การคัดเลือกและระบุชนิดของแบคทีเรียชอบเกลือสูงที่สร้างโปรติเนส และสารระเหยในน้ำปลา

นางสาวสิริญญา ป้องจันลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SELECTION AND IDENTIFICATION OF EXTREMELY HALOPHILIC BACTERIA PRODUCING PROTEINASES AND VOLATILE COMPOUNDS IN FISH SAUCE

Sirinya Pongjanla

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Food Technology

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SELECTION AND IDENTIFICATION OF EXTREMELY HALOPHILIC BACTERIA PRODUCING PROTEINASES AND VOLATILE COMPOUNDS IN FISH SAUCE

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

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สิริญญา ป้องจันลา : การคัดเลือกและระบุชนิดของแบคทีเรียชอบเกลือสูงที่สร้างโปรติเนสและสารระเหยในน้ำปลา (SELECTION AND IDENTIFICATION OF EXTREMELY HALOPHILIC BACTERIA PRODUCING PROTEINASES AND VOLATILE COMPOUNDS IN FISH SAUCE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 117 หน้า.

วัตถุประสงค์ของการศึกษานี้คือเพื่อศึกษาถึงความสามารถของแบคทีเรียชอบเกลือสูงที่คัดแยกได้จากกระบวนการหมักน้ำปลาต่อการย่อยสลายโปรตีนเนื้อปลาและการสร้างสารระเหยในระหว่างกระบวนการหมักน้ำปลา จากการคัดแยกแบคทีเรียชอบเกลือสูงจากบ่อหมักน้ำปลาช่วงเดือน 1-12 จำนวน 344ไอโซเลท คัดเลือกได้ 54 ไอโซเลท ที่แสดงกิจกรรมของเอนไซม์โปรติเนสในการย่อยสลายโปรตีนนมและโปรตีนเนื้อปลาในอาหารแข็งที่เติมเกลือโซเดียมคลอไรด์เข้มข้น 25% แบคทีเรียชอบเกลือสูง 24 จาก 54 ไอโซเลทที่แสดงกิจกรรมการย่อยสลายโปรตีนนมสูงสุดถูกคัดเลือกมาศึกษาความสามารถในการย่อยสลายโปรตีนจากปลากะตัก และศึกษาการสร้างไบโอ - จีนิกเอมีนรวมถึงสารระเหยในกระบวนการหมักน้ำปลาในระดับห้องปฏิบัติการ ที่ระยะเวลาการหมัก 30 วัน พบว่า จำนวนแบคทีเรียเริ่มต้นลดลงจาก 4-6 Log CFU/กรัม เป็น 1-4 Log CFU/กรัม ตัวอย่างที่เติมกล้าเชื้อมีปริมาณกลุ่มแอลฟา -อะมิโนเทียบเท่ากับตัวอย่างควบคุม ทุกไอโซเลทไม่สร้างสารไบโอจีนิกเอมีนในกระบวนการหมักน้ำปลาในระดับห้องปฏิบัติการ ไอโซเลทที่สร้างสาร 2-เมธิลบิวทานาล (2-Methylbutanal), และ 3-เมธิลบิวทานาล (3-Methylbutanal) ในตัวอย่างปลาหมักสูงสุดคือ J-1-S4, J-1-S22 และ 2m-40-15-R2 ส่วนไอโซเลท P-1-S8 และ J-1-S13 แสดงปริมาณไดเมธิลไดซัลไฟด์และไดเมธิลไตรซัลไฟด์สูง จึงคัดเลือกไอโซเลทเหล่านี้เพื่อนำมาศึกษาบทบาทในกระบวนการหมักน้ำปลารวมถึงระบุชนิดและสายพันธุ์

กล้าเชื้อแบคทีเรียชอบเกลือสูง 5 ไอโซเลทที่เติมลงในปลากะตักผสมกับเกลือสมุทร ในระดับ 25% มีเชื้อเริ่มต้นประมาณ 7 Log CFU/กรัม และลดลงจากวันเริ่มต้นประมาณ 2 Log CFU/กรัม ในระยะเวลา 60 วัน และไม่พบการเจริญของกล้าเชื้อ ที่เวลาการหมักมากกว่า 120 วัน ตัวอย่างที่เติมกล้าเชื้อแบคทีเรียชอบเกลือสูงหมักเป็นระยะเวลา 180 วัน มีปริมาณกลุ่มแอลฟา - อะมิโน 917-1,029 มิลลิโมลาร์ ซึ่งสูงกว่าตัวอย่างควบคุม (P<0.05) น้ำปลาที่เติมกล้าเชื้อและหมักที่ระยะเวลา 180 วัน มีปริมาณไนโตรเจนทั้งหมด 2.1-2.4% และมีปริมาณไบโอจีนิกเอมีนใกล้เคียงกับตัวอย่างควบคุม (P>0.05) สารระเหยเด่นที่มีผลต่อการเกิดกลิ่นเนื้อต้มในน้ำปลาที่เติมกล้าเชื้อคือ 3-เมธิลบิวทานาล โดยพบในตัวอย่างน้ำปลาที่เติมกล้าเชื้อไอโซเลท P-1-S8 และ 2m-40-15-R2 ในปริมาณที่สูงกว่าตัวอย่างอื่น (P<0.05) ตัวอย่างที่เติมกล้าเชื้อทุกตัวอย่างพบปริมาณไดเมธิลได - ซัลไฟด์ซึ่งเป็นสาเหตุของกลิ่นอุจจาระ (Fecal note) ต่ำกว่าตัวอย่างทางการค้า (P<0.05) จากการ

ประเมินคุณภาพทางประสาทสัมผัสพบว่า การยอมรับโดยรวม (Overall acceptance) ของน้ำปลาที่เติมกล้าเชื้อแบคทีเรียชอบเกลือสูงที่หมักเป็นระยะเวลา 6 เดือน ไม่มีความแตกต่างจากน้ำปลาทางการค้าที่หมักเป็นระยะเวลา 12 เดือน (P>0.05)

การระบุชนิดของแบคทีเรียโดยอาศัยลักษณะทางสัณฐานวิทยา และสรีรวิทยา พบว่าเป็นแบคทีเรียแกรมลบ รูปร่างท่อน ทุกไอโซเลทเจริญในสภาวะที่มีเกลือโซเดียมคลอไรด์ในระดับ 15-30% และเจริญได้ดีที่โซเดียมคลอไรด์เข้มข้น 20-25% การวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene พบว่าแบคทีเรียชอบเกลือสูง ที่คัดเลือกทั้ง 5 ไอโซเลท มีความเหมือนของลำดับนิวคลีโอไทด์กับ *Halobacterium salinarum* DSM3754^{T} ในระดับ 99.2-100% ดังนั้นทั้ง 5 ไอโซเลท คือ *Halobacterium salinarum*

สาขาวิชาเทคโนโลยีอาหาร
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SIRINYA PONGJANLA : SELECTION AND IDENTIFICATION OF EXTREMELY HALOPHILIC BACTERIA PRODUCING PROTEINASES AND VOLATILE COMPOUNDS IN FISH SAUCE. THESIS ADVISOR: ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 117 PP.

FISH SAUCE/α-AMINO GROUP CONTENT/STARTER CULTURE/VOLATILE COMPOUNDS/EXTREMELY HALOPHILIC BACTERIA

The objective of this study was to elucidate the ability of extremely halophilic bacteria isolated from fish sauce fermentation to hydrolyze fish proteins and to form volatile compounds during fish sauce fermentation. Three hundred and forty-four isolates of extremely halophilic bacteria were isolated from fish sauce fermented for 1-12 months, of which 54 isolates showed proteinase activity on skim milk salt agar and fish agar containing 25% NaCl. Twenty-four out of 54 isolates exhibiting the highest proteolytic activity towards skim milk were selected to investigate their ability to hydrolyze anchovy proteins and to produce biogenic amines as well as volatile compounds in the laboratory scale fish sauce fermentation. At day 30 of fermentation, the initial microbial counts were decreased from 4-6 Log CFU/g to 1-4 Log CFU/g. α-Amino group contents of all inoculated samples were comparable to the control (P>0.05). All isolates did not produce biogenic amines in the laboratory scale fish sauce fermentation. Samples inoculated with isolate J-1-S4, J-1-S22, and 2m-40-15-R2 exhibited the highest content of 2-methylbutanal and 3-methylbutanal. Isolate P-1-S8 and J-1-S13 appeared to generate the highest content of dimethyl disulfide and dimethyl trisulfide. Therefore, isolate J-1-S4, J-1-S22, 2m-40-15-R2, P-1-S8 and

J-1-S13 were chosen for further study of their role in fish sauce fermentation and bacterial identification. Starter cultures prepared from 5 isolates were added to anchovy mixed with 25% of solar salt with approximate cell count of 7 Log CFU/g. Cell counts were decreased about 2 Log CFU/g at day 60 and undetected after 120 days of fermentation. α-Amino group contents of 180-day-old fish sauce samples were 917-1,029 mM, which were higher than the control (P<0.05). The total nitrogen content of all samples was 2.1-2.4% and biogenic amine contents were comparable to that of the control after 180 days of fermentation (P>0.05). Major volatile compounds contributing to meaty note in the starter culture inoculated samples were 3-methylbutanal. Fish sauce inoculated with P-1-S8 and 2m-40-15-R2 contained 3-methylbutanal in the highest amount (P<0.05). Dimethyl disulfide, a compound contributing to fecal note, was detected in all inoculated samples with a lower amount than that in commercial fish sauce (P<0.05). Based on sensory evaluation, fish sauce inoculated with all starter cultures fermented for 6 months showed similar overall acceptance to the commercial fish sauce fermented for 12 months (P<0.05).

Bacterial identification based on morphological and physiological characteristics revealed that these isolates were Gram-negative rods. All isolates grew in 15-30% NaCl and grew best in the medium containing 20-25% NaCl. The result of 16S rRNA gene sequences revealed that 5 selected isolates showed 99.2-100% similarity to *Halobacterium salinarum* DSM3754^{T}. Therefore, five selected isolates were identified as *Halobacterium salinarum*.

School of Food Technology

Academic Year 2011

Student's Signature_____

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Sirinya Pongjanla

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LIST OF ABBREVIATIONS

α	=	Alfa
BLAST	=	Basic local alignment search tool
bp	=	Base pair
CFU	=	Colony forming unit
cm	=	Centrimeter
°C	=	Degree Celsius
dNTPs	=	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DNA	=	Deoxyribonucleic acid
et al.	=	et alia (and others)
(m, μ) g	=	(milli, micro) Gram
h	=	Hour
(m, μ) l	=	(milli, micro) Liter
(m, μ) M	=	(milli, micro) Molar
min	=	Minute
(m, μ) mol	=	(milli, micro) Mole
%	=	Percentage
s	=	Second
sp.	=	Species

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce (nam-pla) is a clear brown liquid traditionally produced through natural fermentation of fish and salt in a ratio of 3:1 and fermented for 12-18 months (Yongsawatdigul, Choi, and Udomporn, 2004). The growth of industry is rather limited due to high capital investment of land and extremely long fermentation time. Acceleration of the fish sauce fermentation process has been investigated. Many attempts have been made to accelerate the process by reducing the amount of salt, lowering the pH, and elevating the fermentation temperature (Akolkar, Durai, and Desai, 2010). However, these approaches have never been successfully adopted by industry. Protein hydrolysis during fermentation mainly relies on the action of endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Yongsawatdigul, Rodtong, and Raksakulthai, 2007). The addition of proteinase-producing bacteria as a starter culture is an alternative means to accelerate protein hydrolysis during fish sauce fermentation. Yongsawatdigul et al. (2007) applied starter culture of *Virgibacillus* sp. SK33 and SK37 and *Staphylococcus* sp. SK1-1-5 with commercial enzymes, Flavourzyme and Alcalase, for fish sauce fermentation. After 4 months of fermentation, inoculated samples showed similar product characteristics to those of the commercial fish sauce fermented for 12 months. Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul (2011)

reported that α-amino group contents of fish sauce inoculated with *Tetragenococcus halophilus* after 6 months was higher than that of control. Moreover, they claimed that this starter culture improved amino acid profiles and volatile compounds as well as reduces biogenic amine content of a fish sauce product. Akolkar et al. (2010) applied *Halobacterium* sp. SP1(1) for acceleration of fish sauce process. They found that fish sauce inoculated with starter culture showed high α-amino group content and total nitrogen content than the control. These studies indicated that the use of proteinase-producing bacteria as a starter cultures could be an effective alternative to reduce fish sauce fermentation time. However, one concern of using bacterial starter culture is biogenic amines formation from a starter culture.

Biogenic amines are basic nitrogenous compounds produced mainly by bacterial decarboxylation activity toward amino acids in food (Zaman, Bakar, Jinap, and Bakar, 2010). Histamine is considered as the most active amine and is related to almost all food amine poisoning incidents (Zaman, Bakar, Selamat, and Bakar, 2011). Histamine poisoning causes nausea, vomiting, diarrhea, headache and severe respiratory distress. The toxicity of histamine appears to be enhanced by cadaverine and putrescine. Histamine was regulated in fish sauce at the maximum level of 20 mg/100 ml by Canada (Brillantes, Pakon, and Totakien, 2002). Thus, an ideal starter culture for fish sauce fermentation should not produce biogenic amines.

Fish sauce contains a high concentration of NaCl, allowing various halophilic microorganisms to thrive (Tapingkae et al., 2008). Extremely halophilic bacteria are predominant in fish sauce fermentation (Thongthai and Siriwongpairat, 1978). Extremely halophilic bacteria are defined as microorganisms that grow best in the medium containing 2.5-5.2 M NaCl and require at least 12% NaCl (2 M) for growth.

Halobacteriaceae within domain Archaea can grow well in high salt condition of 25% NaCl which is similar to fish sauce fermentation (Grant et al., 2001). It has been reported that some strains can produce proteinases, such as *Halobacterium salinarum* strain ORE, *Halobacterium* sp. SP1(1), and *Halogeometricum* sp. TSS101. Proteinases produced by these organisms are active at high salt condition. The enzyme required 1-4 M NaCl (5.8-23.3%) for catalytic activity and stability. Extremely halophilic archaea accumulate K^+ in the cell in order to thrive at high salt environment, while halophilic or halotolerant eubacteria accumulate compatible solutes for high salt tolerance, and usually their enzymes tend to denature at high salt content (Vidyasagar, Prakash, Litchfield, and Sreeramulu, 2006). Therefore, using halophilic enzymes from extremely halophilic bacteria could be more advantageous due to their optimum activities at high salt concentrations.

The addition of proteinase-producing bacteria might not only increase the rate of protein solubilization, but also contribute to flavor development during fermentation (Yongsawatdigul et al., 2007). The unique flavor of fish sauce is often considered as a quality indicator. Three distinctive notes contributed to odor of fish sauce are ammoniacal, cheesy, and meaty (Fukami et al., 2002). These flavors were produced by volatile compounds derived from chemical reaction and/or microbial fermentation. Fukami, Funatsu, Kawasaki, and Watabe (2004) reported that addition of *S. xylosus* into fish sauce mash reduced dimethyl disulfide and dimethyl trisulfide which contribute to fecal note in the finished product. Udomsil et al. (2011) found that fish sauce inoculated with *T. halophilus* MS33 and MRC 10-1-3 showed lower contents of dimethyl disulfide than the control. However, volatile compounds produced by extremely halophilic bacteria have not been widely investigated.

1.2 Research objectives

The objectives of this research were to:

(1) Isolate, screen, and select extremely halophilic bacteria producing proteinases that do not produce undesirable volatile compounds and produce low concentration of biogenic amines in fish sauce fermentation process,

(2) Identify the selected extreme halophiles,

(3) Apply the selected extreme halophiles for fish sauce fermentation and investigate their roles on volatile compounds production.

1.3 Research hypotheses

(1) Some extremely halophilic bacteria isolated from fish sauce fermentation were able to produce proteinases to increase protein hydrolysis during fish sauce fermentation.

(2) Extremely halophilic bacteria could play an important role in volatile compounds production in fish sauce.

1.4 Scope of the study

Extremely halophilic bacteria were isolated from commercial fish sauce samples fermented at 1, 3, 5, 7, 9, 12 months, fish juice and the fourth fish sauce extract. Medium containing 25% NaCl was used for isolation. These isolates were tested for proteinase secretion in conjunction with volatile compounds and biogenic amines production. Isolates showing high proteinase secretion, low formation of sulfur-containing compounds and biogenic amines were selected for starter culture development. The identification of extremely halophilic bacteria was carried out by

morphological, physiological, biochemical and genomic characteristics. Genomic analyses were achieved using 16S rRNA gene sequence analysis amplified by polymerase chain reaction (PCR) technique. Changes of volatile compounds and chemical characteristics of fish sauce inoculated with selected extremely halophilic starter cultures were also studied.

1.5 Expected results

Extremely halophilic bacteria that could produce proteinases and desirable volatile compounds in fish sauce were selected and identified. They could be further developed for starter culture for fish sauce fermentation to accelerate protein hydrolysis and/or to control flavor/aroma characteristics of the product. Knowledge regarding extremely halophilic bacteria in fish sauce fermentation would be obtained. Formation of biogenic amines and volatile compounds of selected isolates in fish sauce fermentation would be illustrated.

CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce

Fish sauce (nam-pla) is a clear brown liquid with unique characteristic, salty taste and fishy odor, traditionally produced through natural fermentation of fish with salt and fermented for 12-18 months in order to complete hydrolysis. Fish sauce is currently popular in Southeast Asia and become popular among Western consumers. Thailand is one of the leading fish sauce producers of the world (Lopetcharat, Choi, Park and Daeschel, 2001). It is commonly used as seasoning for cooking and dipping seafood and oriental food. Moreover, this is an important source of protein, amino nitrogen, iron, calcium and B vitamins. It is called by different names depending on the country, such as nampla (Thailand), patis (Philippines), yu-lu (China), shotshuru (Japan), budu (Malaysia), noucnam (Vietnam), and bakasang (Indonesia) (Yuen, Yee, and Anton, 2009). Fish sauce is usually produced from small pelagic fishes, such as anchovy (*Stolephorus* spp.), mackerel (*Ristrelliger* spp.), and herring (*Cirrhinus* spp.) and solar salt as primary raw materials. Mixing fish and salt is the first step in making fish sauce. The ratio of fish and solar salt varies from 2:1 or 3:1 depending on the country (Lopetcharat et al., 2001). The high salt concentration (28-30% NaCl) can prevent the growth of spoilage microorganisms (Thongthai, Mcgenity, Suntinanalert, and Grant, 1992). Then, the salt-mixed fish is transferred to a fermentation tank where a bamboo mat is laid on top of the fish and loaded with heavy weight to keep the fish

flesh in the brine during fermentation. Natural fermentation is allowed to proceed under uninterrupted condition at ambient temperature (30-40^{o}C) for 12-18 months. After 12-18 months of fermentation, the mature fish sauce obtained by filtration is bottled as finished goods or blended with some ingredients to obtain finished products. The solid residues left from filtering were further added with brine salt. The content eluted from the residue was blended with good quality fish sauce and other ingredients to obtain second grade fish sauce (Wichaphon, Thongthai, Assavanig, and Lertsiri, 2012). Caramel color and other additives, which are not harmful for consumers, are also added to improve color and flavor qualities of fish sauce. The standard of Thai fish sauce is officially graded into first and the second grade based on total nitrogen content above 20 g/l and 15 g/l, respectively. Moreover, the premium grade as claimed by some manufacturers is the product with nitrogen content above 20 g/l and fermented for more than 18 months (Wichaphon et al., 2012). pH is between 5.0-6.5 for a traditional product and salt (NaCl) content is not less than 200 g/l.

2.1.1 Roles of proteinases in fish sauce fermentation

The major change during the fermentation period is the conversion of proteins to small peptides and free amino acids. Protein hydrolysis during fermentation mainly relies on action of endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Yongsawatdigul et al., 2007). Generally, fish visceral enzymes are believed to be responsible for early proteolysis of fish sauce. Proteinases found in viscera include pepsin, trypsin, chymotrypsin, carboxypeptidase and aminopeptidase (Siringan, Raksakulthai, and Yongsawatdigul, 2006). However, the majority of proteinase activity was found in the

liquid surrounding the fish, rather than in the fish residue (Akolkar et al., 2010). Thongthai et al. (1992) revealed that halophilic bacteria found in fish sauce also produce proteolytic enzymes. Moreover, the activity was stable at 25% NaCl, one of the characteristics of halobacterial preteases, whereas the proteinase from viscera of fish was inhibited at high salt concentration. This indicated the role of halobacterial proteinases in the fermentation process. When fish protein is hydrolyzed by both endogenous and exogenous proteinases, several amino acids and small peptides are accumulated. Apart from protein hydrolysis, various biochemical and chemical changes (i.e. lipid degradation, breakdown of carbohydrates, amino-carbonyl reaction) and microbial metabolism occur (Wichaphon et al., 2012). The process of making fish sauce is simple, but the resulting products have various distinctive odors and flavors according to the fish species used, the process of fermentation, and microorganisms surviving during fermentation.

2.1.2 Bacteria in fish sauce fermentation

Microorganisms vary depending upon season, place, transportation, species, storage, and catching methods. In fresh marine fish, there are about 10^2–10^7 cells/cm^2 on the mucus on fish skin and about 10^3–10^9 cells/g in fish intestine (Lopetcharat et al., 2001). Fish sauce contains a high concentration of NaCl of 28-30%, most of spoilage bacteria originally present in fish will die off quickly after mixed with salt. The osmotic effect of salt kills or retards microbial growth because of plasmolysis of the microbial cells. Lowering water activity (*A*w) reduces water for all metabolic activities causing a longer lag phase. In a very high salt environment, enzymes are easily denatured and inactivated. Thus, metabolism of bacteria cannot function properly or totally stops (Jay, 2000). This condition allows various halophilic

microorganisms to thrive (Tapingkae et al., 2008). Many microorganisms belonging to *Bacillus, Staphylococcus*, *Streptococcus*, *Pediococcus*, *Tetragenococcus*, *Lentibacillus*, other halophilic bacteria such as *Virgibacillus*, and extremely halophilic bacteria, such as *Halobacterium, Halococcus* are found in fish sauce. (Lopetcharat et al., 2001, Kimura, Konagaya, and Fujii, 2001, Yongsawatdigul et al., 2007, and Thongthai et al., 1992). The important roles of bacteria in fish sauce are protein degradation and flavor formation. Among bacteria in fish sauce, proteinases-producing halophilic bacteria could play the major role in protein hydrolysis, resulting in an increase of amino acids during fermentation process. There are several groups of proteolytic bacteria including halophilic bacteria, halotolerant, lactic acid bacteria and extremely halophilic bacteria. Numerous studies showed that bacteria were isolated from fish sauce can produce proteinases. Namwong, et al (2006) showed that moderately halophilic bacteria, *Halobacillus* sp. SR5-3 isolated from fish sauce produced halophilic serine proteinase. *Bacillus licheniformis* RKK-04 produced halotolerant proteinases (Toyokawa et al., 2010). *Virgibacillus* sp. SK37 produced salt-activated extracellular proteinases (Sinsuwan, Rodtong, and Yongsawatdigul, 2007). *Tetragenococcus halophilus* and *Halobacterium salinarium* was also reported to produce proteinases (Udomsil, Rodtong, Tanasupawat, and Yongsawatdigul, 2010; Thongthai et al., 1992). *Halococcus thailandensis* isolated from Thai fish sauce produced extracellular proteinases and catalyzed hydrolysis of gelatin at NaCl concentration up to 30% (Chaiyanan et al, 1999). Most of proteinases produced by these bacteria are active at high salt concentration where other enzymes usually tend to denature. These studies indicated that halophilic bacteria might contribute to protein hydrolysis during fish sauce fermentation.

2.1.3 Biogenic amines in fish sauce

Biogenic amines are low molecular weight basic nitrogenous compounds occurring in many foods, mainly due to the amino acid decarboxylation activities of certain microbes (Zaman et al., 2010). These amines are designated as biogenic because they are formed by the action of living organisms (Shalaby, 1996). The presence of biogenic amines in foods is an indication of food spoilage which is dependent upon the availability of free amino acids and, the presence of decarboxylase positive microorganisms (Halasz Barath, Sarkadi, and Holzapfel, 1994). Proteolytic enzymes play an important role in releasing of free amino acids which serve as substrates for decarboxylation (Zaman, Abdulamir, Bakar, Selamat, and Bahar, 2009). Increment of free amino acids in fish sauce fermentation indicates that fermentation process has the potential to produce biogenic amines. Histamine, putrescine, cadaverine, tyramine, tryptamine, spermine, and spermidine are considered to be the most important biogenic amines occurring in foods. Their presence is undesirable because of their toxicological effects. Often fish sauces are known to contain high levels of histamine. This compound is vasoactive and can cause changes in blood pressure, severe headache, hypertension, renal intoxication, intracerebral hemorrhage and scombroid. Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists. Except for the gamma irradiation, no other food processing methods are available for histamine degradation. Therefore, histamine, if present, is difficult to destroy and posts a risk of food intoxication. (Tapingkae, Tanasupawat, Parkin, Benjakul, and Vissanguan, 2010). The toxicity of histamine appears to be enhanced by cadaverine and putrescine because they inhibit the histamine-detoxifying enzyme. (Stratton, Hutkins, and

Taylor, 1991). Tyramine can cause migraine and when it reacts with monoamine oxidase inhibitor drugs may lead to hypertensive crisis (Zaman, et al., 2011). Moreover, putrescine, cadaverine, spermine, and spermidine can react with nitrites in food to form carcinogenic nitrosamines (Shalaby, 1996). However, biogenic amines can be degraded through oxidative deamination catalyzed by amines oxidase with the production of aldehyde, ammonia and hydrogen peroxide. The potential role of microorganisms with amine oxidase activity has become interest. Zaman et al. (2011) reported that *Staphylococcus carnosus* FS19 and *Bacillus amyloliquefaciens* FS05 reduced biogenic amines by 15.9% and 12.5%, respectively. Yongsawadigul et al. (2007) reported that *Virgibacillus* sp. SK33 was able to reduce histamine content of fish sauce more than 50% during 4 months of fermentation. Tapingkae et al. (2010) reported that *Natrinema gari* HDS3-1 isolated from fish sauce exhibited the ability to degrade histamine in hypersaline condition.

2.1.4 Flavor formation in fish sauce

The unique flavor of fish sauce is often used as a quality index for a fish sauce product, but is measured somewhat subjectively by consumers. The characteristic flavor-aroma formation in the fish sauce depends on the manner of production employed, as well as raw materials and strains of microorganism used (Saisithi, Kasermsarn, and Dollar, 1966). These odors are derived from protein hydrolysate and lipid oxidation product brought about by either autolytic or microbial activity or fish enzymes (Fukami et al., 2002). Most proteins in fish, except connective tissue and other stroma proteins, are hydrolyzed into small peptides and amino acids. The small peptides, free amino acids and trimethylamine (TMA) contribute to the specific aroma and flavor in fish sauce (Lopetcharat et al., 2001).

Dougan and Haward (1975) reported that three distinctive notes contributing to the odor of fish sauce are ammoniacal, cheesy, and meaty note. The ammoniacal note is contributed from ammonia, amines, and other basic nitrogen-containing compounds. Among nitrogen-containing compounds, trimethylamine contributed to the major proportion and might impart fishy odor to fish sauce products. Giri, Osako, Okamoto, and Ohshima (2010) reported that trimethylamine usually described as "rotten fish, ammonical", had an odor threshold of 0.051 μg/l and existed in different fish sauce. Trimethylamine was also assumed as a single most compound responsible for "ammoniacal" organoleptic perception of a fish sauce product. The cheesy note is mainly due to low molecular weight volatile fatty acids, such as formic, acetic, and *n*-butanoic acids (Fukami et al, 2002). These low molecular weight volatile fatty acids were produced from the autoxidation of polyunsaturated acids and by bacterial action on amino acids, which are used as a carbon source (Lopetcharat et al., 2001). Mclver, Brooks, and Reineccius (1982) reported that acetic acid is a prominent acid in Thai fish sauce. In addition, Giri et al. (2011) revealed that Vietnamese fish sauce contains a large amount of methyl ketones which probably account for cheesy odor. The volatile ketones were most likely the products of lipid and/or amino acid degradation, however, ketones did not have much effect on fish sauce flavor because of their high threshold value (Lopetcharat et al., 2001). The meaty note is more complicated than other two notes. Meaty odor was produced by the oxidation of a substance that can be extracted entirely from fish sauce with isopropanol. Glutamic acid also contributes to meaty taste in nampla (Lopetcharat et al., 2001). Giri et al. (2010) reported that the result of 2-ethylpyridine together with 2-methylpropanal and 2-methylbutanal could be responsible for the

meaty note. Shimoda, Rossana, Peralta, and Osajima (1996) reported that nitrogen-containing compounds together with aldehydes were responsible for the meaty note of fish sauce. Aldehydes have low threshold values and are important aroma compounds in different food stuffs. The odor description indicated that the volatile aldehydes contribute to desirable aroma as well as rancid odor and flavor to food products. Linear and branched chain aldehydes generally provide herbaceous, grassy and pungent aroma, while unsaturated aldehydes are linked with vegetal and fishy notes (Giri et al., 2010). The aldehydes in fermented meat paste products are generally derived from the Strecker degradation of amino acids (Giri et al., 2010). In addition, another major note in fish sauce is sulfur-containing compounds, such as dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide, which are responsible for fecal note (Shimoda et at., 1996). The sulfur-containing compounds have previously been reported in different fermented bean and fish paste products (Landaud, Helinck, & Bonnarme, 2008) and can be originated either from raw materials or during the fermentation process from free, peptidic and proteinic sulfur-containing amino acids as well as glutathione pool in the fish tissue (Giri et al., 2010). Demethiolation of methionine results in methanethiol, which subsequently is autoxidized to dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide. Sulfur-containing compounds are considered to be potent odorants because of their low threshold values (Devos, Patte, Roualt, Laffort, Gemert, 1995). Shimoda et al. (1996) founded 124 volatile compounds, including 20 nitrogen-containing compounds, 20 alcohols, 18 sulfur-containing compounds, 16 ketones, 10 aromatic hydrocarbons, 8 acids, 8 aldehydes, 8 esters, 4 furans, and 12 miscellaneous compounds. Large amounts of acids, aldehydes, and ketones of 15, 55, and 21%, respectively, of the total peak area were

detected in fish sauce sample. One hundred and fifty five volatile compounds were also identified from fish sauce by Peralta, Shimode, and Osajima (1996), including, 17 nitrogen-containing compounds, 10 sulfur-containing compounds, 14 acids, and 36 carbonyls, and large amount of acids were detected. However, it is not every compound that contributes to flavor in fish sauce. Shimoda et al. (1996) reported that furans could not have contributed to fish sauce odor because of their quite high odor threshold value. Sulfur-containing compounds have low threshold value and seemed to be the most potent contributors to fish sauce odor.

The use of bacterial starter culture has been reported to improve aroma characteristic of fish sauce. Fukami et al. (2004) reported that addition of *Staphylococcus xylosus* into fish sauce mash reduced 2-ethylpyridine, butanoic acid, dimethyl disulfide and dimethyl trisulfide which contribute to fecal note in the finished product. Based on sensory evaluation, the rancid note was significantly reduced by bacteria treatment. In addition, the inoculated sample had higher contents of 2-methylpropanal and 2-methylbutanal which contribute to meaty note. Yongsawatdigul et al. (2007) reported that fish sauce inoculated with *Staphylococcus* sp. SK1-1-5 showed higher levels of volatile fatty acids that contributed to cheesy notes in fish sauce. Udomsil et al. (2011) found that fish sauce inoculated with *Tetragenococcus halophilus* MS33 and MRC 10-1-3 showed lower contents of dimethyl disulfide than the control (without *T. halophilus*), implying that *T. halophilus* MS33 and MRC 10-1-3 could reduce undesirable odor in fish sauce. Volatile compounds of fish sauce are produced by nonenzymatic reactions of various components (amino acids, lipids, and sugars) and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation

(Fukami et al., 2004). However, volatile compounds produced by extremely halophilic bacteria have not been widely investigated.

2.1.5 Acceleration of fish sauce fermentation

Traditional fish sauce fermentation process takes about 12-18 months to complete. The growth of industry is rather limited due to high capital investment of land and extremely long fermentation time. Acceleration of the fish sauce fermentation process has been investigated. Many attempts have been made to accelerate the process of fish fermentation by reducing the amount of salt, lowering the pH, and elevating the temperature (Akolkar et al., 2010). Gildberg, Hermes, and Orejana (1984) accelerated fish sauce fermentation process of anchovies (*Stolephorus* spp.) by adjusting the initial pH to 4 and reducing salt concentration to 5%. After 2 months of fermentation, acceptable fish sauce was obtained. However, fish sauce from this method had a lower level of volatile bases and acids. Lopetcharat and Park (2002) increased temperature of Pacific whiting fish sauce fermentation to 50^{o}C and they found that total nitrogen content equivalent level of commercial fish sauce was obtained within 15 days. Beddows and Ardeshir (1979) used bromelain, papain and ficin to reduce fermentation time. Bromelain showed better result than others within 18-21 days, yielding fish some with comparable quality to traditional fish sauce. Based on these approaches, low salt concentration may favor growth of pathogenic microorganisms, high temperatures also increased cost of process, whereas the use of enzymes resulted in fish sauce with little aroma.

The addition of proteinase-producing bacteria as a starter culture is alternative to accelerate protein hydrolysis during fish sauce fermentation. Yongsawatdigul et al. (2007) used starter culture of *Virgibacillus* SK33 and SK37 and

Staphylococcus sp. SK1-1-5 with commercial enzymes, Alcalase and Flavourzyme. After 4 months of fermentation, inoculated samples showed similar patterns to that commercial fish sauce fermented for 12 months. Udomsil et al. (2011) reported that fish sauce inoculated with *Tetragenococcus halophilus* after 6 months showed higher α-amino group content than the control. Moreover, they claimed that this starter culture improves amino acid profiles and volatile compounds as well as reduces biogenic amine content of a fish sauce product. Akolkar et al. (2010) applied *Halobacterium* sp. SP1(1) to accelerate the simulation of fish sauce process. They found that simulated fish sauce inoculated with starter culture showed high α-amino group content, protein and total nitrogen content than control.

2.2 Halophilic bacteria

Halophilic bacteria is microorganisms that require salt to grow. They are able to grow over a wide range of salt concentration and found in high salt environment. Vreeland and Hochstein (1993) suggested that halophilic bacteria can be classified by the level of salt needed for growth as nonhalophiles, slight, moderate, and extreme halophiles which are those that grow best in media containing <2, 2-5, 5-20, and 20-30% salt, respectively. Bacteria able to grow in the absence of salt and in the presence of high salt concentration are designated as halotolerant (or extremely halotolerant if growth extends above 15% NaCl).

All halophilic microorganisms have two fundamentally different strategies used by halophilic microorganisms to balance their cytoplasm osmotically with their medium (Oren, 2006). The first strategy of haloadaptation based on the biosynthesis and/or accumulation of organic osmotic solutes. Cells that use this strategy exclude

salt from their cytoplasm as much as possible. The high concentrations of organic compatible solutes do not greatly interfere with normal enzymatic activity. Such organisms can often adapt to a surprisingly broad salt concentration range. The organic compounds that have been shown to serve as osmotic solutes in halophilic prokaryotes as well as eukaryotes include amino acids and amino acid derivatives, sugar, or sugar alcohols. Most are either uncharged or zwitterionic (Oren, 2008). Accumulation of compatible solutes helps them to maintain an environment isotonic with the growth medium. These substances also help to protect cells against stresses (Gomes and Steiner, 2004). For moderately halophilic and halotolerant organisms, potassium ions (K^+) is accumulated an early response to an increase in external NaCl. However, the increased K^+ is often transient and is superseded by the accumulation of organic solutes. The second strategy involves accumulation of molar concentrations of potassium and chloride. *Halobacteriaceae*, halophilic archaea have high intracellular concentrations of K^+ for all life time. This strategy requires extensive adaptation of the intracellular enzymatic machinery to the presence of salt, as the proteins should maintain their proper conformation and activity at near-saturated salt concentrations. Proteins of such organisms are highly acidic and denature when suspended in low salt (Oren, 2008). Remarkably, K^+ is the major cation accumulated. The saline cytoplasm of these microorganisms requires that most of their enzymes are enriched in acidic amino acids and at the same time they are strictly dependent on K^+ and/or Na^+ for activity (Empadinhas and da Costa, 2008).

Halophiles are found in many saline environments. They can thrive in hypersaline environments. Hypersaline environments originated from thalassohaline (seawater) or athalassohaline (nonseawater) environments. Composition of

thalassohaline is similar to that of seawater: sodium and chloride are the dominating ions, and the pH is near neutral to slightly alkaline, such as found in saltern crystallizer ponds (Oren, 2002). In contrast to thalassohaline environments, dominant ions in athalassohaline environments are potassium, magnesium, or sodium, such as Dead Sea, Great Salt Lake (Ventosa, 2006; Oren, 2002). In addition, halophilic bacteria can be isolated from salt or salt deposits and from a variety of salted products as well as other materials, such as salted animal hides.

2.3 Extremely halophilic bacteria

Extremely halophilic bacteria or extreme halophiles are a diverse group of the family *Halobacteriaceae*, order *Halobacteriales*, class *Halobacteria*, phylum Euryarchaeota, domain Archaea (or *Archaeobacteria*). Extreme halophiles are fundamentally different from other known prokaryotes as they lack a peptidoglycan cell wall, require high salt concentrations for growth and structural stability with unusual pigmentation (Oren, 2012).

These extreme halophilic bacteria require salt at least 12% (2 M) NaCl for growth and grow best in the medium containing 2.5-5.2 M NaCl (Oren, 2006). Cell walls, enzymes, and ribosomes are stabilized by Na^+. The extreme halophiles are strictly aerobic. They adapt to the high salt environment by the development of purple membrane, actually patches of light-harvesting pigment in the plasma membrane. The pigment is bacteriorhodopsin containing 25% lipids and 75% protein. It reacts with light, resulting in a proton gradient on the membrane just as in the case of the respiratory chain allowing the synthesis of ATP. Accordingly, the extreme halophiles can efficiently produce ATP by respiration and bacteriorhodopsin.

The first respresentatives of the family *Halobacteriaceae* were isolated more than a hundred years ago, and currently (April 2012) the family encompasses 36 genera with 129 species (Oren, 2012), including *Haladaptatus*, (*Hap*.); *Halalkalicoccus*, (*Hac*.); *Halarchaeum*, (*Hla*); *Halobellus*, (*Hbs*.); *Haloarcula*, (*Har*.); *Halobacterium*, (*Hbt*.); *Halobaculum*, (*Hbl*.); *Halobiforma*, (*Hbf*.); *Halococcus*, (*Hcc*.); *Haloferax*, (*Hfx*.); *Halogeometricum*, (*Hgm*.); *Halogranum*, (*Hgn*.); *Halolamina*, (*Hlm*.); *Halomarina,* (*Hmr*.); *Halomicrobium*, (*Hmc*.); *Halonotius,* (*Hns*.); *Halopelagius,* (*Hpl*.); *Halopiger*, (*Hpg*.); *Haloplanus*, (*Hpn*.); *Haloquadratum*, (*Hqr*.); *Halorhabdus*, (*Hrd*.); *Halorubrum*, (*Hrr*.); *Halosarcina*, (*Hsn*.); *Halosimplex*, (*Hsx*.); *Halostagnicola*, (*Hst*.); *Haloterrigena*, (*Htg*.); *Halovivax*, (*Hvx*.); *Natrialba*, (*Nab*.); *Natrinema*, (*Nnm*.); *Natronoarchaeum*, (*Nac*); *Natronobacterium*, (*Nbt*.); *Natronococcus*, (*Ncc*.); *Natronolimnobius*, (*Nln*.); *Natronomonas*, (*Nmn*.); *Natronorubrum*, (*Nrr*.); and *Salarchaeum*, (*Sar*.).

Extreme halophiles contain a variety of morphological types, from rod and cocci to flat extremely pleomorphic type, square flat cells, and triangular cells (Oren, 2006). *Halobacteriaceae* have a cell wall (often referred to as an S-layer) that consists of subunits of a large glycoprotein. The glycoprotein is essential for maintaining the rod shape of *Halobacterium* and the flat disk or triangular shape of *Haloferax* and *Haloarcula* species. The glycoprotein cell wall requires high salt concentrations for stability. The wall protein denaturates when suspended in distilled water, leading to lysis and cell death (Oren, 2006). *Halococcus* species possess a thick sulfated heteropolysaccharide cell wall that do not depend on high salt for structural stability.

The primary structure of the protein backbone of the extreme halophiles and the mode of its glycosylation vary among different species. The cytoplasmic

membrane of extreme halophiles is composed of lipids and proteins. It contains all the functions needed for respiratory electron transport, nutrients, inward and outward transport of ions, and other compounds, sensors that provide information about the extracellular environment and their transducers, and many other components. The members of the *Halobacteriaceae* have archaeal-type lipids based on branched 20-carbon (phytanyl) and sometimes also 25-carbon (sesterterpanyl) chains, bound to glycerol by ether bonds. Oren (2002) revealed that the diether core lipid that forms the basis for most polar lipid structures present in the family *Halobacteriaceae* is 2,3-di-*O*-phytanyl-sn-glycerol (C_{20}, C_{20}). Certain species, however, contain in addition the asymmetric 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol (C_{25}, C_{20}) in different amounts. A thin layer chromatographic procedure has been developed to separate the C_{20}, C_{20} and C_{25}, C_{20} lipid species. The C_{25}, C_{20} core lipid is found in many of the alkaliphilic types, and the neutrophilic *Natrialba asiatica* (Kamekura and Dyall-Smith, 1995), and in the genera *Natrinema* (McGenity, Gemmell, and Grant, 1998) and *Halococcus*, and in *Halobacterium halobium* IAM13167, which may be a *Halobacterium salinarum* strain (Morita, Yamaguchi, Eguchi, and Kakinuma, 1998). The lipids are expected to form 'zip' type bilayer membranes as exemplified in Figure 1.1.

A great variety of polar lipids, including phospholipids, sulfolipids, and glycolipids, is encountered in the different representatives of the *Halobacteriaceae*. All known species contain diether derivatives of phosphatidylglycerol (PG), phosphatidylglycerosulfate (PGS), and methyl ester of phosphatidylglycerophosphate (Me-PGP). This is a useful as an important characteristic in the taxonomic classification of strains.

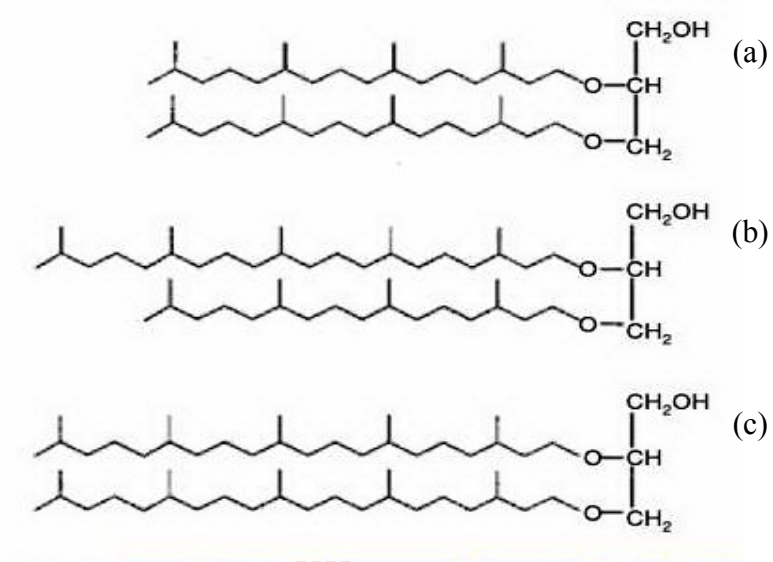

Figure 2.1 Core lipids of halophilic Archaea of the family Halobacteriaceae: 2,3-di-*O*-phytanyl-*sn*-glycerol (C_{20}, C_{20}) (a), 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol (C_{25}, C_{20}) (b), and 2,3-di-*O*-sesterterpanyl-*sn*-glycerol (C_{20} C_{25}) (c) (Oren, 2006).

Most of the family *Halobacteriaceae* are brightly red-orange, colored by a high content of carotenoid pigments in their cell membrane. However, white mutants of species, such as *Halobacterium salinarium*, can easily be isolated. Most caratenoids of the extreme halophiles are C_{50} straight-chain derivatives of α-bacterioruberin. C_{40} Caratenoids are generally found in small amounts (Oren, 2006).

2.3.1 Bacteria in the genus *Halobacterium*

Halobacterium cells are rod shaped (0.5-1.2×1.0-6.0 μm). Pleomorphic forms are common (bent and swollen rods, clubs, spheres). The cell divides by constriction. Resting stages are not known. They are Gram-negative, motile by tufts of polar flagella. Some strains have gas vacuoles. Most strains are strict aerobes, but some exhibit facultatively anaerobic growth. They are oxidase- and catalase-positive,

extremely halophilic with growth occurring in media containing 3.0-5.2 M NaCl. Most strains grow best at 3.5-4.5 M. Optimum growth is 35-50^{o}C with maximum at 55^{o}C; minimum: 15-20^{o}C. pH range for growth was 5.5-8.5. Amino acids are required for growth. Most strains are proteolytic. DNA is usually composed of a major component and minor component. The latter makes up 10-30% of the total DNA (minor component). Strains that have a minor component usually harbor a large plasmid (144 kb). The mol% G+C of the DNA of the major component is 67.1-71.2% mol (T_d, T_m), and that of the minor component, 57-60% mol (minor component) (Grant, 2001).

Currently, the genus *Halobacterium* contains three recognized species: *Halobacterium salinarum* (Ventosa and Oren, 1996), *Halobacterium jilantaiense* (Yang, Cui, Zhou, and Liu, 2006) and *Halobacterium noricense* (Gruber et al., 2004).

2.3.1.1 *Halobacterium salinarum*

Halobacterium salinarum cells are rod-shaped (0.5-1.0×1.0-6.0 μm or more in length), but display a multitude of involution form, especially in deficient media or at elevated temperatures. Some strains contain gas vacuoles, are motile by tufts of polar flagella. They are basically aerobic, but may grow anaerobically in light, when bacteriorhodopsin is present, or fermentatively in the dark in the presence of arginine. Best growth occurs at 3.5-4.5 M NaCl; good growth occurs in saturated NaCl (5.2 M); no growth occurs below 3 M NaCl. Temperature range for growth is 20-55^{o}C with the optimum temperature at 50^{o}C. Mg^{2+} requirement for growth is 0.005-0.05 M. pH range for growth is 5.5-8.0. Amino acids are required for growth. Carbohydrates are not utilized, but stimulation of growth is observed in the presence of glycerol. Starch is not hydrolyzed. Gelatin is hydrolyzed. Media

become alkaline as a result of the deamination or decarboxylation of amino acids. They are commonly found in proteinaceous products heavily salted with crude solar salt. The mol% G+C of the major component of the DNA is 66-70.9, and that of the minor component is 57-60.

2.3.1.2 *Halobacterium jilantaiense*

Halobacterium jilantaiense cells are slender rods (0.5-1.0×1.0-3.0 μm) and Gram-negative. Colonies are red, elevated and round. Growth occurs at 2.7-5.2 M NaCl and 0.05-0.3 M Mg^{2+} at pH 5.5-8.5 and at 22-55^{o}C. Optimal NaCl concentration, Mg^{2+} concentration, pH and temperature for growth are 3.1-3.5 M, 0.1-0.2 M, pH 7.0-7.5 and 40^{o}C, respectively. They are catalase- and oxidase-positive. They grow anaerobically in the presence of L-arginine, nitrate and DMSO. Nitrate reduction to nitrite is observed and H_2S is produced from cysteine. Indole formation is positive. Tween 80 and starch are not hydrolyzed. Casein and gelatin are hydrolyzed. Amino acids are required for growth. Starch is not hydrolyzed. They are susceptible to anisomycin and novobiocin and resistant to penicillin, ampicillin, rifampicin, chloramphenicol, gentamicin, kanamycin, streptomycin, tetracycline, bacitracin and erythromycin. Polar lipid structures are 2,3-di-*O*-phytanyl-sn-glycerol (C_{20}, C_{20}). Cells contain phosphatidylglycerol (PG), methylated phosphatidylglycerol phosphate (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated triglycosyl diether (S-TGD-1), sulfated triglycosyl diether (S-TeGD) and sulfated tetraglycosyl diether (TGD-1). The DNA G+C content is 64.2 mol%. The type strain was isolated from the salt lake of Jilantai in Inner Mongolia, China (Yang, et al. 2006).

2.3.1.3 *Halobacterium noricense*

Halobacterium noricense cells are rods shaped (1.2-2.0 μm in

length), growing in liquid media as single cells. The bacterium is Gram-negative, motile, lyses in water, aerobic, facultatively anaerobic in the presence of L-arginine or nitrate, but not DMSO. Yeast extract and casamino acids support growth. It requires at least 12.5% NaCl for growth in the presence of 0.6 M $MgCl_2$. Optimal NaCl concentration, Mg^{2+} concentration, pH and temperature for growth are 15.0-17.5% NaCl, 0.7-0.8 M, pH 5.2-7.0 and 37-45^{o}C, respectively. They exhibit small colonies (0.4 mm diameter after 18 days of incubation at 37^{o}C) on a complex medium of neutral pH with light red, circular, entire margins. They are catalase-positive and oxidase-negative. Nitrate is not reduced to nitrite. Gelatin and starch is not hydrolyzed. They are susceptible to anisomycin and novobiocin and resistant to ampicillin, bacitracin, chloramphenicol, gentamycin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin. Main polar lipids are 2,3-di-*O*-phytanyl-sn-glycerol (C_{20}, C_{20}). derivatives of PG, PGP- Me, PGS, triglycosyl diether (TGD) and S-TeGD. Menaquinones MK-8 and MK-8[H_2] are the main components and MK-7, MK-7[H_2] and MK-9 are present in traces. G+C content is 54.3-54.5 mol%. At least two plasmids of approximate sizes 200 kbp and 400 kbp are present. The type strain was isolated from a dry bore core of Permian rock salt from the salt mine in Altaussee, Austria, approximately 470 m below surface. (Gruber et al., 2004).

Table 2.1 Comparison of shows some characteristics of *Halobacterium* spices.

Characteristic	Type culture strain		
	Hbt. salinarum[a]	***Hbt. jilantaiense***[b]	***Hbt. noricense***[c]
Habitat	NA	salt lake	salt mine
Cell morphology	Small rods	Slender rods	Small rods
Cell size (μm)	0.5-1.0×1.0-6.0	0.5-1.0×1.0-3.0	1.2-2.0
Pigmentation	Red	Red	Light red
Optimum [NaCl] for growth (M)	3.5-4.5	3.1-3.5	2.5-3.0
Optimum [$MgCl_2$] for growth (M)	0.05-0.1	0.1-0.2	0.6-0.9
Temperature range for growth (^oC)	20-55	22-55	28-50
Optimum growth temperature (^oC)	50	40	45
pH range for growth	5.5-8.0	5.5-8.5	5.2-7.0
Oxidase	+	+	-
Urease	-	+	-
Starch	-	-	-
Tween80	-	-	NA
H_2S from $S_2O_3^-$	NA	+	NA
Indole production	NA	+	NA
Esterase	-	-	+
Nitrate reduced	+	-	-
Gelatin liquefaction	+	+	-
Anaerobic growth with DMSO	+	+	-
Anaerobic growth with nitrate	-	+	+
Anaerobic growth with L-arginine	+	+	+
DNA G+C content (mol%)	67.1-71.2	64.2	54.3-54.5
Alkaline phosphatase	+	NA	-
Cystine arylamidase	-	NA	+
Valine arylamidas	-	NA	+
Acidic phosphatase	+	NA	-
Naphthol-AS-BI phosphohydrolase	+	NA	-

Note; [a], Data from Yang, et al. (2006) and Grant, (2001); [b], data from Yang, et al. (2006); [c], data from Gruber, et al. (2004); +, Positive; -, negative; NA, not available.

CHAPTER III

MATERIALS AND METHODS

3.1. Screening and selection of extremely halophilic bacteria producing proteinase

3.1.1 Isolation of extremely halophilic bacteria

Extremely halophilic bacteria were isolated from fish sauce samples fermented at 1, 3, 5, 7, 9, 12 months, fish juice and the fourth extract obtained from Rayong Fish Sauce Industry Co., LTD. (Rayong, Thailand). In addition, cultures from the Culture Collection at Suranaree University of Technology were included. Samples were plates on agar plate of JCM No.169 medium (Appendix 2.2), Halophilic medium (Appendix 2.3) and plate count agar (PCA) containing 25% NaCl and incubated at 35^{o}C for 15-20 days. Colonies with different morphological characteristics were randomly selected. A pure culture was obtained by streaking on the same medium.

3.1.2 Screening of extremely halophilic bacteria for proteinase production

Proteinase-producing ability of selected isolates was tested by point inoculation in JCM No.169 agar plates containing 1% skim milk (casamino acid was omitted), incubated at 35^{o}C for 7 day. The presence of a clear zone surrounding the colonies in skim milk agar indicated proteolytic activity. Proteolytic activity on fish protein of positive isolates on skim milk agar was also tested using fish agar. Fish agar was prepared by boiling 1 part of anchovy (*Stolephorus* spp.) with 2 parts of

distilled water for 20 min. Fish slurry was filtered through cheesecloth and filtrate was collected and pH was adjusted to 7.0. (Udomsil et al., 2010). NaCl (25%) and agar powder (2%) were added into fish broth. The medium was autoclaved at 121^{o}C for 15 min, and then poured into petri dishes. After solidification of the medium, a sterile 6 mm-diameter cork-borer was punched into fish agar. Twenty-five µl of 4-day cultured broth of each isolate was transferred into each well. Plates were incubated at 35^{o}C for 7 days. After incubation, agar plates were stained using 0.05% (w/v) Coomassie brilliant blue R-250 in methanol:acetic acid:distilled water (50:10:40) according to Mauriello, Casaburi, and Villani (2002). Clear zone surrounding wells indicated proteolytic activity. The extent of hydrolysis on skim milk agar were also measured and expressed as the ratio of clear zone to colony diameter.

3.2 Selection of extremely halophilic bacteria for fish sauce fermentation

Twenty-four isolates showing the highest proteolytic activity on skim milk salt agar were selected to evaluate their potential for being used as a starter culture for fish sauce fermentation.

Frozen anchovies (*Stolephorus* spp.) were minced and mixed with 25% NaCl. pH was adjusted to 7.5 using 1 N NaOH. Subsequently, the mixtures (30 g) were filled into a glass bottle (3-cm dia×5-cm height) and inoculated with 10% of starter culture. The inocula were prepared in a 125-mL Erlenmeyer flask containing 25 ml JCM No.169 broth at pH 7.0 and incubated at 35^{o}C for 4 days with a shaking speed of 120 rpm to attain an approximate cell count of 6-7 Log CFU/ml. The control was prepared by adding 10% JCM No.169 broth without cultures. All samples were

incubated at 35°C for 30 days. The samples (5 g) were taken for analyses of microbiological changes on JCM No.169 agar and changes of α-amino group content at 0, 14 and 30 days of fermentation. Volatile compounds and biogenic amines of all samples were also determined at day 30th.

3.2.1 Microbiological changes

The samples were taken aseptically from the fermentation glass bottles, and enumerated for extremely halophilic bacteria population by spread plate method using JCM No.169 agar and incubated at 35°C for 7-14 days.

3.2.2 α-Amino group contents

Soluble peptide contents of samples were measured as α-amino group contents using trinitrobenzensulfonic acid (TNBS) method (Adler-Nissen 1979). Fermented fish (3 g) was homogenized in 30 ml cold 5% (w/v) trichloroacetic acid. The mixture was centrifuged at 10,000×g (Legend™ MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 10 min at 4°C. The supernatant was collected for trichloroacetic acid (TCA)-soluble oligopeptides determination. One hundred µl of sample was mixed with 1 ml of 0.2125 M phosphate buffer (pH 8.2). One ml of 0.05% TNBS solution was added and thoroughly mixed and incubated in a water bath at 50°C for 1 h. Two ml of 0.1 N HCl was added to stop reaction and samples were left at room temperature for 30 min. Absorbance was measured at 420 nm (SmartSpecTM Plus, Bio-Rad Laboratories, California, USA) using leucine as a standard.

3.2.3 Volatile compounds

Volatile compounds of fermented fish samples were analyzed using solid phase microextraction-gas chromatrography mass spectrometry (SPME-GC-

MS). Volatile compounds were extracted from headspace of the sample vial using a 3-phase SPME fiber (1 cm-50/ 30 µm StableFlex Divinyl-benzene/Carboxen/PDMS, Supelco, PA.) that was stabilized at 50^{o}C for 30 min in a heating block. Volatile compounds were desorbed for 3 min at the gas chromatography injection port at 250^{o}C. Separation of the desorbed volatile compounds were achieved using GC-MS (Varian Inc., Walnut Creek, CA., U.S.A.) connected to a capillary column (DB-WAX, 60 m×0.25 mm×0.25 µm Agilent Technologies, Redwood, CA., U.S.A.). The oven temperature was increased from 25 to 200^{o}C at 15^{o}C/min. Volatile compounds were identified using a quadrupole mass detector (Mass spectrometer 1200L quadrupole, Varian Inc., Walnut Creek, CA., U.S.A.). Mass spectra of volatile compounds were obtained by electron ionization (EI) at 70 eV. The content of volatile compounds was from the area of an individual peak calculated as a percentage of the total areas recorded for all peaks in the chromatogram. Identification of volatile compounds was performed by comparing retention time and mass spectral data with mass spectral libraries (National Institute of Standards; NIST data).

3.2.4 Biogenic amines

Determinations of biogenic amines (tryptamine, cadavarine, tyramine, putrecine, histamine, spermidine, and spermine) was carried out using high performance liquid chromatrography (HPLC) according to the method of Dadakova et al. (2009) with slight modifications. Biogenic amines of fermented fish were extracted by adding 10 ml of 0.4 M perchloric acid to 5 g of homogenized sample and centrifuged at 5000×g for 10 min. The supernatant was collected and filtered through filter paper (Whatman® No.1). Ten ml of 0.4 M perchloric acid was added into the pellet and centrifuged again. Total volume of supernatant was adjusted to 25 ml using

0.4 M perchloric acid and kept at -20^{o}C until use. One ml of samples (if necessary, they were diluted with 0.4 M perchloric acid) were mixed with 1.5 ml of carbonate buffer pH 11. Two ml of dansyl chloride solution (10 mg/ml) prepared in acetone were added to the mixture, and incubated at 50^{o}C for 30 min. Residual dansyl chloride was removed by addition of 600 µl of 30% ammonia. After 30 min at room temperature, the mixture was adjusted to 5 ml with deionized water (DI), followed by 3-time extraction with 3 ml of heptane. Nine ml of the extract was dried at 50^{o}C under a stream of nitrogen. The dry residue was dissolved in 1.5 ml of acetonitrile. Samples were filtered through a 0.45-µm regenerated cellulose membrane filter (Agilent Technologies Inc., Palo Alto, CS., U.S.A.).Ten µl of the solution was injected into HPLC (HP 1100, Agilent Technologies Inc., Palo Alto, CS., U.S.A). A Zorbax Eclipse-XDB-C18 column (4.6×150 mm, 5 µm, Agilent Technologies Inc., Palo Alto, CA., U.S.A.) was used. Chromatographic separation was carried out using a gradient elution of (A) acetonitrile (100%), (B) acetonitrile (50%) as follows: 0-5 min, A 70%; 5-10 min, A 70-60%; 10-15 min, A 60–20%; 15-20 min, A 20-70% at column temperature of 28^{o}C. The flow rate was set at 0.8 ml/min. Detection wavelength was set at 254 nm and reference wavelength at 550 nm.

3.3 Roles of selected extremely halophilic bacteria on fish sauce fermentation

Five strains were selected based on volatile compound formation from 4.2.3 to study their roles in fish sauce fermentation. Strains producing desirable volatile compounds were J-1-S4, J-1-S22 and 2m-40-15-R2, while isolate P-1-S8, J-1-S13 represented those producing undesirable volatile compounds.

3.3.1 Fish sauce fermentation

The inocula were prepared in a 500-ml Erlenmeyer flask containing 125 ml JCM No.169 broth and incubated at 37^{o}C for 4 days with a shaking speed of 120 rpm to attain an approximate cell of 7-8 Log CFU/ml. Fish sauce fermentation was prepared using Indian anchovy (*Stolephorus* spp.). Fish were kept in ice for 5 days before fermentation. Solar salt used in the fish sauce industry was collected from a fish sauce plant. Fish and salt were mixed thoroughly at a ratio of 3:1. A portion of the mixture (1.5 kg) was filled in a glass jar (8.7-cm dia×17-cm height). Each bacterial starter culture was then added at 10% inoculum size (w/w) and mixed. The control was added 10% JCM No.169 broth without culture. All samples were incubated at room temperature (~27-32^{o}C). Fish sauce mash (50 g) was aseptically taken from the glass jar for determination of microbial changes, α-amino group content, pH and color at each time interval (0, 30, 60, 90, 120, 150 and 180 days). When the fermentation time reached 6 months, samples were filtered through cheese cloth and filter paper (Whatman® No.1). The filtrates were collected and analyzed for physico-chemical properties.

3.3.2 Microbiological analysis

Ten grams of fish sauce mash was aseptically taken (they were diluted with 25% NaCl sterile solution). Bacterial growth was enumerated using spread-plate technique on plate count agar (PCA), JCM No.169 containing 15 and 25% NaCl. All plates were incubated at 37^{o}C for 2-14 days.

3.3.3 Physico-chemical analysis

Fish sauce mash was filtered through filter paper (Whatman® No.1). Filtrates were measured for α-amino group content using the nitrobenzenesulfonic

acid (TNBS) method as described in 3.2.2. pH value of fish sauce mash was monitored using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland). The filtrate was diluted with distilled water at a ratio of 1:4. The mixtures were mixed and absorbance was measured at 440 nm.

3.3.4 Quality parameters of finished product

3.3.4.1 Chemical properties

Fish sauce samples fermented for 6 months were analyzed for total nitrogen (TN) using the micro-Kjeldahl method, ammonical nitrogen (AN) and salt content using Volhard titration method according to AOAC (2000).

3.3.4.2 Biogenic amines

Biogenic amines of fish sauce samples were carried out by HPLC as described in 3.2.4. Fish sauce samples were determined directly without extraction.

3.3.4.3 Volatile compounds

Fish sauce samples fermented for 6 months were analyzed for volatile compounds using gas chromatography mass spectrometry (GC-MS) equipped with purge and trap (Texmar velocity XPT^{TM}, Teledyne Tekmar, Mason, OH., U.S.A.). Sample of 10 ml with 0.1 ml of 100 ppm cyclohexanol as an internal standard was taken into purge and trap glass sample tube. Helium gas purge was carried out at 60^{o}C for 20 min at a flow rate of 40 ml/min. Volatile compounds were desorbed from column at 225^{o}C for 4 min. Separation of the desorbed volatile compounds was carried out using gas chromatography coupled to a mass spectrometry (Varian Inc., Walnut Creek, CA., U.S.A.) with a capillary column (DB-WAX, 60 m × 0.25 mm × 0.25 µm Agilent Technologies, Redwood, CA., U.S.A.).

The oven temperature was programmed from 25 to 200^{o}C at a rate of 15^{o}C/min. The volatile compounds were identified using a quadrupole mass detector (Mass spectrometer 1200L quadrupole, Varian Inc., Walnut Creek, CA., U.S.A.). The ionization energy (EI) for analysis were 70 eV. The content of volatile compounds were determined from peak area relative to the internal standard (cyclohexanol). Identification of volatile compounds was based on database in library search (NIST spectral library 2005). Kovats retention index (RI) of each compound was also calculated and compared with those reported in the literatures.

3.3.4.4 Sensory evaluation

Sensory evaluation of fish sauce samples was conducted using a hedonic-rating test. Nine expert panelists from industry who deal with fish sauce tasting on the regular basis as a quality control or production operators were used to judge samples. Samples included those inoculated with culture, control, and the commercial fish sauce fermented for 12 months. Panelists were asked to give acceptance scores for 4 attributes: color, odor, flavor and overall acceptance in the 7-point hedonic scale (Yongsawatdigul et al., 2007).

3.4 Identification of extremely halophilic bacteria

3.4.1 Morphological characteristics

Morphological characteristics of selected isolates were observed by light microscopy with cells grown on JCM No.169 at 37^{o}C for 7 days. Gram staining was performed by suspended in sterile 20% NaCl. After being air-dried, the slide was fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. (Dussault, 1955).

3.4.2 Physiological and biochemical characteristics

3.4.2.1 Biochemical characteristics

A Oxidase test

Tetramethyl-p-phenylenediamine dihydrochloride at 1% (Appendix 1.11) was dropped onto filter paper. Cell grown on JCM No.169 was streaked on the filter paper. Dark purple color colony indicated the positive result.

B Catalase test

Cells grown on JCM No.169 were smeared on the slide glass. Hydrogen peroxide at 3% (Appendix 1.2) was dropped. The bubble indicated the positive result.

C Hydrolysis of starch

Hydrolysis of starch was tested by point inoculation in JCM No.169 agar plates containing 1% starch (Appendix 2.9), incubated at 35^{o}C for 14 days. The presence of a clear zone surrounding colonies after flooded with iodine solution indicated the positive result.

D Hydrolysis of skim milk

Hydrolysis of casein was tested by point inoculation in JCM No.169 agar plates containing 1% skim milk (Appendix 2.8), incubated at 35^{o}C for 7-14 days. The presence of a clear zone surrounding the colonies indicated the positive result.

E Hydrolysis of Tween 80

Hydrolysis of Tween 80 was tested by point inoculation in JCM No.169 agar plates containing 0.2% Tween 80 (Appendix 2.10), incubated at 35^{o}C for 7-14 days. The presence of a clear zone surrounding the colonies indicated

the positive result.

F Hydrolysis of gelatin

Hydrolysis of gelatin was tested by point inoculation method in JCM No.169 agar plates containing 1% gelatin (Appendix 2.1), incubated at 35^{o}C for 7-14 days. The presence of a clear zone surrounding the colonies after flooded with 10% trichloroacetic acid indicated the positive result.

G Hydrolysis of L-arginine

Hydrolysis of L-arginine was tested by inoculating bacteria into L-arginine agar medium. (Appendix 2.5), incubated at 35^{o}C for 7-14 days. Red color of the agar indicated the positive result.

H Indole test

Cultures were inoculated into indole broth (Appendix 2.4) and incubated at 35^{o}C for 7 days. Kovac's reagent was added at 0.5 ml. A pink color at upper layer of broth indicated the positive result.

I Nitrate reduction

Cultures were inoculated into nitrate reduction broth (Appendix 2.6) and incubated at 35^{o}C for 7 days. After incubation, 1 drop of solution A (Appendix 1.10) was added. Development of pink color in 5 minutes indicated the positive result. Samples with negative result was added zinc powder, development of red color indicated the presence of nitrate in the medium.

J Anaerobic growth on arginine, nitrate and dimethyl sulfoxide (DMSO)

Pure cultures were inoculated into JCM No.169 broth supplemented with 0.5% of arginine, nitrate or DMSO. Cultures were incubated in the

dark at 35°C for 7 days in an anaerobic chamber (SHEL LAB, Sheldon Manufacturing Inc., IA., U.S.A.). Growth was measured at 660 nm. (Oren, Ventosa, and Grant, 1997)

3.4.2.2 Effects of salt concentration, temperature, pH, and Mg^{2+} on growth

Growth at different NaCl concentrations, temperatures, pHs, and Mg^{2+} concentrations of seven isolates were tested in JCM No.169. Inoculum size of 2% (approximately 10^6 CFU/ml) was added into 5 ml of JCM No.169 broth. Growth was measured at 600 nm. Growth at all conditions was monitored in 3 consecutive cycles. Positive (+) was given to conditions allowing growth in all 3 cycles.

For the effect of NaCl: cultures were added into 5 ml of JCM No.169 broth containing 0, 5, 12, 15, 18, 20, 25, and 30% NaCl and incubated at 35°C for 7-28 days.

For the effect of temperature was carried out by inoculating cultures into 5 ml of JCM No.169 broth containing 25% NaCl, pH 7.0. Cultures were incubated at various temperatures of 5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60°C for 7-28 days.

For the effect of pH, cultures were added into 5 ml of JCM No.169 broth containing 25% NaCl at pH 4.0, 5.0, 5.5, 6.0, 7.0, 8.0, 8.5 and 9.0 and incubated at 35°C for 7-28 days.

For the effect of Mg^{2+}, cultures were added into 5 ml of JCM No.169 broth containing 25% NaCl supplemented with 0, 0.005, 0.05, 0.1, 0.5 and 1 M $MgCl_2$, and incubated at 37°C for 7-28 days.

3.4.3 Ribosomal RNA gene sequence

16S Ribosomal RNA gene sequence of the selected extremely halophilic bacteria was analyzed.

3.4.3.1 Genomic DNA extraction

Chromosomal DNA was extracted from cells grown on JCM No.169 agar for 14 days according to the method of Yachai et al (2008). Cells were harvested and dissolved with 1 ml of saline-EDTA (pH 8.0) (Appendix 1.8). Cell suspension was added 0.75 mg/ml lysozyme and incubated at 37^{o}C for 1 h followed 5-min incubation at 60^{o}C with 250 µl of Tris-NaCl (pH 9.0) (Appendix 1.13) and 125µl of 10% SDS (Appendix 1.9). Subsequently, proteins were removed by addition of 500 µl phenol chloroform (1:1) and centrifuged at 10,000×g at 4^{o}C for 15 min. The upper layer of the mixture was collected. Chromosomal DNA was precipitated using cold absolute ethanol and gently spooled using the glass rod. Residual salts were removed with 70% and 95% ethanol and air dried. DNA were dissolved with 0.1×SSC (pH7.0). RNA and proteins were removed from crude DNA by adding RNase and proteinase K solution (Appendix1.5-1.6) and incubated at 37^{o}C for 1 h. Purified chromosomal DNA was stored in 0.1×SSC. The ratio of absorbance at 260 and 280 nm was measured to assess the purity of DNA. The purified DNA was stored at -20^{o}C until use.

3.4.3.2 Amplification of 16S r RNA gene

A total volume of 50 µl contained 1 µl of genomic DNA, 5 µl of 10X PCR buffer (200mM Tris-HCl, pH 8.0, 500 mM KCl; Invitrogen^{TM} Life Technologies, Foster, CA., U.S.A.), 2 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs mixture, 1 µl of 10 pmole of each primer (Table 3.1), 0.3 µl of 5U *Taq* DNA

polymerase (Invitrogen^{TM} Life Technologies, Foster, CA., U.S.A.), and 38.70 µl of MilliQ water. PCR product size was approximately 1,500 bp. Polymerase Chain Reaction (PCR) was performed using Thermoelectron corporation P×2 Thermal Cycle (Bioscience Technologies Division, San Francisco, CA., U.S.A.) with a temperature profile of primary heating step for 3 min at 95^{o}C, followed by 30 cycles of denaturation for 30 s. at 95^{o}C, 15 s at 55^{o}C (primer annealing), 1 min at 72^{o}C (polymerization), and a final extension for 5 min at 72^{o}C. The PCR amplified products were analyzed by electrophoresis in 1.0% agarose gel electropholysis. Agarose gel was stained in ethidium bromide solution (1 mg/ml) and examined under UV-transilluminator to visualize the amplified 16S rRNA gene bands.

3.4.3.3 Cloning of 16S rRNA gene

Bacterial 16S rRNA genes from PCR amplification were purified using Wizard Gel/PCR product kit (Promega Corporation, Madison, WI., U.S.A.) and ligated into pGEM-T easy vector (Promega Corporation, Madison, WI., U.S.A.) according the manufacturer's protocol. The recombinant vector was transformed into *E. coli* JM109 (Promega Corporation, Madison, WI., U.S.A.) by chemical transformation. The transformed *E. coli* JM109 was selected by blue-white selection method (Sambrook and Russell, 2001). The plasmid vector was purified using Wizard DNA purified kit (Promega Corporation, Madison, WI., U.S.A.).

3.4.3.4 Analysis of 16S rRNA gene sequence

The pGEM plasmid was used for sequence analysis. T7 and SP6 primers were used for sequencing. Nucleotide data were obtained from DNA sequencing software of ABI 377 Automated DNA. The sequences were compared to local alignment search of GenBank database using the BLAST version 2.2.9 program

of the National Center for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment was performed using CLUSTAL_X. Phylogenetic tree was constructed by the Maximum Pasimony method with software MEGA version 4.0 (Kumar, Tamura, Jakobsen, and Nei, 2004). The robustness of relationships was evaluated by a bootstrap analysis through 1,000 bootstrap replications.

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

Primer	Primer sequence (5′ to 3′)	Target region	Reference
16S rRNA gene amplification			
D30F	5′-ATTCCGGTTCATCCTGC-3′	6-22	Namwong et al. (2007)
D56R	5′-GYTACCTTGTTACGACTT-3′	1492-1509	Namwong et al. (2007)
B36R	5′-AGGACTACCAGGGTATCTA-3′	789-806	Namwong et al. (2007)
Nucleotide sequencing			
T7	5′-TAATACGACTCACTATAG GG -3′	53-72	Hans et al. (2002)
SP6	5′-TAATACGAC TCACTATAG GG -3′	2896-2916	Hans et al. (2002)

3.5 Statistical analyses

All chemical experiments and fish sauce fermentation were carried out in duplicates with different lots of fish. Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were used to determine differences between mean at $P<0.05$. Principal component analysis (PCA) was applied to the volatile compounds of fish paste sample, to reduce the large set of

variables into a smaller set and to group these sets of variables in order to explain the characteristic of selected isolates. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening and selection of extremely halophilic bacteria producing proteinase

In samples collected from fish sauce factory, population of halophilic bacteria of fish sauce samples fermented for 1-3 months was approximately 2-3 Log CFU/ml and that of samples fermented for 5-12 months was less than 30 CFU/ml (Table 4.1). Halophilic bacterial counts of the fourth fish sauce extracts were 2 Log CFU/ml on JCM No.169 agar. Fish juice is a liquid obtained after the salting process and showed viable halophilic bacterial counts of 4 Log CFU/ml (Table 4.1). It is well known that extremely halophilic bacteria are commonly found in solar salt. pH of fish sauce and fish juice samples ranged from 5.5-6.0 and 6.0-6.9, respectively. High amount of extremely halophilic bacteria was found in fish juice and the first three months of fermentation, then it decreased as the fermentation time proceeded. Thongthai et al. (1992) isolated extremely halophilic bacteria from Thai fish sauce and reported that extremely halophilic bacteria reached maximum density in the liquor after 3 weeks and persisted throughout the fermentation period.

Five hundred and sixty one colonies with different morphological characteristics were randomly selected. Halotolerant bacteria typically died after the first subculture, while moderately halophilic bacteria exhibited less growth rate, forming small colonies and died in the second subculture or later. Subculturing in

high salt medium (25% NaCl) is a means for selection extremely halophilic bacteria. Most of halotolerant and moderately halophilic colonies were isolated from PCA agar, while extremely halophilic bacteria were obtained from halophilic agar and JCM No.169.

A total number of 344 isolates of extremely halophilic bacteria were collected from 3 different media and tested for proteolytic activity on skim milk agar containing 25% NaCl. Fifty nine isolates showed proteinase activity on skim milk salt agar. However, skim milk is a milk protein and most proteolytic enzymes are substrate specific (Fransen, O'Connell, and Arende, 1997). Therefore, screening of proteolytic activity for the purpose of fish sauce fermentation should be carried out using fish protein. Fifty four out of 59 isolates exhibited proteinase activity on fish agar containing 25% NaCl. Representative results of 2 different protein substrates are shown in Figure 4.1.

Most of extremely halophilic bacteria producing proteinase were obtained from the first month of fermentation. Twenty four out of 54 isolates exhibiting the highest proteolytic activity towards skim milk salt agar were selected to investigate the ability to hydrolyze anchovy proteins, to produce biogenic amines and volatile compounds by inoculating into salted anchovy (Table 4.2).

Table 4.1 Bacterial counts (CFU/ml) of various fish sauce samples collected from a factory.

Sample for bacterial isolation	Halophilic bacterial counts (CFU/ml)			No. of isolate	
	PCA agar	Halophilic agar	JCM No.169 agar	Total selected isolate	Proteinase-producing isolate
1 mo-fermented fish sauce	7.39×10^3	6.18×10^2	4.93×10^2	131	34
3 mo-fermented fish sauce	2.18×10^2	1.63×10^3	1.25×10^3	73	8
5 mo-fermented fish sauce	<30 (10)	<30 (22)	50	2	0
7 mo-fermented fish sauce	<30 (6)	31.5	<30 (10)	6	0
9 mo-fermented fish sauce	33.5	46.5	46.5	8	0
12 mo-fermented fish sauce	30	nd	37	7	0
Fish juice	2.65×10^4	1.01×10^4	7.38×10^3	60	0
Fourth fish sauce extract	<30 (14.5)	<30 (13)	2.75×10^2	41	1
Labolatory scale-fermented fish sauce	NA	NA	NA	16	16
Total				344	59

nd, Not detected; NA, not available

4.2 Selection of extremely halophilic bacteria for fish sauce fermentation

4.2.1 Changes of cell counts and ability to hydrolyze fish protein

The initial microbial counts after inoculation of 24 isolates were approximately 5-7 Log CFU/g and cell counts decreased to approximately 1-2 Log CFU/g within 14 days. At day 30 of fermentation, cell counts were decreased to about 1-4 Log CFU/g. Isolate J-1-5, J-1-S6, J-771-S6 and J-1-S27 showed lower counts than 30 CFU/g, while counts of other 7 isolates were not detected. No growth was observed in the control without inoculation (Figure 4.2).

Table 4.2 Proteolytic activity of selected isolates of extremely halophilic bacteria on skim milk agar and fish agar containing 25% NaCl.

Bacterial isolate code	Isolation source	Isolation medium[a]	Colony color	Protein hydrolysis	
				Skim milk agar[b]	Fish agar
Samples from fish sauce fermentation					
P-1-S2	1st month	P25	red-orange	1.37±0.02	+
P-1-S8	**1st month**	**P25**	**red**	**4.98±0.00**	**+**
H-1-4	**1st month**	**HM**	**orange**	**3.22±0.82**	**+**
H-1-S4	**1st month**	**HM**	**orange**	**1.45±0.23**	**+**
H-1-S7	1st month	HM	orange	+	+
H-1-S9	1st month	HM	yellow	+	+
H-1-S11	1st month	HM	yellow	1.37±0.00	-
H-3-7	3rd month	HM	orange	+	+
J-1-1	1st month	J169	pink	1.99±0.16	+
J-1-5-1	**1st month**	**J169**	**red**	**5.20±0.20**	**+**
J-1-5-2	1st month	J169	+	1.71±0.00	+
J-1-7	1st month	J169	pink	+	+
J-1-8	**1st month**	**J169**	**pink**	**3.63±0.72**	**+**
J-1-10	**1st month**	**J169**	**orange**	**3.05±0.69**	**+**
J-1-17	1st month	J169	red-orange	+	+
J-1-23	1st month	J169	orange	1.31±0.11	+
J-1-28	1st month	J169	pink	1.54±0.02	+
J-1-S2	1st month	J169	yellow	+	-
J-1-S3	**1st month**	**J169**	**orange**	**1.52±0.22**	**+**
J-1-S4	**1st month**	**J169**	**white**	**3.00±0.68**	**+**
J-1-S5	1st month	J169	pink	1.29±0.06	+
J-1-S6	**1st month**	**J169**	**pink**	**3.98±0.73**	**+**
J-1-S9	1st month	J169	orange	1.49±0.35	+
J-1-S10	1st month	J169	pink	1.43±1.25	+
J-1-S13	**1st month**	**J169**	**pink**	**1.94±0.29**	**+**
J-1-S15	1st month	J169	orange	1.27±0.00	-
J-1-S22	**1st month**	**J169**	**red**	**2.08±0.14**	**+**
J-1-S23	1st month	J169	red	1.47±0.00	+
J-1-S24	1st month	J169	orange	+	-
J-1-S27	**1st month**	**J169**	**pink**	**3.36±1.69**	**+**
J-1-S28	**1st month**	**J169**	**pink**	**3.68±0.81**	**+**
J-1-S31	**1st month**	**J169**	**pink**	**3.20±0.63**	**+**

Table 4.2 (Continued)

Bacterial isolate code	Isolation source	Isolation medium[a]	Colony color	Protein hydrolysis	
				Skim milk agar[b]	Fish agar
J-1-S32	**1st month**	**J169**	**orange**	**2.57±0.37**	**+**
J-1-S34	1st month	J169	purple	+	+
J-1-S36	1st month	J169	orange	+	+
H-3-16	**3rd month**	**HM**	**yellow**	**3.68±0.12**	**+**
H-771-7	**3rd month**	**HM**	**orange**	**2.47±0.90**	**+**
J-771-4	**3rd month**	**J169**	**orange**	**2.30±0.00**	**+**
J-771-6	**3rd month**	**J169**	**orange**	**2.85±0.34**	**+**
J-771-8	**3rd month**	**J169**	**red**	**2.72±0.42**	**+**
J-771-S3	**3rd month**	**J169**	**pink**	**5.02±0.02**	**+**
J-771-S6	**3rd month**	**J169**	**orange**	**2.64±0.00**	**+**
J-F42-4	4th fish sauce extract	J169	red	1.51±0.02	+
E8	4th fish sauce extract	J25	red-orange	+	+
G4-1	**4th fish sauce extract**	**J25**	**red**	**3.17±0.10**	**+**
G8	4th fish sauce extract	J25	red	+	+
T11	4th fish sauce extract	J25	red	+	+
T12	4th fish sauce extract	J25	red-orange	1.04±0.56	+
S1	4th fish sauce extract	J25	red	1.38±0.00	+
S2	4th fish sauce extract	J25	red	+	-
Samples from laboratory scale fish sauce fermentation					
cont 2w-CR3	2nd week	J25	orange	1.19±0.00	+
2w-40-R1	2nd week	J25	orange	1.36±0.00	+
2w-40-15-R1	2nd week	J25	red-orange	2.28±0.00	+
2w-40-15-Y1	2nd week	J25	yellow	1.41±0.22	+
2mo-CRB2	2nd month	J25	pink	+	+
2mo-40-15-R2	**2nd month**	J25	**red-orange**	**1.65±0.09**	**+**
2mo-50-15-R2	2nd month	J25	orange	1.34±0.00	+
2mo-50-RB2-2	2nd month	J25	red-orange	+	+
6m-50-R4	2nd month	J25	orange	1.22±0.42	+

[a]P25, PCA+25% NaCl; HM, Halophilic medium; J169, JCM No.169 medium, J25; JCM No.168+25%NaCl; [b], the ratio of clear zone diameter to colony diameter; Bold indicates selected isolates; +, positive result.

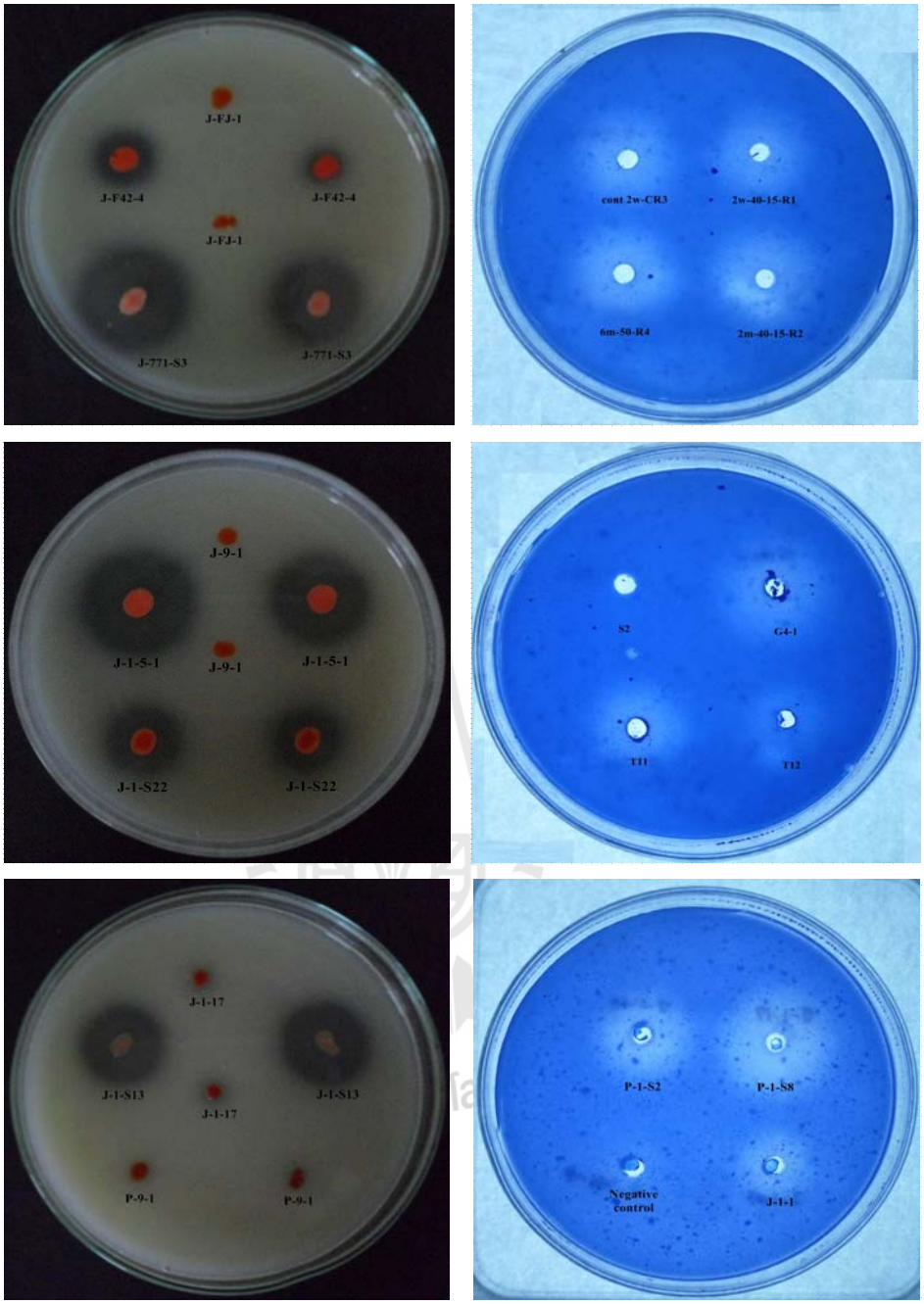

Figure 4.1 Representative colonies of extremely halophilic bacteria isolated from fish sauce fermentation and grown on skim milk agar (left) and fish agar (right) incubated at 37^{o}C for 7 days.

An increase in α-amino group content indicated the extent of proteolysis during fish sauce fermentation as it reflected the formation of oligopeptides and/or amino acids (Adled-Nissen, 1979). Amount of α-amino group contents of all inoculated samples was comparable to that of control (P>0.05) (Figure 4.2). Akolkar et al. (2010) reported that cell counts of *Halobacterium* sp. SP1(1), when used as a starter culture for fish sauce fermentation was approximately 5 Log CFU/ml at day 30th. In addition, α-amino group contents of the inoculated sample (15.4 mM/l) were higher than that of the control (12.3 mM/l) at day 30th. However, in that study fish sauce fermentation was prepared by mixing homogenized fish with brine solution (25% NaCl) in the ratio of 1:1. In that case, protein concentration in their system was half diluted. In our study, some isolates were unable to grow in fish fermentation system. It was hypothesized that high protein/peptide content in the fermentation may suppress the growth of those strains. Isolate P-1-S8 and J-1-S4 were tested in a series of varied peptide concentrations of fish sauce and found that their growth was inhibited in the presence of peptide content higher than 750 mM (Appendix E1). High protein/peptide could presumably have affected growth and proteinase production of some selected strains and resulted in insignificant difference of α-amino group content between some samples and the control.

4.2.2 Biogenic amine content

For application of starter cultures, the selected isolates should not produce biogenic amines in the fermentation process. Tryptamine, cadaverine, histamine, spermidine and spermine were found in fermented samples, while putrescine and tyramine were not detected (Table 4.3). Eight out of 24 fermented fish samples showed histamine content lower than the control.

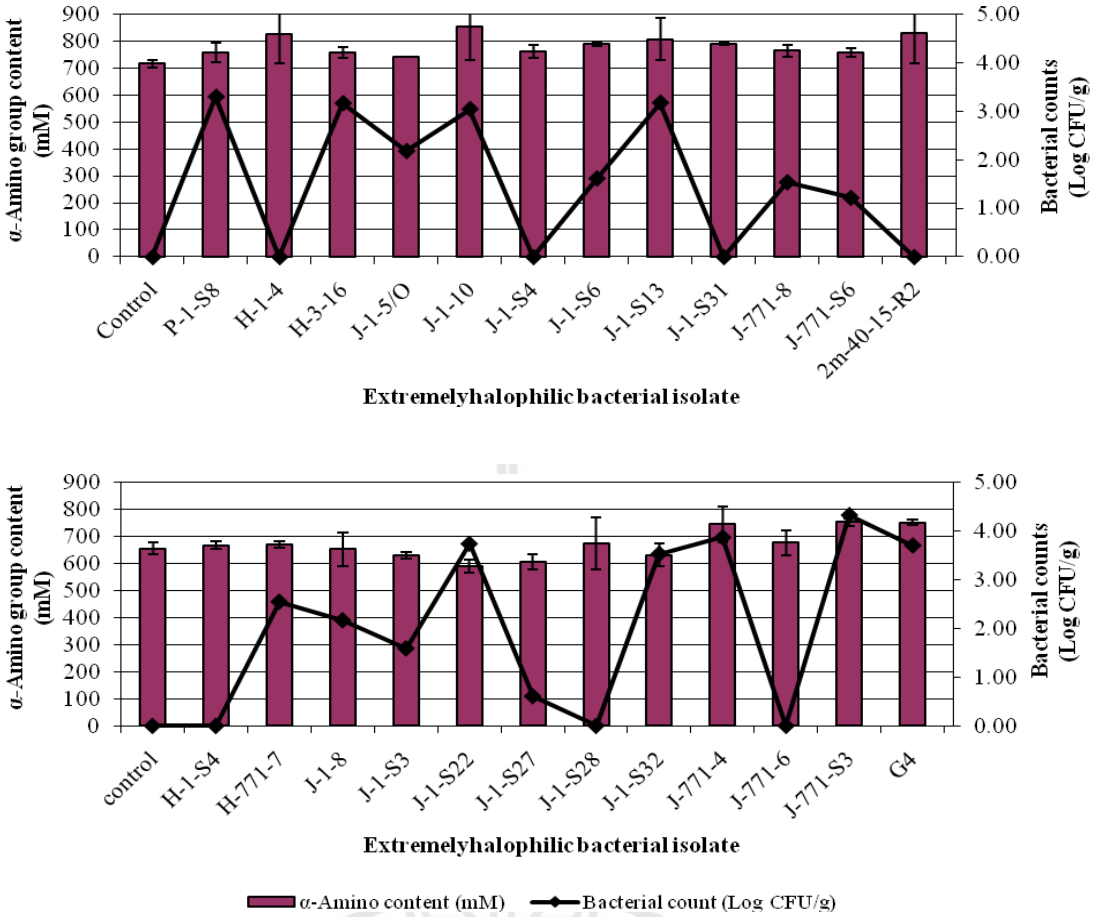

Figure 4.2 α-Amino group content and bacterial counts of anchovies mixed with 25% NaCl inoculated with various extremely halophilic bacterial isolates and incubated at 35°C for 30 days.

However, the amount of histamine in all samples was far below the allowable limit of 20 mg/100ml (CFIA, 2011). Typtamine was present in some samples. Cadaverine, spermine and spermidine have no adverse health effect on health, but they may react with nitrite to form carcinogenic nitrosoamines and are proposed as indicators of spoilage (Zaman et al., 2010). They were founded in trace amounts of 0.38-2.17 mg/100g cadaverine, 3.76-7.15 mg/100g spermidine and 1.12-2.80 mg/100g spermine (Table 4.3). Biogenic amine contents of all inoculated sample

appeared to be comparable to the control. These results indicated that these isolates did not increase biogenic amines during fish sauce fermentation.

Table 4.3 Biogenic amines (mg/100g) of fermented fish samples inoculated with selected extremely halophilic bacterial isolates and fermented at 35°C for 30 days.

Sample	Tryptamine	Cadaverine	Histamine	Spermidine	Spermine
control	nd	0.98	1.41	7.41	1.60
P-1-S8	nd	nd	0.97	4.52	1.57
H-1-4	nd	nd	2.83	4.03	2.07
H-3-16	nd	nd	2.76	4.18	1.92
J-1-5	nd	nd	2.33	3.93	1.71
J-1-10	nd	nd	3.56	5.42	2.80
J-1-S4	nd	nd	1.34	4.55	2.20
J-1-S6	nd	nd	2.48	7.15	2.17
J-1-S13	nd	nd	1.38	4.71	2.07
J-1-S31	1.86	nd	2.22	6.79	1.35
J-771-8	nd	nd	2.00	5.77	1.26
J-771-S6	nd	nd	1.09	6.73	1.33
G4	nd	nd	2.15	6.92	1.12
2m-40-15-R2	nd	nd	1.30	3.78	1.38
H-1-S4	1.41	0.50	2.09	7.04	1.26
H-771-7	1.49	1.13	1.57	4.21	1.48
J-1-8	1.75	nd	2.09	4.38	1.50
J-1-S3	2.11	1.18	2.09	4.88	1.12
J-1-S22	2.18	1.39	1.35	3.91	1.66
J-1-S27	nd	1.27	1.56	5.12	1.50
J-1-S28	nd	nd	1.39	4.29	1.49
J-1-S32	2.23	1.10	1.27	4.78	1.39
J-771-4	2.08	1.48	1.94	6.05	1.57
J-771-6	1.98	1.43	1.92	5.28	1.91
J-771-S3	1.22	nd	1.23	4.15	1.18

nd, Not detected

4.2.3 Formation of volatile compounds

Based on SPME-GC-MS analysis, nine major volatile compounds detected at day 30th in the laboratory-scale fish sauce samples. There were 2-methylbutanal and 3-methylbutanal (meaty note); benzaldehyde and 2,6-dimethyl pyrazine (sweet note); dimethyl disulfide and dimethyl trisulfide (fecal note); 3-methyl butanoic acid and hexanal (rancid note); and 1-butanol (pungent note). Based on principal component analysis (PCA), they were separated into 3 components. The first principal component (PC) explained 34.7%, while the second component explained 20.4% of total variance (Table 4.4).

Table 4.4 Principal components analysis of major volatile compounds of fish sauce samples inoculated with various extremely halophilic starter cultures.

Volatile compound	Odor description	Component		
		PC1	PC2	PC3
2-Methyl butanal	meaty	0.806		
3-Methyl butanal	meaty	0.858		
Dimethyl disulfide	fecal	-0.786		
Dimethyl trisulfide	fecal	-0.830		
Hexanal	rancid		0.799	
Benzaldehyde	sweet		0.941	
1-Butanol	pungent			0.744
2,6 Dimethyl pyrazine	sweet			0.705
3-Methyl butanoic acid	rancid			0.618
Eigenvalues		3.706	1.949	1.129
% of varance		34.694	20.441	20.239

The control and samples inoculated J-1-S22, J-1-S4, 2m-40-15-R2, J-1-5 and J-1-S3 appeared to show high content of 2-methylbutanal and 3-methyl butanal, compounds contributing to the meaty note (Figure 4.3). In addition, they contained less amount of dimethyl disulfide and dimethyl trisulfide which have been reported to be responsible for a fecal note. (Fukami et. al., 2002; Udomsil et. al., 2010).

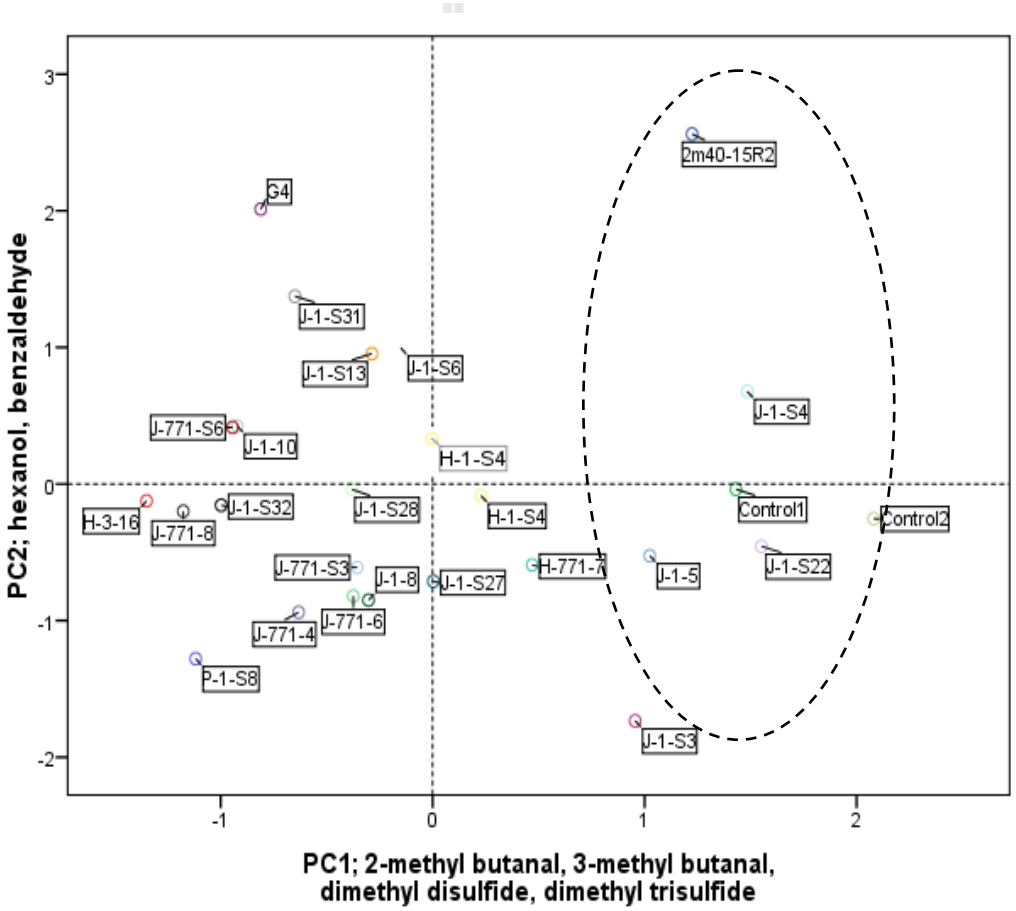

Figure 4.3 Principal component analysis of volatile compounds of laboratory scale fish sauce fermentation inoculated with 24 selected isolates at 30-g fermentation.

Samples inoculated H-3-16, J-771-8, J-771-S6, P-1-S8, J-1-S23 and G4 appeared to produce undesirable flavor of dimethyl disulfide and dimethyl trisulfide. Isolate J-1-S4, J-1-S22, 2m-40-15-R2, H-771-7, J-1-5 and J-1-S3 tended to produce meaty note and found in the same cluster as the control (Figure 4.3). Isolate J-1-S4, J-1-S22, and 2m-40-15-R2 were chosen from the group of isolates that produced desirable volatile compounds, while isolate P-1-S8 and J-1-S13 were chosen as representatives of undesirable flavor-producing isolates. It should be noted that isolate 2m-40-15-R2 produced high amount of hexanol and benzaldehyde as compared to other selected isolates that produced desirable volatile compounds. All of selected isolates showing ability to hydrolyze fish protein and low histamine producers in small scale fish sauce fermentation.

4.3 Roles of extremely halophilic bacteria on fish sauce fermentation

4.3.1 Microbiological changes

The initial count of inoculated samples was approximately 7 Log CFU/g at day 0 and decreased about 2 Log CFU/g at day 60 (Figure 4.4a). The number of inoculated cultures did not increase throughout the course of fermentation. At day 90, cell count of the sample inoculated with J-1-S22 was undetected, while those of others were 2-4 Log CFU/g. However, fish sauce samples inoculated with P-1-S8, J-1-S4 or 2m-40-15-R2 showed different colony characteristics from the inoculated strains. At day 120, cell counts of the sample inoculated with 2m-40-15-R2 was undetectable, while those of samples inoculated with P-1-S8, J-1-S4 and J-1-S13 were detected at about 1-3 Log CFU/g. Different colony characteristics were observed

on the agar plates of J-1-S4, implying that detected colonies were unlikely to be the inoculated J-1-S4. The sample inoculated with P-1-S8 also showed different morphology from the inoculated strain, but the sample inoculated J-1-S13 still exhibited the same colony morphological characteristics as the original at 120 days. At day 150, inoculated cultures of J-1-S4 and J-1-S13 appeared to completely diminish. P-1-S8 did not increase their numbers with the counts of less than 30 CFU/g at day 180. It should be concluded that inoculated cultures of P-1-S8, J-1-S4 and 2m-40-15-R2 appeared in the fish sauce fermentation up to 3 months, while J-1-S13 showed longer survival rate in the system for 120 days. When fermentation proceeded, extremely halophilic bacterial counts decreased in the inoculated samples, while the control showed different patterns. The initial extremely halophilic bacterial count of the control was 2 Log CFU/g and increased to 5 Log CFU/g after 30 days. It remained constant at 5 Log CFU/g until the 180^{th} day of fermentation. The results of control was in agreement with Thongthai et al (1992) who reported that extremely halophilic bacteria reached maximum density in the liquor after 3 weeks and persisted throughout the fermentation period. Zaman et al. (2010) revealed that bacteria have to complete for survivor with indigenous bacterial flora during fermentation. Some species of bacteria could stop growing when other bacterial species reach the maximum population. It might be possible that high amount of starter culture can inhibit the growth of extremely halophile microflora (from raw materials such as, solar salt and fish).

Initial microbial counts on JCM No.169 containing 15% NaCl and PCA of all samples were 3 and 4 Log CFU/g, respectively, and decreased to less than 30 CFU/g at day 30 (Figure 4.4b, c).

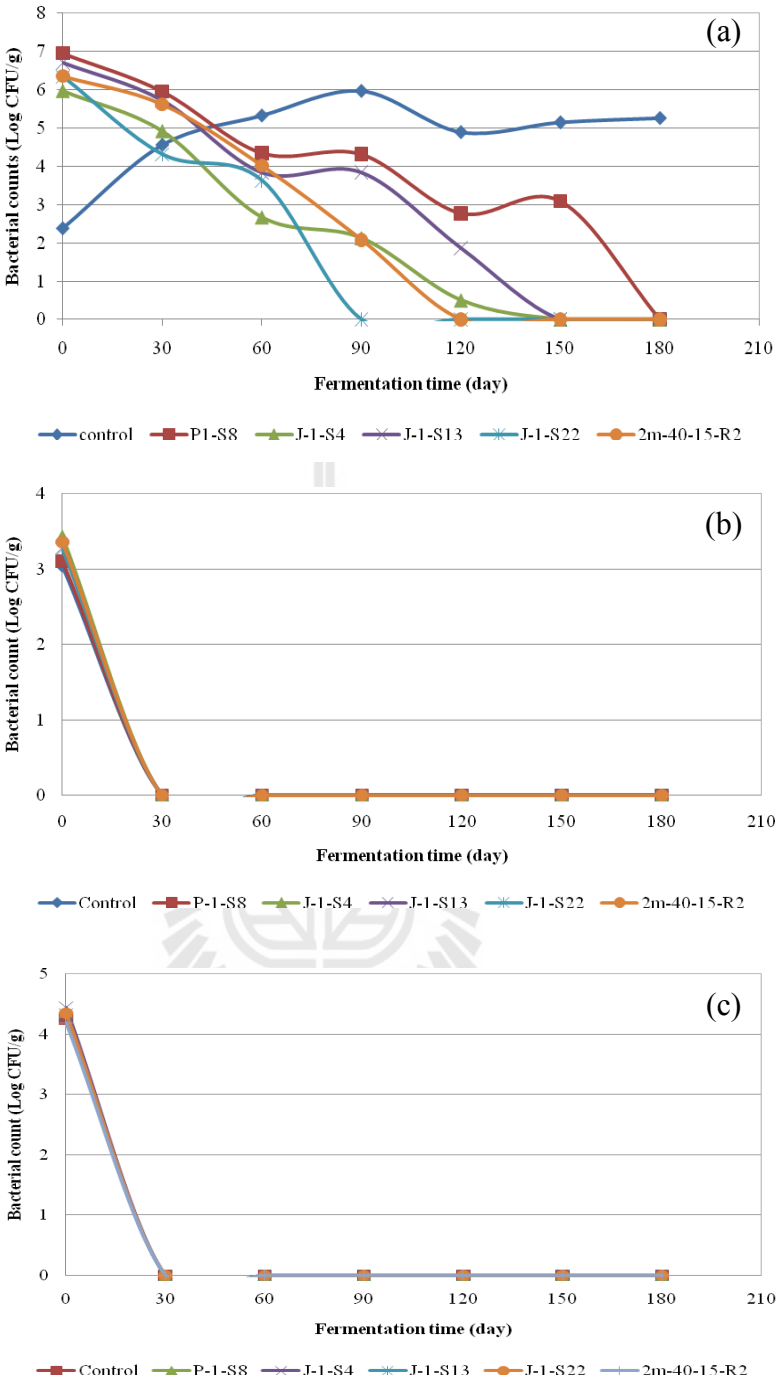

Figure 4.4 Changes of extremely halophilic bacteria on JCM No.169 (a); JCM No.169 added 15%NaCl (b) and non-halophilic bactrial count on PCA (c) of fish sauce samples incubated at room temperature for 180 days.

JCM No.169 containing 15% NaCl was used to detect halophiles and PCA was used to detect non-halophilic bacteria. Non-halophilic bacterial counts rapidly decreased when the fermentation time increased, possibly due to high concentrations of salt and reduced pH. Thus, microorganisms found during fish sauce production are generally classified as halophiles. Such a high salt content also inhibits other groups of microorganisms. The osmotic effect of salt kills or retards microorganisms, causing cell plasmolysis. Yongsawatdigul et al. (2007) reported that population of halophilic bacteria of samples collected from various fish sauce plants were relatively low (<30 CFU/g) during the fermentation period of 3 to 12 months, which were in agreement with this study.

4.3.2 Changes of α-amino group content

The initial α-amino group contents of all samples were approximately 138-160 mM and increased during fish sauce fermentation (Table 4.5). The conversion of protein to small peptides and free amino acids takes place during the fermentation. α-Amino group content drastically increased during the first three months, corresponding to the remaining population of inoculated cultures. In addition, α-amino group contents of samples, especially the control, increased during fermentation due to endogenous proteinases in fish muscle and digestive tract. However, proteinases activity from fish declined gradually due to high salt of 28-30% and relatively acidic condition at pH 5.0-6.5 (Youngsawatdigul et al., 2007). Therefore, proteinases produced from halophilic bacteria are likely to play an important role in protein hydrolysis during fish sauce fermentation. α-Amino group content after 180 days of all inoculated samples were 917-1029 mM, which were higher than that of control (831 mM) (P<0.05). Samples inoculated with J-1-S22 and

2m-40-15-R2 showed the highest α-amino group content, while the sample inoculated P-1-S8 showed the lowest content throughout the course of fermentation. These results suggested that extremely halophilic starter cultures could produce proteinase hydrolyzing anchovy proteins in the fermentation process. Therefore, the addition of proteinase-producing extremely halophilic bacteria, especially J-1-S22 and 2m-40-15-R2, as a starter culture could be an alternative means to accelerate protein hydrolysis during fish sauce fermentation. Although isolate J-1-S13 showed long survival rate up to 4 months in the fermentation system, the extent of protein hydrolysis was not predominant. Although high counts were detected in the control at day 60 and thereafter, the extent of protein hydrolysis was less than those inoculated extremely halophilic bacterial cultures. These implied that extremely halophilic microflora detected in the control did not mainly contribute to protein hydrolysis during fish sauce fermentation.

4.3.3 Quality parameters of finished products

4.3.3.1 Physico-chemical properties

pH values of all finished products were 5.68-5.77 (Table 4.6). pH of inoculated samples (pH 6.00-6.10) was higher than the control (pH 5.70; $P<0.05$) at day 30 and 60. Grant et al (2001) revealed that culture media became alkaline as a result of the deamination or decarboxylation of amino acids by extreme halophiles. pH of inoculated samples at day 90 and thereafter decreased and became comparable to the control. This result indicated that extremely halophilic starter cultures did not affect pH of the finished product. Most nitrogenous compounds in fish sauce are free amino acids and small peptides, which contribute to brown color development. Liquid obtained from samples inoculated with J-1-S22 and 2m-40-15-

R2 showed the highest α-amino group content after 180 days of fermentation (Table 4.5) and exhibited the greater extent of browning as compared to others (P<0.05; Table 4.6).

Table 4.5 Changes of α-amino group contents (mM) of fish sauce inoculated with extremely halophilic bacterial cultures and incubated at room temperature for 6 months.

Fermentation time (day)	Control	P-1-S8	J-1-S4	J-1-S13	J-1-S22	2m-40-15-R2
0	138.06±25.86	137.88±46.21	150.38±51.16	149.92±32.33	159.15±20.54	160.87±18.73
30	404.72±9.43^{a}	464.68±6.12^{b}	500.91±1.62	504.51±7.80^{c}	530.86±7.42^{c}	518.66±28.46^{c}
60	568.85±40.35	610.46±4.31	610.89±68.47	636.06±51.97	659.33±53.08	701.53±55.46
90	714.96±31.96	628.76±5.63	715.91±67.62	741.91±55.79	725.68±17.00	719.52±7.54
120	687.64±33.93^{ab}	659.42±26.35^{a}	791.78±24.29^{bc}	786.51±96.39^{bc}	816.59±27.30^{c}	818.85±7.94^{c}
150	716.90±125.48	799.21±58.26	795.88±92.46	859.87±69.87	867.63±82.07	876.84±92.90
180	798.13±45.78^{a}	855.51±87.42^{ab}	967.86±37.93^{bc}	978.97±39.18^{bc}	1009.95±70.72^{c}	1003.22±35.92^{c}

Different superscripts within a row indicate significant differences (P<0.05)

Salt content of all fish sauce samples was comparable to the control (P>0.05). Changes of salt content during fermentation were minimal (Yongsawatdigul et al., 2007; and Zaman et al., 2011). Total nitrogen (TN) has been the only indicator for fish sauce quality. The increased nitrogen content suggested an increased hydrolysis of protein. All products contained over 2% total nitrogen and, therefore, they can be classified as the first grade fish sauce (http://www.tisi.go.th). The samples inoculated with J-1-S4, J-1-S13, and J-1-S22 showed the highest TN after 180 days of fermentation. Generally, TN results corresponded with α-amino

group content except the sample inoculated with isolate 2m-40-15-R2. The sample inoculated with 2m-40-15-R2 showed the highest α-amino group content, but did not show the highest TN content (Table 4.6). TN value reflects the amount of protein nitrogen compounds and non-protein nitrogen compounds, such as ammonia, free amino acids, nucleotides, peptides, urea and trimethylamine (Shahidi, Synowiecki, Dunajski, and Chong, 1993). Ammoniacal nitrogen content of sample inoculated J-1-S4, J-1-S13 and J-1-S22 were higher than that of 2m-40-15-R2 (Table 4.7). It is possible that the highest TN value of J-1-S4 and J-1-S13 might be resulted from non-protein nitrogen compounds. Therefore, TN of samples inoculated with J-1-S4 and J-1-S13 were higher than 2m-40-15-R2 despite the fact that its α-amino group content was lower than the sample inoculated 2m-40-15-R2. All inoculated samples showed TN and ammoniacal nitrogen higher than control (P<0.05).

Table 4.6 Physico-chemical properties of fish sauce samples inoculated with extremely halophilic starter culture and incubated for 6 months.

Bacterial isolate	pH	Abs @440 nm	Total nitrogen (%)	Amonical nitrogen (%)	NaCl (%)
Control	5.68±0.22	0.362±0.01^{a}	2.10±0.01^{a}	0.207 ± 0.01^{a}	26.25±1.90
P-1-S8	5.73±0.18	0.466±0.00^{bc}	2.22±0.09^{ab}	0.224 ± 0.00^{b}	25.96±0.00
J-1-S4	5.71±0.02	0.494±0.03^{cd}	2.43±0.18^{c}	0.247 ± 0.02^{cd}	26.82±1.22
J-1-S13	5.69±0.03	0.448±0.00^{b}	2.44±0.23^{c}	0.265 ± 0.00^{d}	26.40±2.11
J-1-S22	5.75±0.03	0.561±0.01^{e}	2.45±0.04^{c}	0.258 ± 0.01^{d}	27.00±2.53
2m-40-15-R2	5.77±0.06	0.525±0.02^{de}	2.30±0.18^{bc}	0.234 ± 0.03^{bc}	25.96±0.21

Different superscripts within a column indicate significant differences (P<0.05)

4.3.3.2 Biogenic amine content in fish sauce

Putrescine, cadaverine, histamine and spermine were found in all fish sauce samples, while tryptamine and tyramine were not detected (Table 4.7).

Table 4.7 Biogenic amine (mg/100g) of fish sauce samples inoculated with starter cultures and fermented at room temperature for 6 months.

Sample	Putrescine	Cadaverine	Histamine	Spermine
Control	0.28±0.11	0.64±0.06	0.68±0.00	1.45±0.33
P-1-S8	0.41±0.06	0.50±0.17	0.71±0.06	1.31±0.18
J-1-S4	0.47±0.01	0.51±0.08	0.83±0.26	1.64±0.70
J-1-S13	nd	0.63±0.16	0.74±0.14	1.39±0.31
J-1-S22	0.49±0.11	0.65±0.09	0.64±0.17	1.53±0.04
2m-40-15-R2	nd	0.55±0.07	0.64±0.14	1.51±0.21

nd, Not detected.

Falb et al. (2008) reported that archaea also utilize arginine for the biosysthesis of polyamines such as putrescine or spermine. Putrescine was not presented in fish sauce inoculated with J-1-S13 and 2m-40-15-R2. Histamine is a common amine found in fishery products and also known as the causative agent of scombroid poisoning (histamine intoxication). The samples contained relatively low putrescine and cadaverine because samples were prepared from fresh anchovy. When fresh fish is chilled immediately, decarboxylase enzymes are unable to function at low temperatures (Brinker, Rayner, and Kerr, 2002). Every biogenic amines detected were found in trace amount. Histamine levels of all samples were found to be very low (0.64-0.83 mg/100g) when compared to other reports and lower than the maximum limit of 20 mg/100 ml (CFIA 2011). Yongsawatdigul et al. (2004) analyzed Thai commercial fish sauce samples and found that histamine ranged between 21-78

mg/100 ml. Falb et al. (2008) reported that histidine is likely to be degraded to glutamate via the urocanate pathway by *Halobacterium salinarium*. This could lead to lower content of histamine. The contents of putrescine, cadaverine, histamine and spermine in all inoculated samples and the control were comparable (P>0.05). Fish sauce fermentation carried out in the laboratory was prepared from fresh anchovy, resulting in relatively low biogenic amine contents. These results indicated that these isolates did not increase biogenic amines during fish sauce fermentation.

4.3.3.3 Volatile compounds of fish sauce

Total 35 volatile compounds were detected in fish sauce fermented for 180 days. Volatiles were classified into 11 alcohols, 12 aldehydes, 3 ketones, 1 esters, 1 acid, 3 sulfur-containing compounds, and 4 nitrogen-containing compounds (Table 4.8). Aldehydes and alcohols were predominantly present. Major aldehydes were 3-methylbutanal and 2-methylbutanal that contributed to meaty note. They have low threshold values of 2.24 to 40.7 ppb (Devos et al, 1995), and contribute to desirable aroma in the fish sauce product. Dougan and Howard (1975) reported that cheesy, ammoniacal, and meaty note are important odor characteristic of Thai fish sauce. Fish sauce samples inoculated P-1-S8 and 2m-40-15-R2 showed higher 3-methylbutanal than the control (P<0.05). Aldehydes are derived from lipid oxidation during fermentation or branched short-chain aldehydes might have resulted from deamination of amino acids (Shimoda et al., 1996). Acetaldehyde was another one of aldehydes that showed high level. J-1-S13 and 2m-40-15-R2 showed higher acetaldehyde than others (P<0.05) but did not seem to have important note in fish sauce. 2-Butenal contributing to pungent odor (Giri et al., 2010) was found in all samples except J-1-S4. Ethanol is a major alcohol found to be the highest level in all

samples. However, its note in fish sauce is insignificant due to its high threshold value. 3-Methyl-1-butanol has the lowest threshold of 4 ppb and contributes to fried fat and rancid odor (Giri et al., 2010). Fish sauce samples added J-1-S4 and J-1-S22 showed lower content of 3-methyl-1-butanol than others and comparable to the commercial fish sauce fermented for 12 months. Fish sauce samples added J-1-S4 and J-1-S22 contained lower ketones, which were comparable to that of the commercial fish sauce fermented for 12 months. Ketones were responsible for the cheesy note, but could not have a major impact on fish sauce odor because of their high odor threshold values. Ethyl acetate was negligible in some samples (P-1-S8, J-1-S13 and commercial fish sauce), and this compound contributed to flowery and sweet odor (Wichaphon et al., 2012). Gari et al. (2010) reported that ethyl acetate was significantly higher in miso sample than fish sauce. Dimethyl sulfide and dimethyl disulfide were found in all samples in this study, while dimethyl trisulfide was found in the commercial fish sauce and the sample inoculated J-1-S13. Dimethyl sulfide contents of inoculated fish sauce samples were comparable to the control and lower than the commercial fish sauce. Threshold value of dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide was 2.24, 0.43 and 1.66 ppb in the vapor phase, respectively (Devos et al., 1995). These compounds can cause unpleasant odor of fecal note in fish sauce (Fukami et al., 2004). J-1-S13 was the only culture found to produce dimethyl trisulfide. Major nitrogen-containing compounds found in samples were trimethylamine. Fish sauce added P-1-S8 exhibited the highest trimethylamine content and was comparable to the commercial fish sauce, while J-1-S4 and J-1-S13 contained the lowest content and comparable to the control (P>0.05). Among the nitrogen-containing compounds, trimethylamine contributes to major proportion and

might impart fishy odor of fish sauce product. Trimethylamine was presumed to be the source of fishy, ammonical odor and somewhat meaty note in fish sauce (Shimoda et al., 1996). Pyridine, 4-methylpyridine and 2,6-dimethyl pyrazine in samples inoculated with cultures were comparable to the control and commercial fish sauce. These compounds contributing to flower, grass, and roasted nuts note, respectively. Fish sauce inoculated P-1-S8 showed lower acetic acid than the control (P<0.05), while fish sauce inoculated J-1-S13 showed higher acetic acid than the control and was comparable to the commercial fish sauce (P<0.05). This compound was associated with cheesy note. Wichaphon et al. (2012) reported that volatile organic acids were found to have high odor intensity in the second grade fish sauce, but low amount in the premium grade samples. However, acetic acid has high threshold value (145 ppb) when compared with other volatile fatty acids, such as propanoic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, with odor threshold values of 35.5, 19.5, 3.89 and 2.45 ppb, respectively (Devos et al., 1995).

During the first three months of fermentation, inoculated samples showed different notes in the sniffing test among samples and control. Thereafter, odor characteristics of all samples changed to be similar to the control. It is possible that different aroma at the first three months was the action of inoculated cultures. However, volatile compounds in fish sauce are not only occurring from microbial metabolism but also formation via chemical reaction in fermentation process. Smit et al (2004) reported that aldehydes can be formed via nonenzymatic reaction including Strecker degradation. Fukami et al (2002) reported that volatile compounds are derived from protein hydrolysate and lipid oxidation products. Isolate P-1-S8, chosen as a representative of undesirable flavor-producing strain from

experiment 4.2.3, produced high content of 3-methylbutanal but low content of dimethyl disulfide, while isolate 2m-40-15-R2 chosen as a representative of desirable flavor-producing strain, produced high content of 3-methylbutanal and dimethyl disulfide.

Different results between this experiment and 4.2.3 might be caused by different fermentation period and forms of samples when analyzed. Fish sauce fermented at 180 days was used in this experiment, while the experiment 4.2.3 was carried out using fish sauce mash (fish residuals and sauce) fermented for 30 days. Therefore, the results reflect that longer fermentation period allows more chemical reaction to occur, resulting in different volatile compounds formation.

4.3.3.4 Sensory evaluation of fish sauce

Odor, taste and overall acceptance of all samples were comparable to those of the control and the commercial fish sauce ($P>0.05$), although volatile compounds analyzed by GC-MS showed different profiles. These results are similar to Giri et al. (2010) who reported that 4 different odor characteristics namely strong cheesy, meaty, fishy and ammoniacal note, were characterized in Thai fish sauce by gas chromatrography-olfactometry, but no significant difference were observed by sensory evaluation. It should be noted that odor, taste and overall acceptance of 6-month-old fish sauce inoculated with starter cultures were equivalent to the commercial sample fermented for 12 months. Addition of starter cultures can accelerate protein hydrolysis during fish sauce fermentation but does not affect overall acceptance of the finished product. Most panelists who were familiar with fish sauce tasting revealed that fish sauce samples in this study exhibited high hedonic score of color than the commercial fish sauce ($P<0.05$, Table 4.9)

Table 4.8 Volatile compounds of fish sauce samples inoculated with starter cultures and fermented at room temperature for 6 months.

Peak no.	RI[a]	Volatile compounds	Relative peak area[b]						
			Control	P-1-S8	J-1-S4	J-1-S13	J-1-S22	2m-40-15-R2	CM[c]
		Alcohols							
11	892	Methyl Alchol	0.186^{b}	0.248^{c}	0.176^{b}	0.356^{d}	0.166^{b}	0.165^{b}	0.037^{a}
14	942	Ethanol	28.795^{cd}	32.005^{d}	18.792^{b}	25.054^{c}	20.382^{b}	26.647^{c}	1.667^{a}
16	1039	1-Propanol	0.201^{ab}	0.302^{bc}	0.141^{a}	0.140^{a}	0.158^{a}	0.340^{c}	nd
20	1103	2-Methyl,1-propanol	0.068	0.081	0.033	0.069	0.022	0.083	0.029
21	1159	1-Butanol	0.103^{abc}	0.129^{c}	0.067^{ab}	0.098^{abc}	0.062^{a}	0.110^{c}	0.109^{bc}
22	1170	1-Penten-3-ol	0.301^{cd}	0.374^{d}	0.151^{ab}	0.117^{a}	0.148^{ab}	0.252^{bc}	0.093^{a}
25	1215	1-Butanol, 3-methyl	0.707^{c}	0.557^{bc}	0.209^{a}	0.447^{b}	0.131^{a}	0.520^{b}	0.157^{a}
26	1263	1-Pentanol	0.031^{bc}	0.049^{d}	0.024^{ab}	0.026^{b}	0.010^{a}	0.043^{cd}	nd
29	1323	2-Penten-1-ol,(E)	0.009	0.013	0.007	0.007	0.006	0.007	nd
30	1329	2-Penten-1-ol,(Z)	0.060^{cd}	0.076^{c}	0.048^{bc}	0.050^{bc}	0.039^{b}	0.051^{bc}	0.009^{a}
32	1365	Hexanol	0.019	0.019	0.009	0.019	0.010	0.009	0.012
		Aldehydes							
2	746	Acetaldehyde	0.334^{a}	0.465^{a}	0.354^{a}	0.742^{b}	0.283^{a}	0.497^{ab}	0.316^{a}
4	796	Propanal	0.055^{bc}	0.093^{d}	0.032^{ab}	0.031^{ab}	0.051^{bc}	0.068^{cd}	0.014^{a}
5	813	2-Methylpropanal	0.054	0.131	0.053	0.091	0.079	0.129	nd
7	837	2-Propanal	0.009^{a}	0.016^{abc}	0.013^{ab}	0.022^{bc}	0.011^{a}	0.022^{c}	0.075^{d}
8	867	Butanal	0.004	0.007	0.003	0.011	0.004	0.006	nd
12	902	2-Methylbutanal	0.151	0.241	0.123	0.176	0.140	0.230	0.162^{a}
13	907	3-Methylbutanal	0.329^{ab}	0.425^{b}	0.237^{a}	0.281^{a}	0.271^{a}	0.453^{b}	0.231^{a}
15	1035	2-Butenal	0.014	0.024	nd	0.016	0.009	0.017	0.009
18	1081	Hexanal	0.010	0.014	0.008	0.007	0.016	0.010	nd
19	1085	2-Methyl-2-butenal	0.012	0.017	0.013	0.018	0.013	0.017	nd
23	1183	Heptanal	0.004	0.007	0.004	nd	0.005	nd	nd

Table 4.8 (continued)

Peak no.	RI[a]	Volatile compounds	Relative peak area[b]						
			Control	P-1-S8	J-1-S4	J-1-S13	J-1-S22	2m-40-15-R2	CM[c]
		Aldehydes s							
35	1529	Benzaldehyde	0.014	0.019	0.018	0.023	0.015	0.020	0.013
		Ketones							
6	814	Acetone	1.788^{c}	1.572^{bc}	0.849^{a}	1.271^{ab}	0.900^{a}	1.483^{bc}	0.823^{a}
10	891	2-Butanone	0.208^{b}	0.250^{b}	0.127^{a}	0.204^{b}	0.125^{a}	0.212^{b}	0.136^{a}
28	1289	Cyclohexanone	0.073^{bc}	0.083^{c}	0.061^{ab}	0.047^{a}	0.063^{ab}	0.082^{c}	0.047^{a}
		Esters							
9	881	Ethyl acetate	nd	0.006	nd	0.001	nd	nd	0.011
		Sulphur-containing compounds							
3	769	Dimethyl sulfide	0.012^{a}	0.015^{a}	0.007^{a}	0.005^{a}	0.008^{a}	0.042^{a}	0.140^{b}
17	1065	Dimethyl disulfide	0.059^{ab}	0.045^{a}	0.037^{a}	0.035^{a}	0.040^{a}	0.085^{b}	0.312^{c}
33	1377	Dimethyl trisulfide	nd	nd	nd	0.001	nd	nd	0.014
		Nitrogen-containing compounds							
1	721	Trimethylamine	0.272^{ab}	0.636^{c}	0.173^{a}	0.149^{a}	0.307^{ab}	0.423^{bc}	0.567^{c}
24	1190	Pyridine	0.003	0.004	0.003	0.004	0.003	0.004	nd
27	1275	4-Methyl,pyrimidine	0.014	0.015	0.013	0.017	0.014	0.015	nd
31	1341	2,6-Dimethyl, pyrazine	0.035	0.027	0.011	0.025	0.012	0.018	0.007
		Acids							
34	1461	Acetic acid	0.005^{b}	0.003^{a}	nd	0.025^{c}	nd	nd	0.026^{d}

Note: nd, Not detected; Different superscripts within a row indicate significant differences (P<0.05).; [a]Retention indices calculated for DB-WAX column using n-alkanes as standards.; [b] The values represent the ratio of the peak area of any compound to that of the internal standard (cyclohexanol).
[c]Commercial fish sauce fermented conventionally for 12 months.

Fish sauce fermented in this study showed dark brownish color, this was likely due to the greater extent of Maillard browning reaction during fermentation. A greater extent of protein hydrolysis induced by proteolytic activity of starter culture could result in higher content of amino acids and peptides, which were subsequently served as substrates for Millard browning reaction. In addition, in this experiment fish sauce samples were thoroughly stirred before sampling at each time interval. This could in turn increase oxidation and the degree of Maillard reaction.

Table 4.9 Hedonic score of color, odor, taste, and overall acceptance of fish sauce samples inoculated with extremely halophilic bacteria starter cultures and commercial sample.

Sample	Attributes			
	Color	Odor	Taste	Overall acceptance
Commercial	3.50±1.62[a]	2.61±1.65	3.78±1.64	3.56±1.40
Control	5.33±0.61[c]	3.50±1.06	3.72±0.91	3.83±0.83
P-1-S8	5.06±0.68[c]	3.94±0.85	3.50±1.20	3.83±0.94
J-1-S4	4.72±0.62[bc]	3.78±1.44	3.78±0.83	3.89±0.93
J-1-S13	5.22±0.62[c]	3.89±1.47	3.78±1.33	3.72±1.03
J-1-S22	4.06±1.13[ab]	4.06±1.13	3.83±0.79	3.61±0.82
2m-40-15-R2	4.56±1.04[bc]	4.22±1.15	3.44±1.01	3.61±0.89

Acceptance score: 7 = extremely like; 4 = neither like nor dislike; 1 = extremely dislike. Different superscripts within a column indicate significant difference ($P< 0.05$).

4.4 Identification of extremely halophilic bacteria

4.4.1 Phenotypic and biochemical characteristics of selected extremely halophilic bacteria

Five extremely halophilic bacterial isolates were selected for identification. All selected isolates (J-1-S4, J-1-S13, J-1-S22 and 2m-40-15-R2) were Gram-negative, rod-shape, while isolate P-1-S8 was cocci. Cell lysis in distilled water was noticed in all selected isolates. Colonies on JCM No.169 agar of all isolates were circular (1-2 mm in diameter) and smooth. Isolates P-1-S8, J-1-S22 and 2m-40-15-R2 were red pigmented. Isolate J-1-S13 was pink and J-1-S4 was white-cream. All selected isolates grew in the medium containing at least 15% NaCl. It grew optimally in the presence of 20-25% NaCl, similar to most extremely halophilic bacteria (Grant, 2001). They grew at temperature ranging from 25-50^{o}C with the optimum growth at 35-45^{o}C. pH range for growth was 5.0-8.5 with the optimum at pH 6.0. Isolate J-1-S4, J-1-S13, J-1-S22 and 2m-40-15-R2 required at least 5 mM $MgCl_2$ for growth. It grew optimally in the presence of 100-500 mM $MgCl_2$, while isolate P-1-S8 did not require. All isolates hydrolyzed skim milk, gelatin and L-arginine but not Tween80 and starch. Catalase, oxidase, urease and indole production were positive. Nitrate was not reduced. They grew anaerobically in the presence of L-arginine and DMSO. Physiological and biochemical properties of all isolates are given in Table 4.10.

4.4.2 Ribosomal RNA gene sequence

The size of amplified DNA fragments was 1439-1440 bp (Figure 4.5). When compared 16S rRNA sequence of 5 isolates to type strains, *Halobacterium salinarum* DSM 3754^{T}, it showed 99% similarity. (Table 4.11). Sequence similarity

with type strains of *Halobacterium jilantaiense* JCM 13558^T and *Halobacterium noricense* A1 were 98%, and 97%, respectively.

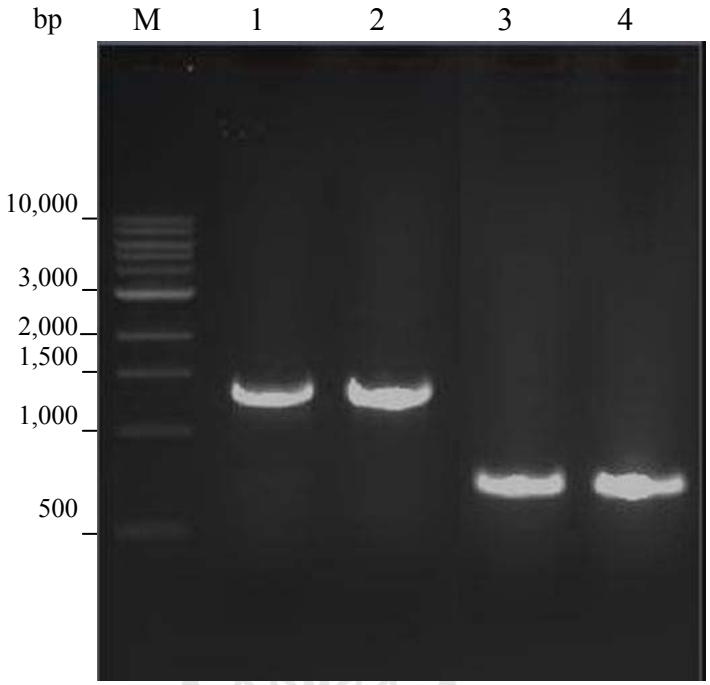

Figure 4.5 Gel electrophoresis of PCR product obtained from the amplification of 16S rRNA gene: Lanes: M, Molecular weight marker (1 kb ladder, Fermentas Life Sciences); 1, isolate J-1-S4 using primers D30F with D56R; 2, isolate J-1-S13 using primers D30F with D56R; 3, isolate J-1-S4 using primers D30F with B36R; 4, isolate J-1-S13 using primers D30F with B36R.

These results demonstrated that 5 isolates appeared to be closely related to the genus *Halobacterium* (*Hbt*). When phylogenetic tree analysis was performed, all five isolates fell in the cluster of *Hbt. salinarum* DSM 3754^T (Figure 4.6). These results demonstrated that all selected isolates appeared to be or closely

related to *Hbt. salinarum*. Tanasupawat et al. (2009) and Thongthai et al. (1992) identified *Hbt. salinarum* in fish sauce. Isolate P-1-S8 is coccid, but cells lysis occurred when suspended in distilled water, which is not a characteristic of *Halococcus.* Grant (2001) revealed that cell of some extremely halophiles such as *Halobacterium* are rods of varying length under optimal conditions in young liquid culture. Pleomorphic and coccid forms may be present in old liquid culture and in agar-grown cultures (Grant, 2001). Therefore, five selected strains of extremely halophilic bacteria were identified as *Halobacterium salinarum.*

Table 4.10 Comparison of characteristics of the selected extremely halophilic bacteria and some closely related genera.

Characteristic	Bacterial isolated from fish sauce sample					Type culture strain[a]			
	J-1-S4	**J-1-S13**	**J-1-S22**	**2m-40-15-R2**	**P-1-S8**	**1**	**2**	**3**	**4**
Source	Fish sauce	Fish sauce	Fish sauce	Laboratory scale fish sauce fermentation	Fish sauce	Pla-ra	Salt mine	Fish sauce	Fish sauce
Cell morphology	rods	rods	rods	rods	Pleomorphic rods	rods	rods	rods	cocci
Pigmentation	white	pink	red	orange	red	red	red	orange	red
Gram stain	-	-	-	-	-	-	-	-	-
NaCl range for growth (%)	15-30	15-30	15-30	15-30	15-30	15-30	18-30	10-30	15-30
NaCl optimum (%)	20-30	25-30	25-30	25-30	25-30	20-25	20-25	15-20	20-30
pH range for growth	5.5-8.5	5-8.5	5.5-8.5	5-8.5	5-8.5	4.5-8.5	5.0-8.5	5.5-8.5	6-10
pH (optimum)	5.5-6	5.5-6	5.5-6	5.5-6	5.5-6	7-7.5	NA	6.0-6.5	6-8
Temperature range for growth (^{o}C)	20-55	20-50	20-55	20-55	20-50	20-60	20-60	20-60	15-45
Temperature (optimum) (^{o}C)	37-45	37-40	37-45	37-45	37-45	37-40	35-50	37-40	37
Mg^{2+} range for growth (mM)	5-500	5-1000	5-500	5-1000	0-500	0-100	5-50	0-1000	not require
Mg^{2+} (optimum) (mM)	100-500	100-500	100-500	100-500	100-500	500-600	500-600	100-200	not require
Anaerobic growth on:									
Nitrate	-	-	-	-	-	+	NA	-	+
L-Arginine	+	+	+	+	+	+	+	-	+
DMSO	+	+	+	+	+	+	+	+	NA

Table 4.10 (Continued)

Characteristic	Bacterial isolated from fish sauce sample					Type culture strain[a]			
	J-1-S4	J-1-S13	J-1-S22	2m-40-15-R2	P-1-S8	1	2	3	4
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Nitrate from nitrite	-	-	-	-	-	-	-	-	+
Hydrolysis of;									
Starch	-	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	-	+	-	-
gelatin	+	+	+	-	+	+	+	+	-
skim milk	+	+	+	+	+	+	+	-	-
Tween 80	-	-	-	-	-	+	-	-	-
L-Arginine	+	+	+	+	+	NA	NA	NA	-

[a]1, *Halobacterium piscisalsi* JCM14661^T (data from Yachai et al.,2008); 2, *Halobacterium salinarum* DSM3754^T (data from Yachai et al., 2008); 3, *Natrinema giri* JCM14663^T (data from Tapingkae et al., 2008); 4, *Halococcus thailandensis* JCM13552^T(data from Namwong et al.,2007); +, Positive; -, negative; NA, not available

Table 4.11 Similarity of 16S rRNA gene sequence of extremely halophilic bacterial isolates compared with other bacteria from NCBI nucleotide sequence database.

Bacterial isolate code	Length of sequence (bp)	Identification results	Nucleotide sequence comparison			
			Closest relative	Length of sequence (bp)	Sequence homology (%)	NCBI Accession no.
J-1-S4	1440	*Hbt. salinarum*	*Hbt. salinarum* DSM3754^T	1473	99.80	AJ496185
			Hbt. noricense DSM15987^T	1433	98.20	NR_028187
			Hbt. jilantaiense JCM 13558^T	1433	96.90	AB477970
J-1-S13	1440	*Hbt. salinarum*	*Hbt. salinarum* DSM3754^T	1473	99.70	AJ496185
			Hbt. noricense DSM15987^T	1433	98.10	NR_028187
			Hbt. jilantaiense JCM 13558^T	1433	96.90	AB477970
P-1-S8	1440	*Hbt. salinarum*	*Hbt. salinarum* DSM3754^T	1473	99.90	AJ496185
			Hbt. noricense DSM15987^T	1433	98.20	NR_028187
			Hbt. jilantaiense JCM 13558^T	1433	97.00	AB477970
2m-40-15-R2	1440	*Hbt. salinarum*	*Hbt. salinarum* DSM3754^T	1473	100.00	AJ496185
			Hbt. noricense DSM15987^T	1433	98.30	NR_028187
			Hbt. jilantaiense JCM 13558^T	1433	97.10	AB477970
J-1-S22	1439	*Hbt. salinarum*	*Hbt. salinarum* DSM3754^T	1473	99.20	AJ496185
			Hbt. noricense DSM15987^T	1433	97.50	NR_028187
			Hbt. jilantaiense JCM 13558^T	1433	96.40	AB477970

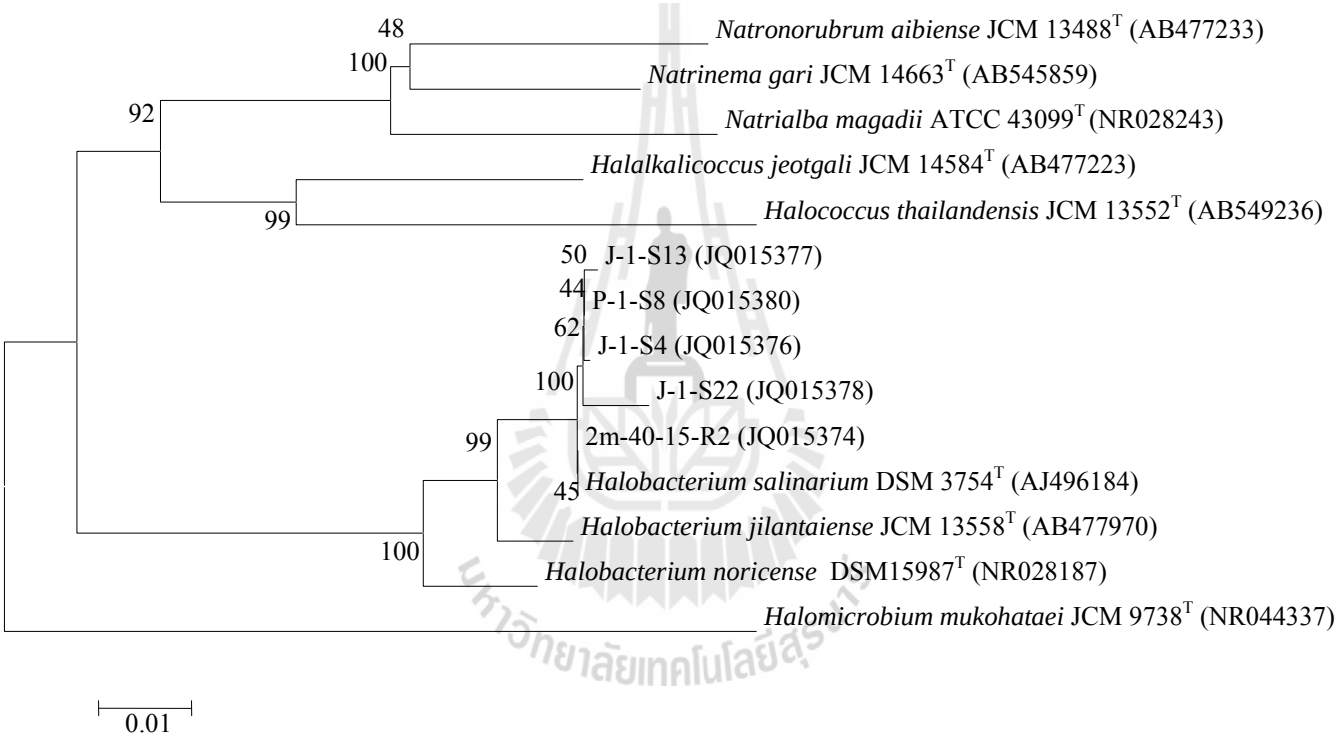

Figure 4.6 Phylogenetic tree of extremely halophilic bacteria isolated from fish sauce based on 16S rRNA gene sequence data. Bar indicates 0.01 substitutions per nucleotide position.

Table 4.12 Similarity of 16S rRNA gene sequences of all extremely halophilic bacterial isolate and related species.

Bacterial code	Bacterial isolate					Type culture strain[a]								
	J-1-S4	**J-1-S13**	**J-1-S22**	**P-1-S8**	**2m-40-15-R2**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
J-1-S4	100													
J-1-S13	99.70	100												
J-1-S22	99.20	99.10	100											
P-1-S8	99.90	99.80	99.20	100										
2m-40-15-R2	99.80	99.70	99.20	99.90	100									
1	99.80	99.70	99.20	99.90	100	100								
2	96.90	96.90	96.40	97.00	97.10	97.10	100							
3	88.80	88.70	88.20	88.90	88.90	88.90	89.90	100						
4	88.20	88.30	87.80	88.30	88.40	88.40	88.50	88.00	100					
5	87.20	87.00	86.80	87.10	87.20	87.20	87.10	85.40	85.90	100				
6	89.40	89.10	88.60	89.30	89.40	89.40	90.70	93.80	88.90	86.20	100			
7	88.50	88.40	88.00	88.60	88.60	88.60	89.40	93.70	89.50	86.10	94.50	100		
8	89.80	89.60	89.20	89.70	89.80	89.80	90.70	90.20	92.40	87.40	91.40	90.70	100	
9	98.20	98.10	97.50	98.20	98.30	98.30	97.30	88.80	88.40	87.00	89.90	88.60	89.80	100

[a]1,*Halobacterium salinarium* DSM 3754$^{T;}$ 2, *Halobacterium noricense* DSM15987^{T}; 3, *Nattrialba magadii* ATCC 43099^{T}, 4: *Halococcus thailandensis* JCM 13552^{T}; 5, *Halomicrobium mukohataei* JCM9738^{T}; 6,*Natrinema gari* JCM 14663^{T}; 7, *Natronorubrum aibiense* JCM 13488$^{T;}$ 8, *Halalkalicoccus jeotgali* JCM 14584^{T}; 9, *Halobacterium jilantaiense* JCM 13558^{T}

CHAPTER V

CONCLUSIONS

Most extremely halophilic bacteria were found in the first three months of fish sauce fermentation and fish juice. Three hundreds and forty four isolates of extremely halophilic bacteria were isolated from fish sauce fermented at various periods. High amount of extremely halophilic bacteria producing proteinase were found in the first month of fermentation. Twenty four isolates exhibiting the highest proteolytic activity towards skim milk were selected to investigate the ability to use as starter culture. At day 30 of fermentation, the initial microbial counts were decreased from 4-6 Log CFU/g to 1-4 Log CFU/g. α-Amino group contents of all inoculated samples were comparable to the control (P>0.05). All isolates did not produce biogenic amines. In addition, isolate J-1-S4, J-1-S22 and 2m-40-15-R2 produced high amount of 2-methylbutanal and 3-methylbutanal contributing to meaty note. Isolate P-1-S8 and J-1-S13 appeared to generate the highest content of dimethyl disulfide and dimethyl trisulfide. Isolate J-1-S4, J-1-S13, J-1-S22, P-1-S8 and 2m-40-15-R2 were selected for use as a starter culture for fish sauce fermentation. Population of inoculated P-1-S8, J-1-S4 and 2m-40-15-R2 appeared in the fish sauce fermentation for 3 months, while J-1-S13 showed longer survival rate in the fermentation system up to 4 months. Halophiles, halotolerant, and non-halophiles were detected at the initial stage of fermentation and decreased to less than 30 CFU/g thereafter. α-Amino group content after 180 days of all inoculated samples were 917-1,029 mM, which were higher than

the control (831 mM) (P<0.05). Samples inoculated with J-1-S22 and 2m-40-15-R2 showed the highest α-amino group content. Although isolate J-1-S13 showed long survival rate up to 4 months in the fermentation system, the extent of protein hydrolysis was not predominant. No biogenic amines were formed by the selected extremely halophilic starter cultures. A total of 35 volatile compounds were detected in fish sauce added extremely halophilic starter cultures including alcohols, aldehydes, ketones, esters, acids, sulfur-containing compounds, and nitrogen containing compounds. Fish sauce samples added P-1-S8 and 2m-40-15-R2 showed higher content of 3-methylbutanal than the control and the commercial fish sauce (P<0.05). Dimethyl sulfide and dimethyl disulfide of inoculated fish sauce samples were lower than the commercial fish sauce. All inoculated samples showed TN greater than 2% and ammonical nitrogen higher than the control (P<0.05). pH values and salt content of all finished samples were comparable to the control (P>0.05). Fish sauce obtained from samples inoculated with J-1-S22 and 2m-40-15-R2 showed the highest α-amino group content after day 180 of fermentation and the greatest extent of browning (P<0.05). All fish sauce samples fermented for 6 months inoculated with starter cultures exhibited similar overall acceptance to the commercial fish sauce fermented for 12 months. Therefore, extremely halophilic bacteria could be potential starter culture for fish sauce fermentation.

Bacterial identification based on physiological characteristic revealed that these isolates were Gram-negative and cell lysis occured in distilled water. Isolate P-1-S8, J-1-S22 and 2m-40-15-R2 were red pigmented, while isolate J-1-S13 and J-1-S4 were pink and white-cream, respectively. All selected isolates grew at 15-30% NaCl. They grew optimally at 20-25% NaCl, 0.1-0.5 M Mg^{2+}, pH 5.0-8.5 and at

25-50^{o}C. The result of 16S rRNA gene sequences revealed that five selected isolates showed 99% similarity to *Halobacterium salinarum* DSM3754^{T}. Therefore, they were identified as *Halobacterium salinarum*.

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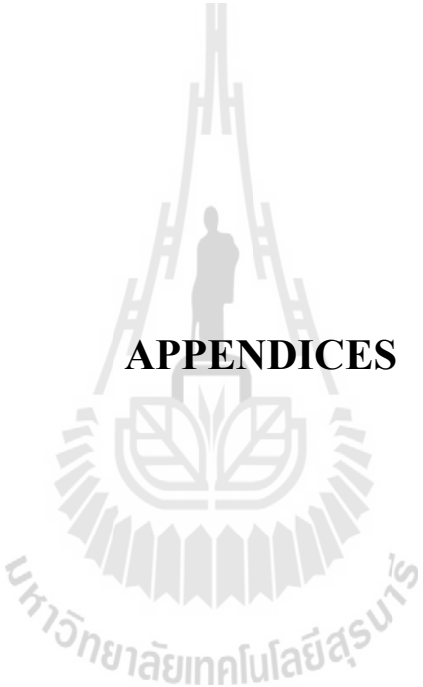
APPENDICES



APPENDIX A

Reagent and culture media preparation

1. Reagents

1.1 Crystal violet (Gram stain)

Crystal violet	2.0	g
Ethanol (95%)	20	ml
Mixed thoroughly		
Ammonium oxalate	80	ml
(1% Aqueous solution)		

1.2 Hydrogen peroxide (3% solution)

Hydrogen peroxide	3.0	g
Distilled water	1000	ml

1.3 Iodine solution (Gram stain)

Iodine	1.0	g
Potassium iodide	2.0	g
Add distilled water and bring volume up to	300.0	ml

1.4 Phenol:Chloroform (1:1 v/v)

Crystalline phenol was liquidified in water bath at 65˚C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a tight bottle.

1.5 Proteinase K

Proteinase K (Sigma)	4	mg
50 mM Tris-HCl (pH 7.5)	1	ml

Use freshly prepared solution.

1.6 RNAsae (10mg/ml)

RNAase	10	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20^{o}C

1.7 Safranin (Gram stain)

Safranin O (0.25% solution in 95% ethanol)	10	ml
Distilled water	90	ml

1.8 Saline-EDTA (0.15 M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA (di-Sodium salt)	37.22	ml

Dissolve the solids in deionized water, adjust to pH 8.0 with 1 N HCl and 1 N NaOH. The final volume was adjusted to 1000 ml with deionized water. Then, the solution was sterilized by autoclaveing for 10 min at 121^{o}C, 15 lb/in^{2}.

1.9 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100	g
Add distilled water and bring volume up to	1,000	ml

1.10 Nitrate reduction test reagent

Sulfanilic acid solution

Sulfanilic acid	0.8	g
5 N Acetic acid	100	ml

Dissolve by gentle heating in a fume hood.

***N*,*N*-dimethyl-1-naphthylamine solution**

N,*N*-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	ml

Dissolve by gentle heating in a fume hood.

1.11 Tetramethyl-p-phenylenediamine dihydrochloride (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1	g
Add distilled water to bring volume up to	1,000	ml

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

Tris-HCl	0.79	g
EDTA (di-soduim salt)	0.37	g
Boric acid	5.54	g
Add distilled water and bring volume up to	1,000.0	ml

1.13 Tris-NaCl

Tris-base	121.14	g
NaCl	5.84	g

Dissolve the solids in deionized water, adjust to pH 8.0 with 1 N HCl and

1 N NaOH. The final volume was adjusted to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

1.14 SSC (20×)

NaCl	175.3	g
Sodium citrate	88.2	g

Dissolve the solids in deionized water, adjust to pH 8.0 with 1 N HCl and 1 N NaOH. The final volume was adjusted to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

2. Culture media

2.1 Gelatin agar

JCM No.169 medium (omitted casamino acid)

Gelatin	1000	ml
Agar	100	g
Dissolve and adjust pH 7.2	20	g

The medium was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

2.2 Halobacterium medium JCM No.169

Yeast extract		
Casamino acid	10	g
Tri-sodium citrate	7.5	g

$MgSO_4 \cdot H_2O$	3	g
KCl	20	g
NaCl	2	g
$FeSO_4 \cdot 4H_2O$	250	g
$MnSO_4 \cdot 4H_2O$	0.05	g
Agar	20	g

Dissolve the solids in deionized water, adjust to pH 7.0±0.2 with NaOH. The final volume was adjusted to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

2.3 Halophilic medium

Yeast extract	10	g
Casamino acid	10	g
Propeose peptone	50	g
Tri-sodium citrate	3	g
$MgSO_4 \cdot H_2O$	25	g
KCl	2	g
NaCl	250	g
Agar	20	g

Dissolve the solids in deionized water, adjust to pH 7.0±0.2 with NaOH. The final volume was adjusted to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

2.4 Indole test

Bacto peptone	10	g
NaCl	10	g
Distilled water	200	g
Adjust pH to 7.0±0.2 with NaOH	1,000	ml

The medium was sterilized by autoclaving for 10 min at 121^{o}C, 15 lb/in^{2}.

2.5 L-arginine agar medium

Peptone	1	g
NaCl	200	g
K_2HPO_4	0.3	g
Phenol red, 1.0% aq. solution	1	ml
L(+)arginine hydrochloride	10	g
Agar	3	g
Distilled water	1,000	ml

Dissolve the solids in the water, adjust to pH 7.2, distribute into tubes or screw-capped (6-mm dia) bottles to a depth of about 16 mm (3.5 ml). The medium was sterilized by autoclaving for 10 min at 121^{o}C, 15 lb/in^{2}.

2.6 Nitrate broth

Beef extract		
Peptone	10	g
NaCl	10	g

Distilled water	200	g
Adjust pH to 7.0±0.2 with NaOH	1,000	ml

The medium was sterilized by autoclaveing for 10 min at 121°C, 15 lb/in^2.

2.7 Pate count agar (PCA)

Tryptone		
Yeast extract	5	g
Dextrose	2.5	g
Agar	1	g
Distilled water	3	g
Adjust pH to 7.0±0.2 with NaOH	1,000	ml

The medium was sterilized by autoclaving for 10 min at 121°C, 15 lb/in^2.

2.8 Skim milk agar

Use JCM No.169 medium (omitted casamino acid) and add 1% Skim milk

Agar	20	g

2.9 Starch agar

Use JCM No.169 medium and add 10% Starch

Agar	20	g

2.10 Tween 80 agar medium

JCM No.169 medium added 2 ml of Tween 80. Dissolve and adjust pH to 7.2, bring volume to 1,000 ml, added 20 g of agar.

APPENDIX B

Nucleotide sequence data

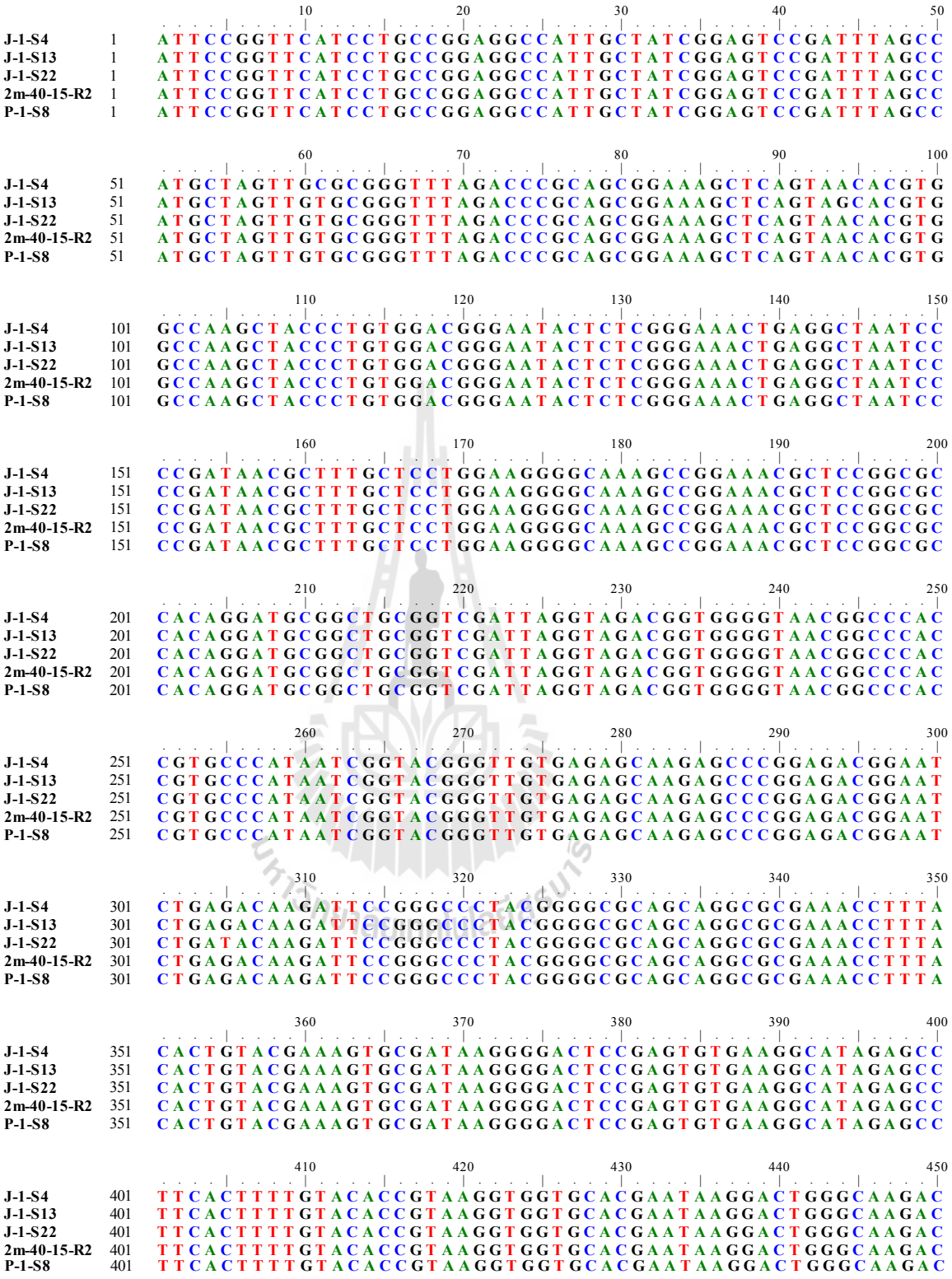


Figure 1 B Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of extremely halophilic bacteria isolated from fish sauce fermentation.

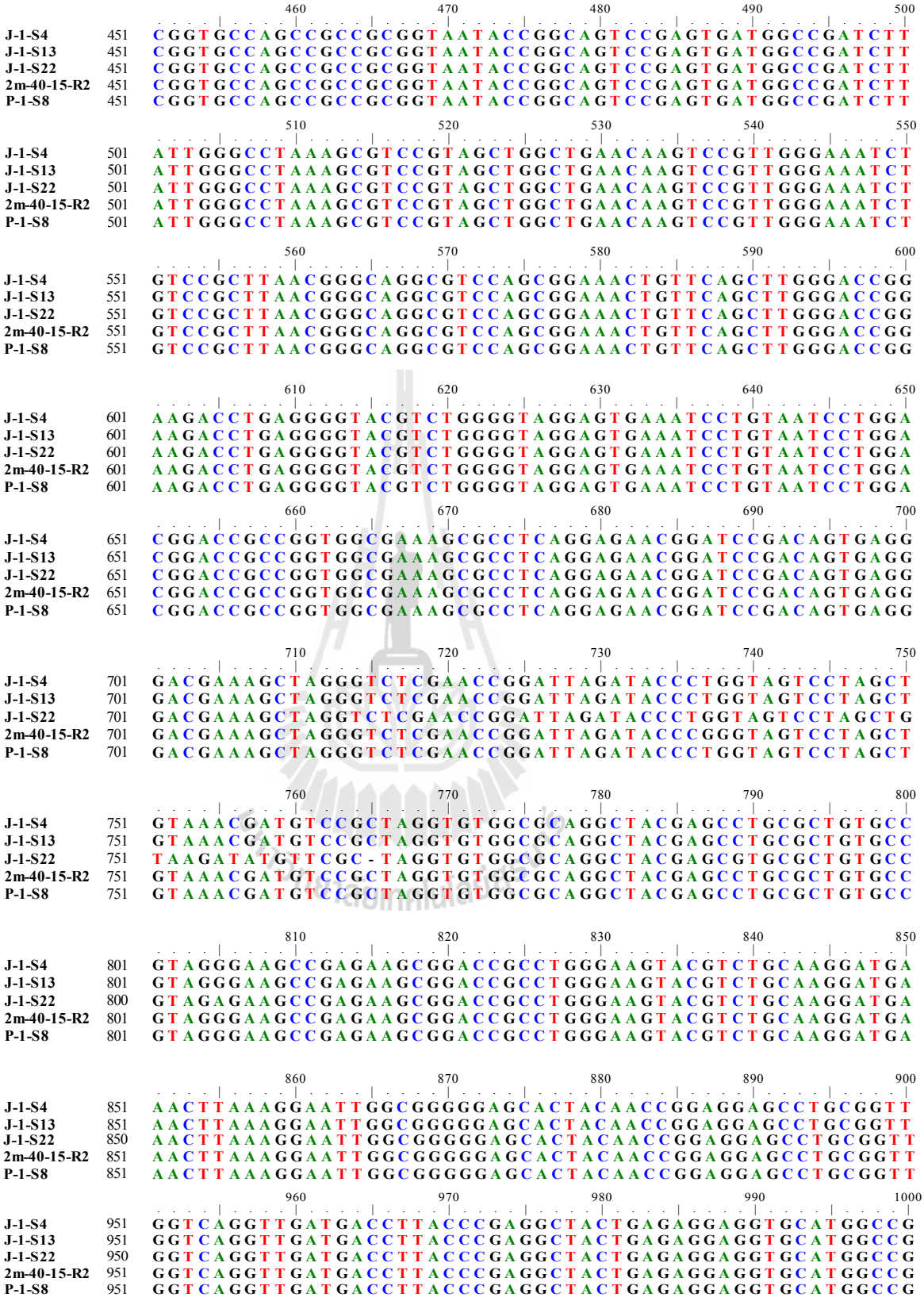


Figure 1 B (Continued)

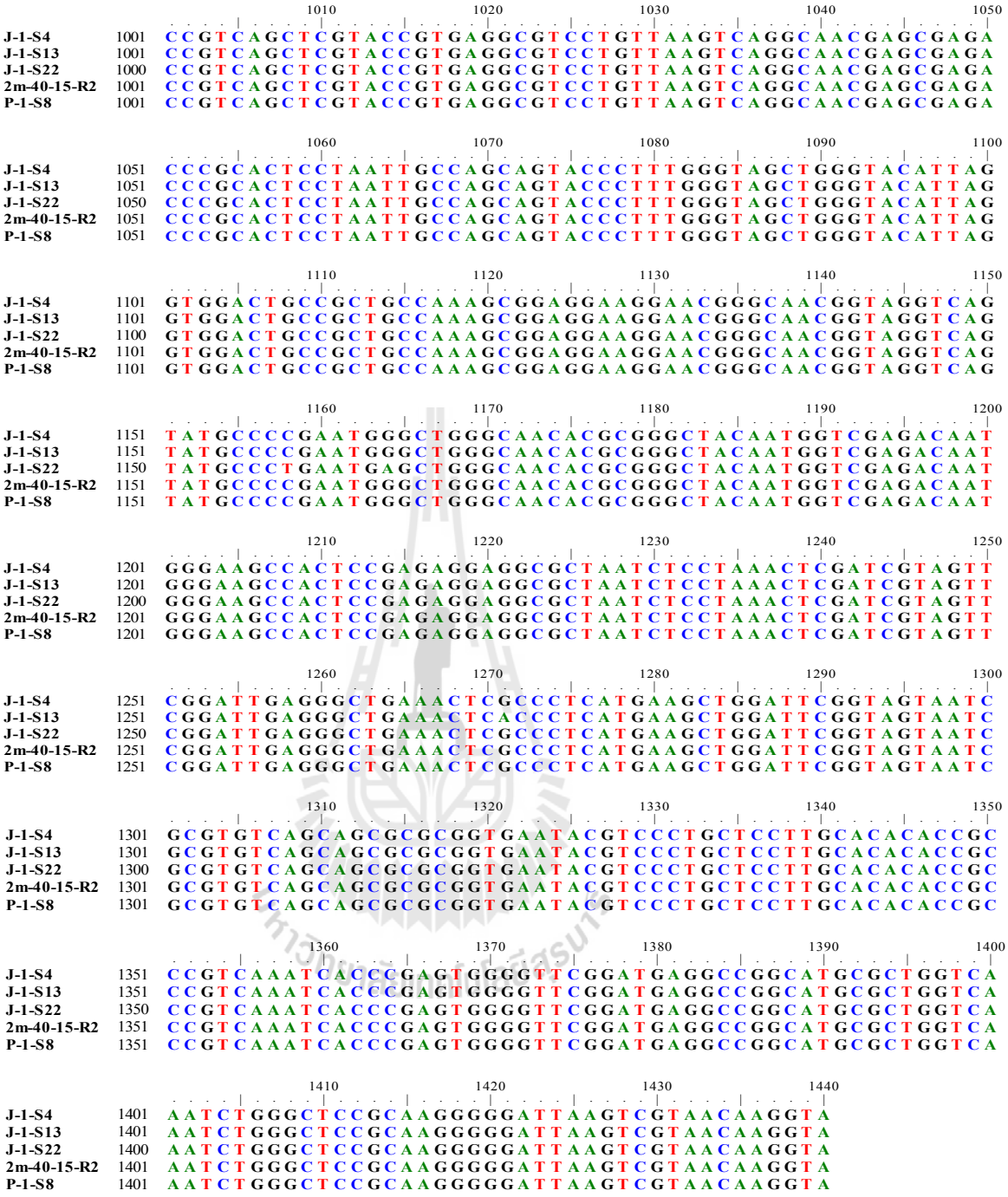


Figure 1 B (Continued)

APPENDIX C

Colony morphology on JCM No.169 agar at 37^{o}C for 7 days and Gram staining of selected extremely halophilic bacteria.

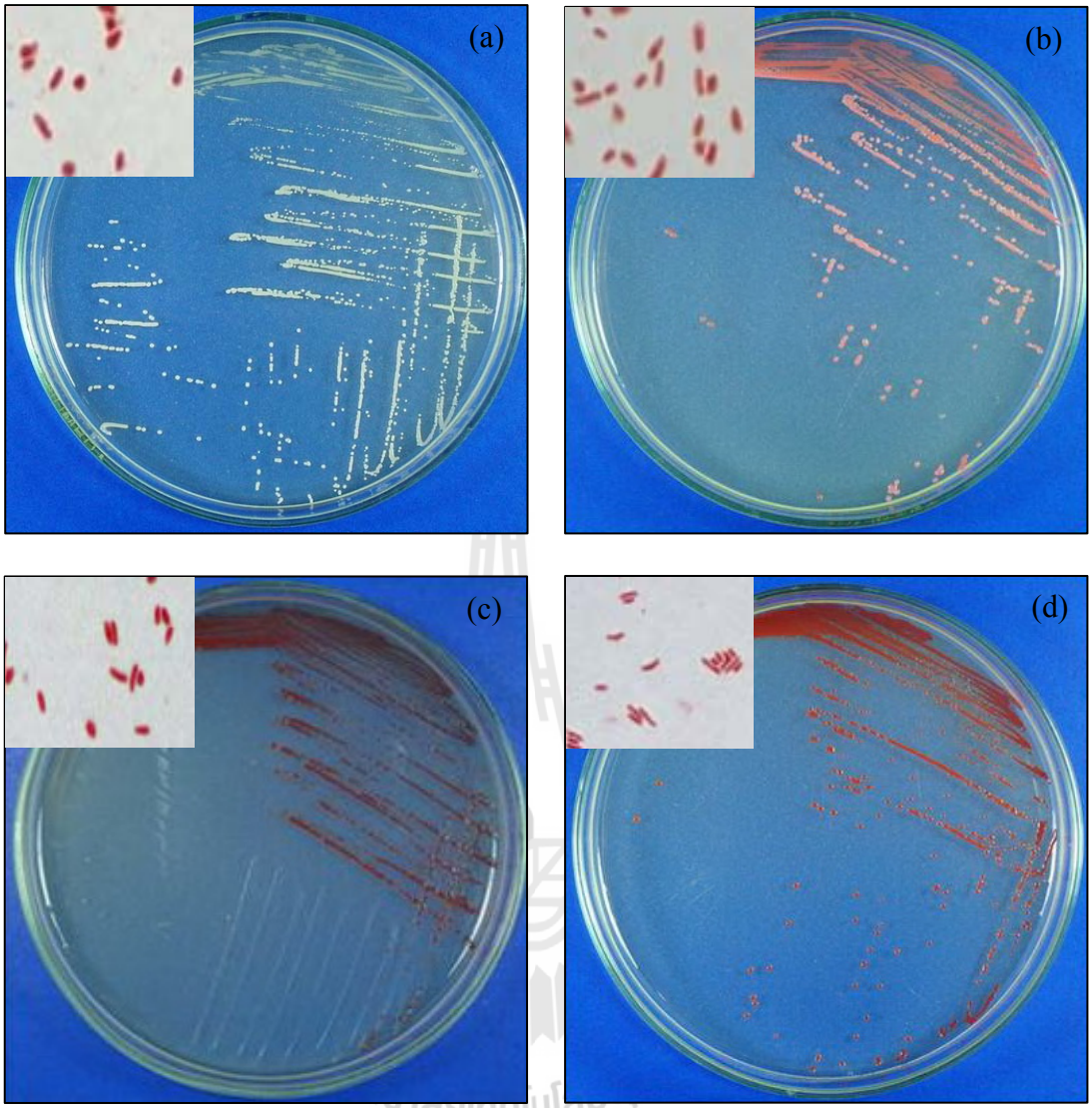


Figure C1 Colony morphology of isolate P-1-S8 (a), J-1-S13 (b), J-1-S22 (c), 2m-40-15-R2 (d) on JCM No.169 agar at 37°C for 7 days and Gram staining.

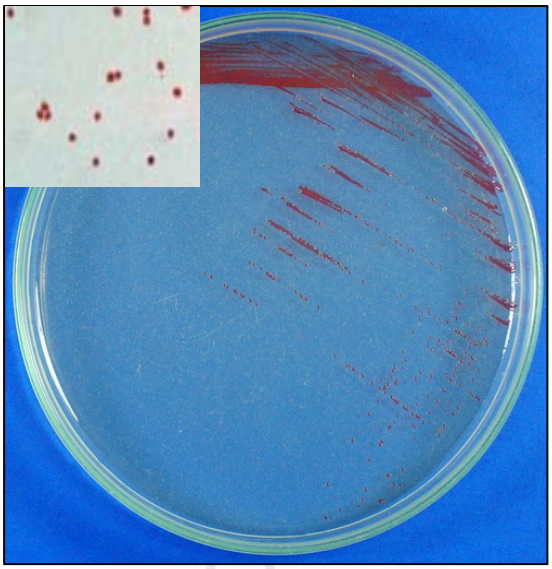


Figure C2 Colony morphology of isolate P-1-S8 on JCM No.169 agar at 37^{o}C for 7 days and Gram staining.

APPENDIX D

Chromatograms of volatile compounds

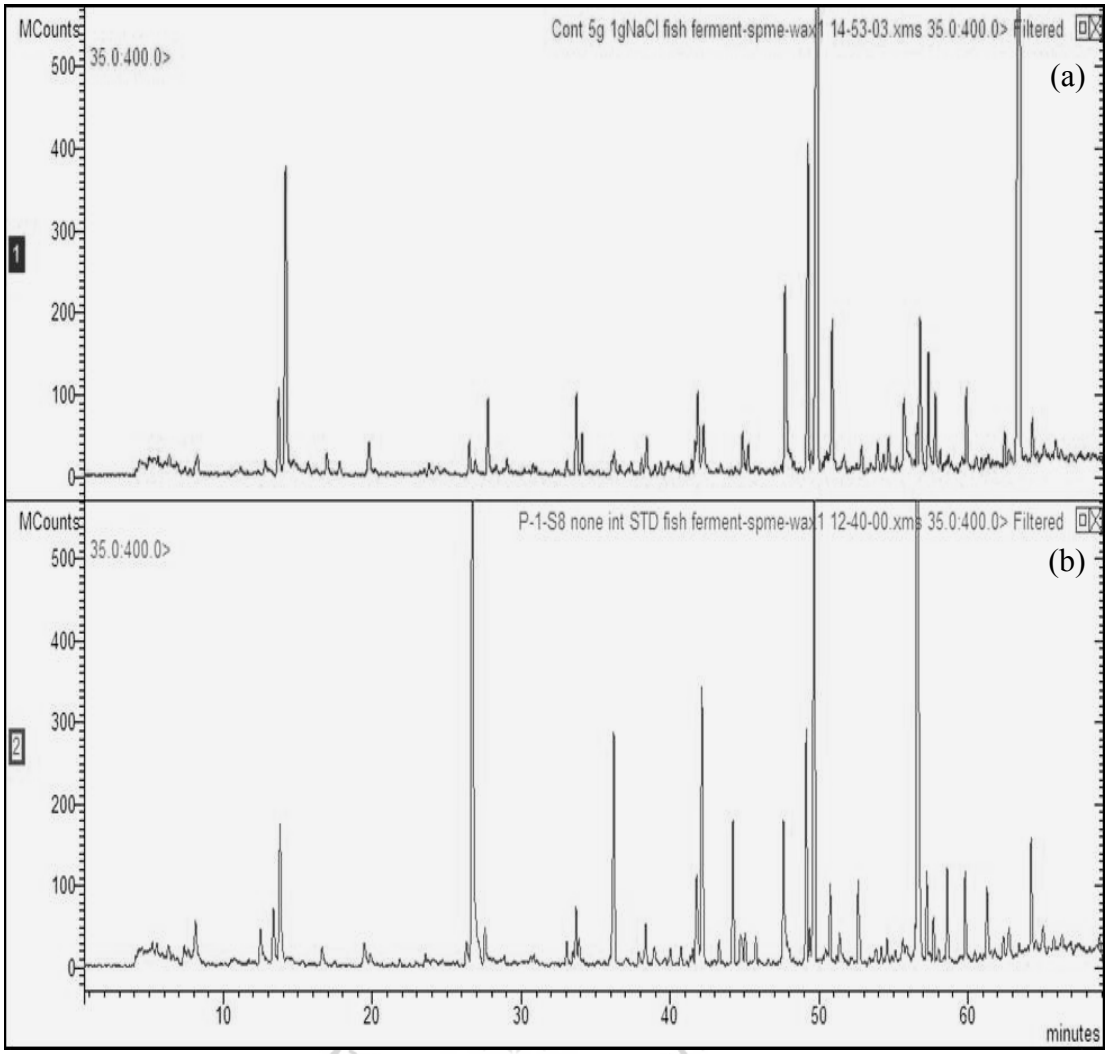


Figure D1 Chromatograms of volatile compounds of control (a) and laboratory scale fish sauce samples inoculated with *Hbt. Salinarium* P-1-S8 (b) fermented for 30 days.

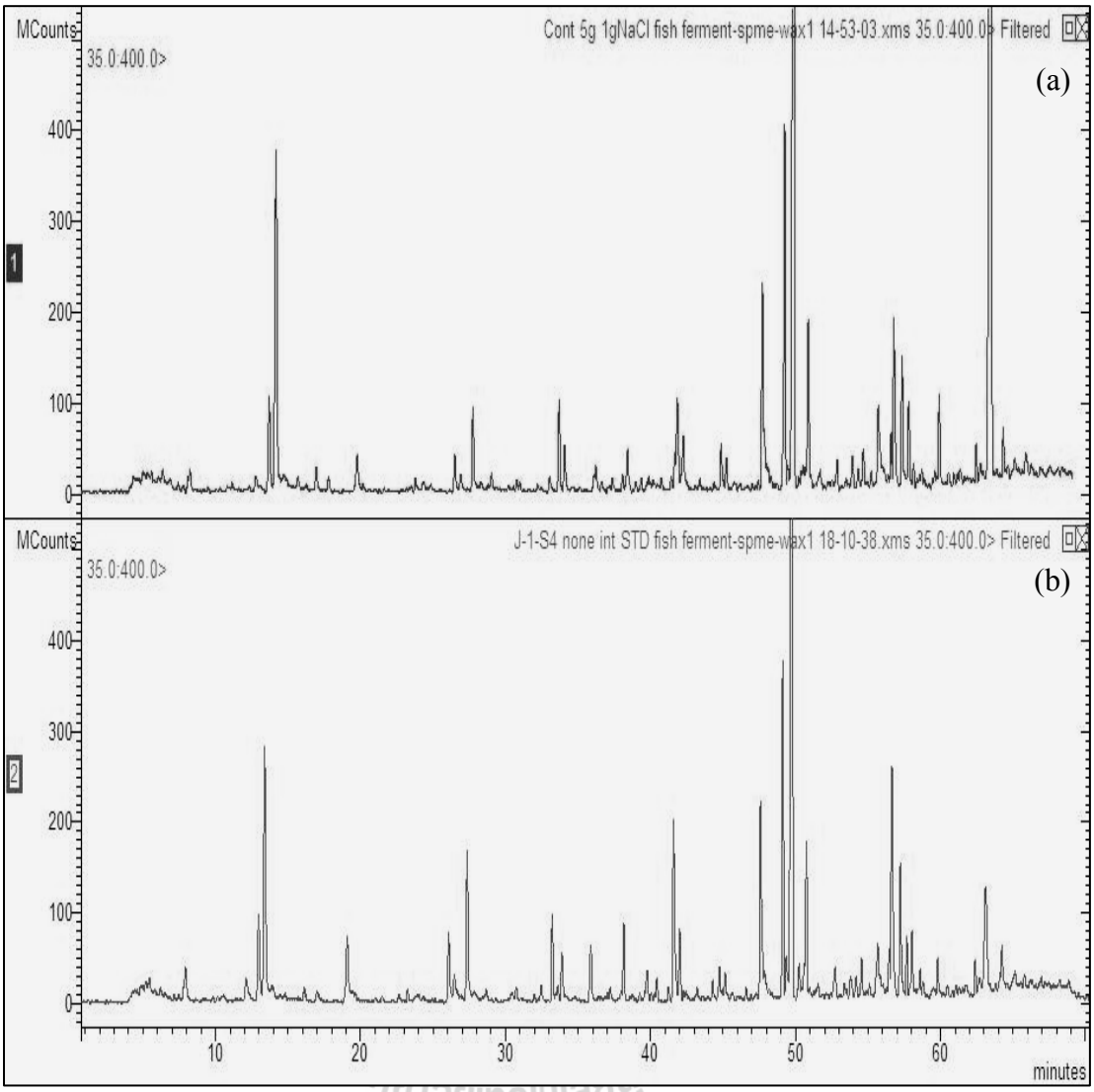


Figure D2 Chromatograms of volatile compounds of control (a) and laboratory scale fish sauce samples inoculated with *Hbt. Salinarium* J-1-S4 (b) fermented for 30 days.

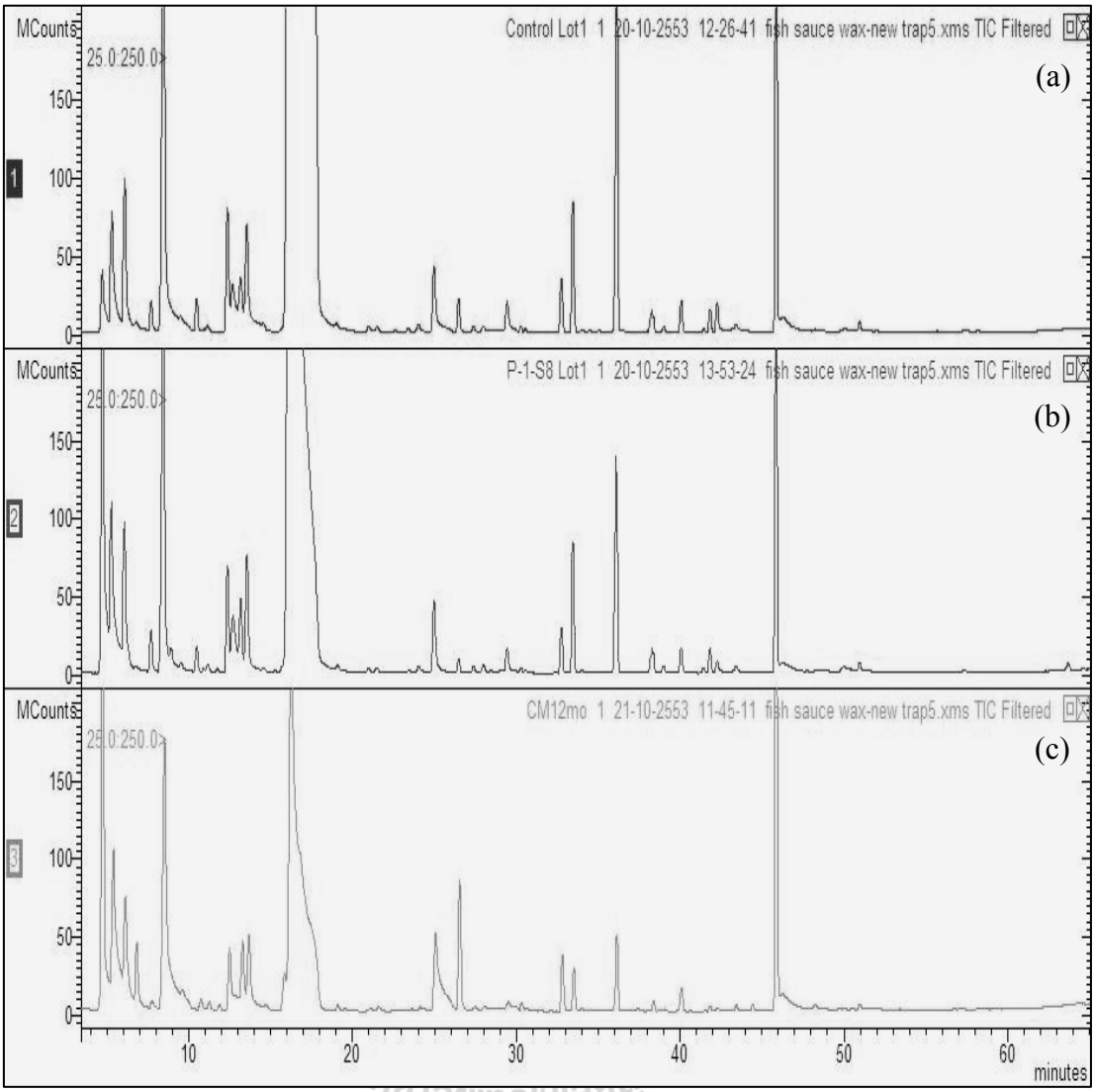


Figure D3 Chromatograms of volatile compounds of control (a), fish sauce sample inoculated with *Hbt. Salinarium* P-1-S8 (b) fermented for 180 days, and commercial fish sauce (c).

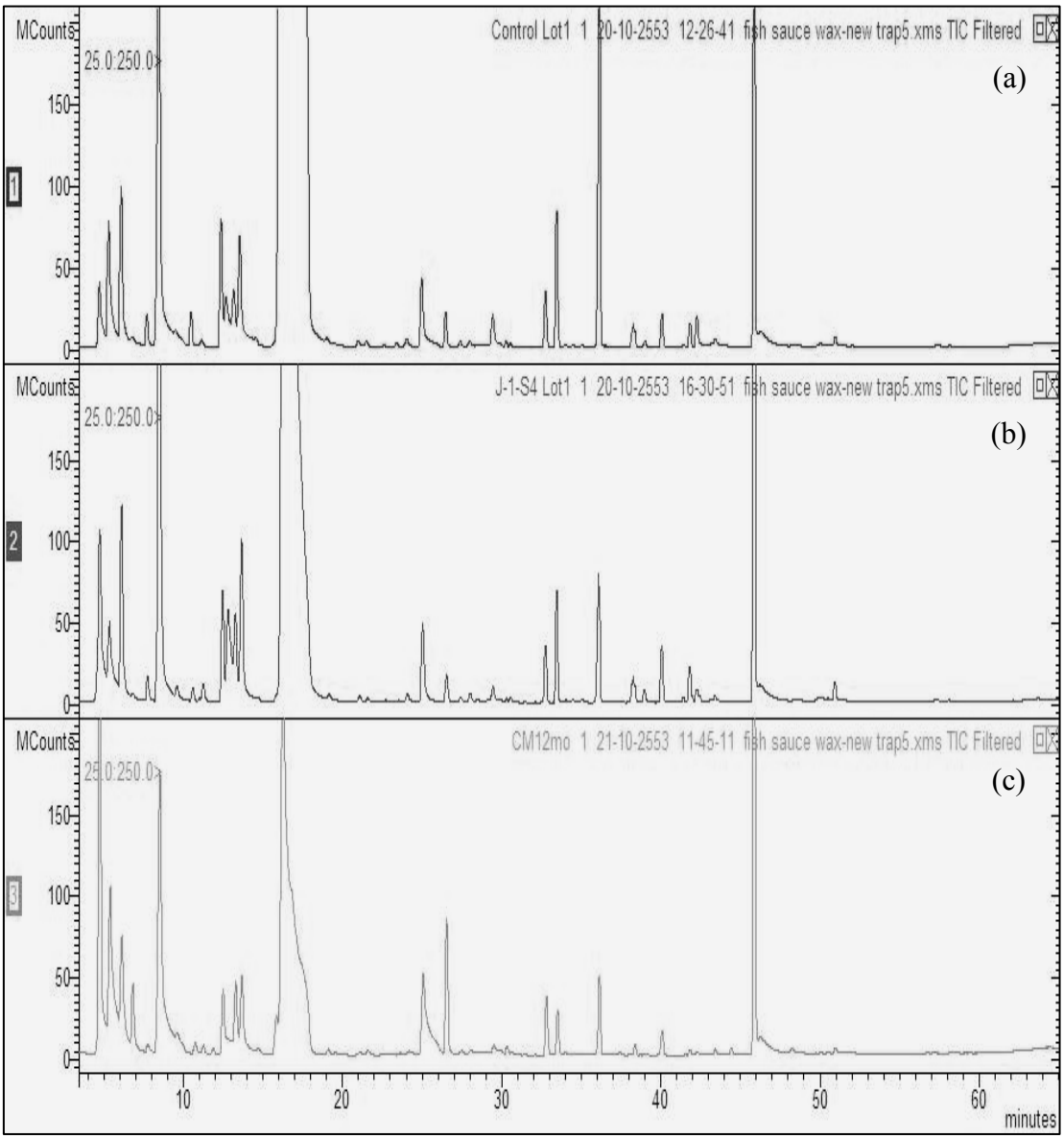


Figure D4 Chromatograms of volatile compounds of control (a), fish sauce sample inoculated with *Hbt. Salinarium* J-1-S4 (b) fermented for 180 days, and commercial fish sauce (c).

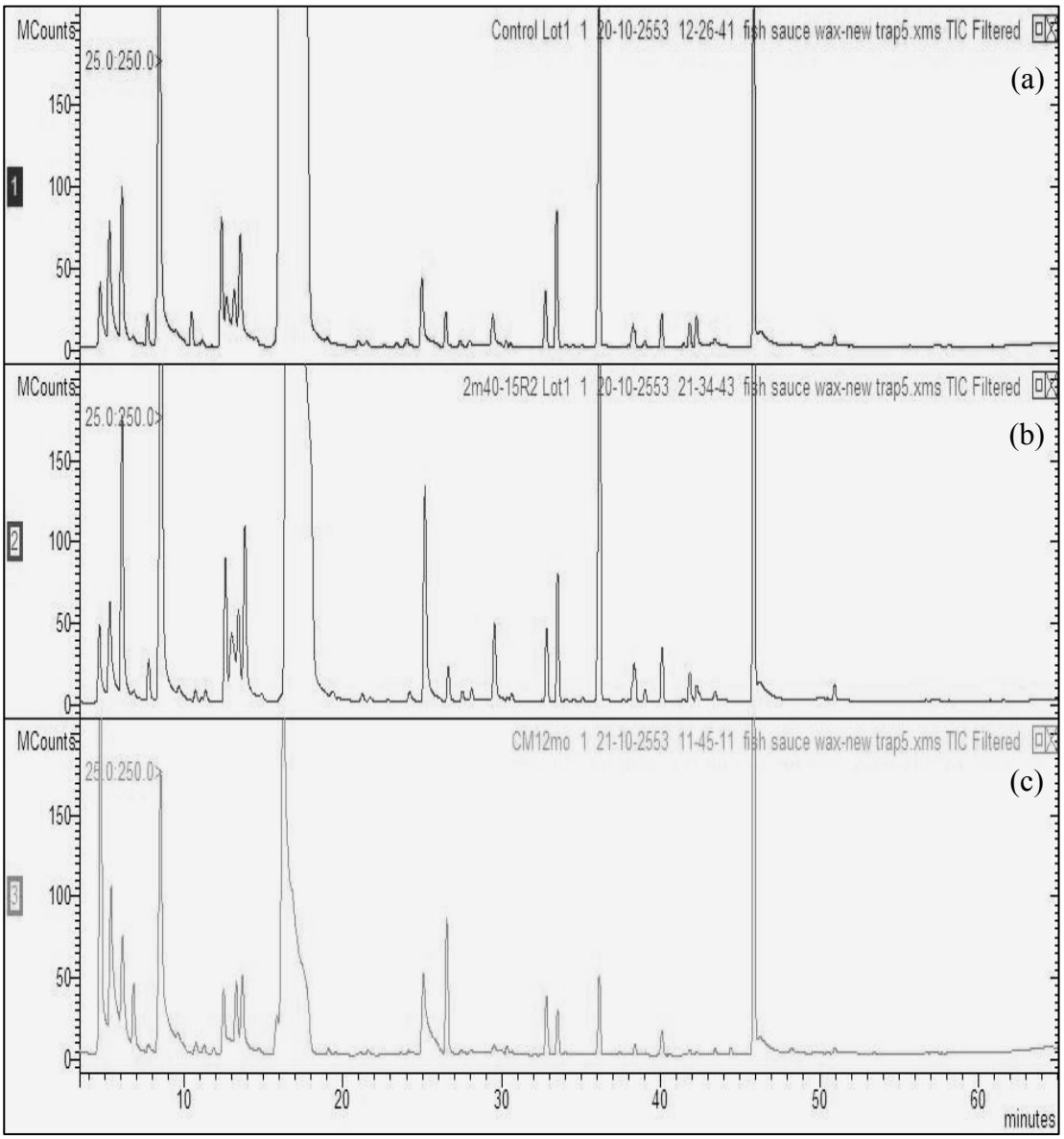


Figure D5 Chromatograms of volatile compounds of control (a), fish sauce sample inoculated with *Hbt. Salinarium* 2m-40-15-R2 (b) fermented for 180 days, and commercial fish sauce (c).

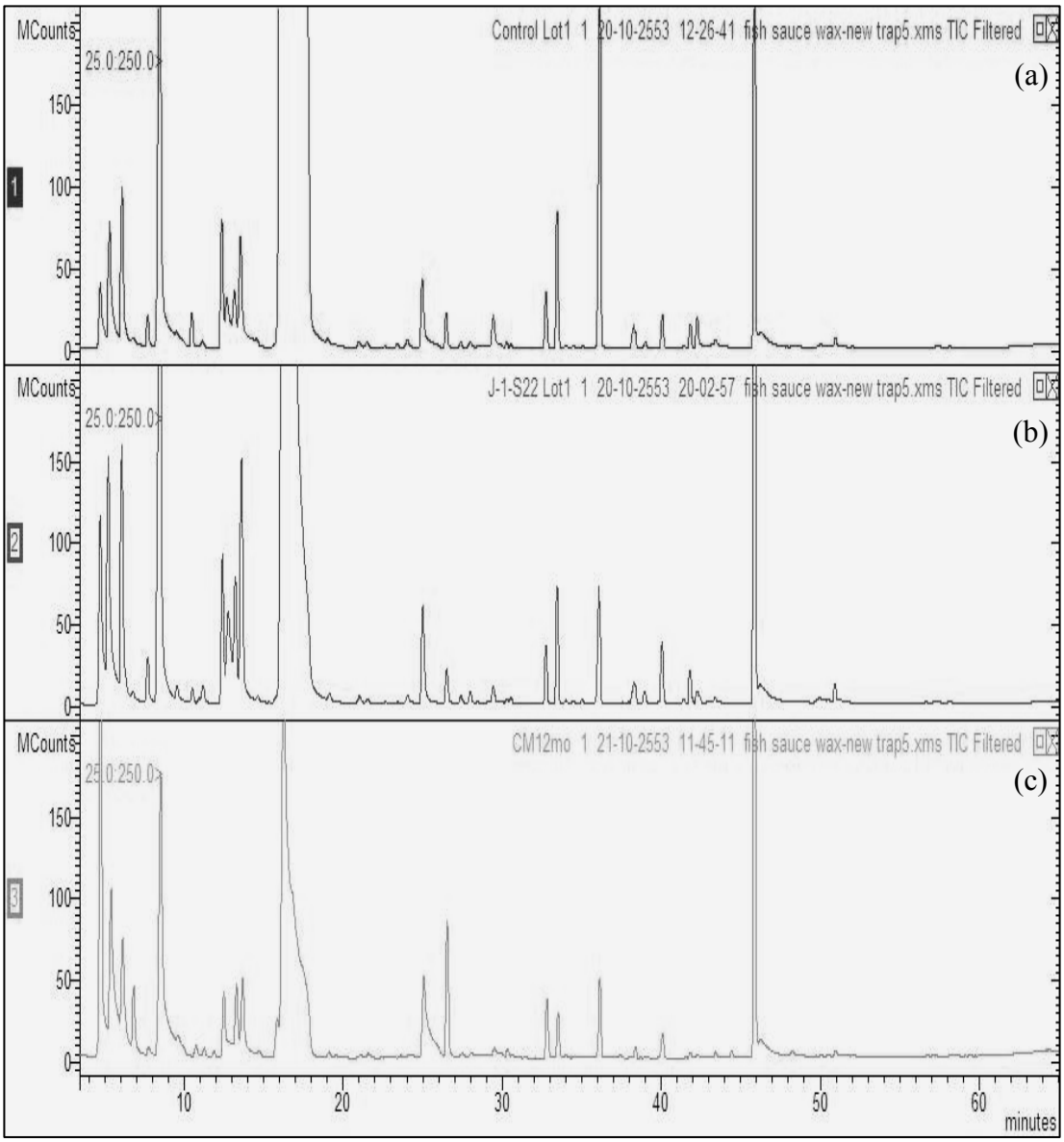


Figure D6 Chromatograms of volatile compounds of control (a), fish sauce sample inoculated with *Hbt. Salinarium* J-1-S22 (b) fermented for 180 days, and commercial fish sauce (c).

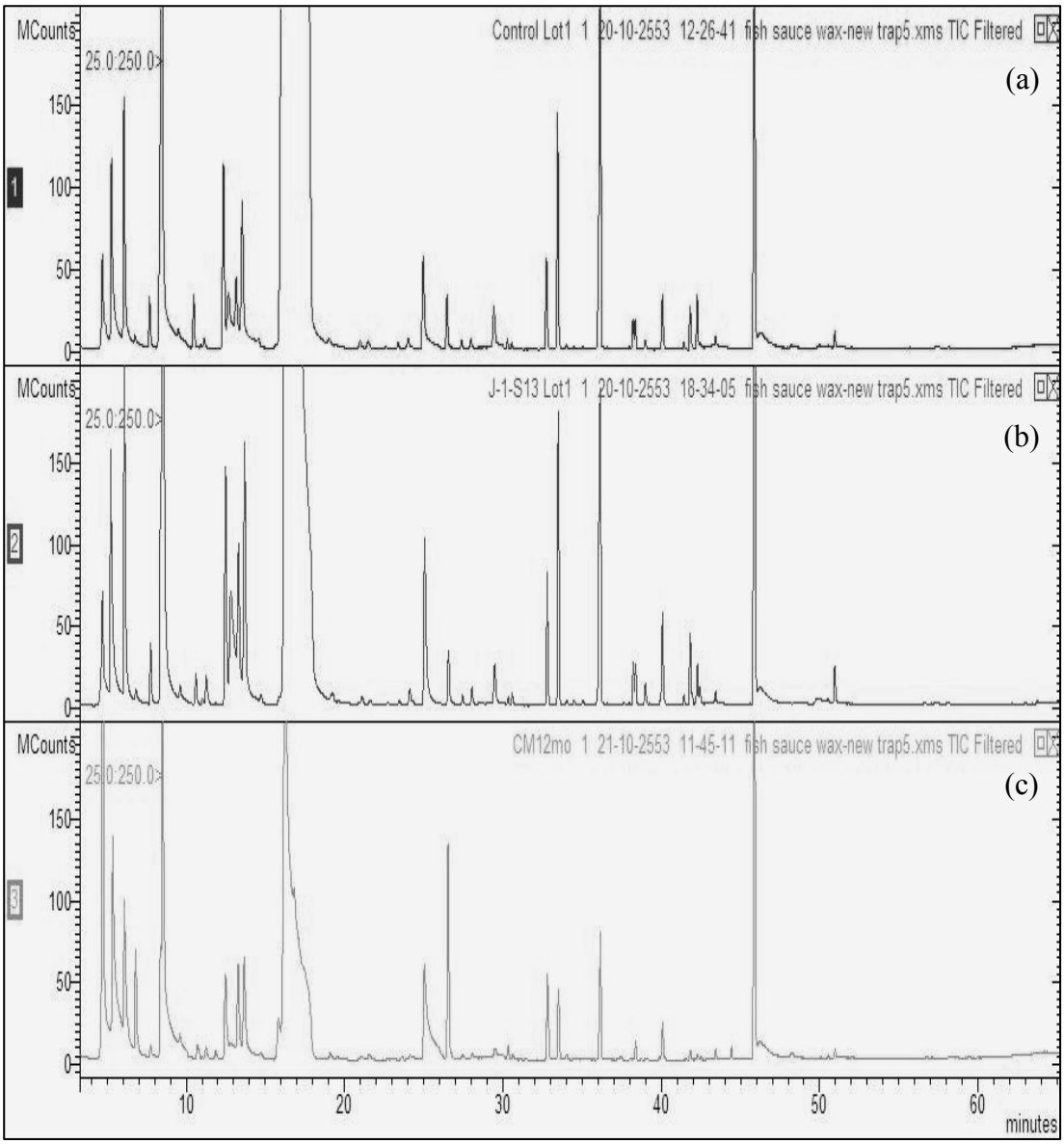


Figure D7 Chromatograms of volatile compounds of control (a), fish sauce sample inoculated with *Hbt. Salinarium* J-1-S13 (b) fermented for 180 days, and commercial fish sauce (c).

APPENDIX E

Supplementary results for chapter IV

Table E1 Changes of extremely halophilic bacterial counts at varied α-amino group contents (supplementary results for 4.2.1).

α-Amino group content (mM)	Bacterial counts (Log CFU/ml)			
	Isolate P-1-S8		Isolate J-1-S4	
	0 day	7 day	0 day	7 day
1314±37.26	4.96	nd	5.31	nd
745.99±46.66	6.93	nd	6.56	nd
519.45±23.00	6.68	5.24	6.75	4.82
239.76±4.92	5.69	9.88	5.48	7.31
113.28±4.63	5.91	9.15	5.19	7.54

nd, Not detected; commercial fish sauce fermented conventionally for 12 months was diluted, inoculated with extremely halophilic bacterial isolates and incubated at 37^{o}C for 7 days.

Table E2 Changes of extremely halophilic bacterial counts and α-amino contents of anchovies mixed with 25% NaCl inoculated with extremely halophilic bacterial isolates and incubated at 35°C for 30 days (supplementary results for Figure 4.2).

Sample code	Bacterial count (Log CFU/g)			α-Amino group content (mM)		
	Fermentation time (day)					
	0	14	30	0	14	30
Control I	nd	nd	nd	256.79±21.53	573.08±17.23	716.31±13.85
P-1-S8	5.88	5.75	3.29	270.28±7.36	585.26±9.51	757.62±36.74
H-1-4	5.79	5.00	nd	258.33±25.90	640.31±37.44	825.97±30.30
H-3-16	6.25	5.35	3.15	276.06±9.54	542.41±36.84	756.55±18.97
J-1-5/O	6.02	5.23	2.17	277.99±6.81	669.72±31.49	740.79±1.51
J-1-10	6.06	5.36	3.03	270.28±8.45	671.40±31.49	854.30±15.54
J-1-S4	5.15	5.22	nd	265.65±1.36	547.45±40.41	760.39±23.19
J-1-S6	5.43	3.87	1.61	258.52±15.81	706.69±39.81	787.00±6.63
J-1-S13	6.12	5.16	3.16	260.64±17.72	676.86±51.40	806.60±77.10
J-1-S31	5.77	3.49	nd	269.70±6.54	786.94±12.78	788.49±3.31
J-771-8	6.87	4.27	1.53	252.93±20.99	681.06±73.38	762.73±23.49
J-771-S6	6.51	4.21	1.21	263.92±1.09	729.38±38.33	756.34±17.47
2m-40-15-R2	5.25	4.33	nd	261.03±18.81	618.88±57.34	828.95±30.90
Control II	0.00	0.00	nd	179.74±11.45	623.19±68.02	655.31±22.00
H-1-4	7.34	3.67	nd	214.43±15.27	652.16±27.85	669.00±13.79
H-771-7	7.07	4.80	2.55	245.27±35.98	767.63±60.64	669.53±12.28
J-1-8	7.60	5.48	2.17	293.85±79.60	718.85±14.76	653.04±60.34
J-1-S3	7.43	4.57	1.59	185.13±26.71	826.61±20.98	631.47±13.22
J-1-S22	8.02	3.86	3.74	191.69±11.72	724.75±80.21	590.24±24.08
J-1-S27	7.55	4.97	0.61	188.99±25.35	622.89±18.29	606.83±26.16
J-1-S28	7.71	5.22	nd	189.37±11.18	722.71±35.61	676.34±95.94
J-1-S32	7.84	6.08	3.54	189.37±15.54	587.73±4.49	631.47±41.93
J-771-4	7.25	4.38	3.87	218.67±3.82	561.64±47.16	746.32±65.35
J-771-6	7.90	5.43	nd	204.02±15.81	645.12±74.11	677.88±46.74
J-771-S3	7.19	3.61	4.33	176.27±5.18	622.89±27.27	753.60±13.88
G4	6.28	5.07	3.71	267.77±10.90	634.00±17.53	752.51±10.84

nd, Not detected

Table E3 Volatile compounds of anchovies mixed with 25% NaCl inoculated with extremely halophilic bacterial isolates and incubated at 35°C for 30 days (supplementary result for 4.2.3).

Sample related to bacterial code	Volatile compounds (% of total)								
	2-methyl butanal	**3 methyl butanal**	**dimethyl disulfide**	**hexanol**	**1-butanol**	**2,6 dimethyl pyrazine**	**dimethyl trisulfide**	**benzaldehyde**	**3-methyl butanoic acid**
Control1	1.369	5.941	0.190	1.167	0.145	0.905	nd	2.832	1.613
P-1-S8	0.096	1.646	11.837	0.669	0.121	1.027	2.852	0.977	30.693
H-1-4	0.643	0.738	1.668	1.490	0.401	0.313	nd	1.463	6.956
H-3-16	0.127	0.799	15.216	1.044	0.447	2.721	3.760	2.103	15.437
J-1-5	1.107	5.239	6.496	1.177	0.434	5.947	1.156	1.840	16.230
J-1-10	0.725	0.889	13.613	1.461	0.292	4.764	2.923	2.330	13.128
J-1-S4	1.655	5.518	0.544	2.524	0.211	0.962	0.382	2.923	4.831
J-1-S6	0.790	2.562	8.555	2.670	0.237	3.979	1.788	2.661	8.817
J-1-S13	0.715	1.863	4.974	1.524	0.224	4.497	1.567	4.050	8.934
J-1-S31	0.565	0.961	5.478	2.231	0.238	3.623	1.937	4.315	11.360
J-771-8	0.816	0.743	11.777	1.100	0.338	0.865	5.194	1.436	15.478
J-771-S6	0.446	1.602	10.481	1.401	0.271	2.638	3.316	2.675	10.562
2m40-15R2	1.647	4.673	0.140	4.153	0.320	1.094	0.180	5.461	3.332
G4	0.765	0.938	7.752	2.676	0.272	0.395	2.130	5.309	8.297
Control2	1.859	7.456	0.100	2.350	nd	nd	nd	0.773	1.852
H-1-S4	0.944	2.302	3.498	1.819	0.000	1.536	0.964	1.302	6.657
H-771-7	0.718	2.315	6.561	1.395	0.394	0.851	0.709	1.131	3.218

nd, Not detected

Table E3 (Continued)

Sample related to bacterial code	Volatile compounds (% of total)								
	2-methyl butanal	**3 methyl butanal**	**dimethyl disulfide**	**hexanol**	**1-butanol**	**2,6 dimethyl pyrazine**	**dimethyl trisulfide**	**benzaldehyde**	**3-methyl butanoic acid**
J-1-8	0.676	1.481	8.808	0.548	0.263	2.073	2.356	0.766	6.579
J-1-S3	0.628	2.226	1.118	0.986	0.456	1.002	0.091	0.394	17.899
J-1-S22	1.106	2.759	0.503	2.526	0.646	1.486	nd	1.018	6.501
J-1-S27	0.274	0.945	3.244	1.457	0.291	1.555	0.971	1.028	9.482
J-1-S28	0.321	1.124	3.267	1.614	nd	1.230	1.119	1.693	5.360
J-1-S32	0.122	0.169	6.820	1.279	nd	0.990	1.861	1.538	6.660
J-771-4	nd	0.968	4.806	0.871	nd	nd	1.569	0.557	5.816
J-771-6	0.187	0.220	2.582	0.533	0.151	1.496	1.128	1.353	5.360
J-771-S3	0.109	nd	2.505	1.050	0.190	0.717	0.945	1.354	3.925

nd, Not detecte

BIOGRAPHY

Ms. Sirinya Pongjanla was born in May 31, 1984 in Nakhonratchasima. She received Bachelor Degree in B.Sc. (Biotechnology) from Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand in 2006. Part of her master thesis work was presented as a poster presentation at Food Innovation Asia Conference, June 17-18, 2010, BITEC, Bangkok, Thailand and poster presentation at The 4th Internation on Fermentation Technology for Value Added Agricultural Products with Joint Sessions from Asia Core Program, August 29-31, 2011, Kosa Hotel, Khon Kaen, Thailand. She reseived a scholarship for her master program from National Science and Technology Development Agency (NSTDA) under the grant No. BT-B-01-FT-19-5014.