	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ornithine decarboxylase	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
Citrate utilization	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
H_2S production	-	-	-	NA	NA	NA	NA	NA	+	NA	-
Urease	-	-	NA	NA	-	+	-	-	+	NA	+
Hemolysis	-	-	NA	NA	-	-	NA	NA	NA	NA	NA
Source	Thai fish sauce	Thai fish sauce	Marine solar saltern of the Yellow Sea in Korea	NA	Yellow Sea in Korea	Thai fish sauce	Yellow Sea in Korea	Thai fish sauce	Great salt lake in Utah, U.S.A	Depth of 1050 m on the Iheya Ride	Dead Sea

d: 11-89% strains positive; delayed reaction, +w: positive to weak, -w: negative to weak
[b]E = Ellipsoidal
[c]C = Central, S = Subterminal, T = Terminal
V = Variable
ND = Not detected
NA = Not available

Table 3.7 Comparison of phenotypic characteristics of the selected Gram-positive coccus and other species.

Characteristics	Bacterial strain							
	SK1-1-5	S. *cohnii*[a]		S. *xylosus*[a]	S. *saprophyticus*[a]	S. *piscifermentans*[b]	*Staphylococcus*	
		subsp.1	subsp.2				*carnosus*[a]	*carnosus*[b]
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Cell size (diameter, μm)	0.5-1.1	0.5-1.5	0.5-1.2	0.8-1.2	0.8-1.5	1.0	0.5-1.5	0.5-1.0
Gram	+	+	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+	+	+
Anaerobic growth	+	d	+	d	+	+	+	+
Catalase test	+	+	+	NA	+	+	+	+
Oxidase test	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-
Growth at pH	4-12	NA	NA	NA	NA	5-9	4.2-4.8	5-9
Growth in NaCl:								
6 %	+	NA	NA	NA	NA	+	NA	+
8 %	+	NA	NA	NA	NA	+	NA	+
10 %	+	10	10	10	10	+	10	+
15 %	+	15 (d)	15 (d)	15 (d)	15 (d)	+	15 (d)	+
Range %	0-30	NA	NA	NA	NA	NA	NA	NA

Table 3.7 (continued)

Characteristics	Bacterial strain							
	SK1-1-5	S. *cohnii*[a]		S. *xylosus*[a]	S. *saprophyticus*[a]	S. *piscifermentans*[b]	*Staphylococcus*	
		subsp.1	subsp.2				*carnosus*[a]	*carnosus*[b]
Growth at temperature:								
10°C	-	NA	NA	NA	+	NA	NA	NA
15°C	+	+	+	+	+	NA	+	NA
18°C	+	NA	NA	NA	+	NA	+	+
40°C	+	NA	NA	NA	NA	NA	+	NA
42°C	+	NA	NA	NA	NA	+	+	+
45°C	+	+(d)	+(d)	-	NA	NA	+	NA
Range °C	15-55	NA	NA	NA	10-40	NA	15-45	NA
Hydrolysis of :								
Starch	-	NA	NA	NA	NA	NA	NA	NA
Lipase	+	May be produced	May be produced	d	NA	+	NA	d
Gelatin	-	NA	NA	NA	NA	-	NA	-
Acid from:	NA	NA	NA	NA	NA	NA	NA	NA
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	-	-	-	-	+	-	+
D-Mannose	+	d	+	+	-	-	+	d

Table 3.7 (continued)

Characteristics	Bacterial strain							
	SK1-1-5	S. *cohnii*[a]		S. *xylosus*[a]	S. *saprophyticus*[a]	S. *piscifermentans*[b]	*Staphylococcus*	
		subsp.1	subsp.2				*carnosus*[a]	*carnosus*[b]
Maltose	+	d	+	+	+	d	-	d
Lactose	+	-	+	+	d	d	d	d
D-Saccharose (Sucrose)	-	-	-	+	+	d	-	d
D-Trehalose	+	+	+	+	+	+	d	+
D-Mannitol	+	d	d	d	d	d	+	d
Xylitol	-	d	d	-	d	-	-	-
D-Melibiose	-	NA	NA	NA	NA	NA	NA	NA
D-Raffinose	-	-	-	-	-	-	-	NA
D-Xylose	-	-	-	+	-	-	-	-
L-Arabinose	-	-	-	+	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-	-
Salicin	-	-	-	d	-	d	-	NA
D-Galactose	-	-	d	d	-	d	d	d
D-Melozitose	-	-	-	-	-	d	-	d
D-Turanose	NA	-	-	d	+	-	-	-

Table 3.7 (continued)

Characteristics	Bacterial strain							
	SK1-1-5	S. *cohnii*[a]		*S. xylosus*[a]	S. *saprophyticus*[a]	S. *piscifermentans*[b]	*Staphylococcus*	
		subsp.1	subsp.2				*carnosus*[a]	*carnosus*[b]
D-Ribose	-	-	-	d	-	d	NA	NA
Methyl-αD-glucopyranoside	-	NA	NA	NA	NA	NA	NA	NA
N-Acetylglucosamine	+	NA	NA	NA	NA	NA	NA	NA
Nitrate reduction	-	-	-	d	-	+	+	+
Alkaline phosphatase	+	-	+w	d	-	+	+	d
Acetyl-methyl-carbinol production	+	NA	NA	NA	NA	-		-
Argininedihydrolase	-	-	-w	-	-w	+	+	+
Urease	+	+	+	+	+	+	-	d
Isolation	Thai fish sauce	NA	NA	NA	NA	Fermented fish	NA	Fermented fish

d: 11-89% strains positive; delayed reaction, +w: positive to weak, -w: negative to weak
[a] Kloos and Schleifer (2001), [b]Tanasupawat et al. (1992)
ND = Not detected
NA = Not available

- Effect of NaCl concentrations on bacterial cell morphology

NaCl concentration (0, 0.5, 3, 5, 10, 15, 18, 20, 25 and 30%) affected cell morphology and topography of all 3 strains (Figures 3.5-3.7). *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 showed normal cell surface at 0.5-18% NaCl (Figures 3.5b-3.5g) and 0.5-20% NaCl (Figures 3.6b-3.6h), respectively. Cells of these two strains appeared to be rough and lysis at 0% NaCl (Figures 3.5a, 3.6a). The cell surfaces of *Virgibacillus* sp. SK33 were rough when treated in 20% NaCl (Figure 3.5h) while those of *Virgibacillus* sp. SK37 were normal. *Virgibacillus* sp. SK37 could tolerate high salt (20% NaCl) to a greater extent than *Virgibacillus* sp. SK33. It can be implied that the *Virgibacillus* sp. SK37 had more salt tolerant ability than *Virgibacillus* sp. SK33. Cells of both strains collapsed at 25-30% NaCl (Figures 3.6i-3.6j and 3.7i-3.7j). This could be due to the damage of cell wall and cell membrane. Based on these results, *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 were not considered to be extremely halophilic bacteria, but could be categorized as moderately halophilic bacteria. Moderately halophilic bacteria require salt and can survive up to 20% NaCl, while extremely halophilic bacteria requires 15-30% NaCl for growth (Ventosa et al., 1998). Kushner (1993) reported that moderate halophiles grew between 0.5-2.5 M NaCl (approximatly 3-15%) and were able to grow in <0.1 M NaCl (about 0.5%). Typically, moderately halophilic bacteria excrete compatible solutes from their cytoplasm or uptake from the medium to achieve osmotic balance (Robert, 2000). Halophilic bacteria adapted well to high salt concentrations. In contrast, non halophiles cannot grow at salt concentration > 0.2 M NaCl (about 1%) (Kushner, 1993). Cell wall of non halophilies breaks down at high salt concentration. High osmotic pressure causes the removal of water from cell, leading to plasmolysis

or collapse of the cell cytoplasm (Tortora et al., 2004)

Cells of *Staphylococcus* sp. SK1-1-5 had normal surface at 0-20% NaCl (Figure 3.7a-3.7h). Although cell surface appeared rough, cell lysis did not take place at 25-30% NaCl. Since *Staphylococcus* sp. SK1-1-5 could grow in the medium without salt, it was classified as an extremely halotolerant bacteria but not extremely halophilic bacteria. Vreeland et al. (1992) reported that extremely halophilic bacteria were able to grow in salt concentration more than 2 M (about 11% NaCl) and cells immediately lysed in the absence of salt. In contrast, halotolerant bacteria, especially extremely halotolerant bacteria, could grow and tolerate high salt concentrations up to 14% NaCl (>2.5 M NaCl).

- Genotypic characterization

The genomic DNA was extracted from 27 bacterial isolates, and used for 16S rDNA amphification. The size of amplified DNA fragments was about 700-800 bp. (Figure 3.8). Results of % similarity and strain homology are shown in Table 3.8.

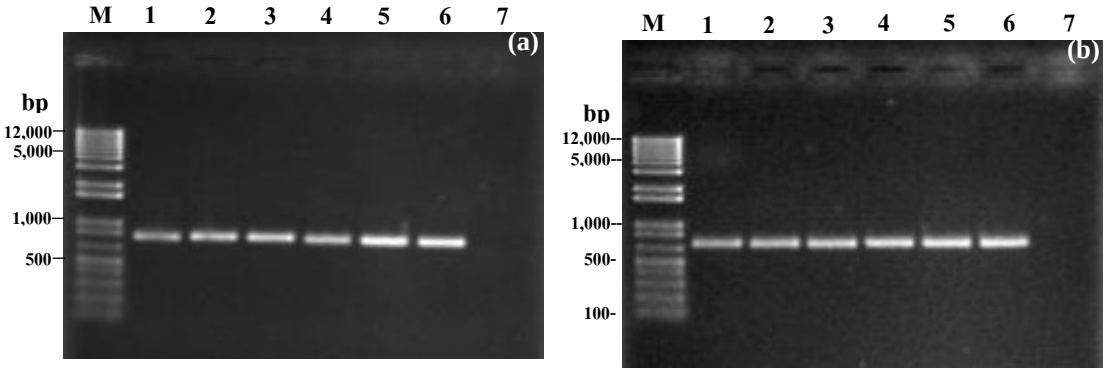

Figure 3.8 Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rDNA using primers POmod/ PC3mod r (a) and P3mod f/PC5 r (b). Lanes M; Molecular weight marker, 1, SK25; 2, SK33; 3, SK37; 4, SK1-1-2; 5, SK1-1-5; 6, SKM20-7-9; and 7, negative control.

Table 3.8 16S rRNA gene sequence similarity and relation of Gram-positive rods.

Isolate	SK33	SK35	SK37	SK1-3-7	SKW19	Haw9	SKW12	SK1-3-19	SK1-3-11	SK1-3-3	SK1-1-9	SKS16	SK39	SK1-1-8	SK32	SK37-1	SK1-1-6
SK33	100																
SK35	71	100															
SK37	82	74	100														
SK1-3-7	78	72	81	100													
SKW19	83	75	85	83	100												
Haw9	57	57	58	57	59	100											
SKW12	55	55	57	55	56	73	100										
SK1-3-19	78	70	82	77	81	58	56	100									
SK1-3-11	75	67	79	75	77	57	54	82	100								
SK1-3-3	68	61	65	65	65	54	52	67	68	100							
SK1-1-9	75	70	76	76	79	57	56	79	76	70	100						
SKS16	76	70	73	76	76	58	56	76	73	74	80	100					
SK39	68	61	72	69	71	54	52	71	71	63	66	63	100				
SK1-1-8	72	65	76	72	76	56	54	73	73	64	70	67	77	100			
SK32	68	62	71	68	71	54	51	71	71	64	67	63	80	77	100		
SK37-1	70	64	73	71	74	55	53	73	73	64	69	65	78	81	76	100	
SK1-1-6	59	63	60	60	62	56	53	58	59	58	61	60	55	58	56	57	100

Table 3.9 16S rRNA gene sequence similarity and relation of Gram-positive cocci.

Isolate	SKW29	SK25	SK1-1-2	SK1-1-5	SKM20	SKW23	SKW24	SK1-1-1	SKS23	SKS20
SKW29	100									
SK25	83	100								
SK1-1-2	82	88	100							
SK1-1-5	85	90	88	100						
SKM20	83	89	88	93	100					
SKW23	65	67	64	66	65	100				
SKW24	86	82	80	83	83	63	100			
SK1-1-1	77	79	79	78	78	67	76	100		
SKS23	76	80	77	78	77	66	76	84	100	
SKS20	78	80	77	80	79	67	77	77	76	100

From a total of 27 isolates studied, 15 isolates were Gram-positive rods, 2 isolates were Gram-negative rods, and 10 isolates were Gram-positive cocci. The percentage of 16S rDNA sequence similarity among isolates were 51-93% (Table 3.8 and 3.9). SK33 and SK37 showed the highest similarity with SKW19 at relation value of 83 and 85%, respectively (Table 3.8). Moreover, DNA sequence of SK33 was similar to those of SK37. The percentages of 16S rDNA sequence similarity of SK33 and SK37 compared to *Virgibacillus halodenitrificans* DSM 10037, were 81 and 82%, respectively (Table 3.10). SK33 and SK37 showed low level of DNA similarity (51-81%) when compared to other isolates. These results demonstrated that SK33, SK37 and SKW19 appeared to closely related with the genus *Virgibacillus*. When phylogenetic tree analysis was performed, these three isolates fell in the same cluster of *Virgibacillus halodenitrificans* (Figure 3.9).

SK1-1-5 showed the highest similarity to SKM20-7-9 at relation value of 93%. 16S rRNA gene sequence of SK1-1-5 was also similar to SK25 and SK1-1-2 at 90 and 88%, respectively. SK1-1-5, SKM20-7-9, SK25 and SK1-1-2 showed low level of DNA similarity (lower than 83%) when compared to other isolates (Table 3.9). However, all of the positive coccus isolates were in the same cluster of the genus *Staphylococcus* (Figure 3.10). Moreover, the percentage of 16S rDNA sequence similarity of SK1-1-5 compared to *Staphylococcus saprophyticus* ATCC 15305 was 94% (Table 3.11).

Based on phylogenetic relationships shown in Figures 3.9 and 3.10, ten isolates were identified as belonging to *Bacillus halodenitrificans* at 95% similarity when compared to *B. halodenitrificans* DSM 10037. *Bacillus halodenitrificans* was transfered to *Virgibacillus halodenitrificans*

Table 3.10 16S rRNA gene sequence similarity of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and related species.

Bacterial	SK33	SK37	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
SK33	100																												
SK37	87	100																											
1	82	84	100																										
2	83	84	96	100																									
3	82	85	96	99	100																								
4	60	61	69	68	68	100																							
5	74	74	86	84	84	67	100																						
6	79	80	94	90	91	68	88	100																					
7	81	82	98	94	94	68	88	96	100																				
8	81	82	98	94	94	68	88	96	99	100																			
9	61	63	69	67	68	78	73	71	71	71	100																		
10	74	74	86	86	86	66	98	89	88	88	72	100																	
11	75	75	87	84	84	66	98	89	89	89	73	98	100																
12	75	75	88	85	85	66	97	89	89	89	72	97	99	100															
13	60	62	69	67	67	89	72	71	71	71	86	72	73	72	100														
14	81	82	98	94	94	68	86	944	97	98	70	87	87	88	69	100													
15	80	81	97	93	94	67	86	98	97	98	69	86	86	87	69	97	100												
16	62	64	71	69	69	79	71	71	71	71	98	71	72	73	85	71	71	100											
17	61	64	69	71	71	93	67	68	67	67	79	67	67	67	91	67	67	81	100										
18	73	73	85	85	85	68	87	87	87	87	70	87	86	85	69	85	85	68	68	100									
19	77	78	93	86	89	67	87	94	95	95	71	88	88	88	71	93	92	70	67	86	100								
20	60	60	67	65	65	60	67	68	69	68	63	67	68	68	63	67	67	62	60	66	69	100							
21	60	60	67	65	65	60	68	68	69	69	64	68	68	67	63	67	67	62	60	67	69	98	100						
22	61	61	68	68	68	61	65	67	67	68	61	66	66	66	62	67	66	62	62	64	67	93	94	100					
23	80	81	96	92	92	67	87	98	94	94	69	87	87	88	69	94	94	71	69	85	92	67	67	68	100				
24	69	70	78	82	82	67	75	76	77	77	63	76	75	76	63	77	76	64	68	79	76	61	61	63	78	100			
25	70	71	80	82	82	67	77	78	78	78	65	78	77	77	65	78	78	67	69	80	78	63	63	65	79	86	100		
26	81	82	98	94	94	67	88	96	99	99	71	88	89	88	71	98	98	71	67	87	94	68	69	67	94	77	79	100	
27	70	72	79	81	81	65	76	77	78	77	65	76	76	77	65	78	77	66	68	78	77	62	62	64	79	89	89	78	100

Note; SK33: *Virgibacillus* sp. SK33, SK37: *Virgibacillus* sp. SK37, 1: *Bacillus* sp. SB J81 (AB167057), 2: *Bacillus* sp. JM-B (AB189318), 3: *Bacillus* sp. Sag-B1 (AB189319), 4: *Corynebacterium* sp. 14III/A0 (AY571697), 5: *Bacillus firmus* CV93b (AJ717384), 6: *Virgibacillus picturae* GSP60 (AY505536), 7: *Bacillus halodenitrificans* SF-121 (AY543168), 8: *Bacillus halodenitrificans* DSM 10037 (AY543169), 9: *Brevibacterium* sp. BH (AY577816), 10: *Bacillus* sp. 6160m-C1 (BAC509006), 11: *Bacillus firmus* 5695m-D2 (BAC509007), 12: *Bacillus firmus* (BFI491843), 13: *Corynebacterium ammoniagene* (X84440), 14: *Virgibacillus halodenitrificans* (DQ089000), 15: *Virgibacillus halodenitrificans* (DQ089678), 16: *Brevibacterium* sp. CHNDP32 (DQ337537), 17: *Corynebacterium* sp. B-5121 (DQ399759), 18: *Filobacillus* sp. RF2-5 (AB191344), 19: *Lentibacillus juripiscarius* (AB127980), 20: *Halomonas* sp. CM4 (AJ306889), 21: *Halomonas halmophila* (AY858696), 22: *Halomonas almeriensis* strain M8 (AY973823), 23: *Oceanobacillus* sp. J23 (AB167055), 24: *Brevibacillus* sp. L67 (DQ192211), 25: *Brevibacillus* sp. PC6 (AY372925), 26: *Virgibacillus halodenitrificans* (AB021186), 27: *Brevibacillus brevis* (D78457)

Table 3.11 16S rRNA gene sequence similarity of *Staphylococcus* sp. SK1-1-5, *Staphylococcus* sp. SKM20-7-9 and related species.

Bacterial	SK1-1-5	SKM20	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SK1-1-5	100																					
SKM20	94	100																				
1	85	85	100																			
2	85	85	99	100																		
3	85	85	99	98	100																	
4	85	84	99	98	99	100																
5	85	84	98	97	97	97	100															
6	85	84	98	97	98	98	99	100														
7	86	86	98	98	98	98	98	98	100													
8	86	86	94	95	94	94	94	94	96	100												
9	86	86	98	98	98	98	98	98	99	96	100											
10	86	86	98	98	98	98	98	98	99	96	99	100										
11	86	86	98	98	98	98	98	98	99	96	99	99	100									
12	86	85	98	97	97	97	97	97	99	95	99	99	99	100								
13	86	85	98	98	97	97	97	97	99	95	99	99	99	99	100							
14	85	85	98	98	97	97	97	97	96	96	99	99	99	96	99	100						
15	76	76	87	87	87	87	88	87	87	87	87	87	87	87	87	88	100					
16	74	74	82	82	82	82	82	82	82	84	82	81	81	82	82	82	89	100				
17	72	72	81	80	81	80	80	80	81	81	81	81	81	81	81	81	80	75	100			
18	67	67	74	74	74	74	75	75	74	75	74	74	74	74	74	74	75	72	72	100		
19	51	51	55	55	55	55	55	55	55	55	55	55	55	55	55	55	55	55	54	57	100	
20	51	51	56	56	56	56	56	56	56	55	56	56	56	56	56	56	56	53	55	57	96	100

Note; SK1-1-5: *Staphylococcus* sp. SK1-1-5, SKM20: *Staphylococcus* sp. SKM20-7-9, 1: *Staphylococcus pasteuri* (AJ717376), 2: *Staphylococcus warneri* (L37603), 3: *Staphylococcus epidermidis* (AY030340), 4: *Staphylococcus* sp. MO28 (AY553115), 5: *Staphylococcus carnosus* (AB009934), 6: *Staphylococcus piscifermentans* (AB009943), 7: *Staphylococcus* sp. BBC1 (AM158917), 8: *Staphylococcus* sp.VM3 (DQ238832), 9: *Staphylococcus cohnii* (AB009936), 10: *Staphylococcus_cohnii* (AJ717378), 11: *Staphylococcus saprophyticus* (D83371), 12: *Staphylococcus arlettae* (AB009933), 13: *Staphylococcus gallinarum* (D83366), 14: *Staphylococcus xylosus* (AF515587), 15: *Salinicoccus siamensis* (AB258358), 16: *Salinicoccus marinus* (AY328901), 17: *Marinococcus halotolerans* (AY817493), 18: *Micrococcus* sp. (DQ491453), 19: *Halococcus* sp. DB5-2 (AB220647), 20: *Halococcus morhuae* (HM016SRD)

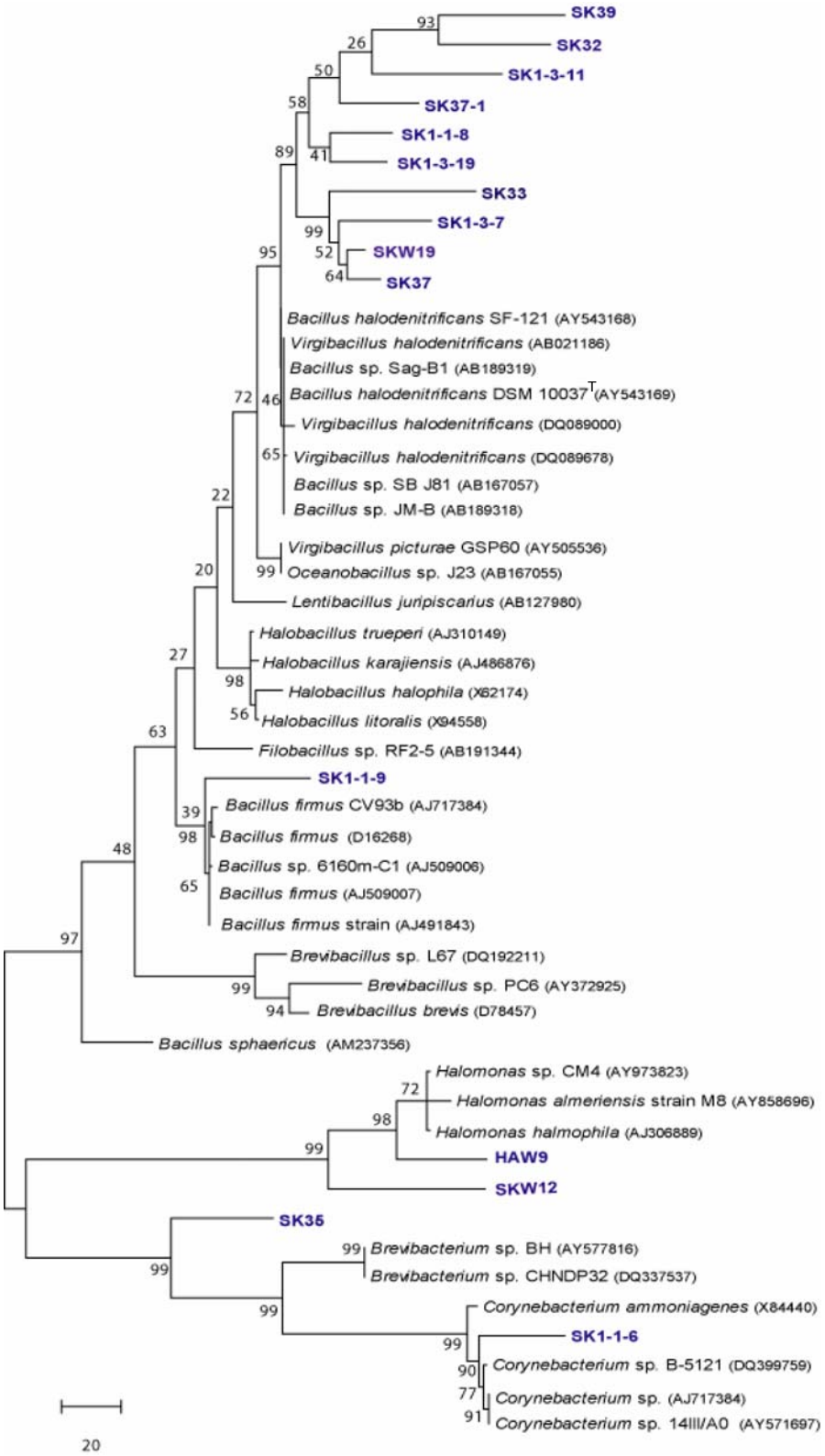

Figure 3.9 Phylogenetic tree of proteinase-producing Gram-positive rods isolated from fish sauce fermentation, based on 16S rRNA gene sequence data. Bar indicates 20 substitutions per nucleotide positions.

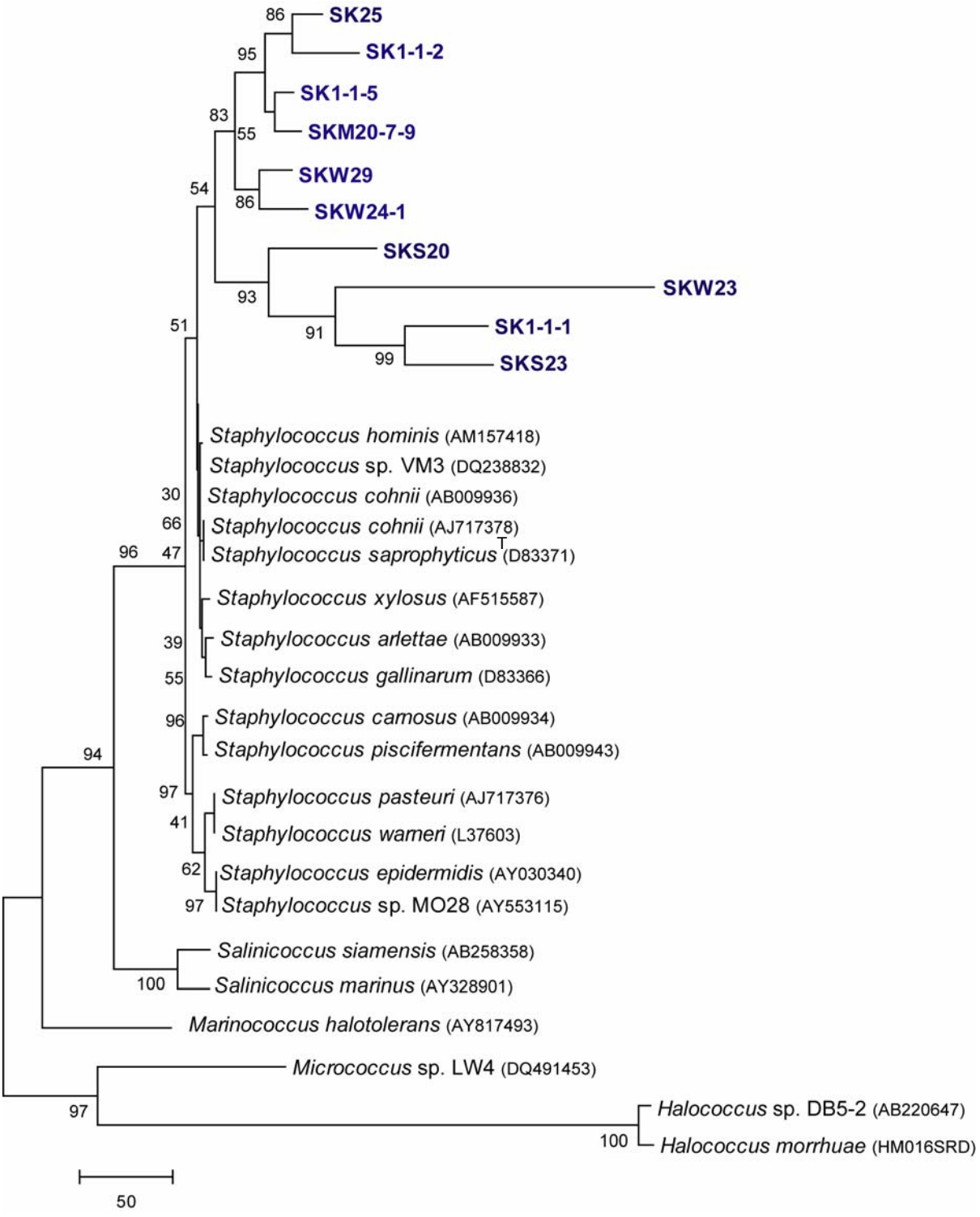

Figure 3.10 Phylogenetic tree of proteinase-producing Gram-positive cocci isolated from fish sauce fermentation, based on 16S rRNA gene sequence data. Bar indicates 50 substitutions per nucleotide positions.

according to Yoon et al. (2004). Only 2 isolates (Haw 9 and SKW 12) belonged to the genus *Halomonas* (98-99% similarity) and other isolates including SK1-1-9, SK35 and SK1-1-6 belonged to *Bacillus firmus* (98% similarity), *Brevibacteium* sp. (99% similarity) and *Corynebacterium* sp. (90%), respectively (Figure 3.9). Ten Gram-positive cocci isolates were classified in the genus *Staphylococcus* (Figure 3.10).

SK33 and SK37 were identified as *Virgibacillus halodenitrificans.* They had 95 and 96% 16S rRNA gene sequences similarity, respectively, when compared to *V. halodenitrificans* DSM 10037. Phylogenetic analyses provided the similar result (Figure 3.9). SK33 and SK37 are clearly separeted from the genera *Brevibacillus, Brevibacterium, Halomonas, Halobacillus, Lentibacillus, and Filobacilllus,* based on phylogenetic analyses. The two isolates were also differentiated from three *Bacillus* species (*B. marisflavi*, *B. aquimaris* and *B. marimortui*) based on ability to ferment carbohydrate as detailed in Table 3.6. In addition, SK33 was differentiated from *B. aquimaris* based on lipid hydrolysis as it contained no lipase enzyme activity. SK33 and SK37 showed optimal salt concentrations for growth at 3-23% NaCl, which was higher than that of *Brevibacillus* sp. (5% NaCl) (Shida et al., 1996). They could be differentiated from *Gracibacillus halotolerans* (Waino et al., 1999) and *Ocenobacillus iheyensis* (Lu et al., 2001). SK33 and SK37 isolates were non-motile and could not hydrolyze lipid while *G. halotolerans* and *O. iheyensis* were motile and lipase-positive. Likewise, the ability to produce urease and motility could be used to differentiate SK33 and SK37 from *Filobacillus* sp. RF2-5 (Hiraage et al., 2005). Furthermore, SK33 and SK37 could not ferment glycerol which was different from *Lentibacillus juripiscarins* (Namwong et al., 2005).

Although 16S rRNA gene sequence and phylogenetic analyses revealed that SK33 and SK37 were *Virgibacillus halodenitrificans*, they showed only 96% of 16S rDNA similarity to members of *V. halodenitrificans* (Table 3.12). In addition, some phenotypic properties, particularly motility and pH range for

Table 3.12 Similarity of 16S rRNA gene sequence of three proteinase-producing bacteria isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence	Nucleotide sequence comparison, identification result and details				
		Closest relative	Length of sequence (bp)	Sequence homology (%)	NCBI Accession no.	Isolation source/ remark of closest relative
SK33	1424	*Bacillus halodenitrificans* SF-121[a]	1521	96.0	AY543168	Marine solar saltern of the Yellow Sea in Korea
		Bacillus halodenitrificans DSM 10037[a]	1521	95.0	AY543169	Marine solar saltern of the Yellow Sea in Korea
		Virgibacillus halodenitrificans strain 23	1525	94.0	DQ089678	Vegetable soil in Nanjing
SK37	1401	*Bacillus halodenitrificans* DSM 10037[a]	1521	96.0	AY543169	Marine solar saltern of the Yellow Sea in Korea
		Bacillus sp. Sag-B1	1429	96.0	AB189319	Sediment in Sagami Trough in Japan
		Virgibacillus halodenitrificans	1520	96.0	DQ089000	Saline wastewater
SK1-1-5	1438	*Staphylococcus saprophyticus* ATCC 15305	1477	95.0	D83371	Clinical isolated from animals
		Staphylococcus sp. VM3	1413	95.0	DQ238832	Contaminated soil
		Staphylococcus saprophyticus M36	1384	95.0	DQ462328	Isolated by Chinese scientists

[a] *Bacillus halodenitrificans* SF-121 and *B. halodenitrificans* DSM 10037 were transferred to *Virgibacillus halodenitrificans* (Yoon et al., 2004)

growth, were different. SK33 and SK37 had optimum pH for growth at pH 4-12, while growth of *V. halodenitrificans* ranged from pH 5.8 - 9.6. Thus, SK33 and SK37 could be new species. SK1-1-5 was identified as *Staphylococcus cohnii* subsp. *urealyticus* according to API-system (99.6% identity). But the 16S rRNA gene sequence indicated its relatedness to *Staphylococcus saprophyticus* ATCC 15305 with 95% homology. Although SK1-1-5 had low similarity to *Staphylococcus*, phylogenetic analysis showed that SK1-1-5 was the member of the genus *Staphylococcus* rather than the genera *Salinicoccus, Marinococcus, Micrococcus* and *Halococcus* (Figure 3.10). Stackebrandt et al. (2002) suggested that the similarity of 16S rRNA gene sequence below 97% could indicate the new species. Thus, SK33 and SK37 should be identified as *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37, while SK1-15 was *Staphylococcus* SK1-1-5. In the previous studies, *V. marismortui* (Arahat et al., 1999), *V. halodenitrificans* (Yoon et al., 2004), *V. dokdonensis* (Yoon et al., 2005) and *V. koreensis* (Lee et al., 2006) were isolated from environments containing salt environments, such as sea, marine solar saltern and salt field. Most of these species were moderately halophilic bacteria and a few species were slightly halophilic bacteria. This is the first report of the genus *Virgibacillus* found in Thai fish sauce. Some species of *Staphylococcus* have also been reported in fish sauce made in Southeast Asian countries, such as *S. xylosus, S. saprophyticus, S. carnosus* and *S. cohnii* (Mura, et al., 2000; Fukami, et al., 2004). However, *Staphylococcus* sp. found in this study was different from those previously reported.

3.5 Conclusions

Most proteinase-producing bacteria were found in fish sauce fermented at 1-3

months. A total of 308 isolates were isolated from fish sauce sampling using skim milk salt agar, standard methods caseinate agar and halobacteium agar media. Only 3 isolates showed the highest proteolytic activity in fish broth and were selected for identification. The information from phenotypic characterization was not sufficient for identification of halophilic proteinase-producing bacteria. Based on the combined morphological, biochemical characteristics and 16S rRNA gene sequence results, 27 isolates were identified as *Virgibacillus*, *Halomonas*, *Bacillus fimus*, *Brevibacteium*, *Corynebacterium*, and *Staphylococcus*. Three isolates including SK33 and SK37 and SK1-1-5 were also identified in the genus *Virgibacillus* (SK33 and SK37) and *Staphylococcus* (SK1-1-5). These 3 non histamine-forming isolates were likely to be a new species which have never been isolated from Thai fish sauce samples.

3.6 References

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CHAPTER IV

OPTIMIZATION OF GROWTH AND PROTEINASE PRODUCTION OF SELECTED BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION

4.1 Abstract

Three selected strains producing the highest proteinases were *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5. Each strain showed different optimal salt concentrations, temperatures and pH for growth and proteinase production in the anchovy extract medium (fish broth). The effect of salt concentrations (0, 0.5, 3, 5, 10, 15, 18, 20, 23, 25, and 30% NaCl) and pH (5.5, 6, 6.5 and 7.5) of fish broth containing 2.5 mg/ml protein and 0.1% glucose, were investigated at various temperatures (15, 25, 30, 35, 40, and 45^{o}C). *Staphylococcus* sp. SK1-1-5, *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 optimally grew at 15, 18, and 20% NaCl, respectively, on fish broth with the initial pH 7 at 35°C. *Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK37 also maximally produced proteinase at 35°C and 5% NaCl, while *Virgibacillus* sp. SK33 optimally produced proteinase at 40°C in the fish broth containing 25% NaCl. *Staphylococcus* sp. SK1-1-5, *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 produced oligopeptides at the optimal condition at 13.10, 45.55 and 26.48 mM, respectively. Yeast extract and glucose were not required for bacterial growth and proteinase production of all

strains. When the aeration was applied, cell concentration increased for approximately 3 log cycles. After fermentation for 120 days, ∝-amino contents of samples inoculated with 3 selected strains were higher than the control. This preliminary study indicated the feasibility of using *Staphylococcus* sp. SK1-1-5, *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 for accelerating fish sauce fermentation.

Keywords: Growth condition, Proteinase production, Fish sauce fermentation

4.2 Introduction

Proteolytic enzymes are produced by many varieties of microorganisms and played an important role during fish sauce fermentation. Several proteinase-producing bacteria found in fish sauce fermentation, including halophilics, halotolerants and lactic acid bacteria. These bacteria hydrolyze fish protein to peptides and amino acids (Lopetcharat et al., 2001). Proteinase-producing bacteria found in fish sauce are *Pseudomonas* sp. (Vermelho et al., 1996), *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Pediococcus* sp., coryneforms (Thongthai and Suntinanalert, 1991; Fukami et al., 2002, 2004; Noguchi et al., 2004), *Halobacillus thailandensis* sp. nov., (Chaiyanan et al., 1999), *Tetragenococcus halophililus* and *T. muriaticus* (Satomi et al., 1997; Tanasupawat et al. 2002; Thongsanit et al., 2002).

In the past, many investigators studied only the optimum condition for growth of proteinase-producting bacteria in fish sauce fermentation (Thongthai and Suntinanalert, 1991; Gasaluck et al., 1996; Noguchi et al., 2004; Namwong et al., 2005, 2006). Few investigators have characterized and purified proteinases from these bacteria (Chaiyanan et al., 1999; Hiraga et al., 2005; Namwong et al., 2006). In addition, parameters affecting growth and proteinase production of proteinase-producing

bacteria isolated from fish sauce have not been thoroughly investigated.

There are several factors affecting growth and proteinase production of proteinase-producing bacteria that should be optimized (Mehrotra, Pandey, Gaur and Darmwal, 1999). The growth of microorganisms and enzyme production are significantly affected by agitation and aeration (Garcia, Tomillo and Nunez, 1991). Rahman, Basri and Salleh (2005) reported that agitation increases the rate of oxygen and nutrient transfer from the liquid medium to the cells. Most halotolerant and halophilic bacteria have been found to grow at a wide pH range of 5-9 and produced proteinaes at pH 7-7.5 (Modern et al., 2000; Porro et al., 2003). Patel et al. (1999) found that *Bacillus* sp. isolated from seawater in Western India showed optimum growth and proteinaese production at pH 8 when cultivated in the medium containing 10% NaCl. *Filobacillus* sp. RF2-5, *Halobacillus* sp. SR5-3, *Halobacillus thailandensis*, *Bacillus subtilis*, and *Bacillus licheniformis* grew well, and could produce proteinases at pH 7-7.2 (Chaiyanan et al., 1999; Rozs, Manczinger, Vagvolgyl and Kevel, 2001; Hiraga et al., 2005; Kim and Kim, 2005 Namwong et al., 2006; Terlabie et al., 2006). pH range of fish sauce fermentation process was about 5-6 (Lopetcharat et al., 2001). Proteinase-producing bacteria suitable for starter culture development should grow and produce proteinases at the fermentation condition. The effect of temperature on growth and proteinase production has been investigated by many investigators. *Bacillus* sp. grew and produced proteinase at 28-37^{o}C (Kanekar, Nilegaonkar, Sarnaik and Kelkar, 2002; Uyar and Baysal, 2004; Ralman et al., 2005; Patel et al., 2006). The addition of carbon and nitrogen sources was also important for growth and proteinase production (Mehrotra et al., 1999; Joo et al., 2002). Kanekar et al. (2002) found that gelatin was a suitable carbon as well as nitrogen source for *Bacillus alkalophilus* isolated from

sediment of Lonar lake in India. Mehrotara et al. (1999) found that 1% glucose stimulated growth and proteinase production of *Bacillus* sp. isolated from saline soils of the Avadh region in India. However, the optimization for growth and proteinase production increased proteolytic activity and was useful in development of starter culture for acceleration the fermentation process. This study aimed at investigating effect of temperature, pH, and salt concentration on bacterial growth, and proteinase production of the selected strains isolated from the traditional process of fish sauce fermentation for the starter culture preparation.

4.3 Materials and methods

4.3.1 Effect of temperature, NaCl concentration and pH

In this experiment, three selected strains including *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were studied. Fish broth was prepared and used as a medium for bacterial growth in order to obtain the similar nutrient to fish sauce process. Fish broth contained fish tissue extract was developed from Okuzumi and Awano (1982) and prepared by mixing 1000 g of fresh anchovy with 2 volumes of water (w/v), boiling at 100^{o}C for 30 minutes and filtering through the cheesecloth. The filtrate was centrifuged at 8,000xg for 15 minutes (GSA rotor RC-5C Sorvall®, American Laboratory Trading**,** Groton, CT, USA). D-glucose was added at a final concentration of 1% as a carbon source before adjusting pH and NaCl concentration.

The effect of temperatures (15, 25, 30, 35, 40, 45 and 55^{o}C) on growth and proteinase production was carried out at 5 and 25 % NaCl, pH 7. For the optimization of NaCl concentration, fish broth was adjusted to the initial pH 7 and NaCl concentration

was varied to 0, 0.5, 3, 5, 10, 15, 18, 20, 23, 25 and 30%. The optimal NaCl concentration for each bacterial strain was used for the study of the optimum pH (5.5, 6, 6.5 and 7.5). Optimization of NaCl and pH was carried out at 35^{o}C.

Bacterial growth was enumerated using standard plate count method with JCM168 medium containing 25% NaCl**.** The proteinase production was observed by determining α-amino contents using TNBS method with leucine as a standard (Field, 1979). Protein content of fish broth was determined using BSA (Bovine serum albumin) as a standard (Bradford, 1976).

4.3.2 Effect of nitrogen and carbon source

The optimum concentration of nitrogen source for bacterial growth and proteinase production were investigated using anchovy extract prepared as previously described. Anchovy extract was diluted with distilled water at a ratio of 100:0, 75:25, 50:50, 25:75, 10:90, 5:95 and 0:100 (% v/v). The effect of 0.8% yeast extract was also elucidated. The experiment was conducted at the optimal NaCl concentration of each selected strain. Bacterial growth and proteinase production were monitored as described in 4.3.1.1.

Optimum concentration of carbon source in the fish broth containing 75% fish extract and 25% NaCl (pH 7) was studied using D-glucose at various concentrations (0, 0.1, 0.75, 1.5, 2 and 3% (w/v)). Samples were enumerated on JCM 168 medium containing 25% NaCl incubated at 35^{o}C for 3-5 days.

4.3.3 Effect of aeration on bacterial growth

Virgibacillus sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were cultured in a 250-ml Erlenmeyer flask containing 50 ml fish broth pH 7.0, at 18, 20 and 15 % NaCl, respectively with an inoculum size of 10% (v/v). The

aeration rate was studied by placing the cultures at 35°C in a rotary shaking incubator with the rotation speed of 100, 200, and 250 rpm (INNOVA™ 4340, Incubator Shaker, New Brunswick Scientific Co., Inc., New Jersey, CA, USA). Growth of *Staphylococcus* sp. SK1-1-5 was observed at 0, 12, 24, 48 and 72 h, while growth of *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 were observed within 5 days. Microbial population was enumerated using JCM168 medium containing 25% NaCl as previously described in 4.3.1.1.

4.3.4 Effect of inoculum size

The inocula of two strains (*Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK33) were prepared in fish broth at pH 7 at their optimum NaCl for growth at 35 °C. *Staphylococcus* sp. SK1-1-5 strain was cultured in 15% NaCl for 24 h, while *Virgibacillus* sp. SK33 was cultured in 18% NaCl for 3 days with a shaking speed of 100 rpm.

Anchovy hydrolysate was prepared as follows: 250 g of anchovy was put into a 400-ml beaker and incubated in a water bath until the core temperature reached 65°C. Then, it was added 0.25% (w/w) Alcalase® (2.4L) and incubated at 65°C for 2 h and cooled to 50°C at room temperature. Flavourzyme® (500L) was subsequently added, and the mixture was further incubated at 50°C for 4 h. Solar salt was added to the hydrolyzed anchovy at 25% (w/v) and starter culture at inoculum size of 1, 5 and 10% (v/w) was subsequently added. All samples were incubated at 35°C. Microbiological changes of all samples were monitored using JCM 168 medium containing 25% NaCl. Changes of α-amino content and microbiological changes were measured at 0, 3, 7, 14, 30, 60, 90, 120 and 150 days of fermentation.

4.3.5 Application of starter cultures for fish sauce fermentation

Starter cultures of three strains, namely *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were prepared at the optimum condition of each strain in a 500-ml Erlenmeyer flask containing 100 ml of fish broth and incubated at 35°C. SK1-1-5 was cultured in 15% NaCl for 24 h while SK33 was cultured in 18% NaCl for 3 days, with shaking speed at 100 rpm. The initial cells were approximately 10^6 CFU/ml. Anchovy hydrolysate was prepared as described in 4.3.1.5. One kilogram of anchovy hydrolysate was placed in the glass jar (8.7 cm diameter and 17 cm height). Inoculum size of 10% was added and incubated at 35°C. Microbiological changes of the inoculated strains were monitored using JCM 168 medium containing 25% NaCl. Changes of pH, α-amino content and microbiological changes were measured at 0, 3, 7, 14, 30, 60, 90 and 120 days.

4.3.6 Statistical analyses

All experiments were performed in duplicates. Statistical analysis was evaluated in Completely Randomized Design (CRD) using Statistical Analysis System (SAS Institute, Inc., 1995). Analysis of Variance (ANOVA) and mean comparison by Duncan's Multiple Range Test (DMRT) were analyzed.

4.4 Results and discussion

4.4.1 Temperature, NaCl concentrations and pH

The effect of temperature was conducted at 5 and 25% NaCl. This was because *Virgibacillus* sp. SK33 exhibited high proteinase activity in a medium containing 5% NaCl (Sinsuwan et al., 2006). In addition, NaCl concentration of fish sauce fermentation was over 25%. *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and

Staphylococcus sp. SK1-1-5 had ability to grow in fish broth at 25-45^{o}C at both NaCl concentrations (Figure 4.1 a-c). Initial cell counts of three isolates were approximately 10^5 CFU/ml. At 15^{o}C, only *Virgibacillus* sp. SK33 and *Staphylococcus* sp. SK1-1-5 grew in 5% NaCl (about 10^3 CFU/ml), whereas no growth was found at 25% NaCl. *Virgibacillus* sp. SK37 could not grow at 15^{o}C. At 5% NaCl, the optimum temperatures for the growth of *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 were at 30^{o}C ($p<0.05$) whereas that of *Staphylococcus* sp. SK1-1-5 was at 35-45^{o}C ($p<0.05$). All strains grew well at 35^{o}C ($p<0.01$) in fish broth containing 25% NaCl (about 10^5-10^6 CFU/ml). All strains could not grow at 55^{o}C. *Virgibacillus* sp. was reported to have optimal temperature for growth between 28-37^{o}C (Heyman et al., 2003). *Virgibacillus halodenitrificans* showed optimum growth at 35-40^{o}C which was similar to that of *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37. Most species of *Staphylococcus* sp. can grow between 18 and 40^{o}C, which is different from *Staphylococcus* sp. SK1-1-5 that grew at 25-45^{o}C.

NaCl concentration appeared to affect the optimal temperature for proteinase production (Figure 4.2). At 5% NaCl, proteinase production of *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 showed maximum oligopeptides production of 26.48 and 13.10 mM, respectively at 35^{o}C ($p<0.05$). Whereas the optimum proteinase production of *Virgibacillus* sp. SK33 was at 40^{o}C ($p<0.05$) at 25% NaCl with the α-amino content of 45.55 mM (Figure 4.2b). Although all strains grew well in fish broth containing 5% NaCl at 30^{o}C, they did not produce proteinase at this temperature. They grew and produced proteinase in 5% NaCl fish broth at 35^{o}C. Hence, optimum proteinase production and growth of these 3 strains as at 5% NaCl, 35^{o}C. Moreover, proteinase production of *Virgibacillus* sp. SK33 produced the

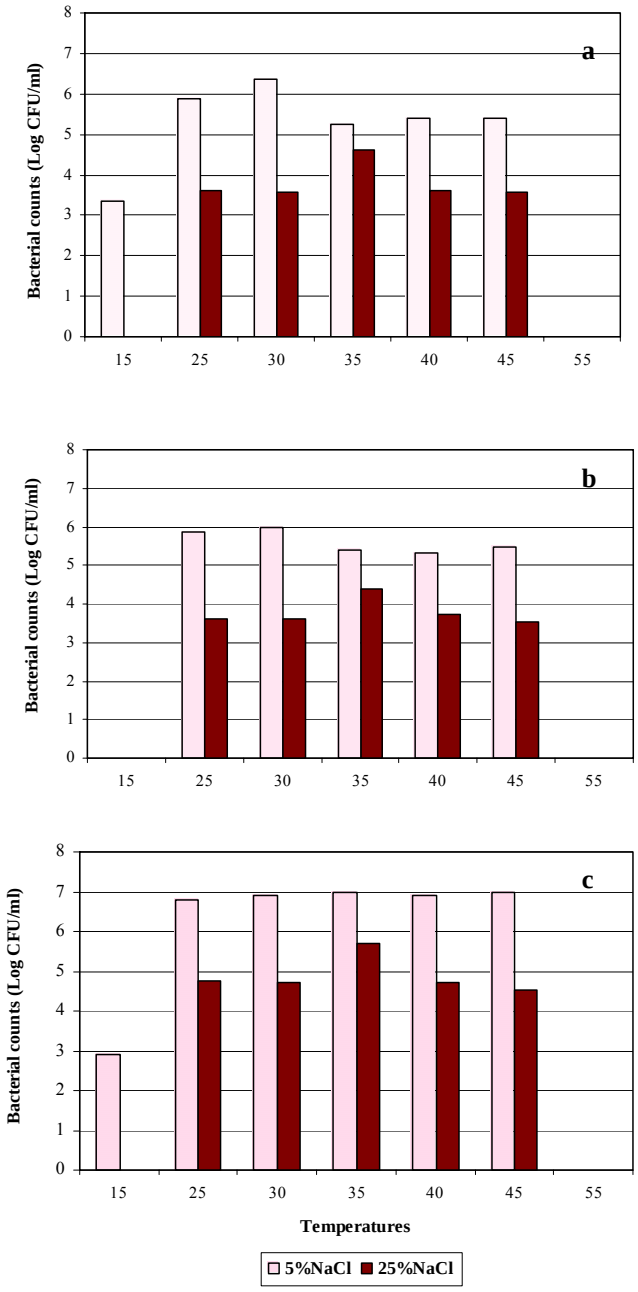

Figure. 4.1 Bacterial counts (Log CFU/ml) of *Virgibacillus* sp. SK33 (a), *Virgibacillus* sp. SK37 (b) and *Staphylococcus* sp. SK1-1-5 (c) in fish broth containing 5% and 25% NaCl, and pH 7, and incubated at various temperatures with initial cell count of about 5 Log CFU/ml

highest proteinase in fish broth containing 25% NaCl at 40°C while *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 did not show proteolytic activity at high salt content (Figure 4.2b). It has been reported that the highest proteinase production of halotolerant/halophilic bacteria were observed at low salt concentrations (~0.5-10% NaCl) at 25-40°C (Kamekura, 1986; Dube et al., 2001; Ralman et al., 2005; Patel et al., 2006). *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3 isolated from fish sauce fermentation optimally produced proteinase when cultivated in modified JCM broth containing 10% NaCl at 30 and 37°C, respectively (Namwong et al., 2006; Hiraga et al., 2005). Kanekar et al. (2002) isolated *Bacillus*, *Staphylococcus*, *Micrococcus*, *Pseudomonas* and *Arthrobacter* from sediment samples of Lonar lake in India showed the optimal proteinase production in Nutrient broth containing 0.5% NaCl at 30°C. Moreover, temperature of 28-37°C was essential for both efficient growth and maximum proteinase secretion of *Bacillus* sp. (Kanekar et al., 2002; Uyar and Baysal, 2004; Ralman et al., 2005; Patel et al., 2006). In contrast, marine bacterium strain PA-43 isolated from the sea in Iceland produced serine proteinase in medium containing 1.5% NaCl at 16.5°C (Irwin, Alfredsson, Lanzetti, Gudmundsson and Engel, 2001). Six strains of *Staphylococcus* (*Staphylococcus xylosus*, *S. saprophyticus, S. equorum, S. simulans* and *S. carnosus*) isolated from Southern Italian fermented sausages showed proteinase activity in BHI broth containing 0.5% NaCl when incubated at 30°C for 24 h (Casaburi et al., 2006). *Staphylococcus* sp. SK1-1-5 produced high proteinase in the medium containing 5% NaCl at 35°C. These results showed that these proteinase-producing bacteria was halotolerant/halophilic bacteria and produced high proteinase at low salt concentration at 25-40°C. Rahman et al. (2005) found that enzyme synthesis and energy metabolism of bacteria was controlled by temperature.

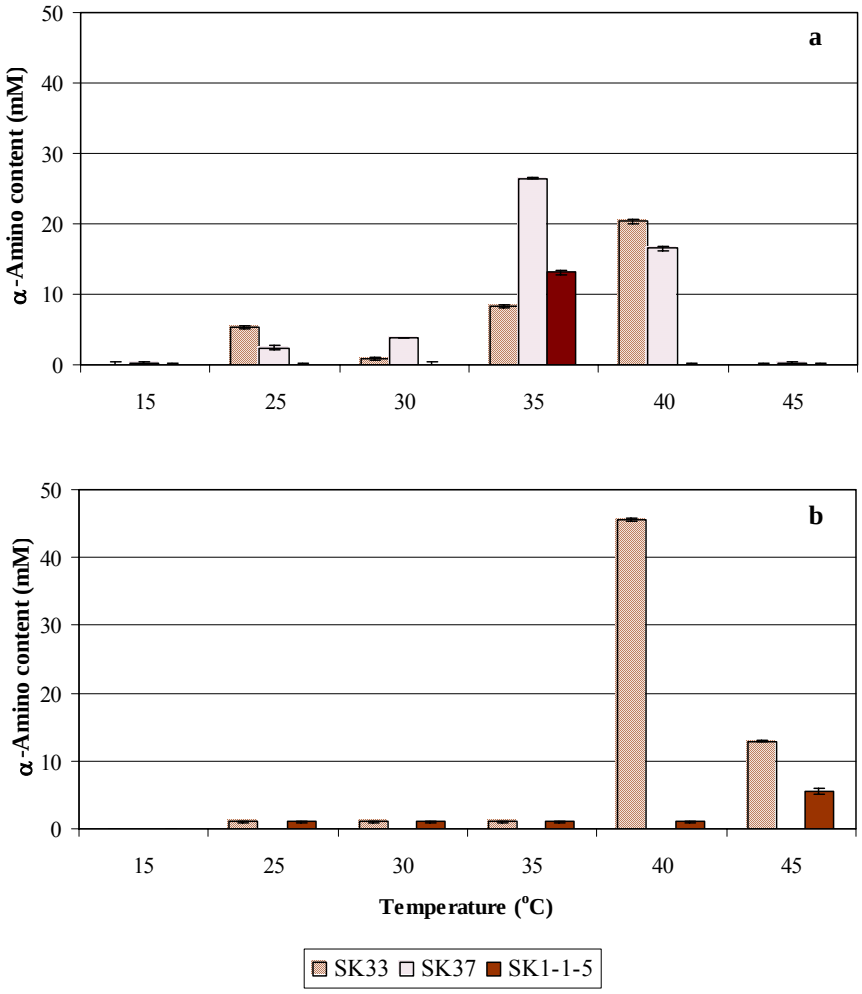

Figure 4.2 α-Amino peptides produced by the selected strains in 5% NaCl (a) and 25% NaCl (b) in fish broth incubated at various temperatures

SK33 = *Virgibacillus* sp. SK33, SK37 = *Virgibacillus* sp. SK37, SK1-1-5 = *Staphylococcus* sp. SK1-1-5

Moreover, Ray et al. (1992) reported that temperature significantly regulated the synthesis and secretion of bacterial extracellular proteinase. Therefore, temperature was a critical parameter that should be controlled in order to obtain the optimum proteinase production.

Based on these studies, the optimal condition for bacterial growth did not correlate with the optimal proteinase production. This means that optimal growth condition might not result the maximum proteolytic activity. From an application point of view, it should be taken into consideration whether proteinase activity or microbial cell is needed for development and acceleration fish sauce fermentation process.

Virgibacillus sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 grew in fish broth containing 0-30% NaCl ($p<0.05$), and the optimal growth varied with strains. *Virgibacillus* sp. SK33 grew well at 3-18% NaCl ($p<0.05$) while *Virgibacillus* sp. SK37 grew well at 3-23% NaCl ($p<0.05$) with the maximum growth at 18 and 20% NaCl, respectively (Figure 4.3). These results were in agreement with those found in *Virgibacillus* sp. (Heyndrickx et al., 1998; Heyman et al., 2003; Yoon et al., 2004). Chandrashekar and Deosthale, (1993) reported that anchovy consisted of several amino acids, such as alanine, arginine, cysteine, leucine lysine, proline, serine especially, glycine. Glycine could have its function as compatible solute (Ventosa et al., 1998; Moral et al., 1999; Robert, 2000; Roebler and Muller, 2001; Margesin and Schinner, 2001). This compatible solute maintained the bacterial cell under high salt concentration (Moral et al., 1999). For this reason, three selected strains could thrive in the high osmolarity environment when cultivated in fish broth. The maximum growth of *Staphylococcus* sp. SK1-1-5 was found at 15% NaCl with a slight growth at 30% NaCl (Figure 4.3C). These data disagreed with Kloos and Schleifer (2001) who reported that *Staphylococcus* sp. grew well in the medium containing up to 10% NaCl and the growth decreased at 15% NaCl. In addition, there are some reports indicating that the maximum NaCl concentration for growth of *S. carnosus* (Tanasupawat et al.,

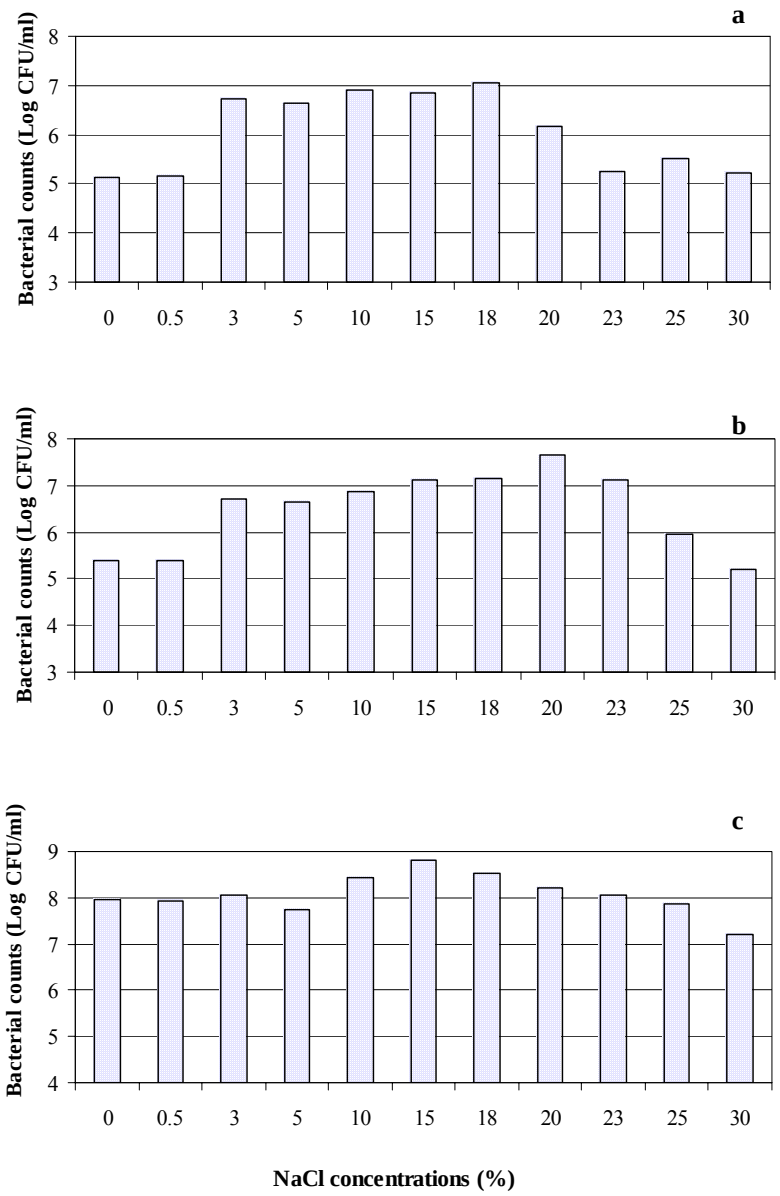

Figure 4.3 Bacterial counts (Log CFU/ml) at various NaCl concentrations of *Virgibacillus* sp. SK33 (a), *Virgibacillus* sp. SK37 (b) and *Staphylococcus* sp. SK1-1-5 (c) in fish broth (initial pH 7) incubated at 35^{o}C with initial cell count of about Log CFU/ml.

1991), *S. piscifermentans* (Tanasupawat et al., 1992) and *S. codimenti* (Andreas et al., 1998) was at 15% NaCl. However, growth of *Staphylococcus* in the medium

containing over 15% NaCl has not been reported. Based on the result of 16S rRNA gene in chapter 3, *Staphylococcus* sp. SK1-1-5 showed 95% homology to *Staphylococcus saprophyticus* ATCC 15305 and belonged to the member of the genus *Staphylococcus* rather than the genus *Salinicoccus, Marinococcus, Micrococcus* and *Halococcus*. It was likely that *Staphylococcus* sp. SK1-1-5 is a new *Staphylococcus* species as it grew in the medium containing higher NaCl (>15%). This is the first report of *Staphylococcus* isolated from fish sauce fermentation process which grew in NaCl concentration higher than 15%.

Virgibacillus sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 could grow at pH 5.5-7.5 (Figure 4.4). Based on this study, the optimum condition for growth of *Virgibacillus* sp. SK33 in fish broth were at 35^{o}C, pH 6.5-7.5 (about 10^6 CFU/ml) while those of *Virgibacillus* sp. SK37 were at pH 7 (about 10^8 CFU/ml). Only *Staphylococcus* sp. SK1-1-5 grew well at pH 5.5-7 (about 10^8 CFU/ml) (Figure 4.4). *Virgibacillus* sp. SK33 could hydrolyze anchovy extract at all studied pH ranging between 5.5 and 7.5 ($p>0.05$) (Figure 4.4). *Virgibacillus* sp. SK37 showed the highest proteinase productions at pH 7 and 7.5 ($p<0.05$). *Staphylococcus* sp. SK1-1-5 appeared to hydrolyze protein at this studied pH. Yoon et al. (2004) reported that *Virgibacillus halodenitrificans* grew at pH between 5.8 and 9.6 with the optimum pH at 7.4-7.5. While the optimum growth of *Virgibacillus* sp. SK33 at 6.5-7.5 and *Virgibacillus* sp. SK37 at 7. Moreover, other proteinase-producing bacteria, such as *Filobacillus* sp. RF2-5, *Halobacillus* sp. SR5-3, *Bacillus subtilis*, and *Bacillus licheniformis* grew well and could produce proteinase at pH 7-7.2 (Rozs et al., 2001; Hiraga et al., 2005; Kim and Kim, 2005 Namwong et al., 2006; Terlabie et al., 2006). *Staphylococcus* sp., such as *Staphylococcus xylosus*, *S. saprophyticus, S. equorum,*

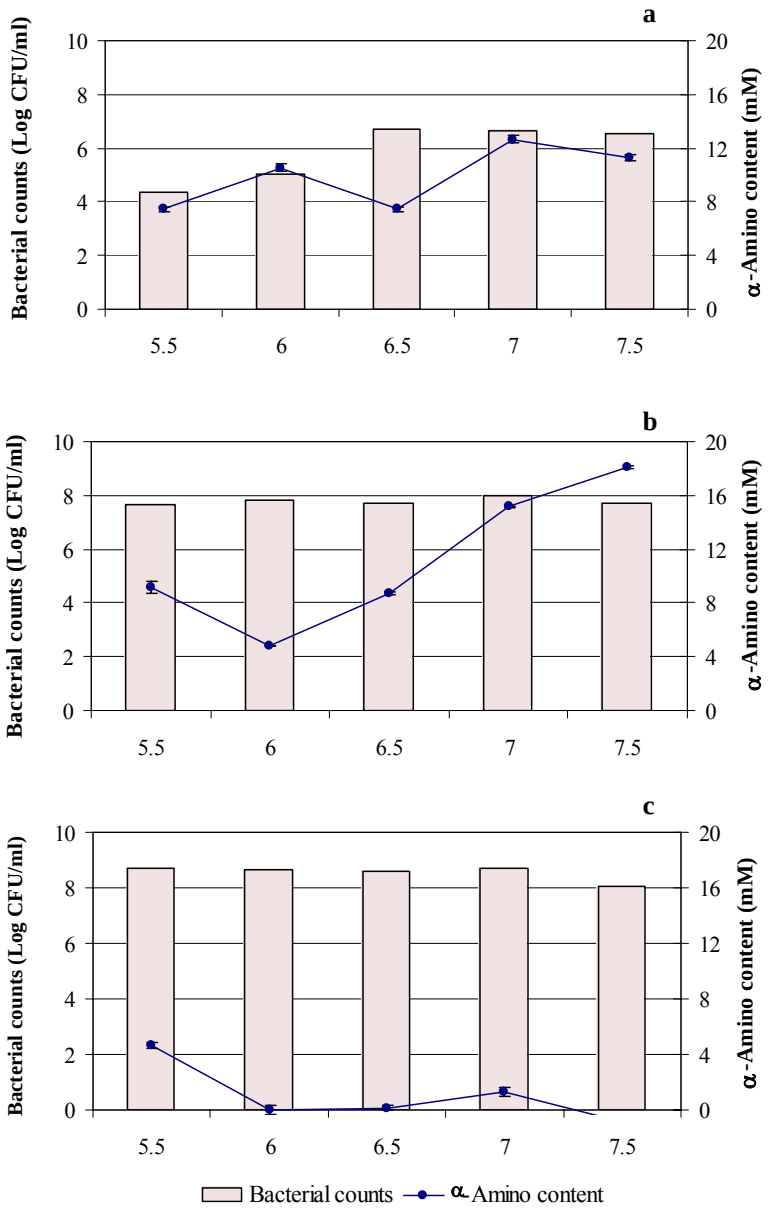

Figure 4.4 Bacterial counts (Log CFU/ml) and α-amino content (mM) of *Virgibacillus* sp. SK33 (+18% NaCl) (a), *Virgibacillus* sp. SK37 (+20% NaCl) (b) and *Staphylococcus* sp. SK1-1-5 (+15% NaCl) (c) in fish broth at various pHs and incubated at 35°C with initial cell count of about 5 Log CFU/ml.

S. simulans and *S. carnosus*, grew well and produced proteinase at pH 7 (Kloos and Schleifer, 2001; Casaburi et al., 2006). The optimum pH range at 5-9 for growth and at 7-7.5 for proteinase production is common among halophilic and halotolerant(Madern et al, 2000; Porro et al, 2003). Three selected strains grew and showed proteolytic degradation of fish broth at 5-6.5 which was the pH range of fish sauce fermentation. Therefore, this study demonstrated that three selected strains could be applicable as starter cultures for fish sauce production.

4.4.2 Nitrogen and carbon source

A. Concentration of nitrogen source

Anchovy extract is the sole nitrogen source for growth and proteinase production of the media**.** Addition of 0.8% yeast extract to fish broth had no effect on the growth of *Staphylococcus* sp. SK1-1-5 ($p>0.05$) but the growth rate of *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 appeared to increase with addition of 0.8% yeast extract (Figure 4.5). Since an increase in cell count was less than 1 log cycle and yeast extract is rather costly, yeast extract might not be needed in the media. Fish broth had enough nutrients for bacterial growth, such as protein, carbohydrates and mineral (Shahidi et al, 1994). Chandrashekar and Deosthale (1993) reported the composition of anchovy (*Thryssa purava*) were 81.5% moisture, 11.4% protein, 6.1% lipid, 0.8% minerals and trace elements. Minerals found were Ca, P, Mg, Fe, Zn, Cu, Mn, Cr and I. The amount of Mg and Mn in anchovy were 45.4 mg/100 g and 64.7 μg/100 g of fish, respectively (Chandrashekar and Deosthale, 1993). These compounds could be sufficient for growth of these 3 strains (Gibbons, 1974; Ventosa et al., 1998; Eunguttanagorn, 2000). Growth of all strains decreased as ratio of protein concentration decreased (Figure 4.5). When carbon source in the media is limited,

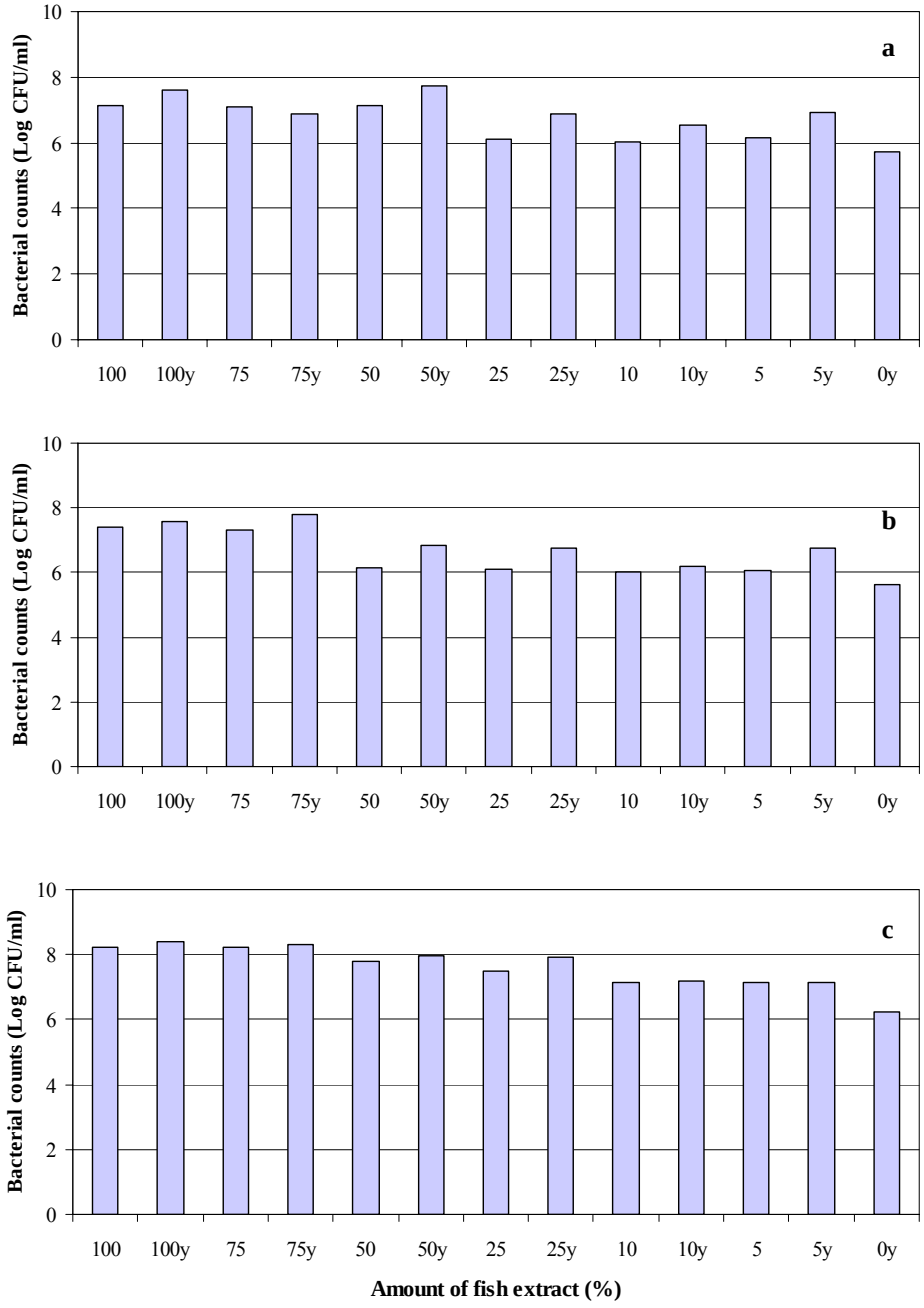

Figure 4.5 Bacterial counts (Log CFU/ml) of *Virgibacillus* sp. SK33 (+18% NaCl) (a), *Virgibacillus* sp. SK37 (+20% NaCl) (b) and *Staphylococcus* sp. SK1-1-5 (+15% NaCl) (c) in fish broth containing various ratios of fish extract and incubated at 35^{o}C pH 7 with initial cell counts of about 5 Log CFU/ml (y = added 0.8% yeast extract).

nitrogen source become essential for bacterial growth. The maximum growth of *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 was achieved at either 75-100% anchovy extract ($p>0.05$), while *Virgibacillus* sp. SK33 showed the optimum growth at 50-100% anchovy extract ($p>0.05$) (Figure 4.5).

α-Amino peptides at various ratios of anchovy extract are shown in Figure 4.6. *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 expressed high proteinase activity in the media containing 50 and 75% anchovy extract, respectively ($p>0.05$). *Staphylococcus* sp. SK1-1-5 could not hydrolyze protein at all varied proportions of anchovy extract. Proteinase production of *Virgibacillus* sp. SK33 in the media containing 75 and 50% anchovy extract were 3.13-3.34 mmole/g protein of fish broth, and those of *Virgibacillus* sp. SK37 were 1.71-1.95 mmole/g protein of fish broth. According to these results, 50% anchovy extract was the optimum condition for proteinase production of *Virgibacillus* sp. SK33. *Virgibacillus* sp. SK37 grew well and produced high proteinase at 75-100% anchovy extract ($p>0.05$). Thus, fish broth containing 50 and 75% anchovy extract without 0.8% yeast extract was the optimal for *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37, respectively. Nitrogen source could induce proteinase synthesis (Moon and Parulekar, 1991; Chu, Lee and Li, 1992). Patel et al. (2006) found that the effect of organic nitrogen sources, especially peptone and yeast extract was pronounced on the proteinase production of *Bacillus* sp. isolated from seawater in Western India. Extracellular alkaline proteinase from *Bacillus horikoshii* was optimally produced when 1% casein was added in the medium (Joo et al, 2002). Recently, Uyar and Baysal (2004) found wheat bran was a good nitrogen source for proteinase production of *Bacillus* sp. The suitable nitrogen source for industrial application should be available with low cost. Furthermore, it should

enhance microbial growth and induce proteinase production. From these results, fish broth was a complex organic nitrogen source containing varied amount of free amino acids and small peptides which could serve as important nutrients for growth and proteinase production. Moreover, fish broth composition is compatible to the fish sauce fermentation process as the same raw material (anchovy) was used. Thus, fish broth was a suitable medium for starter culture preparation of these strains.

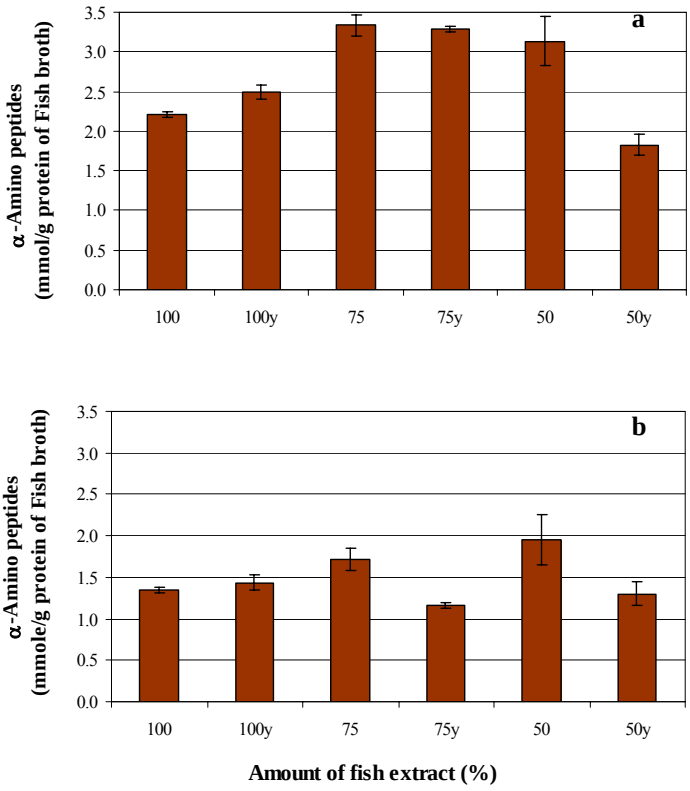

Figure 4.6 α-Amino peptides of media containing anchovy extract at varied proportions and inoculated with *Virgibacillus* sp. SK33 (+18% NaCl) (a) and *Virgibacillus* sp. SK37 (+20% NaCl) (b).

B. Concentration of carbon source.

Virgibacillus sp. SK37 and *Staphylococcus* sp. SK1-1-5 grew in fish broth without glucose to a greater extent than in fish broth added glucose ($p<0.05$) (Table 4.1). *Virgibacillus* sp. SK33 showed maximum growth at 3% glucose, while growth at 0, 0.75 and 2% glucose was not different ($p>0.05$). Thus, glucose was not required for the growth for all studied strains. *Virgibacillus* sp. SK37 grew well at 0-2% glucose ($p>0.05$). The similar study showed that the growth of *Bacillus* sp. Po2 was not different in the medium containing 0-1% glucose (Patel et al., 2006). The growth of *Staphylococcus* sp. SK1-1-5 was inhibited at 3% glucose as cell counts were reduced from 10^6 to 10^4 CFU/ml. This might be due to higher osmotic pressure outside the cell (plasmolysis), This phenomenon occurs when water is drawn out of the cell. Based on these results, glucose was not necessary for the bacterial growth. Fish broth might contain enough carbon sources to support bacterial growth. Several compounds presented in fish broth, such as glycogen derived from fish muscle and liver, can serve as a carbon source for all 3 strains.

Table 4.1 Bacterial counts (Log CFU/ml) of all strains cultured in fish broth containing various glucose concentrations and incubated at 35°C and pH 7 with initial cell counts of about 5 Log CFU/ml

Bacterial isolate	Bacterial counts (Log CFU/ml)					
	Glucose concentration (%)					
	0	0.10	0.75	1.50	2.00	3.00
Virgibacillus sp. SK33	6.08^{ab}	5.57^{c}	6.08^{ab}	5.74^{c}	6.07^{ab}	6.25^{a}
Virgibacillus sp. SK37	6.49^{a}	6.34^{a}	6.41^{a}	6.41^{a}	6.47^{a}	5.95^{b}
Staphylococcus sp. SK1-1-5	6.04^{a}	6.08^{a}	5.71^{bc}	5.82^{b}	5.67^{c}	4.78^{d}

Letters indicate significant difference within the same row ($p<0.05$)

4.4.4 Effect of aeration

Bacterial growth were significantly affected by shaking speed ($p<0.05$) as shown in Table 4.2. Growth of *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 increased about 3 log cycle at all studied shaking speeds of 100-250 rpm ($p>0.05$). *Staphylococcus* sp. SK1-1-5 showed the maximum growth at 250 rpm ($p<0.05$). The growth of *Staphylococcus* sp. SK1-1-5 at 100 rpm was

Table 4.2 Growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 cultured in fish broth (initial pH 7) incubated at 35°C, at various shaking speeds.

Bacterial isolate	Inoculation time	Bacterial counts (Log CFU/ml)		
	(day)	100 rpm	200 rpm	250 rpm
Virgibacillus sp. SK33	0	4.64	4.64	4.64
	3	**7.81[a]**	**7.81[a]**	**7.85[a]**
Virgibacillus sp. SK37	0	5.23	5.23	5.23
	4	**8.22[a]**	**8.21[a]**	**8.16[a]**
Staphylococcus sp. SK1-1-5	0	6.41	6.41	6.41
	1	**9.09[b]**	**9.20[b]**	**9.42[a]**

Letters indicate significant difference within the same row ($p<0.05$)

lower than that at 250 rpm ($p<0.05$) but the difference in the cell count was less than 1 log cycle. Therefore, starter cultures of these strains could be prepared by shaking speed at 100 rpm. The higher shaking speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium (Feng et al., 2003). Oxygen was an important factor for bacterial growth because bacteria used oxygen to produce energy (Tortora, Funke and Case, 2004). Concentration of dissolved oxygen was found to have a significant effect on growth rate of *Bacillus licheniformis* with noticeable changes in biomass formation (Feng et al., 2003). The cell of *B. licheniformis* was

increased about 10% when shaking speed increased from 450 rpm to 600 rpm. Shioya, Morikawa, Kajikhara and Shimizu (1999) found that *Streptomyces virginiae* grew well when shaking at 450 rpm. Milner, Martin and Smith (1996) also reported that bacterial growth was limited when concentration of dissolved oxygen was below a critical level.

4.4.5 Effect of inoculum size

The amount of inoculum used to culture the bacteria could affect the growth and proteinase production of *Virgibacillus* sp. SK33 and *Staphylococcus* sp. SK1-1-5. The maximal growth of *Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK33 were

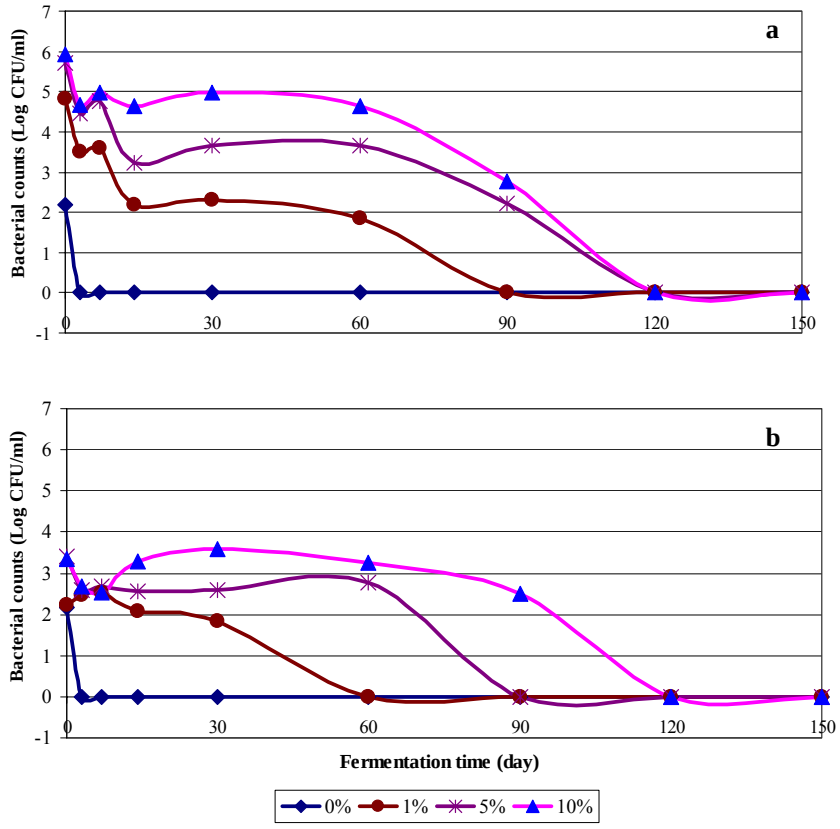

Figure 4.7 Effect of inoculum size on growth (Log CFU/ml) of *Staphylococcus* sp. SK1-1-5 (a) and *Virgibacillus* sp. SK33 (b) on JCM 168 medium (initial pH 7) containing 25%NaCl, incubated at 35°C.

observed at 10% inoculum ($p<0.05$) (Figure 4.7). Growth of inoculated bacteria diminished after 60 days of fermentation. No growth of all strains was found at 120-150 days.

α-Amino acid content produced by 1% inoculum was not different from that of control ($p>0.05$) (Figure 4.8), while addition of 10% inoculum yielded the maximum α-amino acid content ($p<0.05$) (Figure 4.8). Thus, 10% inoculum size increased fish protein hydrolysis during fish sauce fermentation. The inoculum level

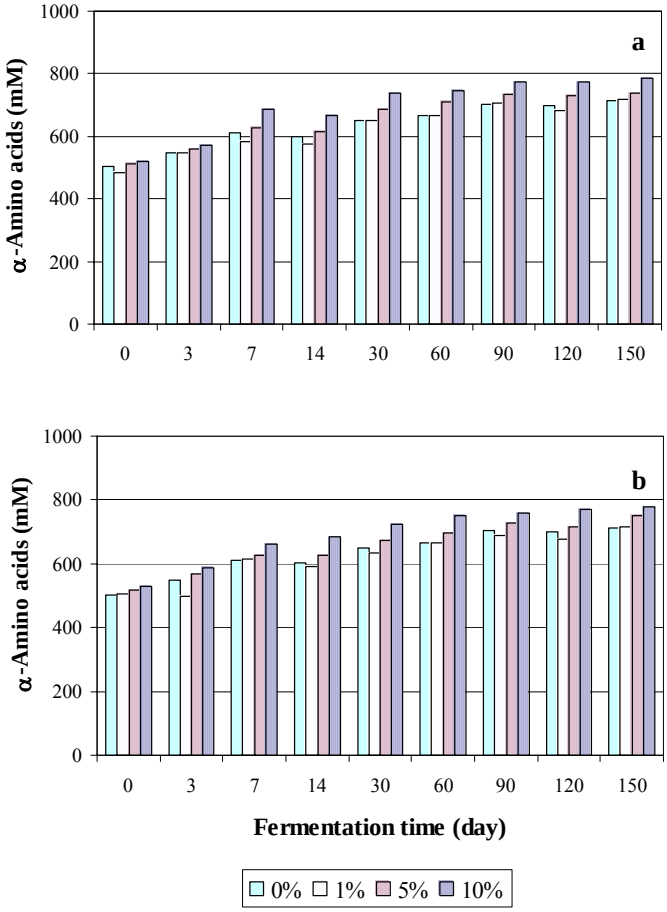

Figure 4.8 α-Amino peptides produced by *Staphylococcus* sp. SK1-1-5 (a) and *Virgibacillus* sp. SK33 (b) at various inoculum sizes.

was an important factor for the production of proteinase (Uyar and Baysal, 2004; Rahman et al., 2005). Garcia et al, (1999) found that the addition of starter culture (*Bacillus subtilis* and *Micrococcus* sp.) at 1% inoculum size accelerated the ripening process of Manchego cheese, which considerably increased the level of nitrogen soluble, consisting mainly of small peptides and amino acids. Eungurranagorn (2000) studied the effect of the inoculum size at 0.5-15% on the growth of *Halobacterium salinarium* on the medium prepared from fish sauce processing waste and mixed with 20-g $MgSO_4$ $7H_2O$, 0.5-g $MnSO_4$ H_2O and 0.2-mg $FeSO_4$ $7H_2O$ (in 1000 ml). *H. salinarium* grew well at 1-15% inoculum size ($p>0.05$). The maximum proteinase production of *Bacillus* sp. isolated from soil was obtained at 20 and 25% inoculumn (Uyar and Baysal, 2004). However, Sen (1995) reported that *Bacillus licheniformis* S40 produced proteinase when 10% inoculum was added. The production of enzyme declined when inoculum level increased, due to exhaustion of available nutrients in the fermentation process. Thus, the level of 10% inoculum should be considered to be optimum for *Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK33 for fish sauce fermentation

4.4.6 Application of starter cultures for fish sauce fermentation

Sinsuwan, Rodthong, Raksakulthai and Yongsawatdigul (2006) reported that *Virgibacillus* sp. SK33 secreted proteinase to a greater extent in the medium containing peptides and amino acids than large protein (fish and casein). Therefore, anchovies were initially hydrolyzed using the commercial proteinases before adding bacterial starter cultures. Based on the preliminary studies, growth of inoculated cultures, in the salted anchovy was minimum and declined after 7 days. This could be due to the limited available nutrients during the first stage of fermentation. To

overcome such a problem, anchovies were initially hydrolyzed using the commercial proteinases to attain sufficient amino acids and peptides before adding bacterial starter cultures.

The growth of bacteria during fermentation was monitored using JCM 168 containing 25% NaCl. The viable cell of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 decreased during the first 14 days of fermentation. This might be the adaptation period of bacterial cells at 25% NaCl. Population of all strains increased and remained constant throughout 120 days. Halophilic and halotolerant bacteria were found within 30 days of the fermentation (Figure 4.9). pH value was about 5.46-5.93 (Figure 4.10) which was similar to fish sauce fermentation process. Kilinc, Cakli, Tolasa and Dincer (2006) found that growth of halophilic and halotolerant in fish sauce fermentation process increased during the first 8 days and then decreased gradually thereafter. Kobayashi and Hayashi (1998)

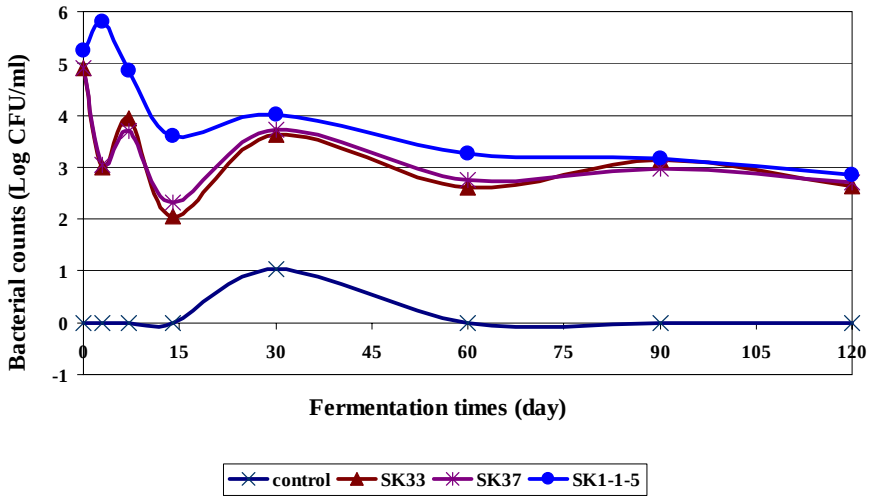

Figure 4.9 Growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 during fish sauce fermentation incubated at 35°C, initial pH 7. (control = sample without starter culture added)

found that growth of microbial contamination was inhibited at 16% NaCl. The growth of yeast, coryneform and other microbial contaminations in Japanese shoyu (soy sauce) and Indonesian kecap (soy sauce) were also inhibited at higher salt concentrations (Roling et al., 1994; Ling and Chou, 1996). Moreover, Ijong and Ohta (1996) reported that salt used in fish sauce fermentation was important because it was bacteriostatic agent for many bacteria including pathogenic and spoilage bacteria.

α-Amino acid content of all treatments were not different during the first 3 days of fermentation ($p>0.05$) (Figure 4.11). Then, it increased in the samples inoculated with starter cultures and became higher than the control throughout 120 days of fermentation ($p<0.05$). An increase of α-amino acid content in this study might be partly due to proteinase activity presenting in starter cultures. Since viable cell count of samples inoculated by starter cultures were greater than the control throughout the fermentation process. So, *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37

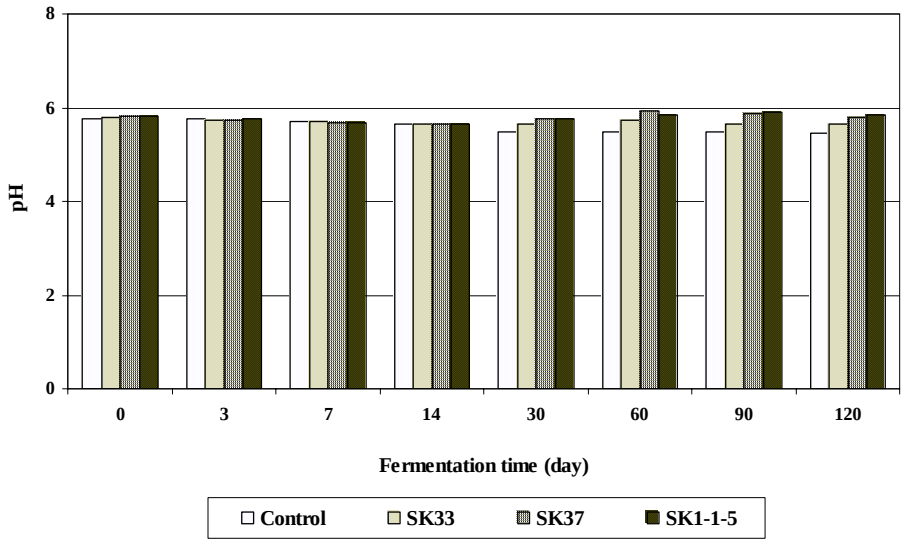

Figure 4.10 Changes of pH during fermentation of samples inoculated by 3 selected strains. SK33=*Virgibacillus* sp. SK33, SK37=*Virgibacillus* sp. SK37, SK1-1-5=*Staphylococcus* sp. SK1-1-5.

and *Staphylococcus* sp. SK1-1-5 hydrolyzed protein in the fermentation process under high salt content and were potential strains for starter culture development. Moreover, colonies observed on JCM168 medium at each time interval showed consistent appearance. Thus, colonies appeared were likely to be an inoculated starter culture. Yin et al. (2005) believed that proteolytic activity in fermentation process was not only caused by the autolysis but also by proteinase activity of bacteria. *Staphylococcus* sp. SK1-1-5 produced the highest α-amino acid content at day 120 ($p<0.05$). These results were similar to those obtained from previous study on the effect of starter culture to fermented soybeans. Terlabie et al. (2006) found that the addition of *Bacillus subtilis* $24BP_2$ as a starter culture showed higher level of proteinase activity than the control (without starter culture). Omafuvbe et al. (2002) also used *B. subtilis*, *B. pumilus* and *B. licheniformis* as a starter culture to accelerate fermentation of soybean. Although *Staphylococcus* sp. SK1-1-5 showed less proteinase activity in fish broth than *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 (Figure 4.4), it showed the highest proteinase activity in fermentation system. Possibly, fish sauce fermentation process consisted of various nutrients that promoted growth and proteinaes production of *Staphylococcus* sp. SK1-1-5 to a better extent than fish broth. Then, three proteinase-producing strains were potential starter cultures for fish sauce fermentation process that showed ability to break down proteins. Protein hydrolysis is reported to be the major biochemical changes occuring during fish sauce fermentation. Addition of starter culture would improve the quality of fish sauce product and reduce the processing time. This was the first report of the addition of proteinase-producing bacteria as a starter culture to accelerate fish sauce fermentation process.

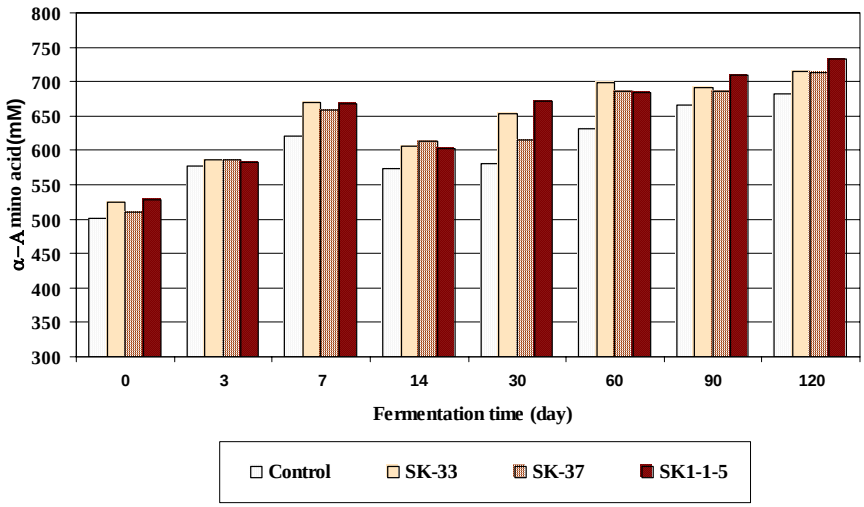

Figure 4.11 α-Amino content of fish sauce inoculated with 3 selected strains. Abbreviations were the same as Figure 4.10.

4.5 Conclusions

Staphylococcus sp. SK1-1-5, *Virgibacillus* sp. SK33, and *Virgibacillus* sp. SK37 showed the optimal salt concentration for growth at 15, 18, and 20% NaCl, respectively, at 35°C and initial pH 7. *Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK37 effectively hydrolyzed protein in fish broth containing 5% NaCl, at 35°C. *Virgibacillus* sp. SK33 showed the highest extent of protein hydrolysis at 40°C in fish broth containing 25% NaCl. Thus, three strains had tendency to hydrolyze protein during fish sauce fermentation. The optimum medium for bacterial growth and proteinase production contained 75% anchovy extract without glucose. This can be of economically feasible because the cost of medium preparation can be reduced. Aeration and 10% inoculum size of starter culture increased the bacterial cell growth and increased fish protein hydrolysis. All strains grew and produced α-amino acid content greater than the control during 120 days of the fermentation process. So,

Staphylococcus sp. SK1-1-5, *Virgibacillus* sp. SK33, and *Virgibacillus* sp. SK37 were potential strains that can be used in fish sauce fermentation.

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CHAPTER V

SUMMARY

Most proteinase-producing bacteria were found in fish sauce fermented at 1-3 months. Twenty seven isolates hydrolyzed anchovy proteins, and were identified as *Virgibacillus*, *Halomonas*, *Bacillus firmus*, *Brevibacteium*, *Corynebacterium*, and *Staphylococcus* based on the combined phenotypic and genotypic characteristics. *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 produced the highest proteinase activity and were likely to be new species.

The optimum NaCl concentration for growth of *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 were 18, 20 and 15%, respectively in fish broth at pH 7. All isolates grew very well at 35°C. *Virgibacillus* sp. SK33 had optimum proteinase production temperature at 40°C in fish broth containing 25% NaCl. Both *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 had optimum proteinase production at 35°C in fish broth containing 5% NaCl. However, three selected strains could grow and produce proteinase under the same condition of fish sauce fermentation which would be practical for fish sauce industry. Yeast extract and glucose were not needed for bacterial growth and proteinase production of all strains tested. The optimum medium for bacterial growth and proteinase production contained 75% anchovy extract without glucose which could be reduced up to 25% anchovy extract. Aeration at shaking speed of 100 rpm and 10% inoculum size of starter culture increased the bacterial cell growth and

increased fish protein hydrolysis during fish sauce fermentation. The three selected strains could be able to accelerate protein hydrolysis of anchovy. ∝-Amino content of salted anchovy inoculated with *Staphylococcus* sp. SK1-1-5, *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 were higher than the control (without starter culture) during 120 days of incubation. Thus, the optimization conditions for growth of three selected strains was useful for increasing bacterial cells. Moreover, the optimal condition of proteinase production should be taken into consideration. Application of these 3 strains to fish sauce fermentation would reduce the extremely by fermentation time and help maintain consistent product quality.

APPENDIX A

Reagent preparation and culture media

1. Reagents

1.1 Acetone alcohol

Alcohol (95%)	700.0	ml
Acetone	300.0	ml

1.2 Bufferfield's buffered phosphate diluent (Phosphate-buffered solution)

Stock solution:

Potassium di-hydrogen phosphate	34.0	g
Distilled water	500.0	ml
1N NaOH (adjusted pH to 7.2)	175.0	ml
Added distilled water and brought volume up to	1,000.0	ml

1.3 Cacodylate buffer (pH 7.2)

Solution A: 0.2M Sodium cacodylate

Sodium cacodylate	42.8	g
Added distilled water and brought volume up to	1,000.0	ml

Solution B: 0.2M Hydrochloric acid

Hydrochloric acid	16.5	ml
Added distilled water and brought volume up to	1,000.0	ml

0.1M Cacodylate buffer

Solution A	50.0	ml
Solution B	4.2	ml

1.4 Crystal violet (Gram stain)

Crystal violet	2.0	g
Ethanol (95%)	20.0	g
Mixed thoroughly		
Ammonium oxalate (1% Aqueous solution)	80.0	ml

1.5 Glutaraldehyde (2%) in 0.1 M Cacodylate buffer

25% Glutaraldehyde	8.0	ml
0.1M Cacodylate buffer	92.0	ml

1.6 Hydrogen peroxide (3% solution)

Hydrogen peroxide (30%)	10.0	ml
Distilled water	100.0	ml

1.7 Iodine solution (Gram's iodine)

Iodine	1.0	g
Potassium iodide	2.0	g
Added distilled water and brought volume up to	300.0	ml

1.8 Malachite green

Malachite green	5.0	ml
Distilled water	100.0	ml

1.9 Ninhydrin (0.5%)

5,6-Dimethoxyninhydrin	0.5	g
Added water-saturated n-buthanol and brought	100.0	ml

1.10 Osmium tetroxide (2%)

Osmium tetroxide	0.5	g
0.1 M Phosphate buffer	25.0	ml

1.11 Safranin (Gram stain)

Safranin O (2.5% solution ใน 95% Ethanol)	10.0	ml
Distilled water	90.0	ml

1.12 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100.0	g
Added distilled water and brought volume up to	1,000.0	ml

1.13 TE buffer (10 mM Tris-HCl, 1 mM EDTA)

Tris-HCl	0.79	g
EDTA (di-Sodium salt)	0.37	g
Boric acid	5.54	g
Added distilled water and brought volume up to	1,000.00	ml

1.14 Tetramethyl-p-phenylenediamine dihydrochloride (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1.0	g
Distilled water	100.0	ml

1.15 Tris-Borate buffer

Tris-Base	10.77	g
EDTA (di-Sodium salt)	0.93	g
Boric acid	5.54	g
Added distilled water and brought volume up to	1,000.00	ml

2. Culture media

2.1 Blood agar (Atlas and Parks, 1997)

Sodium chloride	5.00	g
Pancreatic digest of casein	14.00	g
Peptone	4.50	g
Yeast extract	4.50	g
Agar	12.50	g
Sheep blood defibrinated	70.00	ml

pH 7.3 $\pm$ 0.2

2.2 Halophilic agar (Atlas and Parks, 1997)

Casamino acids	10.0	g
Yeast extract	10.0	g

Proteose peptone	5.0	g
Tri-Sodium citrate	3.0	g
Potassium chloride	2.0	g
$MgSO_4.7H_2O$	25.0	g
Sodium chloride	250.0	g
Added distilled water and brought volume up to	1,000.0	ml
Agar	20.0	g

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.3 JCM 168 broth

Casamino acids	5.00	g
Yeast extract	5.00	g
Sodium glutamate	1.00	g
tri-Sodium citrate	3.00	g
Potassium chloride	2.00	g
$MgSO_4.7H_2O$	20.00	g
Sodium chloride	250.00	g
$FeCl_2.4H_2O$	36.00	mg
$MnCl_2.4H_2O$	0.36	mg
Added distilled water and brought volume up to	1,000.00	ml

pH 7.0 + 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.4 JCM 168 Medium

The components were similar with JCM 168 broth and added agar 20 g/L

2.5 Motility test medium (Atlas and Parks, 1997)

Tryptose	10.0	g
Sodium chloride	250.0	g
Added distilled water and brought volume up to	1,000.0	ml
Agar	3.0	g

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.6 MØller Broth (Rodriguez-Jerez et al., 1994)

L-Lysine	4.00	g
L-Histidine	4.00	g
L-Ornithine	4.00	g
L-Tyresine	4.00	g
Glucose	0.50	g
Peptone	5.00	g
Beef extract	5.00	g
Sodium chloride	250.00	g
Bromcresol purple	0.01	g
Cresol red	5.00	mg
Added distilled water and brought volume up to	1,000.00	ml

The medium was autoclaved at 121^{o}C for 15 min.

2.7 Nutrient gelatin (Atlas and Parks, 1997)

Beef extract	2.4	g
Peptone	4.0	g
Gelatin	96.0	g
Sodium chloride	250.0	g
Added distilled water and brought volume up to	1,000.0	ml

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.8 O-F test medium (Atlas and Parks, 1997)

Sodium chloride	5.00	g
Pancreatic digest of casein	2.00	g
di-Potassium hydrogen phosphate	0.30	g
Bromthymol blue	0.03	g
Agar	2.50	g
Glucose solution	100.00	ml

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.9 Plate count agar (PCA) (Atlas and Parks, 1997)

Tryptone	5.0	g
Yeast extract	2.5	g
Dextrose	1.0	g

Agar	15.0	g
Added distilled water and brought volume up to	1,000.0	ml

The medium was autoclaved at 121^{o}C for 15 min.

2.10 Skim milk salt agar (Prasad and Seenayya, 2000)

1) Salt solution

$MgSO_4.H_2O$	10.0	g
KNO_3	2.0	g
Sodium chloride	250.0	g
Ferric citrate	trace	
Neopeptone	5.0	g
Glycerol	10.0	ml
Agar	20.0	g
Added distilled water and brought volume up to	900.0	ml

pH 7.0 $\pm$ 0.2

The componentswas autoclaved at 121^{o}C for 15 min.

2) Reconstituted skim milk (10% solids)	100.0	ml

ant autoclaved at 118^{o}C for 10 min.

Mixed Reconstituted skim milk (10% solids) and Salt solution

2.11 Skim milk salt broth

The components were similar with Skim milk salt agar and without agar

2.12 Standard methods caseinate agar (Atlas and Parks, 1997)

Sodium caseinate	10.0	g
Tryptone	5.0	g
Sodium chloride	250.0	g
Yeast extract	2.5	g
Dextrose or Glucose	1.0	g
Calcium chloride (1.0M solution)	20.0	ml
Added tri-Sodium citrate solution (0.015M solution) and brought volume up to	1,000.0	ml
Agar	20.0	g

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.13 Starch agar (Atlas and Parks, 1997)

Soluble starch	2.0	g
Beef extract	3.0	g
Peptone	5.0	g
Sodium chloride	250.0	g
Agar	20.0	g
Added distilled water and brought volume up to	1,000.0	ml

pH 7.0 $\pm$ 0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.14 Trypticase (tryptic) soy broth (TSB) (Atlas and Parks, 1997)

Tryptone (Pancreatic digest casein)	17.0	g
Phytone (Papaic digest soya meal)	3.0	g
Sodium chloride	5.0	g
di-Potassium hydrogen phosphate	2.5	g
Glucose	2.5	g
Added distilled water and brought volume up to	1,000.0	ml

pH 7.3±0.2

The medium was autoclaved at 121^{o}C for 15 min.

2.15 Tween–80 Agar (Lipase test medium) (Atlas and Parks, 1997)

Peptone	10.0	g
Sodium chloride	250.0	g
Calcium chloride	0.1	g
Yeast extract	2.5	g
Tween-80	10.0	ml
Added distilled water and brought volume up to	1,000.0	ml
Agar	20.0	g

pH 7.0 ± 0.2

The medium was autoclaved at 121^{o}C for 15 min.

APPENDIX B

Proteinase-producing bacteria characterization

APPENDIX C

Publication in GenBank (U.S.A.)

Table 1C Publication in GenBank (U.S.A.)

Isolation source	Bacterial isolate code	Nucleotide sequence submission		
		Identification result	Length of sequence	NCBI Accession no.
Sample from fish sauce fermentation process at 1st month	1. SK25	*Staphylococcus* sp. SK25	1402	DQ910836
	2. SK32	*Virgibacillus* sp. SK32	1459	DQ910837
	3. SK33	*Virgibacillus* sp. SK33	1424	DQ910838
	4. SK35	*Brevibacterium* sp. SK35	1382	DQ910839
	5. SK37	*Virgibacillus* sp. SK37	1401	DQ910840
	6. SK37-1	*Virgibacillus* sp. SK37-1	1453	DQ910841
	7. SK39	*Virgibacillus* sp. SK39	1445	DQ910842
	9. SK1-1-2	*Staphylococcus* sp. SK1-1-2	1426	DQ910843
	10. SK1-1-5	*Staphylococcus* sp. SK1-1-5	1438	DQ910844
	11. SK1-1-6	*Corynebacterium* sp. SK1-1-6	1466	DQ910845
	12. SK1-1-8	*Virgibacillus* sp. SK1-1-8	1421	DQ910846
	13. SK1-1-9	*Bacillus* sp. SK1-1-9	1421	DQ910847
Sample from fish sauce fermentation process at 3st month	1. SK1-3-7	*Virgibacillus* sp. SK1-3-7	1443	DQ910848
	2. SK1-3-11	*Virgibacillus* sp. SK1-3-11	1445	DQ910849
	3. SK1-3-19	*Virgibacillus* sp. SK1-3-19	1441	DQ910850
Sample from fish sauce fermentation process at 7st month	1. SKM20-7-9	*Staphylococcus* sp. SKM20-7-9	1407	DQ910851
Fish juice	1. Haw9	*Halomonas* sp. Haw9	1486	DQ910853
	2. SKW12	*Halomonas* sp. SKW12	1349	DQ910854
	3. SKW19	*Virgibacillus* sp. SKW19	1423	DQ910855
	4. SKW23	*Staphylococcus* sp. SKW23	1402	DQ910856
	5. SKW24-1	*Staphylococcus* sp. SKW24-1	1466	DQ910857
Solar salt	1. SKS20	*Staphylococcus* sp. SKS20	1404	DQ910852

Table 1B Comparison of phenotypic characteristics of 27 proteinase-producing bacteria

Isolation source	Bacterial isolate code	Gram stain	Cell shape/size (μm)	Catalase	Oxidase	O/F test	Motility	Gelatin liquefaction	Starch hydrolysis	Tween 80 hydrolysis /Precipitate zone diameter (mm)	Identification result according to biochemical characteristics[a]	
											Closest relative	Identity (%)
Sample from fish sauce fermentation process at 1st month	1. SK25	+	Cocci/ 0.6-1.0	+	-	+/+	-	+	-	-	*Staphylococcus warneri*	55.0
	2. SK32	+	Rods, Spore-forming/ 0.4-1.0 x 4.5-9.3	+	+	+/+	-	+	-	-	*Brevibacillus brevis*	NR
	3. SK33	+	Rods, Spore-forming/ 0.5-0.7 x 2.1-4.1	+	-	+/+	-	+	-	-	*Brevibacillus brevis*	98.9
	4. SK34	+	Rods, Non-spore-forming/ 0.5-0.9 x 1.9-7.0	+	-	+/+	-	+	-	-	*Bacillus sphaericus*	95.3
	5. SK35	+	Rods, Spore-forming/ 0.6-0.9 x 3.8-6.4	+	-	+/+	-	+	-	-	*Brevibacillus brevis*	NR
	6. SK37	+	Rods, Spore-forming/ 0.6-0.7 x 3.0-6.6	+	-	+/+	-	+	-	-	*Brevibacillus brevis*	99.3
	7. SK37-1	+	Rods, Spore-forming/ 0.5-0.7 x 2.4-8.3	+	-	+/+	-	+	-	-	*Bacillus sphaericus*	83.0
	8. SK39	-	Rods, Spore-forming/ 0.5-0.8 x 2.6-10.9	+	-	+/+	-	+	-	-	*Brevibacillus brevis*	NR

Table 1B (continued)

Isolation source	Bacterial isolate code	Gram stain	Cell shape/size (μm)	Catalase	Oxidase	O/F test	Motility	Gelatin liquefaction	Starch hydrolysis	Tween 80 hydrolysis /Precipitate zone diameter (mm)	Identification result according to biochemical characteristics[a]	
											Closest relative	Identity (%)
	9. SK1-1-1	+	Cocci/ 0.9-1.1	+	-	+/+	-	+	-	12.0	*Staphylococcus epidermidis*	98.1
	10. SK1-1-2	+	Cocci/ 0.9-1.2	+	+	+/+	-	+	-	-	*Staphylococcus hominis*	81
	11. SK1-1-5	+	Cocci/ 0.5-1.1	+	-	+/+	-	+	-	4.0	*Staphylococcus cohnii* subsp. *urealyticus*	99.6
	12. SK1-1-6	+	Rods, Non spore-forming/ 0.7-0.9 x 2.0-5.8	+	-	+/+	-	+	-	-	ND	ND
	13. SK1-1-7	+	Cocci/ 0.8-1.1	+	-	+/+	-	+	-	1.0	*Micrococcus* sp.	99.9
	14. SK1-1-8	+	Rods, Spore-forming/ 0.5-0.9 x 1.7-3.3	+	-	-/-	-	+	-	11.0	*Bacillus sphaericus*	NR
	15. SK1-1-9	+	Rods, Spore-forming/ 0.6-1.0 x 2.5-5.0	+	+	+/+	-	+	-	15.0	*Bacillus firmus*	97
Sample from fish sauce fermentation process at 3rd month	1. SK1-3-7	+	Rods, Spore-forming / 0.6-0.9 x 2.0-4.0	+	+	+/+	-	+	-	-	*Bacillus sphaericus*	NR
	2. SK1-3-11	+	Rods, Spore-forming / 0.6-0.9 x 1.7-6.3	+	-	+/+	-	+	-	-	*Brevibacillus brevis*	NR

Table 1B (continued)

Isolation source	Bacterial isolate code	Gram stain	Cell shape / size (μm)	Catalase	Oxidase	O/F test	Motility	Gelatin liquefaction	Starch hydrolysis	Tween 80 hydrolysis /Precipitate zone diameter (mm)	Identification result according to biochemical characteristics[a]	
											Closest relative	Identity (%)
	3. SK1-3-19	+	Rods, Spore-forming/ 0.5-0.7 x 2.0-6.4	+	-	+/+	-	+	-	-	*Bacillus sphaericus*	NR
Sample from fish sauce fermentation process at 7th month	1. SKM20-7-9	+	Cocci/ 0.5-1.0	+	-	-/-	-	+	-	+/7.0	*Staphylococcus xylosus*	NR
Fish juice	1. SKW12	-	Rods, Non-spore-forming/ 0.7-1.1 x 1.3-2.2	+	-	+/+	-	+	-	+/8.0	ND	ND
	2. SKW19	+	Rods, Spore-forming/ 0.9-1.2 x 2.1-3.5	+	-	+/+	-	+	-	-	*Bacillus sphaericus*	NR
	3. SKW23	+	Cocci/ 1.0-1.4	+	-	+/+	-	+	-	-	*Staphylococcus cohnii* subsp. *urealyticus*	NR
	4. SKW 29	+	Cocci/ 0.6-1.2	+	-	+/+	-	+	-	-	*Staphylococcus cohnii* subsp. *urealyticus*	NR
	5. SKW24-1	+	Cocci/ 0.8-1.2	+	-	+/+	-	+	-	-	*Staphylococcus cohnii* subsp. *urealyticus*	NR

Table 1B (continued)

Isolation source	Bacterial isolate code	Gram stain	Cell shape / size (μm)	Catalase	Oxidase	O/F test	Motility	Gelatin liquefaction	Starch hydrolysis	Tween 80 hydrolysis /Precipitate zone diameter (mm)	Identification result according to biochemical characteristics[a]	
											Closest relative	Identity (%)
	6. Haw9	-	Rods, Non-spore-forming/ 0.6-1.1 x 1.1-3.2	+	-	+/+	-	+	-	-	ND	ND
Solar salt	1. SKS20	+	Cocci/ 0.8-1.3	+	-	+/+	-	+	+	+/12.0	*Staphylococcus epidermidis*	NR
	2. SKS23	+	Cocci/ 0.8-1.2	+	-	+/+	-	+	-	-	*Staphylococcus warneri*	NR

[a]API system (API STAPH for cocci belonging to genera *Staphylococcus* and *Micrococcus*, and API 50 CH/B for bacilli having spores; bioMérieux)
ND = Not determined
NR = Not reported

CURRICULUM VITAE

Siriwan Nawong was born in Udonthani, Thailand. She attended Suranaree University of Technology, Thailand and received her Bachelor's degree in food technology in 2001. In 2001, she worked at Royal Home Food Products Co., Ltd. as a production supervisor. In 2002, she worked as research assistant in factors affecting histamine formation in fish sauce fermentation project under the grant No. SUT3-305-42-29-02. In 2003, she attended Suranaree University of Technology, Thailand and she was granted a scholarship by the National Center for Genetic Engineering and Biotechnology (BIOTEC) under the grant No. BT-B-06-FG-19-4603.

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