LACTIC ACID BACTERIA AND THE DEVELOPMENT OF FLAVOR IN FISH SAUCE

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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LACTIC ACID BACTERIA AND THE DEVELOPMENT OF FLAVOR IN FISH SAUCE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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น้ำปลาได้จากกระบวนการหมักที่มีแบคทีเรียหลายกลุ่มเกี่ยวข้องต่อผลิตภัณฑ์และกลิ่นรส จากการศึกษาแบคทีเรียกรดแล็กติกที่คัดแยกได้จากกระบวนการหมักน้ำปลาระดับการค้า จำนวน 288 ไอโซเลต พบว่ามี 38 ไอโซเลต ที่สามารถสร้างสารกลิ่นรสในอาหารเหลวประกอบด้วยกลูโคส สารสกัดจากยีสต์ และทริปโตน ที่เติมเกลือโซเดียมคลอไรด์และลิวซีนความเข้มข้นร้อยละ 5 และ 1 ตามลำดับ เมื่อคัดเลือกแบคทีเรียกรดแล็กติกจำนวน 4 ไอโซเลต คือ 3MC10-11, 3MR10-3, 6MR10-7 และ PMC-11-5 จากความสามารถในการเจริญที่ความเข้มข้นเกลือสูงและไม่สร้างสารไบโอจีนิกเอมีนหรือสร้างในปริมาณต่ำ ที่เมื่อระบุชนิดของแบคทีเรียด้วยลักษณะสัณฐาน สรีรวิทยา ลำดับนิวคลีโอไทด์ของ 16S rRNA gene และแบบแผนแถบดีเอ็นเอที่วิเคราะห์ด้วยเทคนิค Restriction fragment length polymorphism ทั้ง 4 ไอโซเลต จัดเป็นชนิด *Tetragenococcus muriaticus* ที่ต่างสายพันธุ์กัน เมื่อเตรียมการหมักน้ำปลาจากปลากะตักสดผ่านการย่อยและไม่ผ่านการย่อยด้วยเอนไซม์เปรียบเทียบกัน ในห้องปฏิบัติการทำนองเดียวกับการหมักแบบดั้งเดิม โดยมีชุดควบคุม (ไม่เติมกล้าเชื้อ) และชุดเติมกล้าเชื้อแต่ละเชื้อแยกกันคือไอโซเลต 3MC10-11, 3MR10-3, 6MR10-7 และ PMC-11-5 ที่มีจำนวนเริ่มต้น 7 Log CFU/มิลลิลิตร ปริมาณร้อยละ 10 ปริมาตรต่อน้ำหนัก บ่มที่ 35 องศาเซลเซียส เป็นเวลา 240 วัน พบว่าชุดการหมักที่เติมกล้าเชื้อทั้ง 4 ไอโซเลต จากที่มีจำนวนแบคทีเรียกรดแล็กติกในวันเริ่มต้นเท่ากันโดยเฉลี่ย 5 Log CFU/กรัม เมื่อหมักได้ 180 วัน ในตัวอย่างหมักจากปลากะตักสดผ่านการย่อยด้วยเอนไซม์ ยังคงมีจำนวนคงที่ 5 Log CFU/กรัม แต่ในตัวอย่างหมักจากปลากะตักสดไม่ผ่านการย่อยด้วยเอนไซม์พบ 3, 5, 4 และ 5 Log CFU/กรัม ตามลำดับ เป็นจำนวนที่ค่อนข้างลดลง ขณะที่ชุดควบคุมที่เตรียมจากปลากะตักสดผ่านและไม่ผ่านการย่อยด้วยเอนไซม์ ไม่พบแบคทีเรียกรดแล็กติกและพบจำนวน 4 Log CFU/กรัม ตามลำดับ ในวันเริ่มต้นหมักของตัวอย่าง แต่ที่ 180 วันของการหมัก พบจำนวน 6 และ 3 Log CFU/กรัม ตามลำดับ และไม่พบแบคทีเรียกรดแล็กติกในทุกชุดของการทดลองเมื่อหมักถึง 240 วัน ในตัวอย่างหมักที่ 240 วัน เมื่อใช้ปลากะตักสดผ่านการย่อยด้วยเอนไซม์พบว่าชุดเติมกล้าเชื้อ 4 ไอโซเลต มีปริมาณแอลฟาอะมิโน 770, 763, 839 และ 850 มิลลิโมลาร์ โอลิโกเพปไทด์ 31, 19, 22 และ 18 มิลลิโมลาร์ ไนโตรเจนทั้งหมดร้อยละ 1.59, 1.70, 1.69 และ 1.71 ตามลำดับ และพบสารระเหยเด่น เบนซัลดีไฮด์และกรดบิวทาโนอิก (ให้กลิ่นอัลมอนด์) และกรด 3-เมธิลบิวทาโนอิก (ให้กลิ่นเนยแข็ง) ขณะที่ชุดควบคุมมีปริมาณแอลฟาอะมิโน 763 มิลลิโมลาร์ โอลิโกเพปไทด์ 21 มิลลิโมลาร์

ไนโตรเจนทั้งหมดร้อยละ 1.63 และพบสารระเหยเด่น ไดเมทิล ไตรซัลไฟด์ซึ่งเป็นสาเหตุของกลิ่นอุจจาระ สำหรับตัวอย่างหมักจากปลากะตักสดไม่ผ่านการย่อยด้วยเอนไซม์ที่หมักได้ 240 วัน พบว่าชุดเติมกล้าเชื้อทั้ง 4 ไอโซเลต มีปริมาณแอลฟาอะมิโน 810, 821, 852 และ 958 มิลลิโมลาร์ โอลิโกเพปไทด์ 20, 21, 17 และ 16 มิลลิโมลาร์ ไนโตรเจนทั้งหมดร้อยละ 1.58, 1.65, 1.62 และ 1.68 ตามลำดับ และพบสารระเหยเด่น เบนซัลดีไฮด์และกรดบิวทาโนอิก และกรด 3-เมธิลบิวทาโนอิก แบคทีเรียสายพันธุ์ PMC-11-5 มีความสามารถในการสร้างสารระเหยที่ให้กลิ่นดีที่สุด ขณะที่ชุดควบคุมมีปริมาณแอลฟาอะมิโน 808 มิลลิโมลาร์ โอลิโกเพปไทด์ 17 มิลลิโมลาร์ ไนโตรเจนทั้งหมดร้อยละ 1.62 และพบสารระเหยเด่น ไดเมทิล ไตรซัลไฟด์ ซึ่งเป็นสาเหตุของกลิ่นอุจจาระในปริมาณสูงกว่าตัวอย่างที่เติมกล้าเชื้อทุกตัวอย่าง จากนั้นได้ศึกษาจีโนมฉบับร่างของกล้าเชื้อที่ใช้ทดลองหมักน้ำปลาแล้วได้กลิ่นรสดี 2 สายพันธุ์ คือ 3MR10-3 และ PMC-11-5 ที่ได้ผลจีโนมขนาด 2.0 และ 2.1 เมกะเบส ประกอบด้วยลำดับรหัสพันธุกรรมของโปรตีน 2,252 และ 2,626 ชนิด ตามลำดับ แบคทีเรียทั้งสองสายพันธุ์มีจีนควบคุมไอออนอนินทรีย์ (โพเทสเซียมและโซเดียม) และสารควบคุมความดันออสโมติกสำหรับการปรับตัวในสิ่งแวดล้อมที่มีเกลือสูง และจีนที่เกี่ยวข้องกับการเกิดกลิ่นรสคือ *adh* และ *aldh* การศึกษาครั้งนี้ยังได้ตรวจจับแบคทีเรียในน้ำปลาไทยระดับการค้าระหว่างการหมักช่วง 1-12 เดือน ด้วยวิธี Automated ribosomal intergenic spacer analysis (ARISA) โดยสกัดดีเอ็นเอของแบคทีเรียโดยตรงจากตัวอย่างหมัก ซึ่งพบแอมพลิคอนทั้งสิ้น 232 ขนาด แต่ละขนาดเทียบได้กับแบคทีเรีย 1 สายพันธุ์ ผลของการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene ได้ลำดับเบสสายสั้น (Read) จำนวน 1,864,163 reads ซึ่งอยู่ในช่วง 23,642 ถึง 87,579 ในแต่ละตัวอย่างน้ำปลา และตรวจพบแบคทีเรียในสกุล *Tetragenococcus* ตลอดกระบวนการหมัก 12 เดือน จากผลการศึกษานี้แสดงถึงแนวโน้มในการใช้ประโยชน์แบคทีเรีย *Tetragenococcus muriaticus* ทั้ง 2 สายพันธุ์ (3MR10-3 และ PMC-11-5) เป็นกล้าเชื้อในกระบวนการหมักเพื่อให้กลิ่นรสที่ดีในน้ำปลา

สาขาวิชาจุลชีววิทยา
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

CHOKCHAI CHUEA-NONGTHON : LACTIC ACID BACTERIA AND THE DEVELOPMENT OF FLAVOR IN FISH SAUCE. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 220 PP.

FISH SAUCE/VOLATILE COMPOUNDS/*TETRAGENOCOCCUS MURIATICUS*/ /DRAFT GENOME SEQUENCES/ARISA/BACTERIAL COMMUNITY

Fish sauce is produced from fish fermentation process involving with several bacterial groups developing unique flavor of the product. Thirty eight out of 288 lactic acid bacterial isolates obtained from commercial-scale fish sauce fermentation, were selected after screening their flavor compound production capability in glucose yeast extract tryptone medium containing sodium chloride and L-leucine. Four isolates, 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5, growing well at high salt concentration and lacking in producing biogenic amines, were chosen as starter cultures for investigating flavor development during fish sauce fermentation. These isolates were identified as belonging to different strains of *Tetragenococcus muriaticus* according to their morphological and physiological characteristics, 16S rRNA gene sequences, and restriction fragment length polymorphism patterns. The fermentation of fish sauce was performed in laboratory scale using fresh anchovy compared to proteinase-digested anchovy, without (control) and with inoculating 10% (v/w, approximately 7 Log CFU/ml) of the selected starter cultures at 35°C for 240 days. Lactic acid bacterial counts were rather constant in all inoculated samples and control (using fresh anchovy) at approximately 3-5 Log CFU/g during 180 day fermentation. At 240 day fermentation of both proteinase-digested and fresh anchovy batches, none of lactic acid bacteria was detected in all samples. The inoculated samples contained the average α-amino at concentrations of 770 and 810, 763 and

821, 839 and 852, and 850 and 958 mM; oligopeptides of 31 and 20, 19 and 21, 22 and 17, and 18 and 16 mM; and total nitrogen of 1.59 and 1.58, 1.70 and 1.65, 1.69 and 1.62, and 1.71 and 1.68%, respectively, whereas controls contained α-amino (763 and 808 mM), oligopeptides (21 and 17 mM), and total nitrogen (1.63 and 1.62%, respectively). Major volatile compounds found in all inoculated samples were benzaldehyde, and butanoic and 3-methylbutanoic acids, contributing to almond and cheesy notes respectively. PMC-11-5 performed the highest volatile compound amounts. Sulfur-containing compounds contributing to fecal note, were easily detected in all control samples. Strains 3MR10-3 and PMC-11-5 were then chosen for draft genome analysis, and the genome sizes of 2.0 and 2.1 Mbp consisting of 2,252 and 2,626 protein coding sequences, respectively, were achieved. These strains contained genes for regulation of inorganic ions (potassium and sodium) and osmoprotectants for adaptation to high saline environments, and genes *adh* and *aldh* involving flavor formation. Bacterial communities in commercial fish sauce samples at 1-12 month fermentation were detected using the automated ribosomal intergenic spacer analysis (ARISA). A total of 232 amplicon lengths (ALs) representing 232 bacterial species, were detected. 16S rRNA gene sequences showed 1,864,163 reads in the range of 23,642 to 87,579 per sample. *Tetragenococcus* was found throughout 12 month fermentation. Results from this study reveal the potential application of the selected strains as starter cultures for development of desirable flavor in fish sauce.

School of Microbiology

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LIST OF ABBREVIATIONS

α	Alpha
ALs	Amplicon lengths
ASAP	A systematic annotation package for community analysis of genomes
ARISA	Automated ribosomal intergenic spacer analysis
bp	Base pair
BLAST	Basic local alignment search tool
CFU	Colony forming unit
ºC	Degree Celsius
cm	Centrimeter
MRS	De Man, Rogosa and Sharpe medium
dNTP	Deoxynucleotide triphosphate
DNA	Deoxythymidine triphosphate
et al.	et alia (and others)
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
GYT	Glucose yeast extract tryptone
(m, μ) g	(milli, micro) Gram
HPLC	High pressure liquid chromatography
HCl	Hydrochloric acid

LIST OF ABBREVIATIONS (Continued)

ITS	Internal transcribed spacer
IMG	Integrated microbial genomes
LAB	Lactic acid bacteria
l	Liter
µl	Microliter
µm	Micrometer
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
ng	Nanogram
NCBI	National Center for Biotechnological Information
%	Percentage
PGM	Personal Genome Machine
pM /pmol	Picomolar
PCA	Principal component analysis
PCR	Polymerase chain reaction
RAST	Rapid annotation using subsystem technology

LIST OF ABBREVIATIONS (Continued)

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transport ribonucleic acid
TNBS	Trinitrobenzenesulfonic acid
UV	Ultraviolet
U.S.A.	United States of America

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce produced from a mixture of fish and salt, is a clear brown liquid seasoning with distinct aroma and flavor (Shimoda, Peralta, and Osajima, 1996). It is mainly produced and consumed in Southeast Asian countries, and gained popularity worldwide (Fukami, Funatsu, Kawasaki, and Watabe, 2004). Due to its favorable taste, it has been used as flavoring agent in various food preparations (Kilinc, Cakli, Tolasa, and Dincer, 2006). Fish sauce has a characteristic aroma which often serves as an indicator to measure the quality of the sauce. Thai fish sauce or "nam-pla", one of the popular fish sauce in the world market, is manufactured through a natural fermentation of several varieties of fish, particularly anchovies (*Stolephorus* spp.), mackerel (*Ristrelliger* spp.), and herring (*Clupea* spp.), at high salt content of 28% to 30% (Lopetcharat and Park, 2002; Yongsawatdigul, Rodtong, and Raksakulthai, 2007). The fermentation is basically carried out in an underground concrete tank at ambient temperature, which considered being a traditional process with a long period of fermentation time, approximately 12-18 months, and uncontrolled products. A number of bacteria have been reported to involve in fish sauce fermentation, such as some species in genera *Halobacterium*, *Corynebacterium* (Thongthai and Suntinanalert, 1991), *Achromobacter*, *Flavobacterium*, *Proteus*, *Halococcus*, *Sarcina* (Saisithi, 1994), *Streptococcus*, *Micrococcus*, *Pediococcus*, *Enterobacter*,

Lactobacillus (Ijong and Ohta, 1996), *Halobacillus* (Chaiyanan *et al*., 1999), *Pseudomonas* (Vihelmsson, Hafsteinwsson, and Kristjansson, 2001), *Staphylococcus* (Fukami *et al*., 2004), *Bacillus* (Uchida *et al*., 2004), *Filobacillus* (Hiraga *et al*., 2005), and *Virgibacillus* (Sinsuwan, Rodtong, and Yongsawatdigul, 2007; 2008). Lactic acid bacteria have been reported to influence flavor of fish sauce. Some lactic acid bacterial strains could be used as starter cultures for fish sauce production. Two species of halophilic lactic acid bacteria, *Tetragenococcus halophilus* and *T. muriaticus*, were found during Thai fish sauce fermentation (Thongsanit, Tanasupawat, Keeratipibul, and Jatikavanich, 2002). It is likely that *Tetragenococcus* found in fish sauce could play a significant role in flavor formation during fermentation. Selected *T. halophilus* strains isolated from Thai fish sauce could improve flavor characteristics in the fish sauce (Udomsil, 2008).

During fish sauce fermentation, fish proteins are hydrolyzed by endogenous proteases in fish muscle and digestive tract as well as exogenous proteases produced by halophilic bacteria (Dissaraphong, Benjakul, Visessanguan, and Kishimura, 2006). The proteolysis of the proteins leads to small peptides and amino acids, which increase gradually until at about 12 months of fermentation. Accumulation of amino acids and peptides during fermentation has a considerable effect on flavor of fish sauce (Peralta, Shimoda, and Osajima, 1996). It has been reported that three distinctive notes contributed to the odor of fish sauce; ammoniacal, cheesy, and meaty notes (Dougan and Haward, 1975). The compounds; 2-methyl-1-propanal, 2-methyl-1-butanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)-propanal, and 3-methyl-1-butanoic acid, are principal contributors to the distinctive odor of fish sauce (Fukami *et al*., 2002). 2-Methyl-1-butanal, which converted from leucine, is

reported to be responsible for a meaty note (Fukami *et al*., 2002) and perceived as malty, chocolate-like (Smit, Engels, and Smit, 2009). In this study, lactic acid bacteria isolated from fish sauce at various fermentation periods were screened for their production of volatile compounds involving fish sauce flavor, and selected for investigating flavor development during fish sauce fermentation. Draft genomes of selected lactic acid bacterial strains involving unique flavor characteristics of Thai fish sauce was analyzed, and ARISA and 16S rRNA gene sequencing was used to detect and investigate the bacterial succession in fish sauce during fermentation.

1.2 Research objectives

1. To select and characterize lactic acid bacteria found from fish sauce fermentation process and involved in unique flavor characteristics of Thai fish sauce.
2. To investigate volatile compounds produced by the selected dominant strains.
3. To study the development of flavor in Thai fish sauce by the selected bacterial strains.
4. To detect bacteria in fish sauce samples obtained from the commercial production plant during fermentation at 1-12 months using automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene sequencing.

1.3 Research hypotheses

Specific compounds influence flavor development in fish sauce could be produced by some lactic acid bacterial strains isolated from fish sauce fermentation process. Selected lactic acid bacterial strains involving unique flavor characteristics of Thai fish sauce may have potential for applying as starter cultures in the manufacture of fish sauce. Genes involving flavor formation in Thai fish sauce could be detected. Both cultured and non-cultured bacteria could be identified in fish sauce during fermentation.

1.4 Scope and limitations of the study

Lactic acid bacteria isolated from Thai fish sauce fermentation process at 1-12 months of fermentation were collected. The capability of these strains to produce unique volatile compounds in modified complex media was investigated. The strains producing the specific compounds were selected and identified. A couple of strains were chosen for draft genome analysis. Volatile compounds produced by the selected lactic acid bacteria were characterized. The development of flavor compounds by selected lactic acid bacterial strains during fish sauce fermentation was conducted. Samples of fish sauce at 1-12 months of fermentation, which were collected from fish sauce factory in Thailand, were used for the investigation of microbial succession.

1.5 Expected results

Dominant strains of lactic acid bacteria isolated from Thai fish sauce fermentation process and involved in unique flavor characteristics of Thai fish sauce were obtained and identified. Draft genome sequences of a couple of the dominant strains were achieved. The genome sequences can be used to predict flavor formation capability and some other biosynthetic significances in fish sauce. Data of specific compounds produced by the selected dominant strains involving flavor in Thai fish sauce was achieved. The possibility to use selected lactic acid bacterial strains for flavor development in fish sauce can be evaluated. These were beneficial to fish sauce industry. Data of bacteria found in Thai fish sauce during fermentation at 1-12 months was obtained. Bacteria community in fish sauce was detected by automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene sequencing, the rapid molecular methods. This information was beneficial to Thai fish sauce industry research in order to understand microbial ecology during fermentation process.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid bacteria and their principle metabolism

Lactic acid bacteria (LAB) are Gram-positive and non-spore-forming bacteria. They are catalase-negative, devoid of cytochromes, anaerobic, microaerophilic or aero-tolerant, fastidious, acid-tolerant, and strictly fermentative. The bacteria occur as cocci or rods and produce lactic acid as the major end product during sugar fermentation. However, under certain conditions, some lactic acid bacteria do not display all of these characteristics. A key feature of lactic acid bacteria that must be emphasized is the inability to synthesize porphyrin groups. They generally lack catalase and cytochromes when grown in laboratory growth media, which lack heme. Therefore, the lactic acid bacteria do not possess any electron transport chain, and rely on fermentation to generate energy (Axelsson, 2004).

Lactic acid bacteria have been classified in the phylum Firmicutes comprising of 21 genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactococcus*, *Lactosphaera*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 2004). Lactobacillus is the largest genus, including around 80 recognized species. The classification of lactic acid bacteria into different genera is largely based on their cell morphology, mode of glucose fermentation, growth at different temperatures,

configuration of the lactic acid produced, ability to grow at high salt concentration, and acid or alkaline tolerance. Chemotaxonomic markers such as fatty acid composition, constituents of the cell wall, and phylogenetic relationships are also used in classification. All lactic acid bacteria produce lactic acid from hexoses. Lactic acid bacteria have been characterized primarily by their ability to form various isomers of lactic acid from the fermentation of glucose. The ratio of L- to D-lactic acid produced by lactic acid bacteria has been employed for classification of the bacteria (Hammes, Weis, and Holzapfel, 1991). The stereoisomer of lactic acid produced by lactic acid bacteria can be classified into 3 types (L-, DL-, and D-forms). The L-type refers to a content of L-lactic acid that is more than 75%. The DL-type indicates the production of 25-75% L-lactic acid. The D-type is the production of D-lactic acid more than 75% (Otsuka, Okada, Uchimura, and Komagata, 1994). A summary of the differentiation of LAB genera with classical phenotypic tests is shown in Table 2.1.

Table 2. 1 Characteristics of different lactic acid bacterial genera.

	Rods		Cocci							
Characteristics	*Carnob.*[a]	*Lactob.*[a]	*Aeroc.*[a]	*Enteroc.*[a]	*Lactoc.*[a] *Vagoc.*	*Leucon.*[a] *Oenoc.*	*Pedioc.*[a]	*Streptoc.*[a]	*Tetragenoc.*[a]	*Weissella*[b]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[c]	-[d]	±	-	-	-	+	-	-	-	+
Growth at 10°C	+	±	+	+	+	+	±	-	+	+
Growth at 45°C	-	±	-	+	-	-	±	±	-	-
Growth at 6.5% NaCl	ND[e]	±	+	+	-	±	±	-	+	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	±	-	+	±	±	+	-	-	±
Growth at pH 9.6	-	-	+	+	-	-	-	-	+	-
Lactic acid[f]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

+: positive; -: negative; ±: response varies between species; ND: not determined.

[a]: *Lactob.*, *Lactobacillus*; *Leucon.*, *Leuconostoc*; *Pedioc.*, *Pediococcus*; *Streptoc.*, *Streptococcus*; *Aeroc.*, *Aerococcus*; *Carnob.*, *Carnobacterium*; *Enteroc.*, *Enterococcus*; *Lactoc.*, *Lactococcus*; *Oenoc.*, *Oenococcus*; *Tetragenoc.*, *Tetragenococcus*; *Vagoc.*, *Vagococcus*.

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

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

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

[e]: No growth in 8% NaCl has been reported.

[f]: Configuration of lactic acid produced from glucose.

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

Source: Axelsson (2004).

2.1.1 Carbohydrate metabolism

Lactic acid bacteria are obligate fermentors and cannot obtain energy by oxidative or respiratory processes. Since they lack functional heme linked electron transport chains and a functional Krebs cycle, they obtain energy via substrate level phosphorylation (Caplice and Fitzgerald, 1999). Hexose (e.g., glucose) fermentation is divided into two major pathways, homolactic fermentation and heterolactic fermentation. The transport and phosphorylation of glucose take place by transport of free sugar and phosphorylation by an ATP-dependent glucokinase or via the phosphoenolpyruvate phosphotransferase system (PTS) (Axelsson, 2004).

For homofermentative lactic acid bacteria, hexoses are metabolized via glycolysis or Embden-Meyerhof-Parnas pathway (Figure 2.1A). One of the key enzymes of this pathway is aldolase, which commits the glucose to pathway by splitting fructose-1,6-diphosphate into the two trios phosphates; dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) that serve as substrates for ATP-generating reaction. This pathway yields two mol of pyruvate and two mol of ATP per mol of hexose (e.g., glucose). Finally, pyruvate is reduced to L- or D- lactic acid by lactate dehydrogenase (LDH). More than 90% of substrate is converted to lactic acid during homofermentative metabolism (Moat, Foster, and Spector, 2002). In glycolysis, the reduced cofactors NADH are reoxidized to NAD^+ and thus a redox balance is obtained (Axelsson, 2004).

Heterofermentative lactic acid bacteria metabolize hexoses via the 6-phosphogluconate/phosphoketolase pathway (Figure 2.1B). The first phosphorylation step of glucose in this pathway is the same as in glycolysis. The key pathway steps are the dehydrogenation of glucose-6-phosphate to 6-phosphogluconate, its

decarboxylation followed by splitting xylulose-5-phosphate into GAP and acetyl-phosphate by phosphoketolase. Glyceraldehyde-3-phosphate is metabolized to lactic acid via the same pathway as in glycolysis, while acetyl-phosphate is in turn reduced to ethanol via acetyl CoA and acetaldehyde (Axelsson, 2004). Approximately equimolar amounts of lactate, acetate, ethanol and CO_2 are produced, along with only one mol of ATP per hexose (Hutkins, 2006).

Lactic acid bacteria may change their metabolism in response to various conditions, resulting in a different end-product pattern. Under certain circumstances, lactic acid bacteria use alternative ways of utilizing pyruvate than the reduction to lactate. Different species may use different pathways, depending on conditions and enzymatic capacity (Figure 2.2).

The diacetyl/acetoin pathway leads to diacetyl (butter aroma) and acetoin/2, 3-butanediol formation, and is very significant technologically in the fermentation of milk. α-Acetolactate is an intermediate formed from pyruvate by acetolactate synthase. Diacetyl is a non-enzymatically compound formed by chemical decomposition of α-acetolactate. Acetoin and/or 2, 3-butanediol are produced in excess of diacetyl, but do not contribute to the aroma (Axelsson, 2004). Pyruvate oxidase uses oxygen to convert pyruvate into acetyl phosphate, CO_2 and H_2O_2, which occurred in pyruvate oxidase pathway. The enzyme has the main role in the aerobic formation of acetic acid (Axelsson, 2004).

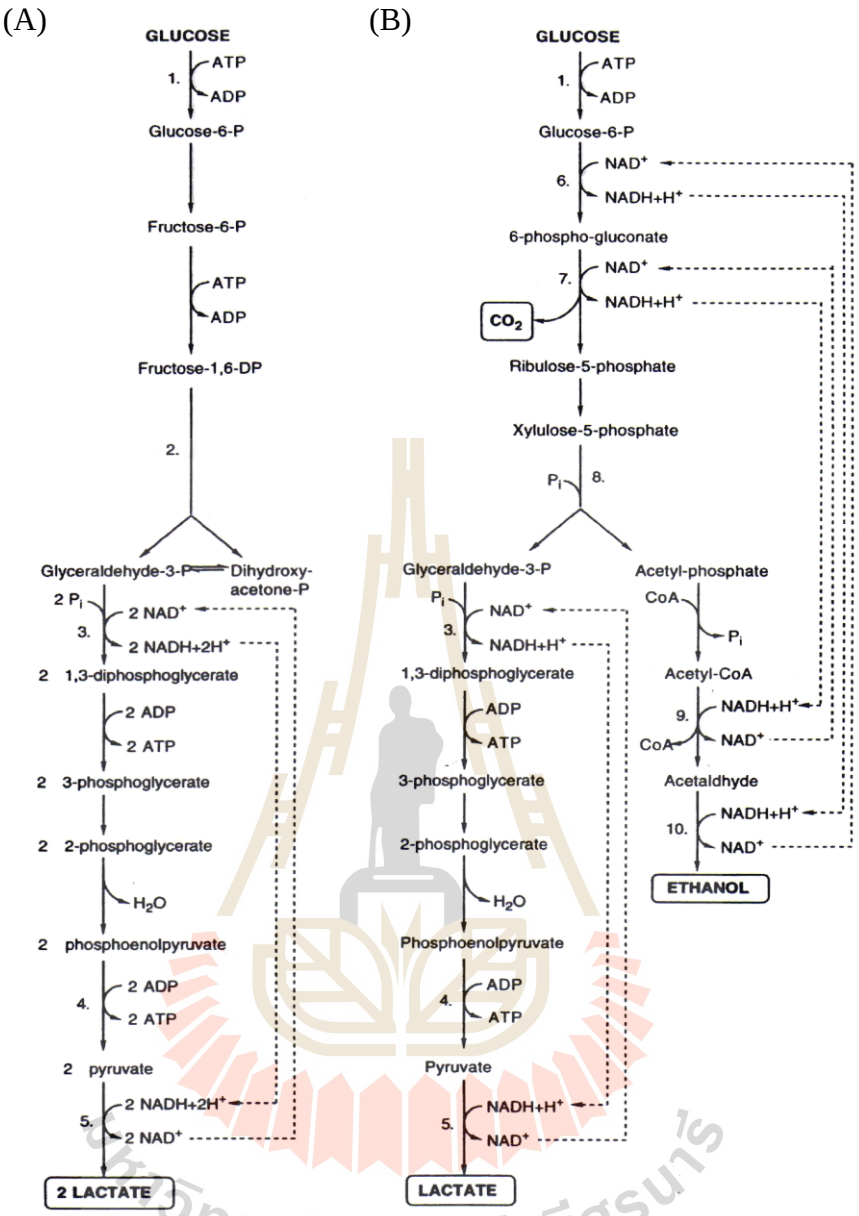

Figure 2.1 Major fermentation pathways of glucose: A, homolactic fermentation (glycolysis pathway); B, heterolactic fermentation (6-phosphorgluconate/phosphoketolase pathway). Selected enzymes are numbered: 1, glucokinase; 2, fructose-1,6-diphosphate aldose; 3, glyceraldehyde-3-phosphate dehydrogenase; 4, pyruvate kinase; 5, lactate dehydrogenase; 6, glucose-6-phosphate dehydrogenase; 7, 6-phosphoglyconate dehydrogenase; 8, phosphoketolase; 9, acetaldehyde dehydrogenase; 10, alcohol dehydrogenase.

Source: Axelsson (2004).

Pyruvate-formate lyase system consists of pyruvate-formate lyase that catalyzes the formation of acetyl CoA and formate from pyruvate and CoA (Figure 2.2). The acetyl CoA can then serve either as an electron acceptor, resulting in ethanol formation, or as a precursor of ATP formation, resulting in acetate. The pyruvate-formate lyase system is oxygen sensitive, and is inactivated in aerobic conditions (Axelsson, 2004). Pyruvate dehydrogenase catalyzes the formation of acetyl CoA under aerobic conditions, and then it can be metabolized further to acetate with the concomitant formation of ATP (Axelsson, 2004).

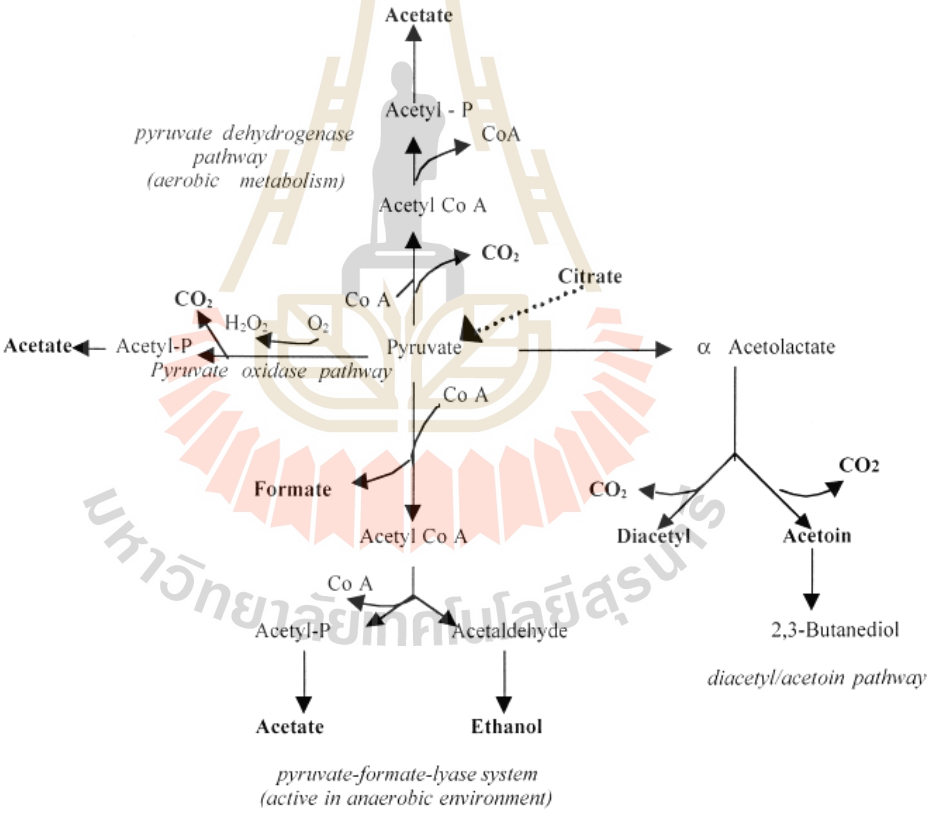

Figure 2.2 Pathways for the alternative fates of pyruvate in lactic acid bacteria. Source: Caplice and-1- Fitzgeralda (1999).

2.1.2 Nitrogen metabolism

Lactic acid bacteria are fastidious microorganisms that require an exogenous source of peptides and amino acids as nitrogen sources through the activities of proteinase which are companied di- and tri-peptide and amino acid transport systems (Axelsson, 2004). Lactococci involved in dairy fermentation is the most extensively studied lactic acid bacteria for proteolytic system, which the milk protein (casein) serves as the primary substrate (Kranenburg *et al*., 2002; Hutkins, 2006).

Degradation of casein by lactic acid bacteria leads to peptides and free amino acids, which can subsequently be taken up by the cells. Casein is degraded by a single cell-wall-bound extracellular proteinase (PrtP) (Smit, Smit, and Engels, 2005) in lactococci (Figure 2.3), that can be either chromosomally or plasmid-encoded (Kranenburg *et al*., 2002). Lactic acid bacteria typically possess only one PrtP but the presence of two PrtPs was reported in strains of *Lactobacillus helveticus* and *Lactobacillus bulgaricus* (Smit *et al*., 2005). The majority end products are large oligonucleotides which most are between four and ten residues (Hutkins, 2006). Peptide uptake occurs via one or two oligopeptide transport systems **(**Opp, Opt) and one or two di-/tri-peptide transporters (DtpT, DtpP) for further degradation into shorter peptides and amino acids by a concerted action of various intracellular peptidases with differing and partly overlapping specificities (Savijoki, Ingmer, and Varmanen, 2006). These peptidases can be divided into endopeptidases, aminopeptidases, di-/tri-peptidases, and proline-specific peptidases (Savijoki *et al*., 2006).

Lactic acid bacteria are likely to respond to changes in nitrogen availability by regulating the activity of the proteolytic system to ensure proper nitrogen balance in the cell. It was reported that di-/tri-peptides with hydrophobic residues act as effector molecules in the transcriptional regulation of the Opp system and thereby impact the whole proteolytic system in *Lactococcus lactis* (Savijoki *et al*., 2006). The transcriptional regulator CodY negatively regulates the expression of several components of the proteolytic system and that the strength of repression is modulated by the intracellular pool of branched-chain amino acids (BCAAs) including isoleucine, leucine, and valine (Savijoki *et al*., 2006) (Figure 2.4).

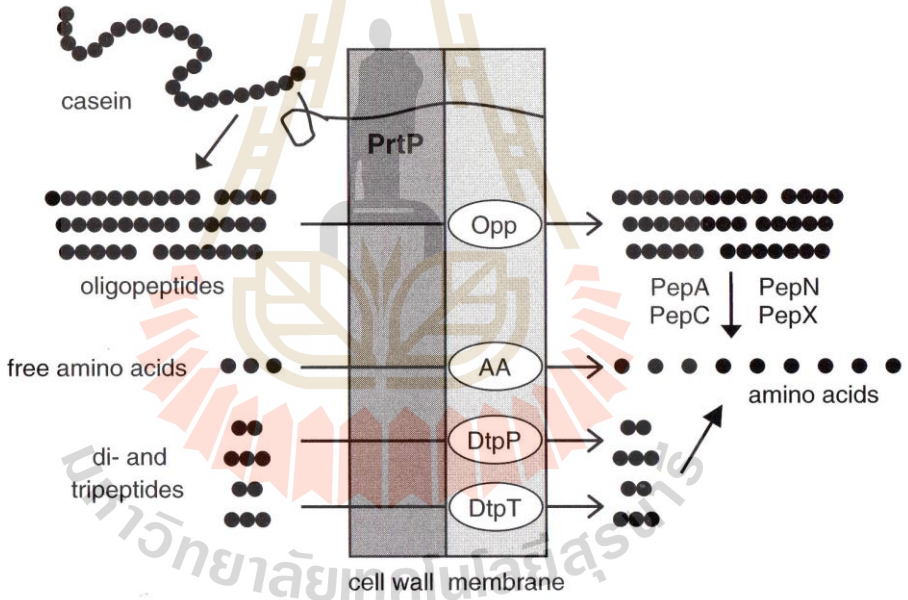

Figure 2.3 Proteolytic system in lactococci. Milk casein is hydrolyzed by a cell envelope-associated proteinase (PrtP) to form oligopeptides. These oligopeptides are then transported across the membrane by the oligopeptide transport system (Opp), di- and tri-peptide transporters (DtpT, DtpP) and amino acid (AA) transporters. The intracellular oligopeptides are then hydrolyzed by cytoplasmic peptidase (e.g., PepA, PepC, PepN, and PepX) to form amino acids.
Source: Hutkins (2006).

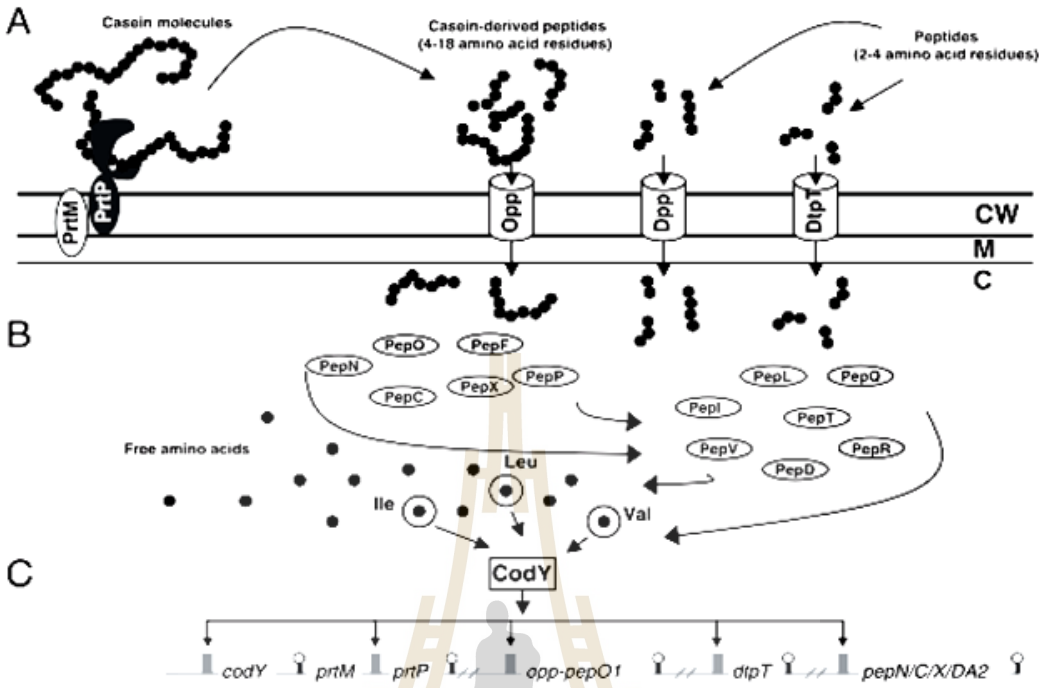

Figure 2.4 The function and regulation of the proteolytic system in lactococci. **A**: PrtP, Cell-envelope proteinase; Opp, oligopeptide permease; DtpT, the ion-linked transporter for di- and tri-peptides; Dpp, the ABC transporter for peptides containing 2 to 9 amino acid residues. **B**: Intracellular peptidases. PepO, and PepF, endopeptidases; PepN/PepC/PepP, general aminopeptidases; PepX, X-prolyl dipeptidyl aminopeptidase; PepT, tripeptidase; PepQ, prolidase; PepR, prolinase; PepI, proline iminopeptidase; PepD, and PepV, dipeptidases D and V. **C**: The transcriptional repressor CodY senses the internal pool of brached-chain amino acids (isoleucine, leucine, and valine); using these residues as cofactors CodY represses the expression of genes comprising the proteolytic system in *Lactobacillus lactis*.

Source: Savijoki *et al.* (2006).

Amino acids are precursors of various volatile compounds. Lactic acid bacteria are equipped with enzyme systems for using the amino acids in their metabolism, and are useful for flavor formation of foods (Ardö, 2006). The amino acids can be converted in many different ways by enzymes such as deaminases, decarboxylases, transaminases (aminotransferases), and lyases (Kranenburg *et al.*, 2002), which can be performed by some lactic acid bacteria, such as lactococci (Figure 2.5). Transamination, the first step of the amino acid catabolism, of each amino acid results in the formation of a specific α-keto acid, which is central intermediate by transaminase (aminotransferase) (Table 2.2). α-Ketoglutarate is typically the preferred amino acceptor for transamination reactions. α-Keto acids can be converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation. Many of these compounds are major aroma components. Direct dehydrogenation of α-keto acids results in the formation of hydroxyl acids, which do not contribute to the flavor of the product. Moreover, the pathway from methionine is the most relevant substrates for cheese flavor development (Kranenburg *et al.*, 2002). Volatile sulfur compounds derived from methionine are regarded as essential components in many cheese varieties (Urbach, 1995).

Branched-chain amino acids, including leucine (Leu), isoleucine (Ile) and valine (Val), are converted into compounds contributing to malty, fruity, and sweaty flavors. Catabolism of aromatic amino acids, such as phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), produces floral, chemical and faecal flavors. Aspartic acid (Asp) is catabolised into buttery flavor and sulfur-containing amino

acids (Methionine (Met), Cystein (Cys) are transferred into compounds contributing to boiled cabbage, meaty and garlic flavors (Ardö, 2006).

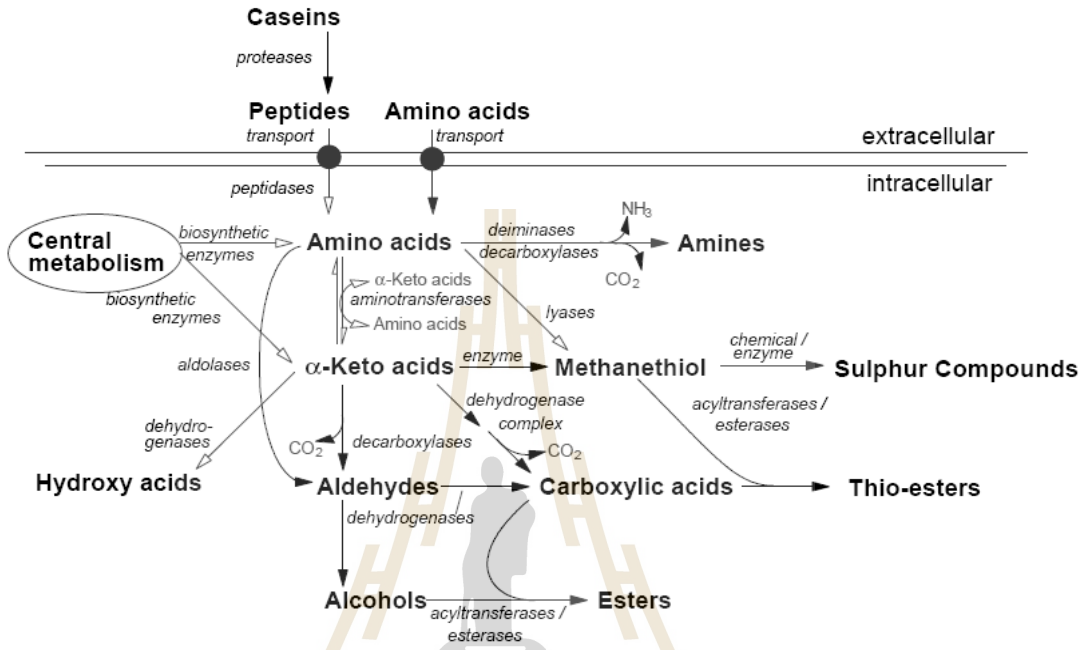

Figure 2.5 Summary of general pathways leading to intracellular amino acids and α-keto acids, and their degradation routes to potential flavor compounds in lactococci.
Source: Kranenburg *et al*. (2002).

Branched-chain aldehydes, such as 3-methylbutanal, 2-methylbutanal and 2-methylpropanal, are potent flavor compounds converted by lactic acid bacteria. Sensorially, they are generally perceived as malty, chocolate-like. Aldehydes are present in low concentrations in food products, however, the taste thresholds of aldehydes are rather low; for 2-methylpropanal and 2- and 3-methylbutanal, they were reported as 0.10, 013, and 0.06 mg/l, respectively. 3-Methylbutanal is an intermediate in the catabolism of leucine, which is converted from α-keto isocaproic acid by branched-chain keto acid decarboxylase activity (Smit *et al*., 2009) (Figure 2.6).

Table 2.2 Volatile compounds produced by amino acid catabolism in lactococci and lactobacilli with aminotransferase activity.

Amino acid	α-Keto acid	Aldehyde	Alcohol	Carboxylic acid	Others
Leu	α-Keto-iso-caproate	3-Methylbutanal	3-Methylbutanol	3-Methylbutanoic acid	-
Ile	α-Keto-β-methylvaleate	2-Methylbutanal	2-Methylbutanol	2-Methylbutanoic acid	-
Val	α-Keto-isovalerate	2-Methylpropanal	2-Methylpropanol	2-Methylpropanoic acid	-
Phe	Phenyl puruvate	Phenylacetaldehyde	Phenylethanol	Phenylacetic acid	-
Tyr	*p*-OH-Phenyl pyruvate	*p*-OH-Phenylacetaldehyde	*p*-OH-Phenyl-ethanol	*p*-OH-Phenylacetic acid	*p*-Cresol
Trp	Indole pyruvate	Indole-3-acetaldehyde	Tryptophol	Indol-3-acetic acid	Skatole
Met	α-Keto-butyrate	3-Methylthiopropanal	3-Methylthio-propanol	3-Methylthiopro-pionic acid	Methanethiol
Asp	Oxaloacetate	-	-	Malate	Diacetyl, acetoin

Leu, leucine; Ile, isoleucine; Val, valine; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Met, methionine; Asp, aspartic acid.
Source: Ardö (2006).

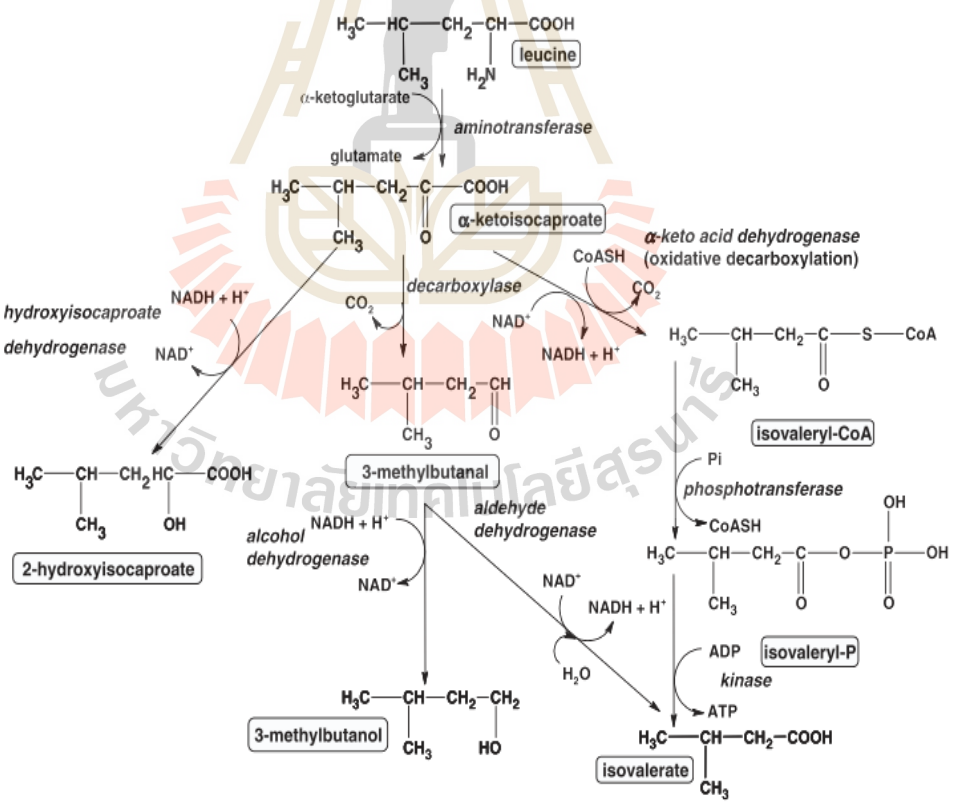

Figure 2.6 Metabolic network around 3-methylbutanal in lactococci and lactobacilli. Source: Marilley and Casey (2004).

2.2 Fish sauce

Fish sauce is a clear brown liquid produced by the fermentation of salted fish (Beddows, 1998). It is not only widely consumed in Southeast Asia but also in Europe and North America (Saisithi, 1994; Klomklao, Benjakul, Visessanguan, Kishimura, and Simpson, 2006), and gained popularlity worldwide (Hariono, Yeap, Kok, and Ang, 2005; Yongsawatdigul, Choi, and Udomporn, 2004). Fish sauce is marketed using various names by different countries: patis in the Philippines, nuoc-mam in Vietnam, shottsuru in Japan, budu in Malaysia, bakasang in Indonesia, aek-jeot in Korea, and nam-pla in Thailand which is the most dominance in the world market (Lopetcharat, Choi, Park, and Daeschel, 2001; Kilinc *et al*., 2006).

Thailand is one of the leading countries in fish sauce production that the annual production is more than 400 million liters (Dissaraphong *et al*., 2006). In 2013, Thailand exports large volume of fish sauce, a total of 46 million liters, mostly to the U.S.A., Japan, Laos, Myanmar, Australia, and Hong Kong, with the value of more than 1,400 million bahts (Thai Customs Deparment, 2013). Fish sauce quality is classified into grade s1 and 2. The quality of Thai fish sauce, requires relative density, acidity-alkalinity, and nitrogen content, is regulated by Thai Industrial Standard Institute (TISI) (Table 2.3).

Fish sauce has been used as a flavor enhancer or salt replacement in various prepared foods with the merit of its characteristic favorable taste and nutritive value. It contains about 20 g/l of nitrogen, of which 80% is in the form of amino acids that they may be considered an important sources of protein, especially in some regions where carbohydrates are the fundamental part of the diet (Fukami *et al*., 2004; Kilinc *et al*., 2006; Klomklao *et al*., 2006; Saisithi, 1994). There are also vitamins and

minerals in fish sauce such as vitamin B12, which is found in large quantities, phosphorus, calcium, magnesium, organic sulfur, and iron (Saisithi, 1994).

Table 2.3 Thai Industrial Standard Institute standard for fish sauce (nam-pla). (Reference No. TIS 3-1983)

Requirement	Grade 1	Grade 2
Relative density at 27ºC, not less than	1.2	1.2
pH	5.0 – 6.0	5.0 – 6.0
Sodium chloride (g/l), not less than	230	230
Total-N (g/100 ml), not less than	20	15
Glutamic acid/ Total-N (G/N)	0.4 – 0.6	0.4 – 0.6
Amino acid (g/l)	10	7.5

Source: Thai Industrial Standard Institute (TISI, 1983).

2.2.1 Characteristics of fish sauce

2.2.1.1 Chemical compositions of fish sauce

Chemical characteristics of fish sauce from Southeast and East Asian countries such as pH, salt contents, moisture, and total nitrogen are shown in Table 2.4. For most samples, the average pH ranged from 5.4 to 5.8 except for fish sauces from Myanmar (6.23), Laos (4.90), and China (6.15) (Park *et al*., 2001). Salt contents were rather low in all samples when compared to the reference fish sauce value of 25.9 g/100 ml (Mizutani, Kimizuka, Ruddle, and Ishige, 1992; Ren, Hayashi, Endo, and Watanabe, 1993). Moisture was 65.9% on average for all samples. The highest content of total nitrogen was in the samples from Vietnam followed by those of Japan and Thailand.

Total organic acid content was high in Vietnamese, Chinese, and Myanmar fish sauces (Table 2.5). Pyroglutamate levels that may come from glutamate during fermentation or heat treatment were very high. Lactate was also high in the fish sauces of most countries which it may be produced by lactic acid bacteria during fermentation or may originate from fish muscle. These organic acids, especially pyroglutamate and lactate may be responsible for the characteristic flavor of fish sauces (Park *et al*., 2001).

The highest content of total amino acids was in the Vietnamese samples followed by the Japanese and Thai samples (Table 2.6). Fish sauces from these three countries showed rather similar amino acid compositions, which had high aspartate, glutamate, alanine, valine, lysine, and histidine contents. Korean fish sauce contained low glutamate and high proline, glycine, and alanine which are sweet amino acids, and had a rather different taste, sweet and mild, compared with those from the other countries (Park *et al*., 2001).

Table 2.4 Chemical characteristics of fish sauce from seven countries in Southeast and East Asia.

	Thailand (n=10)	Vietnam (n=20)	Myanmar (n=7)	Laos (n=2)	China (n=2)	South Korea (n=9)	Japan (n=11)	Mean ± S.D. (n=61)
pH	5.63 ± 0.17^{b}	5.57 ± 0.26^{b}	6.23 ± 0.91^{a}	4.90 ± 0.11^{cd}	6.15 ± 0.38^{ab}	5.49 ± 0.45^{bd}	5.54 ± 0.42^{bc}	5.70 ± 0.50
NaCl[1]	21.4 ± 1.2^{ac}	20.2 ± 1.1^{bc}	22.7 ±1.9^{a}	15.7 ± 0.9^{d}	22.0 ± 1.1^{ab}	22.2 ± 1.5^{a}	18.0 ± 4.5^{d}	20.5 ± 2.8
Moisture[1]	63.7 ± 1.9^{c}	61.4 ± 2.8^{c}	70.0 ± 4.0^{b}	79.2 ± 0.4^{a}	66.0 ± 4.1^{bc}	67.4 ± 1.9^{b}	69.2 ± 5.3^{b}	65.8 ± 5.3
TTL-N[1,2]	1.68 ± 0.24^{b}	2.59 ± 0.51^{a}	0.97 ± 0.75^{cd}	0.35 ± 0.08^{bc}	1.49 ± 0.44^{d}	1.27 ± 0.20^{c}	1.80 ± 0.31^{b}	1.79 ± 0.77
N-recovery[3] (%)	64.3 ± 5.5^{ac}	61.6 ± 13.2^{bcd}	45.6 ± 9.8^{e}	42.5 ± 6.6^{e}	57.8 ± 17.7^{ade}	68.2 ± 5.0^{ab}	70.4 ± 9.9^{a}	61.8 ± 12.8

[1]Values are expressed in g/100 ml for NaCl, moisture, and TTL-N contents; [2] Total nitrogen; [3] Nitrogen recovery.

Mean values in the same horizontal row with different superscript letters are significantly different (P<0.05 or less).

Source: Park *et al*. (2001).

Table 2.5 Organic acid compositions of fish sauce (mg/100 ml).

Organic acid	Thailand (n=10)	Vietnam (n=20)	Myanmar (n=7)	China (n=2)	South Korea (n=9)	Japan (n=11)	Mean ± S.D. (n=59)
Pyroglutamate	317 ± 95^{b}	690 ± 237^{a}	287 ± 227^{b}	252 ± 18^{b}	300 ± 125^{b}	218 ± 110^{b}	409 ± 246
Lactate	314 ± 69^{bc}	470 ± 129^{ab}	135 ± 88^{d}	252 ± 190^{bcd}	419 ± 214^{ac}	535 ± 292^{a}	401 ± 213
Acetate	266 ± 91^{b}	251 ± 221^{b}	704 ± 519^{a}	734 ± 571^{a}	182 ± 104^{b}	93 ± 102^{b}	287 ± 308
Formate	23 ± 11^{a}	21 ± 21^{a}	1 ± 3^{bc}	22 ± 3^{ab}	—^{c}	12 ± 12^{ac}	14 ± 16
Malate	1 ± 2^{a}	4 ± 8^{a}	3 ± 5^{a}	—^{ac}	1 ± 1^{a}	1 ± 4^{a}	2 ± 5
Citrate	22 ± 30^{a}	5 ± 6^{bcd}	8 ± 10^{ac}	—^{ade}	12 ± 26^{ab}	5 ± 10^{bcd}	9 ± 18
Succinate	74 ± 23^{ac}	70 ± 69^{bc}	106 ± 97^{ab}	159 ± 57^{a}	41 ± 23^{c}	30 ± 45^{c}	66 ± 62
Total	1,044 ± 125^{bc}	1,510 ± 267^{a}	1,245 ± 702^{ac}	1,428 ± 422^{ab}	955 ± 233^{bc}	958 ± 349^{bc}	1,187 ± 410

Mean values in the same horizontal row with different superscript letters are significantly different (P<0.05 or less).

—, not detected.

Source: Park *et al*. (2001).

Table 2.6 Amino acid compositions of fish sauce made from fish species as raw materials (mg/100 ml).

Amino acid	Thailand (n=10)	Vietnam (n=20)	Myanmar (n=7)	Laos (n=2)	China (n=2)	South Korea (n=9)	Japan (n=11)
Taurine	119 ± 27^{ce}	171 ± 70^{ac}	142 ± 127^{bcd}	37 ±0de	117 ± 1^{ade}	192 ± 48^{ab}	233 ± 73^{a}
Aspartate	583 ± 129^{bc}	1002 ± 369^{a}	287 ± 331de	54 ± 59de	511 ± 321^{bcd}	349 ± 108^{ce}	657 ± 255^{b}
Threonine	384 ± 82^{b}	584 ± 249^{a}	131 ± 150^{c}	28 ± 13^{c}	285 ± 258^{bc}	207 ± 54^{c}	420 ± 128^{b}
Serine	233 ± 74^{bc}	483 ± 260^{a}	11 ± 15de	24 ± 10^{ce}	95 ± 81^{ce}	128 ± 64^{cd}	369 ± 104^{ab}
Glutamate	1489 ± 327^{ab}	1548 ± 691^{a}	560 ± 456^{cd}	31 ± 34^{d}	1164 ± 913^{abe}	550 ± 144de	1088 ± 343^{b}
Proline	135 ± 53^{cd}	322 ± 146^{a}	67 ± 81^{d}	18 ± 2^{d}	127 ± 109^{bd}	181 ± 75^{bc}	230 ± 71^{b}
Glycine	267 ± 50^{bd}	461 ± 182^{a}	237 ± 248^{bef}	43 ± 2^{df}	265 ± 150^{acde}	346 ± 127^{ab}	298 ± 100^{bc}
Alanine	574 ± 97^{b}	985 ± 320^{a}	469 ± 316^{bd}	179 ± 11^{cd}	597 ± 269^{bc}	715 ± 234^{b}	611 ± 130^{b}
Cysteine	17 ± 24^{a}	45 ± 48^{a}	13 ± 18^{a}	— ^{a}	125 ± 83^{b}	36 ± 7^{a}	23 ± 47^{a}
Valine	478 ± 98^{b}	709 ± 200^{a}	289 ± 200^{cd}	32 ± 45^{e}	493 ± 222^{abc}	427 ± 83^{bd}	517 ± 136^{b}
Methionine	222 ± 44^{a}	230 ± 68^{a}	75 ± 57^{b}	32 ± 5^{b}	260 ± 71^{a}	197 ± 31^{a}	237 ± 95^{a}
Isoleucine	334 ± 49^{a}	374 ± 87^{a}	184 ± 114^{b}	72 ± 22^{b}	348 ± 73^{a}	347 ± 57^{a}	392 ± 84^{a}
Leucine	439 ± 60^{b}	427 ±108^{b}	271 ± 145^{c}	143 ± 42^{c}	471 ± 38^{ab}	544 ± 84^{a}	541 ± 113^{a}
Tyrocine	91 ± 23^{ac}	128 ± 62^{a}	64 ± 49^{bcd}	5 ± 7^{d}	88 ± 51^{ad}	110 ± 26^{ab}	128 ± 71^{a}
Phenylalanine	323 ± 57^{bc}	415 ± 120^{a}	110 ± 62^{d}	36 ± 6^{d}	328 ± 135^{ac}	257 ± 40^{c}	374 ± 123^{ab}
Tryptophan	— ^{be}	50 ± 62^{a}	1 ± 2^{bd}	— ^{acde}	— ^{acde}	12 ± 15^{bc}	24 ± 45^{ab}
Lysine	767 ± 148^{b}	1269 ± 419^{a}	405 ± 368^{cd}	137 ± 17^{d}	653 ± 490^{bd}	655 ± 129^{bc}	801 ± 205^{b}
Histidine	275 ± 60^{b}	370 ± 159^{a}	12 ± 12^{c}	9 ± 1^{c}	191 ± 158^{bc}	103 ± 45^{c}	318 ± 143^{ab}
Arginine	3 ± 10^{c}	217 ± 334^{ab}	8 ± 12^{c}	7 ± 4^{ac}	— ^{ac}	50 ± 74^{bc}	280 ± 186^{a}
Total	6732 ± 1253^{bc}	9826 ± 3107^{a}	3335 ± 2674de	869 ± 110^{e}	6061 ± 3257^{bd}	5406 ± 3257^{bd}	7532 ± 1551^{b}

Mean values in the same horizontal row with different superscript letters are significantly different (P<0.05 or less).
—, not detected.

Source: Park *et al*. (2001).

2.2.1.2 Color and flavor

Although fish sauce is normally a clear brown liquid, the degree of darkening depends on variety of fish and the conditions of fermentation. The brown color comes from Maillard's reaction between amino acid and reducing sugar (Saisithi, Kasermsarn, Liston, and Dollar, 1996).

The unique flavor (aroma and taste) of fish sauce is the most important factor in consumer acceptability of traditionally produced sauces (Beddows, 1998). Dougan and Haward (1975) reported that three distinctive notes contributed to the aroma of fish sauce, those being ammoniacal, cheesy, and meaty notes. They are derived from protein hydrolysate and lipid oxidation products brought about by either autolytic or microbial activity or fish enzymes (Saisithi *et al*., 1996; Beddows, Ardeshir, and Daud, 1980). The ammoniacal note is attributed to ammonia, amines, and other basic nitrogen-containing compounds (Saisithi *et al*., 1996; Dougan and Haward, 1975; Yurkwski, 1965). The cheesy note is mainly due to low molecular weight volatile fatty acids (Saisithi *et al*., 1996; Dougan and Haward, 1975; Sanceda, Kurata, and Arakawa, 1986) and methyl ketone (Grosch, 1993). Shimoda *et al*. (1996) pointed out 2-methylpropanoic acid as the major contributor to cheesy and stinging notes. The meaty note is much more complicated, but it was believed that it could be produced by atmospheric oxidation of precursors that were still present in mature fish sauces (Dougan and Haward, 1975).

Various volatile compounds including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds, are formed during fermentation and thought to be responsible for the distinct aroma of fish sauce (Peralta *et al*., 1996; Fukami *et al*., 2002). It is assumed that the volatiles of fish sauce

are produced by non-enzymatic reactions of various components (that is, amino acids, lipids, and sugars) and enzymatic reactions by endogenous enzymes of fish origin and those of indigenous microorganisms (Fukami *et al*., 2004). Low molecular weight volatile fatty acids (VFA) were produced from the autoxidation of polyunsaturated acids and by bacterial action on amino acids, which are used as a carbon source (Dougan and Harward, 1975).

The volatile compounds of patis (Peralta, Shimoda, and Osajima, 1997), shottsuru (Michihata, Yano, and Enomoto, 2002), nam-pla, and nuoc-mam (Giri, Osako, Okamoto, and Ohshima, 2010) were identified by chromatography-mass spectrometry (GC-MS) listed in Table 2.7. Four fish sauces revealed aroma different from each other. Acids, alcohols, nitrogen-containing compounds, sulfur-containing compounds, ketones, esters, furans, and aromatic compounds were among the main groups of volatile compounds identified. The differences in aroma of these fish sauces were thought to be due to the differences in the level of concentrations of the major acids. Moreover, some differences in kinds of minor volatile compounds such as pyrazines could contribute to the burnt flavor; lactones may have an influence on the sweet aroma, were possible contributing factors in the differences of total aromas.

Park, Watanabe, Endoh, Watanabe, and Abe (2002a) found 11 compounds from nuoc-mam identified to be the taste-active components, which consisted of glutamic and aspartic acids, threonine, alanine, valine, histidine, proline, tyrosine, cysteine, methionine, and pyroglutamic acid. The most effective compound for recreating the characteristic flavor of fish sauce was glutamic acid, followed by pyroglutamic acid and alanine. Many of these components contribute to umami,

sweetness, and overall taste of fish sauce. Moreover, the large amounts of peptides produced during the long-term fermentation of fish sauce are also responsible for the complicated taste of the sauce. They enhanced sweetness and umami as well as sourness and bitterness of the fish sauce, and increased several flavor characteristics including continuity, first taste and after taste (Park *et al*., 2002b).

2.2.1.3 Distinctive volatile compounds in fish sauce

A number of reports revealed that volatile acids were the most abundant group of volatile compounds in fish sauce (Truong, 1952; Saisithi *et al*., 1996). Patis, nuoc-mam, nam-pla and shottsuru contained straight and branched-chain volatile acids (Peralta *et al*., 1997; Michihata *et al*., 2002; Giri *et al*., 2010). Shimoda *et al*. (1996) and Peralta *et al*. (1997) reported that 2-methylpropanoic acid and 2,2-dimethylpropanoic acid were found to be abundant in Taiwanase and Philippine fish sauce. Butanoic acid was identified as the major acid in patis (Peralta *et al*., 1997). 3-Methylbutanoic acid was the most abundant in shottsuru (Michihata *et al*., 2002) and was one of potent distinctive volatile fatty acids detected in nam-pla, and contributed to a rancid note (Fukami *et al*., 2002). Moreover, acetic acid was also abundant in Thai fish sauce (Park *et al*., 2001; Yongsawatdigul *et al*., 2007).

2-Methyl-1-butanal was reported to be one of distinctive volatile compounds of nam-pla (Yongsawatdigul *et al*., 2007) and responsible for a meaty note (Fukami *et al*., 2002). Japanese fish sauce had high levels of 2-methyl-1-propanal, 2-methyl-1-butanal, 3-methyl-1-butanal, and benzaldehyde (Michihata *et al*., 2002). Aldehydes have been derived from lipid oxidation during fermentation, and branched, short chain aldehydes or aromatic aldehydes might have resulted from deamination of amino acids (McIver, Brooks, and Reneccius, 1982).

Fukami *et al*. (2002) aimed to make clear some possible contributors to distinctive odorants of fish sauce, and found that four compounds contributing to the distinctive odor of fish sauce were 2-methyl-1-propanal, 2-methyl-1-butanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)-propanal, and 3-methylbutanoic acid. 2-Ethylpyridine and dimethyl trisulfide were found to contribute to the fishy, sweety and fecal notes. 2-Ethylpyridine in addition to 2-pentanone and volatile acids was essential to the cheesy note. 2-Ethylpyridine together with 2-methyl-1-propanal and 2-methyl-1-butanal was responsible for the meaty note. The burnt note was developed in the presence of 2-ethylpyridine and dimethyl trisulfide and also in the presence of 2-methyl-1-propanal and 2-methyl-1-butanal.

2.2.2 Fish sauce fermentation

Fish sauce fermentation is a common practice as a means of preserving and producing value-added products from underutilized fish species (Klomklao *et al*., 2006). Fish sauce is made from various types of fish, from both freshwater and marine fish species (Beddows, 1998) particularly small pelagic species such as mackerel (*Ristrelliger* spp.), herring (*Clupea* spp.) (Lopetcharat and Park, 2002), and anchovies (*Stolephorus* spp.) (Saisithi *et al*., 1996; Saisithi, 1994; Beddows, 1998; Lopetcharat *et al*., 2001; Klomklao *et al*., 2006).

The processing method for fish sauce can be different in each country, but the basic principle is quite similar. There are two major ingredients in fish sauce production, fish and salt. The ratio between salt and fish is very different, depending on the country, ranging from 1:6 to 1:2 (w/w) (Table 2.8). Traditionally, Thai fish sauce is produced by mixing one part of salt with two or three parts of uneviscerated

fish on a concrete floor (Saisithi *et al*., 1996; Lopetcharat and Park, 2002). The fish/salt mixture is then placed into fermentation tanks which are generally constructed of concrete, built into the ground, and have already had salt spread on the bottom (Saisithi, 1994; Saisithi *et al*., 1996). The mixture does not fill above the top of the tank but instead is placed about 50 cm below the edge of the tank and another layer of salt is spread on the top of fish (Saisithi, 1994) and left to ferment at ambient temperature (30^{o}C to 40^{o}C) for 12-18 months (Lopetcharat and Park, 2002; Saisithi *et al*., 1996). The fermentation process is normally continued for a long time, to ensure the solubilisation as well as the flavor and color development of fish sauce (Klomklao *et al*., 2006). After fermentation is completed, the liquid is drained off and saturated brine is added to the residue to extract the left-over soluble matters (Saisithi, 1994). The first and the second liquids are combined to produce first quality fish sauce (Figure 2.7).

Salting is used to prevent spoilage bacteria during fermentation by reducing water activity (a_w). The maximum amount of salt (NaCl) to prevent the growth of spoilage microorganisms is 15% (Lopetcharat *et al*., 2001). The amount of salt used for fish sauce production is usually between 20 and 30% (Saisithi, 1994; Lopetcharat *et al*., 2001). Most of spoilage bacteria originally present in fish will die off quickly after contacting with high salt concentration.

Table 2.7 Volatile compounds identified in patis, shottsuru, nam-pla, and nuoc-mam.

Compounds	Samples			
	Patis[a]	Shottsuru[b]	Nam-pla[c]	Nuoc-mam[c]
Acids				
Acetic acid	+	+	+	+
2-Methyl propanoic acid	+	-	+	+
Butanoic acid	+	+	+	+
2-Methyl butanoic acid	-	-	+	+
3-Methyl butanoic acid	+	+	+	+
Alcohols				
2-Propanol	-	-	+	+
Ethanol	-	+	+	+
1-Propanol	-	-	+	+
3-Methly-2-butanol	-	-	+	+
2-Methly-propanol	+	+	+	+
3-Pentanol	+	-	+	+
1-Butanol	-	+	+	+
1-Penten-3-ol	-	+	+	+
3-Hexanol	-	-	+	+
2-Methyl-1-butanol	-	+	+	+
3-Methyl-1-butanol	+	+	+	+
2-Hexanol	-	-	+	+
1-Pentanol	-	-	+	+
3-Methyl-1-pentanol	-	-	+	+
2-Ethyl-1-butanol	-	-	+	+
Cyclopentanol	-	-	+	+
2-Heptanol	-	-	+	+
(E)-2-Penten-1-ol	+	-	+	+
3-Methyl-3-buten-1-ol	-	-	+	+
1-Hepten-3-ol	-	-	+	+
Hexanol	-	-	+	+
3-Octanol	-	-	+	+
(E)-2-Hexen-1-ol	-	-	+	+
(Z)-2-Hexen-1-ol	-	-	+	+
1-Octen-3-ol	-	-	+	+
Heptanol	-	-	+	+
2-Ethyl hexanol	-	+	+	+
2-Nonanol	-	-	+	+
2,3-Butanediol *(levo)*	-	-	+	+
Octanol	-	-	+	+
2,3-Butanediol *(meso)*	-	-	+	+
Nonanol	-	-	+	+

Table 2.7 (Continued) Volatile compounds identified in patis, shottsuru, nam-pla, and nuoc-mam.

Compounds	Samples			
	Patis[a]	Shottsuru[b]	Nam-pla[c]	Nuoc-mam[c]
Aldehydes				
Acetaldehyde	+	-	+	+
Propanal	+	-	+	+
2-Methylpropanal	+	+	+	+
2-Methylbutanal	+	+	+	+
3-Methylbutanal	+	+	+	+
Pentanal	-	-	+	+
2-Butenal	-	-	+	+
Hexanal	-	-	+	+
(E)-2-Methyl-2-butenal	-	+	+	+
Heptanal	-	-	+	+
(Z)-2-Hexenal	-	-	+	+
4-Heptenal	-	-	+	+
Octanal	-	-	+	+
Nonanal	-	-	+	+
(EZ)-2,4-Heptadienal	-	-	+	+
(EE)-2,4-Heptadienal	-	-	+	+
(EZ)-2,6-Nonadienal	-	-	+	+
(EZ)-2,4-Nonadienal	-	-	+	+
Esters				
Ethyl acetate	+	+	+	+
Ethyl isobutyrate	-	-	+	+
2-Methylpropyl acetate	-	-	+	+
Ethyl butanoate	-	-	+	+
Ethyl-2-methylbutanoate	-	-	+	+
Ethyl-3-methylbutanoate	-	-	+	+
Butyl acetate	-	-	+	+
Isobutyl isobutanoate	-	-	+	+
Isoamyl acetate	-	-	+	+
Ethyl pentanoate	-	-	+	+
Ethyl hexanoate	-	-	+	+
3-Methylbutyl butanoate	-	-	+	+
2-Methylbutyl-2-methylbutanoate	-	-	+	+
Isoamyl isovalerate	-	-	+	+
Ethyl heptanoate	-	-	+	+
Ethyl octanoate	-	-	+	+
Ethyl decanoate	-	-	+	+
Ketones				
2-Propanone	-	-	+	+
2-Butanone	-	+	-	+
2-Pentanone	-	+	+	+
2,3-Butanedione	-	-	+	+

Table 2.7 (Continued) Volatile compounds identified in patis, shottsuru, nam-pla, and nuoc-mam.

Compounds	Samples			
	Patis[a]	Shottsuru[b]	Nam-pla[c]	Nuoc-mam[c]
2,3-Pentanedione	-	+	+	+
3-Octanone	-	-	+	+
2-Octanone	-	-	+	+
6-Methyl-5-hepten-2-one	-	-	+	+
(EE)-3,5-Octadien 2-one	-	-	-	-
2-Undecanone	-	-	+	+
Furans				
2-Ethylfuran	-	-	+	+
2-*n*-Butylfuran	-	-	+	+
2-Pentylfuran	-	-	+	+
2-Furaldehyde	-	-	+	+
2-Acetylfuran	-	-	+	+
5-Methylfurfural	-	-	+	+
Furfuryl alcohol	-	-	+	+
Ethyl-3-(2-furyl) propanoate	-	-	-	-
Sulfur-containing compounds				
Dimethyl disulfide	+	+	+	+
2,4,5-Trimethyl thiazole	-	-	+	+
Dimethyl trisulfide	+	-	+	+
3-(Methylthio) propanal	-	-	+	+
2-(Methylthio) ethanol	-	-	-	-
Ethyl-3-(methylthio) propanoate	-	-	+	+
2-Ethoxy thiazole	-	-	+	+
3-(Methylthio) propanol	-	+	+	+
Nitrogen-containing compounds				
Trimethylamine	+	+	+	+
Methyl pyrazine	+	+	+	+
2,6-Dimethyl pyrazine	+	+	+	+
2,4-Dimethyl pyrazine	-	-	+	+
Ethyl pyrazine	+	+	+	+
2,3-Dimethyl pyrazine	-	+	+	+
2-Ethyl-3-methyl pyrazine	-	-	+	+
2,3,5-Trimethyl pyrazine	-	-	+	+
3-Ethyl-2,3-dimethyl pyrazine	-	-	-	-
1,3-Dimethyl 1H-pyrazole	-	-	-	-
Tetramethyl pyrazine	-	-	+	+
3,5-Diethyl-2-methyl pyrazine	-	-	-	-
2-Acetyl pyrrole	-	-	+	+
Aromatic compounds				
Ethyl benzene	-	-	+	+
p-Xylene	-	-	+	+

Table 2.7 (Continued) Volatile compounds identified in patis, shottsuru, nam-pla, and nuoc-mam.

Compounds	Samples			
	Patis[a]	Shottsuru[b]	Nam-pla[c]	Nuoc-mam[c]
o-Xylene	-	-	+	+
Cymene	-	-	+	+
Propyl benzene	-	-	+	+
p-Cymene	-	-	-	+
Phenyl ethyne	-	-	-	-
Benzaldehyde	+	+	+	+
Benzene acetaldehyde	-	-	-	-
Acetophenone	-	-	+	+
Ethyl benzoate	-	-	+	+
4-Ethyl benzaldehyde	-	-	+	+
Ethylphenyl acetate	-	-	+	+
2-Phenylethyl acetate	-	-	+	+
p-Guaiacol	-	-	-	-
Benzyl alcohol	-	-	+	+
Phenylethyl alcohol	-	-	+	+
Benzene acetaldehyde α-ethylidiene	-	-	-	-
Phenol	-	-	+	+
4-Vinyl guaiacol	-	-	+	+
3-Ethoxy benzaldehyde	-	-	-	-
4-Ethyl guaiacol	-	-	+	+
2-Methyl phenol	-	-	+	+
4-Ethyl phenol	-	-	+	+

+: detected; -: not detected

[a] : Peralta *et al*. (1997); [b] : Michihata *et al*. (2002); [c] : Giri *et al*. (2010).

Table 2.8 Varieties of fish sauce produced in Asia countries.

Country and local name	Species of fish	Conditions and time of fermentation	Reference
Thailand			
Nam-pla	*Stolephorus* spp., *Ristrelliger* spp., *Sardinella* spp., *Cirrhinus* spp.	2:1-3:1 fish:salt (12-18 months)	Saisithi (1994), Beddows (1998)
Vietnam			
Nouc-mam	*Stolephorus* spp., *Ristrelliger* spp., *Engraulis* spp., *Decapterus* spp., *Dorosoma* spp., *Clupea* spp.	3:1-3:2 fish:salt (4-12 months)	Beddows (1998)
Philippines			
Patis	*Stolephorus* spp., *Clupea* spp., *Decapterus* spp., *Leionathus* spp.	3:1-4:1 fish:salt (3-12 months)	Beddows (1998)
Indonesia			
Bakasang or Ketjap-ikan	*Stolephorus* spp., *Clupea* spp., *Leionathus* spp., *Osteochilus* spp., *Puntius* spp., *Ctenops* spp.	5:1 fish:salt (6 months)	Ijong and Ohta (1996), Beddows (1998)
Malaysia			
Budu	*Stolephorus* spp.	3:2 fish:salt (6-12 months)	Beddows, Ardeshir, and Daud (1979)
Myanmar			
Ngapi	Various	5:1 fish:salt (3-6 weeks)	Beddows (1998)
Japan			
Shottsuru	*Astrocopus japanicus* (sandfish), *Clupea pilchardus* (sardine), *Omnastrephis sloani* (squid), *Omnastrephis paeificus*	5:1 fish:salt+malted rice and koji (3:1) (6 months)	Beddows (1998)
Korea			
Jeot-kal	Various	4:1 fish:salt (6 months)	Beddows (1998)

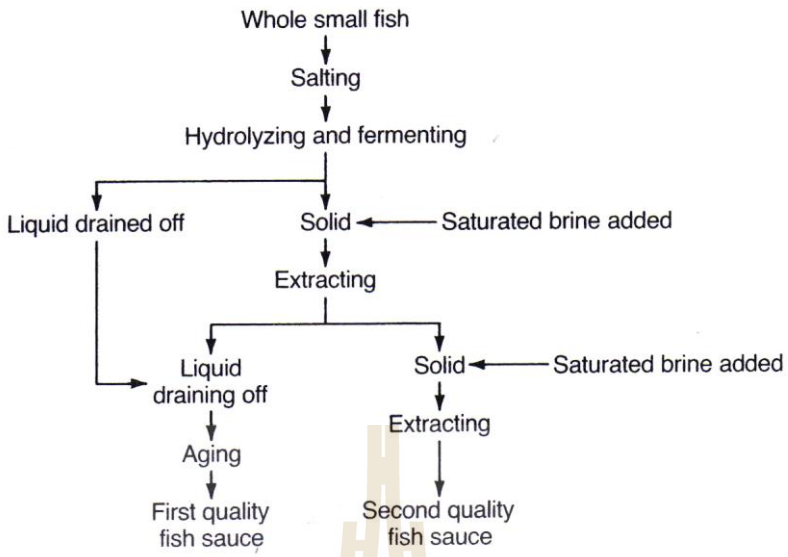

Figure 2.7 Sequential operation of Thai fish sauce.
Source: Saisithi (1994).

2.2.2.1 Biochemical changes during fish sauce fermentation

Protein is the major component of fish and proteolytic digestion is considered to be the most important reaction which converts insoluble fish protein into a soluble or suspended form (Beddows, 1998). Fish protein is induced by endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria during fish sauce fermentation (Orejana and Liston, 1982; Saisithi, 1994; Dissaraphong *et al*., 2006; Hjalmarsson, Park, and Kristbergsson, 2007).

Endogenous fish enzymes are primarily responsible for the degradation of muscle proteins during the fish sauce manufacturing process. Trypsin-like proteinases from digestive tract of anchovy (*Stolephorus* spp.) were responsible for protein hydrolysis during the first 20 weeks of fermentation (Orejana and Liston, 1982). Tissue enzymes such as cathepsin B, A, and C, are the most active in the

beginning of hydrolytic process (Haard, 1994). An, Weerasinghe, Seymour, and Morrissey (1994) reported that cathepsin B was the most active cysteine protease in Pacific whiting fish fillets during fish sauce fermentation, while serine proteases and trypsin-like enzymes had a minor role in hydrolyzing by-products. Cathepsin B is both an endo- and exo-peptidase enzymes, therefore, it might be one of the important enzymes for flavor development of fish sauce during the fermentation period. Cathepsins induce proteolytic hydrolysis in muscle tissue up to the salt level exceeding 15% and tryptic enzyme activity is also reduced, due to the high salt content and endogenous enzyme inhibitors (Orejana and Liston, 1982).

Noda, Van, Kusakabe, and Murakami (1982) identified a number of proteinases from sardines, one acid and one alkaline proteinases, were stable in strong salt solutions and were liberated in the later stages of sauce manufacture. Heu *et al*. (1991) isolated two alkaline α-chymotrypsin-likes proteinases that were still active in the fermentation of anchovy. In addition, the hydrolysis of protein to amino acids was contributed from aminopeptidase from sardines which showed the enzyme activity to be quite high during the early period of fermentation, then gradually decreased and reached the minimum within 6 months of fermentation (Vo, Kusakabe, and Murakami, 1983; 1984).

Besides the involvement of endogenous proteinases, extracellular proteinases produced by halophillic bacteria were also suggested to participate in protein hydrolysis during fish sauce fermentation. *Halobacillus thailandensis* isolated from Thai fish sauce produced extracellular proteinases that catalyzed hydrolysis of gelatin at NaCl concentration up to 30% (Chaiyanan *et al*., 1999). Uchida *et al*. (2004) isolated *Bacillus subtilis* CN2 from a Vietnamese fish

sauce that produced alkaline proteinase. *Filobacillus* sp. RF2-5 isolated from nam-pla produced a serine proteinase that was activated and stable at high NaCl content (15% to 25%) (Hiraga *et al*., 2005). Sinsuwan *et al.* (2007; 2008) reported salt-activated extracellular proteinase from *Virgibacillus* sp. SK37 and *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. The enzyme activity retained at 20% to 25% NaCl. Thongthai, McGenity, Suntinanalert, and Grant (1992) isolated an extremely halophilic archaeobacterium, *Halobacterium salinarium*, from nam-pla that produced a salt-stable extracellular proteinase (4 M NaCl). This may not be the main agent of proteolysis, but it could contribute to the development of particular peptides and amino acids involved in flavor at the end stage of ripening (Voskresensky, 1965).

The release of water-soluble proteins from cells by osmotic pressure and degradation of muscle proteins to peptides and amino acids by proteolytic enzymes resulted in an increased nitrogenous compound content (Saisithi 1994). Uyenco, Lawas, Briones, and Taruc (1953) reported that organic and amino nitrogen increased up to the fourth or fifth month in traditionally vat-fermented nuoc-mam. At about this time, the soluble nitrogen reached a maximum and levels off. The brine was saturated by the soluble products of the fish until reached equilibrium (Table 2.9).

Table 2.9 Chemical compositions of liquid products drained at regular intervals during the processing of nuoc-mam.

Fermentation time (day)	Nitrogen (g/l)				
	Total	Organic	Formal	Ammoniacal	Amino acid
1	5.3	4.6	-	0.7	-
3	9.0	7.8	-	1.1	-
10	11.2	9.9	5.2	1.3	3.8
15	14.0	12.4	7.6	1.6	5.9
20	16.7	13.1	8.2	1.7	6.5
25	16.8	14.9	8.4	1.9	6.5
30	18.6	16.6	9.5	2.0	7.5
35	18.6	16.5	11.0	2.1	8.8
40	19.9	17.7	11.3	2.2	9.1
45	20.3	18.1	11.3	2.2	9.1
50	21.0	18.6	11.9	2.4	9.5
55	21.3	18.8	12.0	2.5	9.5
60	21.8	19.2	12.2	2.7	9.6
70	23.8	20.9	12.2	2.9	9.3
80	22.1	19.2	11.9	3.0	8.9
90	22.7	19.6	13.1	3.1	10.0
105	23.0	19.7	13.1	3.3	9.9
120	23.8	20.4	13.9	3.4	10.5
135	24.1	20.7	14.7	3.4	11.3
150	23.8	20.2	14.8	3.6	11.1
165	24.1	20.4	14.5	3.7	10.8
180	23.8	19.0	14.7	4.8	9.9

Source: Uyenco *et al*. (1953).

Changes in the chemical constituents of Thai fish sauce as fermentation progress involve with pH, NaCl, total nitrogen, ammonia nitrogen, total acids, volatile acids, and volatile bases (Table 2.10). The pH remains relatively stable in the vicinity of 6.2 to 6.6. The concentration of salt remains close to 30%. Total nitrogen increased from 56 to 140 mmoles/100 ml during 6 to 12 months of fermentation, and ammonia nitrogen increased to the maximum of 14 mmols/100 ml by 6 months of fermentation. There was only a slight increase in volatile acid during 12 months of fermentation, while the volatile base increased to a maximum of 14.7

meq/100 ml at 9 months of fermentation and decreased sharply toward the 12 months to 3 meq/100 ml.

Table 2.10 Chemical analysis of Thai fish sauce during fermentation.

Fermentation period (months)	pH	NaCl (%)	Total nitrogen[a]	Ammonia nitrogen[a]	Total acid (as lactic)[a]	Volatile acid (as acetic)[a]	Volatile base[b]
1	6.4	30.1	49	8	6.8	4.3	4.01
3	6.2	30.3	52	7	8.0	3.3	6.61
6	6.6	30.2	56	14	5.2	8.7	10.21
9	6.2	30.2	130	15	5.9	4.3	14.71
12	6.4	27.9	140	15	15.8	6.3	3.01

[a] : mmoles/100 ml ; [b] : meq/100 ml

Source: Saisithi *et al*. (1996).

Thongthai and Okada (1980) reported that total nam-pla fermentation period can be divided into three stages, early, middle and late stages. In the early stage, from the beginning to the fifth week, three measured value, total nitrogen, free and total amino acids increased rapidly and reached 60 to 70% of the maximum value at the end of this stage. The middle stage, from the fifth to the twenty week, was characterized by slight increase in free amino acid and moderate rate of increase in total amino acid. In the late fermentation, after the twenty week, no more increase in total amino acids and little increase in free amino acids.

Beddows *et al*. (1979) reported that during the production of commercial budu, total soluble nitrogen increased with time (Table 2.11). The total percent conversion contribution of free amino acids to total amino-nitrogen increased to 66.3%. Ethanoic acid was found to be a major acid, increased from 1.3 to 2.15

mg/cm^3 in 156 days. Volatile free fatty acids, ethanoic acid, propanoic acid, n-butanoic acid, and iso-pentanoic acid contributed to the typical flavor of budu (Beddows *et al*., 1980) (Table 2.12).

Table 2.11 Percentage of total nitrogen in supernatant liquor during budu fermentation.

Fermentation time (day)	Percentage of total nitrogen in supernatant (fish sauce)			
	Amino nitrogen	Volatile nitrogen	Protein nitrogen	Polypeptide nitrogen
1	36.3	10.5	1.23	52.0
2	40.8	8.9	1.26	49.0
3	39.1	10.1	1.17	49.6
4	44.6	10.7	0.92	43.8
5	47.1	11.1	1.22	41.6
6	47.2	11.3	0.75	40.7
7	47.3	9.9	1.01	41.8
14	51.9	9.1	0.95	36.2
30	56.6	8.9	0.79	33.7
62	60.1	7.1	0.80	32.0
92	62.1	6.6	0.50	30.8
154	66.3	6.6	0.56	26.5

Source: Beddows *et al*. (1979).

Table 2.12 The change in volatile fatty acid concentration with time in budu fermentation.

Time (day)	Ethanoic acid (mg/cm^3)	Propanoic acid (mg/cm^3)	n-Butanoic acid (mg/cm^3)	Iso-pentanoic acid (mg/cm^3)
1	1.29	0.14	0.20	0.04
28	1.70	0.12	0.20	0.08
60	1.70	0.13	0.22	0.07
91	1.81	0.12	0.23	0.06
156	2.15	0.12	0.23	0.07

Source: Beddows *et al*. (1980).

2.2.2.2 Microorganisms in fish sauce fermentation

Fish sauce fermentation process contains very high concentration of salt (25-30%). Most of the microorganisms found in fish sauce are classified as halotolerants and extreme halophiles. These are microflora from fish, solar salt and fermentation tank (Lopetcharat *et al*., 2001) and have been identified as the species of *Halobacterium*, *Corynebacterium* (Thongthai and Suntinanalert, 1991), *Achromobacter*, *Flavobacterium*, *Proteus*, *Halococcus*, *Sarcina* (Saisithi, 1994), *Streptococcus*, *Micrococcus*, *Pediococcus*, *Enterobacter*, *Lactobacillus* (Ijong and Ohta, 1996), *Halobacillus* (Chaiyanan *et al*., 1999), *Pseudomonas* (Vihelmsson *et al*., 2001), *Staphylococcus* (Fukami *et al*., 2004), *Bacillus* (Uchida *et al*., 2004), *Filobacillus* (Hiraga *et al*., 2005), and *Virgibacillus* (Sinsuwan *et al*., 2007 and 2008).

Ijong and Ohta (1996) found that *Streptococcus*, *Micrococcus*, and *Pediococcus* were dominant microorganisms during the first 10 days of bakasang (Indonesian fish sauce) fermentation. After 40 days of fermentation, *Enterobacter*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Micrococcus*, and *Pediococcus* were isolated from bakasang. In addition, Xu, Yu, Xue, Xue, and Ren (2008) reported that a continuously decrease in microbiological counts occurred during the first 10 days of low salt fish sauce fermentation and then increased a little during 10-20 days of fermentation and decreased thereafter. With regard to the changes of bacterial counts during fermentation, it should be explained that non-halotolerant bacteria decreased in the initial fermentation. But after 5 days fermentation, halophiles, such as lactic acid bacteria, began growth and propagated and led to increase in microbiological counts.

Tran and Nagano (2002) found the principal species of microorganisms isolated from Vietnamese fish sauce and Laotian fish sauce was *Bacillus subtilis*. Noguchi *et al*. (2004) isolated *Bacillus vietnamensis* sp. nov. which was moderately halotolerant from Vietnamese fish sauce. Other genera of Bacilli have been isolated from Thai fish sauce, such as *Lentibacillus salicampi*, *Lentibacillus juripiscarius* sp. nov., *Lentibacillus halophilus* sp. nov., *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-*3* (Namwong *et al*., 2005; 2006; Hiraga *et al*., 2005; Tanasupawat *et al*., 2006).

Proteolytic bacteria played an important role during fish sauce fermentation. There are several groups of proteolytic bacteria in fish sauce fermentation, including halophilic bacteria, halotolerants and lactic acid bacteria. These bacteria hydrolyze fish protein to peptides and amino acids (Lopetcharat *et al*., 2001). Some amino acids can be used as substrates for lactic acid bacteria. Proteinase-producing bacteria found in fish sauce were *Pseudomonas* sp. (Vihelmsson *et al*., 1996), *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Pediococcus* sp., *Corynebacterium* sp. (Thongthai and Suntinanalert, 1991; Fukami *et al*., 2004; Noguchi *et al*., 2004), *Halobacillus thailandensis* sp. nov., (Chaiyanan *et al*., 1999), *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* (Satomi, Kimura, Mizoi, Sato, and Fujii, 1997; Tanasupawat, Thongsanit, Okada, and Komagata, 2002; Thongsanit *et al*., 2002).

2.2.2.3 *Tetragenococcus* species found in fermented foods

Several species of lactic acid bacteria belonging to the genus *Tetragenococcus* are reported to be found in fermented foods. The genus has named since 1990 after reclassification of *Pediococcus halophilus* and *Tetragenococcus*

halophilus (Lee *et al*., 2005). *Tetragenococcus*, halophilic lactic acid bacteria, require sodium chloride (NaCl) for growth, and are tolerant to NaCl concentration more than 18% (Holzapfel, Franz, Ludwig, Back, and Dicks, 2006). The bacteria are Gram-positive cocci (0.5-1.0 μm in diameter) and cell form is tetrads or pairs. Colonies on GYP/sodium acetate/mineral salts agar plate at pH 7.0 are smooth, low convex, circular, opaque, matt and white in colour (Lee *et al*., 2005). The bacteria have been found in a variety of fermented foods, including Thai fish sauce (Thongsanit *et al*., 2002), soy sauce (Hanagata, Shida, and Takagi, 2003), Indonesian soy mash (Kecap) (Roling and Verseveld, 1996), kimchi and fermented ham (Lee *et al*., 2005), salted anchovies (Villar, Ruiz-Holgado, Sanchez, Trucco, and Oliver, 1985), sourdough bread (Gül, Özçelik, Sağdıç, and Certel, 2005), fermented mustard (Chen, Yanagida, and Hsu, 2006), and shrimp paste (Kobayashi *et al*., 2003). To date, the genus *Tetragenococcus* comprises of four species, including *T. halophilus*, *T. muriaticus*, *T. koreensis*, and *T. solitarius*. Recently, the species *T. halophilus* includes strains isolated from both salt rich and sugar rich environments and are further referred to as halophilic and osmophilic strains, respectively. Consequently, the new subspecies *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* have been proposed and described, respectively (Justé *et al*., 2012). Additionally, a fifth species called *T. osmosphilus* has been described recently by Justé *et al*. (2012). This latest species has been found in concentrated sugar thick juice, an intermediate in the production of beet sugar (Justé *et al*., 2012). A summary of the characteristics of these species including growth at various temperatures, pH values, and NaCl concentrations, gas production from glucose fermentation, and acid production from carbohydrates is shown in Table 2.13.

Table 2.13 Important characteristics of species in the genus *Tetragenococcus*.

Characteristics	*T. halophilus* subsp. *halophilus*[a] ATCC 33315[T]	*T. halophilus* subsp. *flandriensis*[b] DSM 23766[T]	*T. muriaticus*[c] JCM 10006[T]	*T. koreensis*[d] DSM 16501[T]	*T. solitarius*[e] DSM 5634[T]	*T. osmophilus*[f] DSM 23765[T]
Optimum temperature (°C)	30	30	25-30	15-30	NA	30
Growth at 37°C	-	+	+	+	+	+
40°C	-	-	+	-	+	-
45°C	-	-	-	-	+	-
Growth range of pH	5.0-9.0	NA	5.0-9.6	NA	NA	NA
Optimum pH	7.5-8.0	7.5-8.0	7.5-8.0	9.0	7.5-8.0	8.0
Range of NaCl (%)	0-25	0-25	1-25	0-8	NA	0-25
Optimum NaCl (%)	5-10	5-10	7-10	2-5	7-10	NA
Growth in sugar thick juice at 69 ºBX	-	+	-	-	NA	+
Acid production from:						
Amygdalin	+	+	-	+	+	-
D-Arabinose	+	+	-	-	-	-
D-Cellobiose	+	+	-	-	+	+
D-Galactose	+	+	-	+	+	-
D-Glucose	+	+	+	+	+	+
Glycerol	+	-	-	-	-	-
D-Lactose	-	+	-	-	-	-
D-Maltose	+	+	-	+	+	+

Table 2.13 (Continued) Important characteristics of species in the genus *Tetragenococcus*.

Characteristics	*T. halophilus* subsp. *halophilus*[a] ATCC 33315^T	*T. halophilus* subsp. *flandriensis*[b] DSM 23766^T	*T. muriaticus*[c] JCM 10006^T	*T. koreensis*[d] DSM 16501^T	*T. solitarius*[e] DSM 5634^T	*T. osmophilus*[f] DSM 23765^T
D-Raffinose	-	+	-	-	-	-
D-Mannitol	-	-	+	+	+	+
D-Mannose	+	+	+	+	+	+
D-Melibiose	-	+	-	-	-	-
D-Sucrose	+	+	-	+	+	+
D-Melezitose	-	-	-	+	+	-
α-Methyl-D-glucoside	-	-	-	+	-	+
D-Ribose	+	+	+	+	-	-
D-Sorbitol	-	+	-	-	-	-
D-Sorbose	-	-	-	-	-	-
D-Trehalose	+	+	-	+	+	+
D-Xylose	+	-	-	+	-	-
Xylitol	-	-	-	+	-	-
D-Turanose	-	+	-	+	+	+
D-Tagatose	-	+	-	-	+	-
D-Arabitol	-	-	-	+	+	-
Gluconate	+	NA	-	+	+	NA
DNA G+C content (mol%)	36.0	36.7	36.5	38.0	38.3	36.7

NA: Not available

Source: [a]: Thongsanit *et al.* (2002); [b,f]: Justé *et al*. (2012); [c]: Satomi *et al.* (1997); [d]: Lee *et al.* (2005); [e]: Ennahar and Cai (2005).

2.3 Flavor and flavor development by lactic acid bacteria in fermented foods

Lactic acid bacteria have been reported to influence the flavor of fermented foods, such as fish sauce, fermented dairy products, and fermented meat, in a variety of ways. In many cases, the most obvious change in lactic acid fermentation is the production of acid and lowering pH that results in an increase in sourness. Since most of the acids produced in fermentation were produced by the metabolism of sugars, sweetness likely decreases as sourness increase. The production of volatile flavor components tends to be the first mechanism considered for the development of flavor specific to a particular fermented food. In addition to this direct mechanism, however, there are less direct ways in which fermentation microorganisms affect flavor. Lowering the pH in lactic acid fermentation may reduce the activity or completely inactivate enzymes in the plant that generate either flavor components or flavor precursor compounds. Finally, the fermentation microorganisms may directly metabolize precursor flavor compounds or flavor components themselves (McFeeters, 2004). Fish sauce has various flavor compounds developed by halophilic lactic acid bacteria (Udomsil, 2008). Flavor development in fermented dairy products is involved with lactic acid bacteria by various processes including the conversions of lactose, citrate, fat, and caseins (Kranenburg *et al*., 2002). In addition, the flavor of fermented sausage is produced by lactic acid bacteria through carbohydrate fermentation.

Fish sauce has a characteristic aroma (as mentioned in section 2.2.1.2) which often serves as an indicator to measure the quality of fish sauce, since the very salty taste tends to overpower the other flavor constituents (Sanceda, Suzuki, and Kurata,

2003). The formation and development of these compounds are formed through various reactions, including lipolysis, Maillard browning reaction, and Strecker degradation (Shimoda *et al*., 1996). In addition, they can be derived from the action of indigenous microorganisms. Halophilic lactic acid bacteria were dominant microorganisms during flavor and color changes of final stage of fish sauce fermentation (Saisithi, 1994). It is likely that *Tetragenococcus* found in fish sauce could play a significant role in flavor formation during fish sauce fermentation. Accumulation of amino acids and peptides during fermentation has a considerable effect on flavor of fish sauce (Peralta *et al*., 1996). Amino acids and peptides were formed by the action of microbial and fish proteinases. Microbial proteinases, especially intracellular aminopeptidases, were the key enzymes responsible for flavor formation (Smit *et al*., 2005). Recently, Udomsil (2008) investigated the role of halophilic lactic acid bacteria isolated from fish sauce fermentation on flavor formation and chemical composition of fish sauce. Lactic acid bacteria were isolated from fish sauce mash fermented at various times ranging from 1 to 12 months. Interestingly, the major volatile compounds identified in the fish sauce inoculated with *T. halophilus* as a starter culture were 2-methyl-1-propanal, 2-methyl-1-butanal, 3-methyl-1-butanal, and benzaldehyde. Therefore, the use of lactic acid bacteria for fish sauce fermentation appeared to improve chemical characteristics and volatile composition of a fish sauce product.

Bacteria in the genus *Tetragenococcus* are halophilic lactic acid bacteria that could be found in fish sauce. Satomi *et al.* (1997) showed that *T. muriaticus* was a new moderately halophilic lactic acid bacteria isolated from squid liver sauce. Moreover, *T. halophilus* and *T. muriaticus* were found in Thai fish sauce (nam-pla)

(Thongsanit *et al*., 2002), Indonesian soy mash (Roling, and Verseveld, 1996), Japanese soy sauce (Nakagawa and Kitahara, 1959), and salted anchovies (Villar *et al*., 1985). Despite of its prevalence, the role of *Tetragenococcus* on chemical characteristics of fermented food has not been systematically investigated.

Flavor development in fermented dairy products is a complex and, in the case of cheese ripening, slows process involving chemical and biochemical conversions of milk components. Flavor compounds are formed by various processes, i.e. the conversions of lactose and citrate (glycolysis and pyruvate metabolism), fat (lipolysis), and caseins (proteolysis) (Kranenburg *et al*., 2002)

Lactic acid bacteria form the main microflora in these dairy products, and they are essential for the biochemical conversions that determine the specific flavor. Although lactose is mainly converted to lactate by lactic acid bacteria, a fraction of the intermediate pyruvate can alternatively be converted to various flavor compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid, some of which contribute to typical yoghurt or butter flavors. An important flavor-generating reaction in some dairy products is the conversion of citrate to diacetyl, which can be performed by some lactic acid bacterial strains (Hugenholtz, 1993).

Lipolysis results in the formation of free fatty acids, which can be precursors of flavor compounds such as methyl ketones, alcohols, lactones and esters (Molimard and Spinnler, 1996). Fat hydrolysis is particularly important in soft cheeses like Camembert and blue cheeses. Collins, McSweeney, and Wilkinson (2003) reported the increase in a number of free fatty acids in Cheddar cheese by autolysis of *Lactococcus lactis* subsp. *cremoris* AM2, suggesting that the influence of autolysis of starter bacteria on cheese enzymology and biochemistry may not be confined to

proteolysis but also lipolysis and other enzyme-mediated flavour reactions. Proteolysis directly contributes to cheese flavors by releasing peptides and amino acids. Amino acids are substrates for transamination, dehydrogenation, decarboxylation and reduction, producing a wide variety of flavor compounds. *Lactobacillus helveticus* has previously been shown to have a potent proteolytic and has been shown to enhance early flavor development in Cheddar cheese (Hannon, Kilcawley, Wilkinson, Delahunty, and Beresford, 2003). The main flavor compounds identified in cheeses are in the groups of alcohols, aldehydes, ketones, esters, lactones, sulfur-containing compounds, nitrogen-containing compounds, fatty acids, furans and phenolic compounds (Table 2.14).

The flavor of fermented sausage is not only influenced by several factors, primarily source, quantity and type of ingredients (e.g. meat, salt, and spices), but also temperature, processing time, smoking, and choice of starter culture. Basic flavor results from the interaction of taste (mainly determined by lactic acid production and the pattern of peptides and free amino acids resulting from tissue-generated proteolysis) and aroma (mainly determined by volatile components derived from bacterial metabolism and lipid autoxidation). The primary contribution of lactic acid bacteria to flavor generation is ascribed to the production of large amounts of lactic acid and some acetic acid, although they also produce volatiles through fermentation of carbohydrates (Molly, Demeyer, Civera, and Verplaetse, 1996). Lactic acid is believed to be the main taste component. However, acetic acid is also present, and is actually needed in small amounts for full dry sausage flavor. Lactic acid bacteria isolated from Greek sausage exhibited high *in vitro* leucine and valine aminopeptidase activities (Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, and Kotzekidou, 2003).

However, lactobacilli and pediococci display low catabolism of branched-chain amino acids (Larrouture, Ardaillon, Pepin, and Montel, 2000) A strain of *Carnobacterium piscicola* has been suggested as a new starter culture of dry fermented sausage because of its high formation of aroma compounds derived from leucine metabolism, similar to the ones formed by staphylococci (Larrouture-Thiveyrat and Montel, 2003).

Table 2.14 Compounds isolated as flavors in various cheeses.

Alcohols	**Aldehydes**	**Ketones**	**Esters**
1,2-Butanediol	Acetaldehyde	Acetoin	Ethyl acetate
2-Butanol	Decanal	Acetone	Ethyl benzoate
Ethanol	Heptanal	2,3-Butanedione (diacetyl)	Ethyl butyrate
2-Ethylbutanol	(*Z*)-4-Heptenal	2-Butanone	Ethyl hexanoate
2-Ethylhexanol	Hexanal	*b*-Damaescenone	Ethyl isobutanoate
2-Heptanol	2-Hexenal	2-Heptanone	Ethyl octanoate
Hexanol	Isohexanal	2-Hexanone	Ethyl 2-methylbutanoate
Isobutanol	2-Methylbutanal	3-Methyl-2-butanone	Ethyl 3-methylbutanoate
2-Methylbutanol	3-Methylbutanal	2-Nonanone	Isobutyl butanoate
3-Methylbutanol	2-Methylpropanal	3-Octanone	3-Methylbutyl acetate
2-Methylpropanol	Nonanal	1-Octen-3-one	Methyl-2-methylbutanoate
2-Nonanol	(*E*,*E*)-2,4-Nonadienal	2-Pentanone	3-Octyl acetate
(*Z*)-1,5-Octadien-3-ol	(*Z*)-2-Nonenal	2-Tridecanone	Pentyl acetate
2-Octanol	(*E*)-2-Nonenal	2-Undecanone	Phenethyl acetate
1-Octen-3-ol	Octanal		Propyl butyrate
2-Pentanol	Pentanal	**Fatty acids**	Pyrazines
Phenylethanol	Propanal	Acetate	2,3-Diethyl-5-methylpyrazine
2-Phenylethanol	Propenal	Butyrate	2-Ethyl,3-5-dimethyl-pyrazine
1-Propanol	Thiophen-2-aldehyde	Caproate	2-Methoxy-3-isopropylpyrazine
2-Propanol		Decanoate	
	***S*-compounds**	Isobutyrate	
Lactones	Dimethyl disulphide	Methyl acetate	**Furans**
δ-Decalactone	Dimethyl sulphide	2-Methylbutyric acid	2-Ethyl-4-hydroxy-5-methyl-3-(2*H*)furanone
γ-Decalactone	Dimethyl trisulphide	3-Methylbutyric acid	
δ-Octalactone	Methanethiol	Octanoate	3-Hydroxy-4,5-dimethyl-2-(5*H*)furanone
δ-Dodecalactone	Methional	Phenylacetate	
(*Z*)-6-Dodecen-g-lactone	Methylene bis(methylsulphide)	Propionate	4-Hydroxy-2,5-dimethyl-3-(2*H*)furanone
	Hexanethiol	Valerate	Tetrahydrofuran
Phenolic compounds		***N*-compounds**	
p-Cresol		2-Acetyl-1-pyrroline	

S: Sulfur; *N*: Nitrogen.
Source: Marilley and Casey (2004).

2.4 Methods for detecting flavor in foods

The progress in food flavor analysis techniques over the last decades has led to long lists of volatile compounds including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds (Peralta *et al*., 1996). The perception of volatile compounds released from foods by human nose depends on the extent of release from the food metrix and on flavor properties of the compound. Only a small fraction of the large number of volatile compounds occurring in food actually contributes to the flavor is indicated. Therefore, the distinction between active flavor compounds and the compounds of a food, in order to associate flavor activity with the eluting compounds and the whole range of volatiles present in a particular food product, is an important task in flavor analysis (Van Ruth, 2001).

Volatile compounds can be detected and analysed by various methods as mentioned below.

2.4.1 Detection of aldehydes

2.4.1.1 Schiff's test

Schiff's test is a method used for determining aldehydes. Aldehydes can be oxidized to carboxylic acids. Schiff's reagent is a selective oxidizing agent that will oxidize aldehydes but not alcohols. A typical composition of Schiff's reagent involves a combination of sulfur dioxide and fuchsin. The sulfur dioxide is typically generated by mixing sodium bisulfite with hydrochloric acid. The reagent contains an oxidized form of the bright pink dye, fuschin. When the Schiff's reagent reacts with an aldehyde, the dye is reduced back to its pink form and the solution turns pink. The absorbance value is measured at 570 nm (Owen, 2008).

2.4.1.2 Thiobarbituric acid (TBA) test

The thiobarbituric acid (TBA) test is widely used to evaluate lipid oxidation in meat products. Malonaldehyde (MDA), a secondary decomposition product of polyunsaturated fatty acids with three or more double bonds, reacts with TBA to form a stable pink chromophore with maximal absorbance at 532 nm. Aldehyde compounds other than MDA may also react with TBA to produce yellow (λ_{max} of 455 nm), orange (λ_{max} of 495 nm) and red (λ_{max} of 532 nm) colours (Sun, Faustman, Senecal, Wilkinson, and Furr, 2001) which can be measured by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) (Alghazeer, Saeed, and Howell, 2008). Sun *et al.* (2001) determined aldehyde compounds in freeze-dried beef patties using TBA test and analyzed by HPLC. Aldehydes known to be products of lipid oxidation in meat at 1.0 mM dissolved in 11% trichloracetic acid, except 0.01 mM for malonaldehyde, were incubated with 20 mM aqueous TBA at 25°C for 22 h and/or 95°C for 20 min. The absorbance values for these solutions were measured at 450 and 532 nm. These filtered aldehyde compounds were injected into HPLC and separated by a Waters Resolve 5 μm C18 column (250 × 3.9 mm i.d) with isocratic elution by a mixture of buffer (30 mM sodium citrate/27.7 mM acetate, pH 4.75) and methanol (1:4) at 0.5 ml/min.

2.4.1.3 Chromatography

An HPLC method has been developed for determining total MDA in vegetable oils, after conversion of the MDA released from its precursor, to the dansyl-pyrazole derivative. In this HPLC method using a μ-Bondapak C18 column with mixed mobile phase of 1% acetic acid–acetonitrile (85 + 15; v/v), the

quantitation of malondialdehyde (MDA) in aqueous distillates from freeze-dried chicken meat was determined at a level of 1.0×10^{-6} mol/l (Tsaknis, Lalas, and Evmorfopoulos, 1999).

Aldehydes (such as 2-methyl-1-propanal, 2-methyl-1-butanal and 3-methyl-1-butanal) of fish sauce were analyzed by dynamic headspace GC-MS (Michihata *et al*., 2002). Gas chromatogram of headspace volatile compounds of fish sauce showed the peak area of 2-methyl-1-propanal (peak 5), 2-methyl-1-butanal (peak 9) and 3-methyl-1-butanal (peak 10) (Figure 2.8).

Recently, gas chromatography and the electronic nose system have been used together for analyzing aldehydes which are volatile food flavor compounds. Kim *et al*. (2004) investigated changes in odor patterns of salted and fermented anchovy sauces caused by gamma irradiation. The differences in flavor analyzed by the electronic nose system could be related to the results from GC-MS flavor analysis and sensory evaluation.

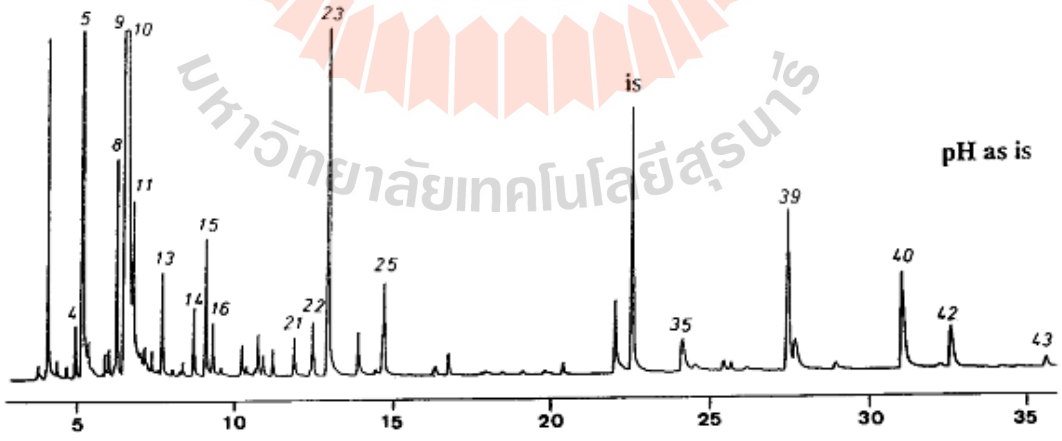

Figure 2.8 Gas chromatogram of volatile compounds of Philippine fish sauce.
Source: Peralta *et al*. (1997).

2.4.2 Detection of diacetyl

Several methods have been used for the detection and quantification of diacetyl found in alcoholic drinks and dairy products, including colorimetric method, steam distillation, gas chromatography-mass spectroscopy, and high performance liquid chromatography. A colorimetric method, based on the Voges-Proskauer reaction, allows the determination of diacetyl which reacts with the guanido groups of creatine in the presence of α-naphthol in an alkaline medium to give a pink colour (measured at absorbance 530 nm) (Dulieu and Poncelet, 1999).

By exploiting the difference in the rate of formation of the pink colour by diacetyl and acetoin, it is possible to obtain a good estimation of their individual contents in the wine sample from the steam distillation of a wine sample, since the steam distillation does not completely separate the diacetyl from acetoin, thus this can lead to an overestimation of the diacetyl content. The colour development is determined after 1 min for the diacetyl concentration and again after 60 min for acetoin (Lewis, Todd, and Stanley, 1997).

Fluorimetry was found to be equivalent but more sensitive than spectrometry and equal to gas chromatography determinations, thus lower concentrations of diacetyl can be measured. HPLC is another method used to detect diacetyl (Zeppa, Conterno, and Gerbi, 2001). Alternative method for diacetyl determination involves gas-chromatography with mass spectroscopy detection (GC-MS). A simplification of the GC-MS method, which involves minimal sample preparation, has been developed for the direct quantification of diacetyl in wine samples (Hayasaka and Bartowsky, 1999). This technique utilises the deuterated form of diacetyl as the internal standard with SPME sampling of the headspace. Additional

advantages are the small wine sample volume used (3 ml) and high specificity is obtained by selective ion monitoring. GC-MS method is a very simple, rapid, accurate and cost-effective technique to determine diacetyl concentrations in wine samples (Hayasaka and Bartowsky, 1999).

2.4.3 Sensory evaluation

Sensory properties are extremely important to food because they directly correlate to product quality and consumer acceptance. The sensory attributes of a food item are typically perceived in the following order: appearance, odor/aroma/fragrance, consistency, texture, and flavor (aromatics, chemical feeling, and taste) (Meilgaard, Civille, and Carr, 2006). Food with sensory defects will poorly affect the quality of further processed products causing consumer rejection. The traditional approach taken by the dairy industry in relation to the sensory evaluation of milk, has been to highly train judges on recognizing milk's defects and on assigning them consistent scores on a system known as score cards (Bodyfelt, Tobias, and Trout, 1998). However, this approach has been criticized for its failure to predict consumer acceptance, its lack of objectivity in quality judgments, and the complexity in assignment of quantitative scores (Chapman, Lawless, and Boor, 2001). Therefore, scaling methods are used to quantify sensory attributes. The types of scales include numerical, verbal, graphical or magnitude estimation scales to quantify food's sensory attributes. The hedonic scale, also called the 9-point hedonic scale, is primarily used for like-dislike judgments and does not require trained panelists (Lawless and Heymann, 1998).

Fukami *et al.* (2004) evaluated the sensory properties of Thai fish sauce by using sniffing and tasting techniques. The panelists were asked to give

acceptance scores for 4 attributes: color, odor, flavor, and overall acceptance, using the 7-point hedonic scale. The odor of fish sauce was divided into 8 attributes (burnt, fishy, sweaty, fecal, rancid, cheesy, meaty, and ammoniacal notes) as the descriptors of quantitative descriptive analysis (Fukami *et al*., 2002). The panelists compared odor by sniffing. Flavor preferences were assessed by tasting fish sauce samples. The panelists were asked to use drinking water and plain cracker for rinsing their mouth before tasting the next sample.

2.5 Genome sequencing technologies

2.5.1 First generation sequencing

Genome sequencing is an experimental process of determining the order of nucleotides present in a given DNA molecules from a short segment of a single molecule, such as a regulatory region or a gene, up to collections of entire genomes. Precise knowledge of the DNA sequence of entire genomes is paramount in identifying downstream genes, transcripts, and proteins, and in the subsequent elucidation of biochemical processes taking place in a given organism. In the early 1970s, first-generation sequencing dominated by chain termination method was developed by Frederick Sanger and colleagues (Sanger, Nicklen, and Coulson, 1977). This was considered as the gold standard for nucleic acid sequencing for the subsequent two and a half decades. In the same year, Gilbert and Maxam developed a method based on chemical modification of the DNA and subsequent cleavage at specific bases (Maxam and Gilbert, 1977). Both techniques greatly increased the throughput of sequencing DNA. The method developed by Gilbert and Maxam, however, was more complex and involved the use of hazardous chemicals. The

Sanger method, on the other hand, was developed after a series of optimizations by introducing four-color Sanger sequencing, using four fluorescently labeled ddNTPs for each DNA base, and enabling optical detection (Smith *et al*., 1986). This semi-automation method in combination with capillary electrophoresis, was the key technology behind the first fully automated DNA sequencing system, the ABI 370, marketed by Applied Biosystems Inc. (Now Life Technologies) in 1986 (Marziali and Akeson, 2001). The first "sequencing by synthesis" method, namely pyrosequencing, was developed. The method utilizes real-time detection of pyrophosphate release upon nucleotide (nt) incorporation (Nyren, 1987). In 1999, the first automated pyrosequencing system was made commercially available by pyrosequencing AB (later Biotage AB, now a part of Qiagen) (Nyren, 2007). The most spectacular achievement, however, was the sequencing of the entire human genome, which took a decade and came at a cumulative cost of $2.7 billion, 13-year-long endeavor, before it was finally completed in 2003 (Grada and Weinbrecht, 2013). Nevertheless, some disadvantages remained with the Sanger method was still rather labour-, reagent- and time-consuming and thus involved major expenses.

2.5.2 Next generation sequencing

The principle of next generation sequencing (NGS) involves DNA molecules, which are sequenced in a massively parallel fashion in a flow cell. The concept of NGS is similar to capillary electrophoresis, which identifies the bases of a small fragment of DNA from signals emitted as each fragment is re-synthesized from a DNA template strand. By virtue of this high-throughput process, however, each single DNA molecule is individually sequenced and can be counted among thousands or millions of sequences generated. The qualitative and quantitative sequence

information generated has allowed advanced genome analyses that technically impossible or cost prohibitive (Metzker, 2010). NGS advances provide a much cheaper and higher-throughput alternative to sequencing DNA than traditional Sanger sequencing. Whole small genomes can now be sequenced in a day. Furthermore, the introduction of several small, personal sequencing devices have contributed to the democratization of the sequencing field. Complex sequencing projects that until recently could have been carried out only in major sequencing centers, can now be performed by the medium-sized research groups or small research facilities (Grada and Weinbrecht, 2013).

Numerous NGS platforms (Pareek, Smoczynski, and Tretyn, 2011) have been launched. The first three platforms, which currently are still the most prevalent technologies in chronological order of their publication, are 454 pyrosequencing (Margulies *et al*., 2005), an array-based pyrosequencing approach, Illumina (Bentley, 2006), another sequencing-by-synthesis method, and SOLiD (Shendure *et al*., 2005), performing sequencing-by-ligation. The 454 Sequencing, Illumina sequencing, and SOLiD™ sequencing are dominating today's NGS technologies market by about 19, 60, and 19%, respectively (Herper, 2010).

2.5.2.1 454 Sequencing

In 2005, the first NGS system, 454 Sequencing, was developed as being the first commercially available platform and marketed by 454 Life Sciences (now Roche) (Margulies *et al*., 2005). The system is based on the same principles as pyrosequencing, hence pyrophosphate detection (Nyren and Lundin, 1985). For sequencing, library construction of template DNA is accomplished by DNA fractionation using nebulization or sonication. Subsequently, DNA fragments

are enzymatically blunt-ended and ligated to adaptor oligonucleotides (Margulies *et al*., 2005). One of the adaptors contains a 5' biotin tag, enabling binding onto streptavidin coated beads. Hereafter, clonal amplification of the DNA fragments is carried out by so-called emulsion polymerase chain reaction (emPCR), where the beads are separated by a water-in-oil emulsion and the amplification occurs in oil droplets containing a PCR reaction mixture (Dressman, Yan, Traverso, Kinzler, and Vogelstein, 2003). Typically, most droplets contain only a single DNA fragment. Clonal amplification generates millions of DNA copies. The emulsion is then broken, and the beads are subsequently treated with denaturant for removal of untethered DNA strands, and finally hybridization-based enrichment of template-carrying beads is performed (Margulies *et al*., 2005). The enriched beads are loaded in a picotiter plate containing 28 μm diameter wells only allowing one bead per well. Small beads containing immobilized sequencing enzymes. At each sequencing cycle, single species nucleotides (4 dNTPs) are flowed across the plate which functions as a flow cell. At strands where the DNA polymerase-catalyzed addition of one or more nucleotides is possible, pyrophosphates are released. This enables oxidization producing localized luminescence, which is detected by a charge-coupled device (CCD) sensor (Ronaghi, Uhlen, and Nyren, 1998). The sequencing cycle is finalized by degradation of unincorporated nucleotides by the action of apyrase (Margulies *et al*., 2005). Image and signal processing occurs as part of a sequencing run, and the end output is flowgrams for individual reads, the base called read sequences. Typically, a single sequencing run can generate around 500 million base pairs (Mb) of sequence data with average read lengths of 400 bases (Voelkerding, Dames, and Durtschi, 2009). A major limitation of the 454 technology is base determination in

homopolymeric regions (Huse, Huber, Morrison, Sogin, and Welch, 2007). Nevertheless, this technology requires a significantly lower coverage rate (10-fold less) for accurate detection of single nucleotide polymorphism compared to other technologies, due to its long read lengths and high accuracy of the reads (Suzuki, Ono, Furusawa, Ying, and Yomo, 2011).

2.5.2.2 Illumina sequencing

The Illumina sequencing technology was launched by Solexa company to the market in 2006. A year later Solexa was acquired by Illumina. Illumina sequencing adopts the technology of sequencing by synthesis (SBS) but opposite to 454 sequencing, it utilizes reversible termination chemistry of nucleotide analogues (Dohm, Lottaz, Borodina, and Himmelbauer, 2008). Its library construction is accomplished like that of 454 sequencing. However, DNA amplification is carried out on the glass surface of a flow cell using solid-phase bridge PCR (Fedurco, Romieu, Williams, Lawrence, and Turcatti, 2006). The flow cell consists of an optically glass slide with 8 individual lanes on the surface. Here, the adaptor fanked DNA fragments are bound to an oligonucleotide covered surface. All amplicons originating from the single molecule template by bridge amplification, are clustered together on the surface. Each cluster consists of approximately clonal 1,000 copies of the template. For sequencing, the amplicons are single stranded, and a sequencing primer is hybridized to one of the adaptors flanking the DNA fragment of interest. During each sequencing cycle, a single base is incorporated with chemically modified nucleotides by a modified DNA polymerase (Dohm *et al*., 2008). These labeled nucleotides serve as a terminator for polymerization. A 3'-O-azidomethyl blocking group ensures that only one base is incorporated, and one of four fluorescent labels

enables detection of the different DNA bases (Turcatti, Romieu, Fedurco, and Tairi, 2008). Subsequently, the sequencing cycle ends with chemical cleavage of the fluorophore and the blocking group, enabling base incorporation at the next sequencing cycle. After subsequent image analysis and base calling, and filtering of poor quality reads, the end output is sequence files in Illumina's FASTQ format. Due to the use of modified polymerase and nucleotides, the most frequent sequence error for Illumina sequencing has been reported to be substitution (Dohm *et al*., 2008). The Read-length of Illumina is limited by several factors, such as incomplete cleavage of the fluorophore or blocking group causing signal decay and dephasing (Whiteford *et al*., 2009). In early 2010, Illumina launched HiSeq 2000 was among the first globally to adopt the HiSeq system. Its output was 200 Gb per run initially, improved to 600 Gb per run currently which could be finished in 8 days (Liu *et al*., 2012).

2.5.2.3 SOLiD™ sequencing

The SOLiD™ (Sequencing by Oligo Ligation and Detection) platform was the first commercially released in October 2007 (Mardis, 2008). Library construction is similar to that of both 454 and Illumina sequencing, and may be constructed in several ways to produce, adaptor-flanked fragments (Shendure and Ji, 2008). Like 454 sequencing, DNA amplification is performed using emulsion PCR (Dressman *et al*., 2003) where DNA fragments are bound to paramagnetic beads. Prior to sequencing, the emulsion is broken, and amplicons bearing beads enriched and immobilized to the surface of a specially treated glass slide (Mardis, 2008), generating a dense, disordered array. The SOLiD technology is also sequencing by synthesis method, but is unique because the synthesis of DNA is not driven by a DNA-polymerase, but a ligase after annealing of a sequencing primer (Shendure *et*

al., 2005). At each step, matching fluorescently labeled octamer or interrogation probe originating from a degenerate set is ligated to the DNA fragment. Fluorophores on the different octamers are correlated to a specific position within the octamer. After image acquisition in four different channels, chemical cleavage of the octamer between the fifth and sixth base is performed removing the fluorophore. Multiple ligation steps enable sequencing of every fifth base of the DNA fragment. Following several rounds of ligation, image acquisition and cleavage, the DNA is denatured, enabling annealing of a new sequencing primer at a different position on the adaptor sequence, and a new set of ligation steps (Shendure *et al*., 2005). The fluorophores are correlated to dinucleotides, and not just a single base. This combined with an alternate use of sequencing primers and octamer sets, where the fluorophores correspond to different positions on the octamer, enables that each base is sequenced twice, and miscalled bases can be corrected (Heinz, 2010). After subsequent analysis, the end output is sequence files in SOLiDs color space format csfasta. However, these can be converted into regular sequence files in FASTA format using dynamic programming (Li and Durbin, 2009). The read length of SOLiD was initially 35 bp reads and the output was 3Gb data per run. A complete run could be finished within 7 days (Liu *et al*., 2012).

2.5.2.4 Paired-end sequencing

All three platforms described above are more or less limited by short read lengths. However, this limitation has been partly overcome by the development of paired-end sequencing, which can be performed using all three sequencing systems. The principle of the Paired-end tags (PETs) strategy is the extraction of only short tag signature information (20-30 base pairs) from the two

ends of target DNA fragments, the pairing of the two tags for sequencing analysis, and then the mapping of the paired tag sequences to reference genomes for demarcating the boundaries of the target DNA fragments in the genome landscape (Fullwood, Wei, Liu, and Ruan, 2009). There are multiple ways of constructing a paired-end library. One is the clone based method, where the target sequence is ligated with adaptors containing MmeI restriction sites immediately next to the target sequence (Ng, Wei, and Ruan, 2007). A second method, target DNA fragments are directly circularized with linker oligonucleotides hereby joining the two ends of the target DNA. The linker sequence contains two restriction sites (e.g. MmeI) flanking the two ends of the target DNA, enabling restriction digestion to release the tag-linker-tag construct for sequencing (Shendure *et al*., 2005). Libraries with long inserts (up to 20 kb) between the two sequence tags can be created, which are often referred to as mate pair libraries (Fullwood *et al*., 2009).

2.5.2.5 Other next generation sequencing platforms

Although the commercial market of DNA sequencing is dominated by NGS technologies described above, other new technologies are being developed. Some have already been commercialized (Zhang, Chiodini, Badr, and Zhang, 2011). In the following, two of these, the Ion Torrent System and the Heliscope will be described.

A. Ion torrent (Life Technologies)

In 2010, Ion Torrent company introduced a sequencer called Personal Genome Machine (PGM) with a novel detection system not based on light emission and optics but ion detection (Golan and Medvedev, 2013). This semiconductor sequencing technology is a pyrosequencing base on the detection of

hydrogen ions by measuring pH changes as nucleotides are incorporated during DNA strand synthesis in picolitre wells. This produces a shift in pH that scales with the number of nucleotides incorporated (Whiteley, Jenkins, Waite, Kresoje, Payne, Mullan, Allcock, and O'Donnell, 2012). For sequencing, library of template DNA is carried out using clonal amplification on beads. Fragments are captured in a dense microwell array containing ion sensitive transistors which are used for measurement of the change in pH. Four nucleotides are sequentially flowed over the array, producing a voltage change where incorporation of one or more nucleotides occurs (Rothberg *et al*., 2011). The technology can generate 100 bp reads with a raw accuracy of 98.90 %, which is similar to other NGS platforms (Whiteley *et al*., 2012). However, base calling in homopolymeric regions is problematic as for 454 sequencing (Rothberg *et al*., 2011). In 2011, Ion Torrent announced the release of the Ion 316™ chip, which can produce 100 Mega bases (Mb) in a 2 hour run. This is a tenfold increase in throughput than the original Ion 314™ chip. In particular, the Ion 318™ chip is able to produce 1 Giga base (Gb), which another tenfold increase. Therefore, semiconductor sequencing has several advantages compared with other high-throughput sequencing platforms, including lack of optics, use of natural, unmodified dNTP molecules and exploitation of ubiquitous semiconductor technology (Whiteley *et al*., 2012).

B. Heliscope™ single molecule sequencer

The major difference between Heliscope™ single molecule sequencer and other NGS platforms is that it utilizes sequencing of single DNA molecules. Instead of undergoing the end-repair, ligation, and amplification process, template material is polyadenylated at the 3′ end and captured on a flow cell

coated with PolyToligonucleotides (Ozsolak *et al*., 2010). Sequencing is performed by hybridizing the DNA fragments to covalently bound PolyToligonucleotides on a flow cell. The flow cell is a 50-channel format that can deliver up to 30,000,000 reads per channel (Metzker, 2010). Sequencing is performed in a similar fashion as Illumina sequencing, with single nucleotide extension followed by detection and cleavage of fluorophores (Harris *et al*., 2008). Heliscope™ Single Molecule Sequencer is comparable with other current NGS sequencing systems with regards to accuracy. However, the system is currently limited by shorter read length (25-55 bp), and lower throughput compared to the Illumina and SOLiD sequencing systems.

2.5.3 Microbial genome overview

Microbial genomes are extremely diverse in terms of their nucleotide composition as well as presence of various forms of sequence repeats and patterns that can affect physical properties of the DNA molecule (Huang and Mrázek, 2014). Recently, public databases contain nearly 2000 complete genomic sequences and several thousands more incomplete sequences of microbial genomes. This valuable resource have been used to study the microbial diversity including comparative analysis of closely related microbes as well as metagenome analysis of various environmental samples, although effective use of this resource is becoming more difficult due to the rapid data accumulation (Uchiyama, Mihara, Nishide, and Chiba, 2013). Integrated Microbial Genomes (IMG), a public sequence database, records primary sequence information, genomic organization in scaffolds and/or contigs, as well as computationally predicted protein-coding sequences, some RNA-coding genes and protein product names. The current version of IMG (as of September 10, 2013) contains >13,300 reference genomes consisting of 8,761 bacterial, archaeal and

eukaryotic genomes, as well as 2,848 viral genomes, 1,198 plasmids and 581 genome fragments, with about 33 million protein-coding genes (Markowitz *et al*., 2013).

2.6 Molecular techniques for determination of microbial community in food

The occurrence and abundance of microbes and genes in a given food ecosystem can be evaluated by studying the microbiome, which refers to the microorganisms and their genomes in the environment in question. The traditional approaches consist of isolating and enumerating microbial groups by growing them on various selective culture media and identifying the predominant populations using phenotypic and molecular techniques (Kirk *et al*., 2004). Interestingly, a number of the bacteria observed microscopically could not be accounted for on regular nutrient media. “The great plate anomaly” as this phenomenon is known, was observed in studies of several environments. Eventually, it would be estimated that more than 99% of bacteria have not been cultured. Nevertheless, this inherent limitation of culture-dependent methods may not diminish their applications, particularly for isolation of microorganisms (Su, Lei, Duan, Zhang, and Yang, 2012).

As a result, the so-called culture-independent techniques are widely applied for analysis of microbial community composition and community structure in food ecosystems. These approaches are based on the direct detection of DNA or RNA in microbial ecosystems. These have enabled the simultaneous characterization of whole ecosystems in food samples and the identification of many key species from these sources, such as strains responsible for fermentation, spoilage or flavor-associated microorganisms (Ercolini, 2013). These techniques provide a more sensitive and rapid

method than culture-dependent analysis with the major benefit of detecting microorganisms which are difficult to culture or uncultivable (Justé, Thomma, and Lievens, 2008). The shift from culture-dependent assessment to culture-independent analysis has led to a revolution in microbial ecology.

As PCR was developed and sequencing became easier, cloning and sequencing of 16S rRNA genes allowed for more in-depth analyses of microbial communities (Giovannoni, Britschgi, Moyer, and Field, 1990). Additionally, the cloning and sequencing of 16S rRNA-encoding genes have been considered the gold standard for the characterization of microbial communities and have been used to describe the composition of a variety of communities. Other culture-independent techniques were also developed. Various forms of *in situ* hybridization that rely on group-specific 16S rRNA probes, such as fluorescent in situ hybridization (FISH), emerged. Molecular fingerprinting techniques, such as polymerase denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), terminal restriction fragment length polymorphism (TRFLP), chain reaction-temporal temperature gradient gel electrophoresis (TTGE), and single stranded conformation polymorphism (SSCP), physically separate fragments of the 16S rRNA genes and detect variations in the sequence among members of communities. Whereas, automated ribosomal intergenic spacera (ARISA) rely on a fluorescent primer to amplify 16S-23S rRNA spacer region. These techniques have been applied to study food microbial communities (Jany and Barbier, 2008). Most PCR-based fingerprinting methods are rapid, easy to use, moderately reproducible, and relatively inexpensive.

Unlike the PCR-based methods, which target a single or a few loci corresponding to a small portion of the genome in microbial communities, metagenomics, a whole genome molecular technique, evaluates the total genomic DNA of a microorganism and traditionally involves direct cloning of DNAs extracted from environmental samples. However, this approach are often laborious, time consuming and costly. Recently, pyrosequencing have been reported the capability to access rare members of several microbial communities simultaneously by amplifying and sequencing the hypervariable regions of the 16S rRNA gene by use of bar-coded pyrosequencing primers (Edwards *et al*., 2006). Barcodes (also known as nucleotide keys) are short runs of nucleotides (typically 3 to 8) incorporated directly 5′ of the primer sequence and are used to differentiate samples within a pyrosequencing run. 454 Life Sciences has scaled this process up to be massively parallel, determining the composition of more than 300,000 sequences at once, for approximately the same price as 96 to 192 sequencing reactions performed using traditional chemistries. In addition to the massive parallelization, the 454 technology does not require cloning of the environmental samples, thus eliminating many of the problems that are associated with this step of metagenomics. Amplicon sequencing data from pyrosequencing is analysed for comparison and analysis of microbial communities by programs, namely canoco, estimateS, mothur, and QIIME (Robinson, Bohannan, and Young, 2010). The Ribosomal Database Project (RDP) has been used for classification of 16S rRNA-encoding genes, which processes sequences and clusters them based on similarity to sequences in the RDP database (a taxonomy-based approach). Additionally, SILVA and greengenes provide comprehensive rRNA gene databases to which 16S rRNA gene data generated by pyrosequencing can be aligned (Robinson *et al*., 2010). These

packages allow for complete analysis of an experiment, from the raw data generated by the sequencer all the way to the generation of figures for publication. Limitation encountered with analyses based on the sequences of the 16S rRNA-encoding gene is that the gene does not contain information that is completely reflective of the remaining genome. Although organisms with similar 16S rRNA-encoding genes typically share similar genomes, they can in fact have vastly different functional capabilities (Quigley *et al*., 2011). However, it is known that the 454 sequencers produce artificially duplicated reads and naturally duplicated reads, which might lead to misleading interpretation of the abundance of species and genes in metagenomic studies. Exact duplicates sometimes were removed before data analyses. Artificial duplicates called artifacts are the reads that begin at the same position but may vary in length or bear mismatches. Exact and nearly identical duplicates were reported that they may make up 11~35% of the raw reads. In particular, artificial duplicates will introduce overestimation of abundance of taxon, gene, and function. Additionally, natural duplicates are the reads from the same origin that start at the same genomic position by chance. Therefore, simply removing all duplicates might also cause underestimation of abundance associated with naturally duplicated reads (Niu, Fu, Sun, and Li, 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

3.1.1 Collection, screening, and selection of lactic acid bacteria for volatile compound production

The microbiological media used for cultivation, screening, and selection were De Man, Rogosa and Sharpe agar (MRS) containing 5% and 10% NaCl (Appendix A.1.1), MRS agar containing 0.5% $CaCO_3$, and 5% and 10% NaCl (Appendix A.1.2), M17 agar containing 5% and 10% NaCl (Appendix A.1.3), and glucose yeast extract peptone (GYP) agar containing 5% and 10% NaCl (Appendix A.1.4).

Chemicals used for medium preparation including D-glucose, yeast extract, tryptone, soy peptone, and calcium carbonate were purchased from Himedia (Himedia Laboratories, Mumbai, India). Sodium acetate, magnesium sulfate, manganese sulfate, and sodium chloride were obtained from Carlo Erba (Montedison group, Milan, Italy). Iron (II) sulfate was a product of BDH (BDH Labolatory supplies, Radnor, PA., U.S.A.). L-Histidine, L-leucine, and L-lysine were ordered from Sigma (Sigma-Aldrich Chemical Company, St. Louis, MO., U.S.A.). L-Ornithine and L-tyrosine were obtained from Fluka (Sigma-Aldrich Chemical Company, U.S.A.). Diaminoheptane, dansyl chloride, and acetonitrile were ordered from Sigma (Sigma-Aldrich Chemical Company).

3.1.2 Identification of the selected lactic acid bacterial isolates

3.1.2.1 Morphological and physiological characterization

Chemicals used for morphological and physiological characterization were crystal violet (POCH, POCH SA, Poland); iodine, potassium iodide, ethanol, safranin O, hydrogen peroxide, paraffin oil, and sodium chloride (Carlo Erba, Montedison group); Tween 80 (ACRŌS organics, Acros Organics, Morristown, NJ., USA); skim milk (Himedia, Himedia Laboratories); and copper(II) sulfate (Sigma, Sigma-Aldrich Chemical Company) and tetramethyl-p-phenylenediamine dihydrochloride (Fluka, Sigma-Aldrich Chemical Company). Soluble starch was purchased from Sanguan Wongse Industries Co., Ltd., (Nakhon Ratchasima, Thailand). Sugars used for sugar fermentation were D-arabinose and D-fructose (Merck, Merck Chemicals, Darmstadt, Germany); D-mannose and D-sucrose (Carlo Erba, Montedison group); D-mannitol (Univar, Ajax Finechem Pty. Ltd., New South Wales, Australia); and D-trehalose and D-maltose (Fluka, Sigma-Aldrich Chemical Company). API 50 CH/CHL medium and strips (bioMérieux, bioMérieux Industry, Marcy l'Etoile, France) were also used for biochemical test.

3.1.2.2 16S rRNA gene sequencing and PCR-RFLP

Chemicals and reagents used for genomic DNA extraction and detection were absolute ethanol and sodium chloride (Merck, Merck Chemicals); ethidium bromide and tris-HCl (Promega, Promega Corporation, Madison, WI., U.S.A.); phenol (BDH, BDH Labolatory supplies); sodium citrate and chloroform (Carlo Erba); sodium dodecylsulfate (SDS) (Fluka, Sigma-Aldrich Chemical Company); lysozyme and ethylenediaminetetraacetic acid (EDTA), RNase and

proteinase K (Sigma, Sigma-Aldrich Chemical Company); and LE Agarose (Seakem, Cambrex Bio Science Rockland Inc., Rockland, ME., USA).

Chemicals and reagents used for polymerase chain reaction (PCR) amplification were 10X reaction buffer, $MgCl_2$ solution, and *Taq* DNA polymerase (Invitrogen^{TM}, Invitrogen life technologies, Foster, CA., U.S.A.); and dNTPs (dATP, dCTP, dGTP, and dTTP) (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA., U.S.A.). The oligonucleotide primers were ordered from the Science Pacific Company, Ltd. (Bangkok, Thailand). The molecular weight marker (1 kb plus DNA ladder) was purchased from Invitrogen^{TM} (Invitrogen life technologies). Restriction endonucleases *Alu*I and *Mbo*I (New England Biolabs, New England Biolabs Inc., Beverly, MA., U.S.A.) were used for restriction fragment length polymorphism (RFLP) analysis.

3.1.3 Development of flavor compounds by the selected lactic acid bacterial strains during fish sauce fermentation

Chemicals and reagents used for fish sauce fermentation were Alcalase 2.4L and Flavouzyme 500L (Sigma, Sigma-Aldrich Chemical Company). Plate count agar (PCA) (Appendix A.1.6) and MRS media were purchased from Himedia (Himedia Laboratories) for determination of viable cell counts. Chemicals used for media preparation including casamino acids, yeast extract, sodium glutamate, and calcium carbonate were purchased from Himedia (Himedia Laboratories). Tri-sodium citrate, magnesium sulfate, manganese sulfate, potassium chloride, and sodium chloride were obtained from Carlo Erba (Montedison group). Iron (II) chloride was a product of BDH (BDH Labolatory supplies).

Chemicals and reagents used for chemical analyses of fish sauce were trinitrobenzenesulfonic acid (TNBS), Folin phenol reagent, hydrochloric acid, sodium hydroxide, and L-leucine (Sigma, Sigma-Aldrich Chemical Company); tri-sodium citrate, sodium carbonate, cyclohexanol, and copper sulfate (Carlo Erba, Montedison group).

3.1.4 Detection of bacteria found in Thai fish sauce samples by ARISA and 16S rRNA gene sequencing

For bacterial enumeration, plate count agar (PCA) and MRS media were purchased from Himedia (Himedia Laboratories). Chemicals used for medium preparation including casamino acids, yeast extract, sodium glutamate, and calcium carbonate were purchased from Himedia (Himedia Laboratories). Tri-sodium citrate, magnesium sulfate, manganese sulfate, potassium chloride, and sodium chloride were obtained from Carlo Erba (Montedison group). Iron (II) chloride is a product of BDH (BDH Labolatory supplies).

Chemicals and reagents for automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene sequencing were dNTPs (New England Biolabs, New England Biolabs Inc.); iProof high-fidelity DNA polymerase (Bio-Rad, Bio-Rad Laboratories Inc.); rhodamine X (Bioventures, TN., U.S.A.); and formamide (Applied Biosystems, Thermo Fisher Scientific Inc., Foster, CA., U.S.A.). Primers were ordered from the Biotechnology Center, University of Wisconsin-Madison, WI., U.S.A.

3.1.5 Draft genomes of the selected lactic acid bacterial strains involving unique flavor characteristics of Thai fish sauce

Chemicals and reagents used for genome analysis of selected lactic acid bacterial strains were dNTPs (New England Biolabs, New England Biolabs Inc.); 5X iProof high-fidelity buffer, and iProof DNA polymerase (Bio-Rad, Bio-Rad Laboratories Inc.); agarose (Seakem); and ethidium bromide (Invitrogen™, Invitrogen life technologies). Primers were ordered from the Biotechnology Center, University of Wisconsin-Madison, WI., U.S.A.

3.2 Instrumentation

Instruments required for cultivation, screening, selection, and identification of lactic acid bacteria, and analysis of volatile compounds, were located at the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Instruments required for study of genomes of the selected lactic acid bacterial strains, and detection of bacteria found in Thai fish sauce by ARISA and 16S rRNA gene sequencing, were located at the Biotechnology Center and Department of Food Science, University of Wisconsin-Madison, Wisconsin, U.S.A.

3.3 Collection of lactic acid bacteria isolated from fish sauce samples

Lactic acid bacteria isolated form Thai fish sauce samples at 3, 6, 8, 9 and 12 months were obtained from the Research Center on Microbial Cultures for Food and

Bioplastics Production, Institute of Science, Suranaree University of Technology. Also, some more isolates were derived from fish sauce samples at 1, 2, 10, 11, and 12 months of fermentation. The samples were collected from commercial fish sauce fermentation plants in Thailand. The bacteria were isolated by spread plate technique using MRS agar containing 5% and 10% NaCl (Appendix A.1.1), MRS agar supplemented with 0.5% $CaCO_3$ containing 5% and 10% NaCl (Appendix A.1.2), M17 agar containing 5% and 10% NaCl (Appendix A.1.3), and GYP agar containing 5% and 10% NaCl (Appendix A.1.4). The plates were incubated at 35°C for 3-14 days in an anaerobic chamber (Shel Lab, Sheldon Manufacturing, Inc., Cornelius, OR., U.S.A.). Bacterial colonies were selected according to the difference in their colony morphology, and purified by streaking on the isolation medium. The purified cultures were screened and selected of lactic acid bacterial isolates capable of producing a unique volatile compound.

3.4 Screening and selection of lactic acid bacterial isolates for volatile compound production

3.4.1 Volatile compound production

3.4.1.1 Development of suitable media for volatile compound production

Aldehyde was firstly chosen as a unique volatile compound for screening lactic acid bacteria capable of producing volatile compound. Suitable composition of cultivation media for this screening step was determined. Components of modified glucose yeast extract tryptone (GYT) media were investigated. *Tetragenococcus halophilus* MS33 and ATCC 33315^T were used as reference strains

for investigation of their abilities to produce volatile compounds in the modified medium (Udomsil, 2008). One loopful of the bacterial strains on MRS agar containing 5% NaCl and 0.5% $CaCO_3$ (Appendix A.1.2) for 3-5 days, was inoculated into 5 ml of modified culture broth and fish broth containing 20% NaCl (FB20) or 25% NaCl (FB25) and incubated at 35°C for 48 h under anaerobic condition. Fish broth was prepared by boiling anchovy (*Stolephorus indicus*) in distilled water (1:2) for 20 min (Yongsawatdigul *et al*., 2007). After incubation, bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). The pH was monitored using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland). Cell pellets in the suitable medium resulted from bacterial growth and pH measurement, were removed by centrifuging at 10,000×g for 10 min at 4°C. The supernatant was collected and analyzed for volatile compounds using purge and trap (Texmar velocity XPTTM, Teledyne Tekmar, Mason, OH., U.S.A.). Ten milliliter of the modified culture broth and fish broth was added with cyclohexanol as an internal standard to contain final concentration of 1 mg/l. The sample was purged for 20 min with helium gas at 40°C at a flow rate of 40 ml/min. The volatile compounds were trapped by Tenax TA column and were desorbed at 250°C for 2 min. Separation and detection of the desorbed volatile compounds were performed using GC-MS (Varian Inc., Walnut Creek, CA., U.S.A.) connected to a capillary column (DBWAX, 60 m × 0.25 mm × 0.25 µm Agilent Technologies, Redwood, CA., U.S.A.). The oven temperature was held at 25°C and then raised to 200°C at 15°C/min. Volatile compounds were identified using a quadrupole mass detector (Mass spectrometer 1200L quadrupole, Varian Inc., Walnut Creek, CA., U.S.A.). The column carrier gas was helium at a constant flow rate of 2 ml/min. Mass spectra of volatile compounds were obtained by

electron ionization (EI) at 70 eV. For each compound, quantitation was performed by measuring the corresponding peak area of the total ion chromatogram and expressed as relative (percent) areas by normalization. Identification of volatile compounds was performed by matching their retention time and mass spectral data against mass spectral libraries (National Institute of Standards; NIST data). Retention indices (RI) of unknown compounds were calculated.

3.4.1.2 Detection method of volatile compounds

Lactic acid bacterial isolates obtained from fish sauce samples were screened for their volatile compound production capability using modified GYT medium and detected by electronic nose (Alpha MOS, Toulouse, France). One loopful of the selected isolates grown on MRS agar containing 5% NaCl for 3-5 days, was inoculated into 5 ml of GYT broth and incubated at 35°C for 48 h. Control consisted of inoculated broth with *T. halophilus* MS33 and commercial fish sauce samples. After incubation, one milliliter of inoculated broth was pipetted into a sterile 10 ml glass vial and incubated at 40°C for 5 min to enhance desorption of volatile compounds matrix into the gas phase for electronic nose measurement. Afterwards, headspace was pumped into Dual flash GC-based electronic nose, which is based on the technology of ultra-fast chromatography and includes two short columns of different polarities (DB5 apolar and DB1701 slightly polar), coupled to two Flame Ionization Detectors (FID). The data obtained from detector was analyzed by principal component analysis (PCA).

3.4.2 Growth in high salt concentration (25% NaCl)

The selected lactic acid bacterial isolates were tested for growth in fish broth containing 25% NaCl (FB25). Fish broth medium containing 25% NaCl (pH

7.0) was autoclaved at 121°C for 15 min. One loopful of each purified isolate was inoculated into 5 ml of MRS broth containing 5% NaCl (Appendix A.1.1) and incubated at 35°C for 48 h. Subsequently, 2% (v/v) of the cultures (approximately 10^5-10^6 CFU/ml) was added to 10 ml of FB25 and incubated at 35°C for 7 days under anaerobic condition. Three cycles of bacterial growth in FB25 were carried out. Bacterial growth was detected by spread plate method using MRS agar containing 5% NaCl added with 0.5% $CaCO_3$ or GYP agar containing 5% NaCl and incubated at 35°C for 3-5 days under anaerobic condition. pH of the cultured broth was monitored using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland).

3.4.3 Biogenic amines formation

The selected lactic acid bacterial isolates were tested for their biogenic amines formation using modified glucose yeast extract peptone (mGYP) medium added with 5% NaCl and fortified with 0.25% of various amino acids (L-lysine, L-histidine, L-ornithine, and L-tyrosine) as precursors (Appendix A.1.5). Biogenic amines were determined by high performance liquid chromatography (HPLC) by the method of Dadakova, Krizek, and Pelikanova (2009) with slight modifications. Inoculum was prepared by culturing two loopfuls of the selected isolates grown on MRS agar containing 5% NaCl added with 0.5% $CaCO_3$ at 35°C for 48 h into 10 ml of mGYP broth . The inoculated broth was incubated at 35°C for 48 h under anaerobic condition without shaking or agitating. After incubation, the viable cell counts (CFU/ml) were determined by plating serial dilutions of bacterial suspension on MRS agar containing 5% NaCl added with 0.5% $CaCO_3$, and incubated at 35°C for 3-5 days. Cells were removed from culture medium by centrifugation at 10,000×g for 15 min at 4°C. Supernatant was collected for biogenic amines determination using

HPLC. One milliliter of collected supernatant contained 10 µl of 1 µg/ml diaminoheptane as an internal standard was mixed with 1.5 ml of carbonate buffer (pH 11). Two milliliters of dansyl chloride solution (10 mg/ml) prepared in acetone were added. The mixture solution was incubated at 50°C for 30 min. Subsequently, it was added 600 µl of 30% ammonia solution to eliminate residual dansyl chloride. After 30 min at room temperature, the mixture solution was adjusted to 5 ml with deionized water (DI) and then extracted three times with 3 ml of heptane. The extract was dried at 50^{o}C for 1 h under a stream of nitrogen. The dry residue was adjusted to 5 ml using acetonitrile and filtered through a 0.45-µm regenerated cellulose membrane filter (Agilent Technologies Inc., Palo Alto, CS., U.S.A.) Ten microliters 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 performed using a gradient elution of (A) acetonitrile (100%), (B) acetonitrile (50%) as follows: 0-8 min, A 70% B 30%; 8-12 min, A 80% B 20%; 12-16 min, A 5% B 95%; 16-20 min, A 70% B 30%; at column temperature of 28^{o}C. The flow rate was set at 0.8 ml/min. Diode array detector was set at 254 nm and 550 nm as a reference wavelength (Yongsawatdigul *et al*., 2004).

3.5 Identification of the selected lactic acid bacterial isolates

Four bacterial isolates were selected due to their good growth in fish broth containing 25% NaCl and ability to produce low concentration of biogenic amines.

3.5.1 Morphological characterization

The selected bacterial isolates were cultured on MRS agar containing 5% NaCl and 0.5% $CaCO_3$, and incubated at 35°C for 48 h under anaerobic condition. Cell morphology and cell arrangement were observed by Gram staining (Cappuccino and Sherman, 1999).

3.5.2 Physiological characterization

3.5.2.1 Catalase test

Bacterial isolates were transferred to the surface of a glass slide and then added with 3% hydrogen peroxide (Appendix A.2.2). Rapid formation of gas bubbles indicated a positive result.

3.5.2.2 Oxidase test

Bacterial isolates were streaked onto filter paper (Whatman no. 4) placed into a sterile petri dish and wet with 0.5 ml of 1% tetramethyl-p-phenylenediamine dihydrochloride (Appendix A.2.5). The appearance of a deep blue color at the inoculation site within 10 seconds indicated a positive result.

3.5.2.3 Gas production

The production of gas from glucose was determined by culturing lactic acid bacterial cells in MRS broth containing 5% NaCl (Appendix A.1.1) with Durham tube for 48 h at 35°C under anaerobic condition. Gas entrapped in Durham tube, represented the positive gas production. Heterofermentatives produced gas but homofermentatives were failure to produce gas.

3.5.2.4 Acid production

Acid production of selected bacterial isolates was assayed by API 50 CH/CHL commercial kit (bioMérieux, bioMérieux Industry, RCS Lyon,

France) according to the manufacturer instructions. Acid formation from some sugars such as D-arabinose, D-fructose, D-maltose, D-mannitol, D-mannose, D-trehalose, and D-sucrose was confirmed by the use of a basal medium of glucose yeast extract peptone (GYPB) broth (Tanasupawat, Okada, and Komagata, 1998) with 5% NaCl.

3.5.2.5 Amylase test

Bacterial isolates were tested for amylase production on MRS agar containing 1% starch supplemented with 5% NaCl (Appendix A.1.8). The agar plate was inoculated with bacterial cells and incubated at 35°C for 3 days under anaerobic condition. The plate was then flooded with iodine solution. Appearance of clear zone around the colony of the test organisms indicated positive amylase production.

3.5.2.6 Lipase test

Lipase production was detected using MRS agar containing 1% Tween 80 supplemented with 5% NaCl (Appendix A.1.9). Bacterial culture was point-inoculated on the agar plate and incubated at 35°C for 3 days. Opaque zone around the colonies indicated the positive reaction of lipase production from bacteria.

3.5.2.7 Proteinase test

Proteinase production was tested by point inoculation of pure culture on MRS agar containing 1% skim milk supplemented with 5% NaCl (Appendix A.1.10). The inoculated plate was incubated at 35°C for 3 days. Clear zone around the colonies indicated the production of proteinase from bacteria (positive test).

3.5.2.8 Stereoisomer of produced lactic acid

Bacterial cells were cultured in 10 ml MRS broth containing 5% NaCl incubated at 35°C for 3-5 days under anaerobic condition. The cells were removed by centrifuging at 10,000×g for 10 min at 4°C. Lactic acid configuration (D/L lactic acid) in filtered supernatant was separated using high performance liquid chromatography (HPLC) (HP 1200, Agilent Technology Inc., U.S.A.) equipped with a Chiral Astec CLC-L column (5 µm, 4.6 mm x 15 cm, Sigma Chemical Co., U.S.A.) (Tanaka *et al*., 2006). Mobile phase (0.005 M $CuSO_4$) was run isocratically at a flow rate of 0.8 ml/min. Injection volume was 10 µl. Lactic acid was detected by UV detector at 254 nm (Manome, Okada, Uchimura, and Komagata, 1998).

3.5.2.9 Temperature, pH, and salt concentration for growth

Bacterial isolates were tested for growth at different temperatures (5, 10, 15, 20, 25, 30, 35, 37, 40, 45, and 50°C), pH (4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0), and salt concentrations (0, 3.0, 6.5, 8.0, 10.0, 15.0, 18.0, 20.0, and 25.0% NaCl), into MRS broth. For optimum temperature test, only cultivation temperature was varied while pH and salt concentration of broth was adjusted to 7 and 5% NaCl respectively. For pH tolerance test, only the pH of the broth was varied while the temperature was fixed at 35°C and salt concentration was 5% NaCl. For bacterial growth at different salt concentration test, only the NaCl concentration of the broth was varied while pH of medium and cultivation temperature were fixed at 7.0 and 35°C respectively. The inoculum size of 2% (approximate 10^6 CFU/ml) was aseptically inoculated into 5 ml of MRS broth. Bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). Each treatment was tested in duplicate.

3.5.3 Ribosomal RNA gene analysis

3.5.3.1 16S ribosomal RNA gene sequencing

Nucleotide sequence of 16S ribosomal RNA (rRNA) gene was determined by genetic characterization involving extraction of genomic DNA, amplification of 16S rRNA gene, and sequencing of 16S rRNA gene.

Genomic DNA was extracted from lactic acid bacterium isolates as described by Weisburg, Barns, Pelletier, and Lane (1991). Bacterial cells cultured on MRS agar containing 5% NaCl and 0.5% $CaCO_3$ at 37°C for 3 days under anaerobic condition were harvested and suspended in 1 ml saline-EDTA (pH 8.0) (Appendix A.2.6). Cell suspensions were added with 0.75 mg/ml lysozyme (Appendix A.2.7), mixed well, and incubated at 37°C for 1 h for cell lysis. Subsequently, the mixture was added with 250 µl of Tris-HCl (pH 9.0) (Appendix A.2.8) and 125 µl of 10% SDS (Appendix A.2.9) and incubated at 60°C for 5 min. Then, phenol:chloroform (1:1) (Appendix A.2.10) was added for protein removal and centrifuged at 10,000×g at 4°C for 10 min. The upper layer was collected and added with cold absolute ethanol to obtain genomic DNA pellet. The DNA pellet was further purified using RNase and 100 µl of proteinase K and incubated at 37°C for 1 h. After incubation, the proteins were removed again with phenol:chloroform (1:1) and top supernatant was collected. DNA was precipitated by cold absolute ethanol, air died and then dissolved in 100 µl of 0.1X saline-sodium citrate (SSC) buffer (pH 7.0) (Appendix A.2.13). Genomic DNA was detected in 0.8% agarose gel electrophoresis (Bio-Rad, Bio-Rad Laboratories Inc.), stained with ethidium bromide (1 mg/ml), and examined under UV transilluminator (Bio-Rad, Bio-Rad Laboratories Inc.). The concentration of DNA was measured by SmartSpecTM 3000 spectrophotometer at

260 nm (Bio-Rad, Bio-Rad Laboratories Inc.) and DNA purity with respect to contaminants, such as protein, was calculated from the ratio of optical density at A260/A280. Then, purified DNA solution was maintained at -20°C until use.

Amplification of 16S ribosomal RNA gene was carried out by polymerase chain reaction (PCR) using fD1 and rP2 primers (Weisburg *et al*., 1991) as forward and reverse primers to obtain approximately 1,500 bp of 16S rRNA gene. The amplification reaction was performed in the total volume of 50 µl of reaction mixture containing 200 ng of bacterial DNA, 5 µl of 10X reaction buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl), 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of dNTPs mixture (dATP, dCTP, dGTP, dTTP), 1.0 µl of 20µM (20.0 pmoles/ µl) of each primer (fD1 and rP2) (Table 3.1), and 0.5 µl of Taq DNA polymerase (5 units/µl), and adjusted volume to 50 µl with deionized water. The program of amplification consisted of 1 cycle of 95°C for 2 min; 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 2 min; and the final cycle of 72°C for 7 min. The PCR reactions were carried out in the automated thermal cycle (Thermo electron corporation Px2 Thermal Cycler, Bioscience Technologies Division, Waltham, MA, U.S.A.). The PCR amplified products were examined by electrophoresis (Bio-Rad, Bio-Rad Laboratories Inc.) using 1% agarose and stained with ethidium bromide. The PCR products were observed under short wavelength UV light and determined size comparison with 1 Kb plus DNA ladder.

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

Primer	Primer sequence (5′ to 3′)	Target region[a]	Reference
fD1 (PCR amplification)	5′-AGAGTTTGATCCTGGCTCAG -3′	8-27	Weisburg *et al.* (1991)
rP2 (PCR amplification)	5′-ACGGCTACCTTGTTACGACTT-3′	1490-1511	Weisburg *et al.* (1991)
Forward primer (Sequencing)	5′-TAACTACGTGCCAGCAGCC-3′	515-533	Udomsil (2008)

[a]: *Escherichia coli* numbering.

Nucleotide sequencing of the 16S rRNA gene was performed using ABI 3730xl DNA analyzer (Perkin Elmer, Applied Biosystems, Thermo Fisher Scientific Inc.) The gene sequences were corrected by manual inspection of chromatograms. All alignments were examined and manually optimized with the BioEdit program (North Carolina State University, U.S.A.). The sequences were compared to nucleotides database provided by the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence information was then imported into the CLUSTAL X software program (Hitachi Software Engineering Co.) for assembly and alignment (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins, 1997). Phylogenetic tree was constructed using the Maximum Pasimony method with software MEGA version 4 (Kumar, Tamura, Jakobsen, and Nei, 2004). The stability relationships were evaluated by a boot strap analysis of 1000 replications.

3.5.3.2 Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rDNA

The amplification of 16S rRNA gene was carried out by PCR method as described in 3.5.3.2. The purified PCR products were separately digested with two restriction endonucleases *Alu*I and *Mbo*I. Approximately 1,000 ng of 16S rDNA was added with 10X reaction buffer and 40 U of *Alu*I or *Mbo*I (New England Biolabs, New England Biolabs Inc.), mixed gently, and spinned down before incubation. The reaction mixture was incubated at 37°C for 16 h or overnight. The digested DNA fragments were separated by 1.5% agarose gel electrophoresis (Bio-Rad, Bio-Rad Laboratories Inc.) and run at 100 V for 1 h. DNA fragment patterns of 4 isolates were compared to those of *Tetagenococcus muriaticus* JCM 10006^{T} (Sitdhipol *et al*., 2013).

3.6 Development of volatile compounds by the selected lactic acid bacteria during fish sauce fermentation

Four lactic acid bacterial isolates were selected to study their roles of flavor compound development during fish sauce fermentation due to their ability to produce low concentration of biogenic amines compared to the control and good growth in fish broth containing 25% NaCl and mentioned above.

3.6.1 Starter culture preparation

One loopful of each selected lactic acid bacterial isolates was cultured in 100 ml of fish broth containing 10% NaCl, pH 7.0 at 30°C for 2-3 days under anaerobic condition to obtain the cells approximately of 10^{6}-10^{7} CFU/ml. Fish broth was prepared according to section 3.4.1.1 (Yongsawatdigul *et al*., 2007).

3.6.2 Fish sauce fermentation

Indian anchovy (*Stolephorus* spp.) were used for fish sauce fermentation which were fresh and enzyme-digested fish. For enzyme-digested fish preparation, frozen anchovy was incubated in a 65°C water bath until temperature at the center of the sample reached 65°C. The samples contained in a glass jar were then added 0.25% (w/w) of Alcalase 2.4L and incubated for 2 h. Subsequently, the samples were cooled to 50°C and added 0.5% (w/w) of Flavouzyme 500L, and incubated at 50°C for 4 h (Yongsawatdigul *et al*., 2007).

One kilogram of fresh and enzyme-digested fish contained in a glass jar was mixed with 25% (w/w) solar salt at a ratio of 3:1 and 10% (v/w) starter cultures. All samples were left at room temperature until the temperature attained 35°C and 25% (w/w) solar salt and 10% (v/w) starter cultures were added. The control was prepared by adding 10% of fish broth without starters. All treatments were incubated at room temperature for 8 months. Microbiological changes, pH, oligopeptide and α-amino content were determined at 0, 14, 30, 60, 90, 120, 180 and 240 days of fermentation. Volatile compounds of the samples were analyzed at 60, 90, 120, 180 and 240 days. Other physico-chemical properties, including salt and total nitrogen contents, were monitored after 240 days of fermentation.

3.6.3 Microbiological changes

Fish sauce mash (25 g) was aseptically collected from the fermentation jar at each time interval of fermentation process (0, 30, 60, 90, 120, 180 and 240 days). Total viable cell counts of all samples were performed by spread plate method using plate count agar (PCA) containing 10% NaCl and incubated at 30°C for 2-3 days under aerobic condition. Halophilic bacteria were enumerated on JCM medium

no.168 containing 20% NaCl and MRS agar supplemented with 0.5% $CaCO_3$ containing 5% NaCl and incubated at 30°C for 3-14 days under anaerobic condition.

3.6.4 Chemical analyses

3.6.4.1 α-Amino content

The α-amino content considered as soluble peptide contents was performed using trinitrobenzenesulfonic acid (TNBS) method with L-leucine as a standard (Field, 1979). Fifty µl of filtered fish sauce fermented at 0, 30, 60, 90, 120, 180 and 240 days was added to 0.5 ml of 0.2125 M phosphate buffer (pH 8.2) (Appendix A.2.14) and 0.5 ml of 0.05% TNBS (Appendix A.2.15). Subsequently, the mixture was incubated at 50°C for 1 h in a water bath. After incubation, the reaction was immediately stopped by addition of 0.1 N HCl (Appendix A.2.16) and left at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer (GBC UV/VIS 916; GBC Scientific Equipment PTY, Ltd., Australia). The data from standard curve are usually linear enough that a straight-line interpolation can be used to determine the concentration of unknowns. □-Amino content was expressed as mM of L-leucine.

3.6.4.2 Oligopeptide content

The oligopeptide content was measured using Lowry's assay method with tyrosine as a standard (Lowry, Rosebrough, Farr, and Randall, 1951). Filtered fish sauce (100 ml) was added with 1 ml of reagent D (Appendix A.2.17) containing reagent A (2% Na_2CO_3 in 0.1 N NaOH) and reagent B (0.5% $CuSO_4.5H_2O$ in 1% $C_6H_5Na_3O_7$). The resulting mixture was mixed and incubated for 10 min at room temperature. After 10 min, 0.1 mL of 1 N Folin phenol reagent (reagent C) (Appendix A.2.18) was added, immediately followed by vortex mixing and incubated

for 30 min at room temperature. After 30 min, the absorbance of the sample was measured at a wavelength of 750 nm in a spectrophotometer (GBC UV/VIS 916; GBC Scientific Equipment PTY, Ltd., Australia). The spectrophotometer had been zeroed using a blank that had been prepared in exactly the same way as the sample, except that it lacked the protein solution. Oligopeptide content was expressed as mM of tyrosine.

3.6.4.3 Other physico-chemical properties

Fish sauce samples (12 months) were analyzed for total nitrogen using the micro-Kjeldahl method and salt content using Volhard titration method (AOAC, 1995). The pH was measured using pH meter (CCMP 510 pH Conductivity Meter, WPA, Biochom Ltd, England).

3.6.4.4 Volatile compound profiles

Fish sauces fermented for 60, 90, 120, 180 and 240 days were analyzed for volatile compounds using a headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC–MS). Ten ml of fish sauce was added with cyclohexanol as an internal standard to contain final concentration of 1 mg/l. Extraction of volatile compounds from headspace of the sample vial was carried out using a 3-phase SPME fiber (1 cm-50/30 μm StableFlex Divinyl-benzene/Carboxen/PDMS, Supelco, PA.) that was exposed at 60^{o}C for 30 min in a heating block. After extraction, the fiber was pulled into the needle sheath and the SPME device was removed from the vial and inserted into gas chromatography injection port for thermal desorption at 250°C for 3 min. Separation and detection of the desorbed volatile compounds were performed using GC-MS as described in 3.4.1.

3.7 Draft genomes of the selected lactic acid bacterial strains involving unique flavor characteristics of Thai fish sauce

The selected lactic acid bacterial strains isolated form Thai fish sauce samples were obtained from the Research Center on Microbial Cultures for Food and Bioplastics Production, Institute of Science, Suranaree University of Technology and brought to Department of Food Science, University of Wisconsin-Madison, Wisconsin, U.S.A. These bacterial strains were isolated from two commercial fish sauce fermentation plants.

3.7.1 Genome sequencing and assembly

Genomic DNA of two selected bacterial isolates was prepared using CTAB method (DOE Joint Genome Institute, CA., U.S.A.), and confirmed its quality by spectrometry and agarose gel electrophoresis. The DNA samples were submitted for genomic sequencing using 454 GS FLX Titanium paired-end sequencing platform (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA., U.S.A.) at the Biotechnology Center, University of Wisconsin-Madison (U.S.A.). Raw data of nucleotide sequences were assembled using the GS *de novo* assembler ("Newbler") (Life Technologies, Thermo Fisher Scientific Inc.).

3.7.2 Genome analysis

Assembled contigs were subsequently annotated by a systematic annotation package for community analysis of genomes (ASAP) (Glasner *et al*., 2006), the Rapid Annotation using Subsystem Technology (RAST) (Meyer *et al*., 2008) and Integrated Microbial Genomes/Expert Review (IMG) (Markowitz *et al*., 2012) pipeline servers for subsystem classification and functional annotation without manual curation. All gene product names used in this study are obtained from the

homologs of *Tetragenococcus muriaticus* DSM 15685^T and *T. halophilus* NBRC 12172^T as showed in the NCBI database (http://www.ncbi.nlm.nih.gov), unless otherwise noted.

3.8 Detection of bacteria found in Thai fish sauce samples by ARISA and 16S rRNA gene sequencing

3.8.1 Collection of Thai fish sauce fermentation samples

Fish sauce samples at 1-12 months of fermentation were collected from a commercial fish sauce fermentation plant located in Rayong Province, Thailand. Samples (approximately 500 ml) were separately taken from two fermentation vats at each fermentation period.

3.8.2 Microbiological analysis by culture-dependent technique

Bacteria in fish sauce samples were isolated by spread plate technique using PCA containing 10% NaCl, MRS agar supplemented with 0.5% $CaCO_3$ containing 5% NaCl, and JCM medium no.168 containing 20% NaCl. The plates were incubated at 30°C for 3-14 days under aerobic condition for aerobe and anaerobic condition for anaerobe. Bacterial counts of the fish sauce samples were expressed as $\log_{10}$ colony forming units (CFU) per ml.

3.8.3 Automated ribosomal intergenic spacer analysis (ARISA)

Total genomic DNA was isolated from 1 ml of fish sauce samples at 1-10 months of fermentation, which were collected from two different fermentation vats, using DNeasy Blood & Tissue Kit (Qiagen, Qiagen Sciences, Germantown, MD., U.S.A.). ARISA-PCR was conducted using primer 1406f, 5′-TGYACACACCGCCCGT-3′, which was labeled with phosphoramidite dye 5-FAM

and primer 23Sr, 5′- GGGTTBCCCCATTCRG-3′. PCR amplification was performed using iProof High-Fidelity DNA polymerase with an iCycler Thermal Cycler (Bio-Rad, Bio-Rad Laboratories Inc.). The PCR condition utilized for amplification of the 16S-23S spacer region was: initial denaturation at 98°C for 30 sec followed by 35 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec, final extension at 72°C for 10 min, and holding at 4°C. A 20 µl reaction with 40 ng of genomic DNA was prepared according to the manufacturer's instructions. One µl of PCR products, 0.4 µl of custom 100- to 2000-bp standard labeled with Rhodamine X, and 10 µl of highly deionized formamide were submitted to capillary electrophoresis analysis using ABI 3700 Genetic Analyzer at the University of Wisconsin-Madison Biotechnology Center. Amplicon sizes were determined by comparison to the internal size standards. Peak area, which was proportional to DNA quantity, was calculated using PeakScanner (Applied Biosystems, Thermo Fisher Scientific Inc.). Peaks with greater than 50 fluorescence units, which were between 300-1,000 bp, were included in the ARISA profiles. The relative amount of each fragment in the PCR product was estimated as the ratio between the fluorescence (peak area) of the fragment of interest and the total fluorescence of all fragments in the profile. The R Statistics Package (R Development Core Team, 2012) was used to compare the relative distributions of peaks in the different months and vats of fish sauce.

3.8.4 Bacterial community analysis by 16S rRNA gene sequencing

The partial 16S rRNA genes were sequenced by using the Ion Torrent Personal Genome Machine (PGM) (Invitrogen™, Invitrogen life technologies) at the University of Wisconsin-Madison Biotechnology Center. Total genomic DNA of fish sauce samples was isolated using DNeasy Blood & Tissue Kit (Qiagen, Qiagen

Sciences). The V1-V2 region of 16S rRNA gene was amplified using bar-coded fusion primers with the Ion Torrent adapters (shown in italics), followed by a unique 13-base barcode sequence (B), and finally the 3' ends of primer 8F (5′-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG BBB BBB BBB BBB BAG AGT TTG ATC MTG GCT CAG-3′; Turnbaugh *et al*., 2009), and primer BSR357 without barcode sequence for unidirectional sequencing (5′-CCT CTC TAT GGG CAG TCG GTG ATC TGC TGC CTY CCG TA-3′; McKenna *et al*., 2008). PCR amplification was performed using iProof High-Fidelity DNA polymerase with an iCycler Thermal Cycler (Bio-Rad, Bio-Rad Laboratories Inc.). The program of amplification consisted of 1 cycle of 98°C for 30 sec; 30 cycles of 98°C for 10 sec, 50°C for 30 sec, 72°C for 30 sec; and the final cycle of 72°C for 30 sec, and holding at 4°C. All PCR reactions were quality-controlled for amplicon saturation by gel electrophoresis. Prior to sequencing, the PCR products were purified with Agencourt® AMPure® XP Reagent (Invitrogen™, Invitrogen life technologies) and then quantified with the Qubit High Sensitivity dsDNA assay kit (Invitrogen™, Invitrogen life technologies). The amplicons from all reactions were pooled at 33.6 ng/sample and then cleaned three times on Axygen beads (Axygen Biosciences, CA., U.S.A.). Diluted library at 12.75 pM was used in the templating reactions. This library was templated and sequenced using the Ion PGM™ Sequencing 400 kit on 314 v2 chips (Life Technologies, NY., U.S.A.). Ion Torrent PGM produced sequencing reads up to 400 bp long. Reads were demultiplexed by the Ion Torrent software. Reads with incomplete barcode sequences were removed. Custom script removed the 5' primer adapter sequence, trimmed the 3' primer adapter sequence (if present), and removed reads with average quality score lower than 20. The reads were further filtered to remove those that are shorter than

250 bp. Thirty samples had combined 1,864,163 reads passed filtering, with number of reads for each sample ranging from 23,642 to 87,579. Further bacterial community analysis is performed by using the QIIME pipeline (Sayess, Saikaly, El-Fadel, Li, and Semerjian, 2013).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Collection of lactic acid bacteria isolated from fish sauce samples

A total of 288 lactic acid bacteria (LAB) were collected from fish sauce samples at 1-12 months of fermentation. One hundred fifty two of the total isolates were obtained from the Research Center on Microbial Cultures for Food and Bioplastics Production, Institute of Science, Suranaree University of Technology. These bacteria were isolated from Thai fish sauce samples at 3, 6, 8, 9, and 12 months from a fish sauce fermentation plant (Plant 1). The rest, one hundred thirty six LAB isolates were isolated from fish sauce samples fermented for 1, 2, 10, 11, and 12 months from a different commercial fish sauce plant (Plant 2) in Thailand. Lactic acid bacteria were found throughout the fermentation process of 12 months at the two fish sauce plants. The bacterial counts from plant 1 were approximately less than 13-10^5 CFU/g, while those isolated from plant 2 were 3-10^4 CFU/g. These results implied that microbial population varied among fish sauce plants depending on raw materials (fish and salt) and fermentation operation. Lactic acid bacteria counts of samples from both places isolated using MRS agar containing 5% NaCl and 10% NaCl supplemented with 0.5% $CaCO_3$ were higher than those from MRS agar containing 5 and 10% NaCl without 0.5% $CaCO_3$. This enumeration technique is considered as a

traditional culture-based approach. Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul (2011) isolated LAB strains from Thai fish sauce and noted that the medium containing $CaCO_3$ showed higher LAB counts than medium without $CaCO_3$, which is appropriated for the selection of LAB in fish sauce mash sample. Halophilic or halo-tolerant LAB are considered as the predominant bacterial group in fish sauce fermentation process (Saisithi, 1994).

4.2 Screening and selection of lactic acid bacterial isolates for volatile compound production

4.2.1 Volatile compound production

4.2.1.1 Development of suitable media for volatile compound production

To obtain the suitable media for screening LAB capable of producing a unique volatile compound, compositions of media enhancing volatile compound production were determined. Components of glucose yeast extract tryptone (GYT) medium were modified as described in Table 4.1. *Tetragenococcus halophilus* MS33 and ATCC 33315^T were selected for evaluating their ability to produce volatile compounds in the optimized media. Both strains grew well in all investigated media. Growth of the two bacterial strains in modified medium no. 1 (Table 4.1) containing 1% glucose as a carbon source, was higher than those of other media at an optical density (600 nm) of 1.209 and 1.464 respectively. L-Leucine, a branched chain amino acids was supplemented in the medium, which could be served as a nitrogen source, growth factor and a precursor for flavor formation, particularly branched aldehydes by the bacteria (Kranenburg *et al*., 2002). Leucine catabolism activity typically results in

3-methyl-1-butanal and subsequently converted to 3-methyl-1-butanol and 3-methyl-1-butanoic acid via reduction and oxidation, respectively (Smit *et al*., 2009). Therefore, aldehyde was chosen as a unique volatile compound contributing to the distinct odor of Thai fish for screening lactic acid bacteria. Three of the modified media (media no. 1, 3, and 5, Table 4.1) were selected for the study of aldehyde production, which contained 1% L-leucine. A total of 8 major aldehydes were detected in modified culture broth samples compared to the controls when analyzed by purge and trap method (Table 4.2). There were acetaldehyde, butanal, 2-methyl-1-butanal, 3-methyl-1-butanal, 2-butenal, 2-methyl-2-butenal, 3-methyl-2-butenal, and benzaldehyde. It was found that isolate MS33 inoculated into modified medium no.1 produced the highest amount of acetaldehyde, 2-methyl-1-butanal, and 3-methyl-2-butenal as compared to other media. 2-Methylbutanal was one of potent distinctive aldehydes of fish sauce (nam-pla) and contributed to a meaty note (Fukami *et al*., 2002). The cultivated medium no.1 inoculated with starter cultures contained the highest amount of 2-methylbutanal. These results suggested that this medium could be suitable for flavor development and selected for screening volatile compound production capability of the selected LAB isolates.

4.2.1.2 Detection method of volatile compounds

Two hundred eighty eight isolates obtained from fish sauce samples were screened for their volatile compound production capability using modified medium no.1 (Appendix A1.10). The cultivated medium was analysed using electronic nose technique compared to six bands of commercial fish sauce. For visualization of 294 data, principal component analysis (PCA) was performed to identify patterns of correlation with produced flavor compounds involved in the

discrimination among the samples. According to the PCA analysis (Figure 4.1), most of the samples could be discriminated. Some samples were located near to five commercial fish sauces and separated from the other sample clusters. This result implied that some LAB isolates are able to produce flavors similar to those of commercial fish sauces. The data from the electronic nose system can be considered a fingerprint, representing the response odor patterns of different, same, or similar odors in different samples (Pisanelli, Qutob, Travers, Szyszko, and Persaud, 1994). Kim *et al*. (2004) reported that different odor patterns were observed among samples of salted and fermented anchovy sauces caused by gamma irradiation using electronic nose system analysis. Nimsuk and Nakamoto (2008) also reported that the result of PCA analysis showed well-separated patterns of fish sauce. Thirty eight isolates of LAB were selected based on different odor patterns.

Table 4.1 Components of modified glucose yeast extract tryptone (GYT) media.

Medium component	Concentration (g/l) of medium no.					
	1	2	3	4	5	6
Glucose	10.00	10.00	5.00	5.00	1.00	1.00
Yeast extract	10.00	10.00	10.00	10.00	10.00	10.00
Tryptone	10.00	10.00	10.00	10.00	10.00	10.00
Sodium acetate	10.00	10.00	10.00	10.00	10.00	10.00
$FeSO_4.7H_2O$	0.01	0.01	0.01	0.01	0.01	0.01
$MgSO_4.7H_2O$	0.20	0.20	0.20	0.20	0.20	0.20
$MnSO_4.H_2O$	0.01	0.01	0.01	0.01	0.01	0.01
L-Leucine	10.00	-	10.00	-	10.00	-
NaCl	50.00	50.00	50.00	50.00	50.00	50.00

Table 4.2 Aldehyde compounds found in glucose yeast extract tryptone (GYT) medium containing 5% NaCl, fish broth containing 20% and 25% NaCl inoculated with *Tetragenococcus halophilus* MS33 and ATCC 33315^{T} and incubated at 35°C for 48 h under anaerobic condition.

No	RI[a]	Volatile compound	Relative peak area[b] of different cultivated media :									
			No.1		No.2		No.3		FB25		FB20	
			MS33	ATCC 33315	MS33	ATCC 33315	MS33	ATCC 33315	MS33	ATCC 33315	MS33	ATCC 33315
1	745	Acetaldehyde	5.650^{a}	2.910^{c}	2.840^{c}	1.610^{d}	2.350^{c}	0.480^{d}	3.286^{b}	3.385^{b}	3.000^{b}	1.500^{c}
2	893	Butanal	0.100^{b}	nd	0.270^{a}	0.060^{c}	nd	nd	nd	nd	nd	nd
3	932	2-Methyl-1-butanal	0.680^{a}	nd	0.450^{b}	0.110^{c}	nd	nd	nd	nd	nd	nd
4	938	3-Methyl-1-butanal	10.000^{a}	5.270^{b}	2.940^{c}	3.310^{c}	0.760^{d}	0.780^{d}	0.300^{d}	0.238^{d}	3.571^{c}	4.846^{b}
5	1064	2-Butenal	0.070^{d}	nd	0.040^{d}	nd	0.380^{a}	0.020^{d}	0.205^{b}	0.094^{d}	0.179^{c}	0.138^{c}
6	1150	2-Methyl-2-butenal	0.040^{a}	nd	0.040^{a}	nd	nd	0.010^{b}	nd	nd	nd	nd
7	1126	3-Methyl-2-butenal	0.280^{a}	nd	nd	nd	nd	nd	nd	nd	0.157^{b}	0.100^{c}
8	1566	Benzaldehyde	0.350^{b}	0.530^{a}	0.500^{a}	0.330^{b}	0.130^{d}	0.080^{e}	0.110^{d}	0.084^{e}	0.179^{c}	0.192^{c}

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).

Thirty eight selected isolates were selected to characterize some morphological and physiological characteristics which were used as the selection criteria associated with a period of fermentation process. These isolates were Gram-positive and identified as belonging to LAB in the genus *Tetragenococcus*. Colonies on MRS agar containing 5% NaCl and 0.5% $CaCO_3$ were 0.25-3.00 mm, low convex, circular, smooth, and white. Cell morphology were cocci and tetrads arrangement with the approximate cell sizes of 0.40-1.09 μm. Taken together with the period of fish sauce fermentation process, seventeen out of 38 isolates were randomly selected to determine the ability to growth at high salt environment. There are 5, 2, 1, 2, 5, and 2 isolates obtained from fish sauce samples fermented for 3, 6, 8, 9, 10, and 11 months, respectively (Table 4.3).

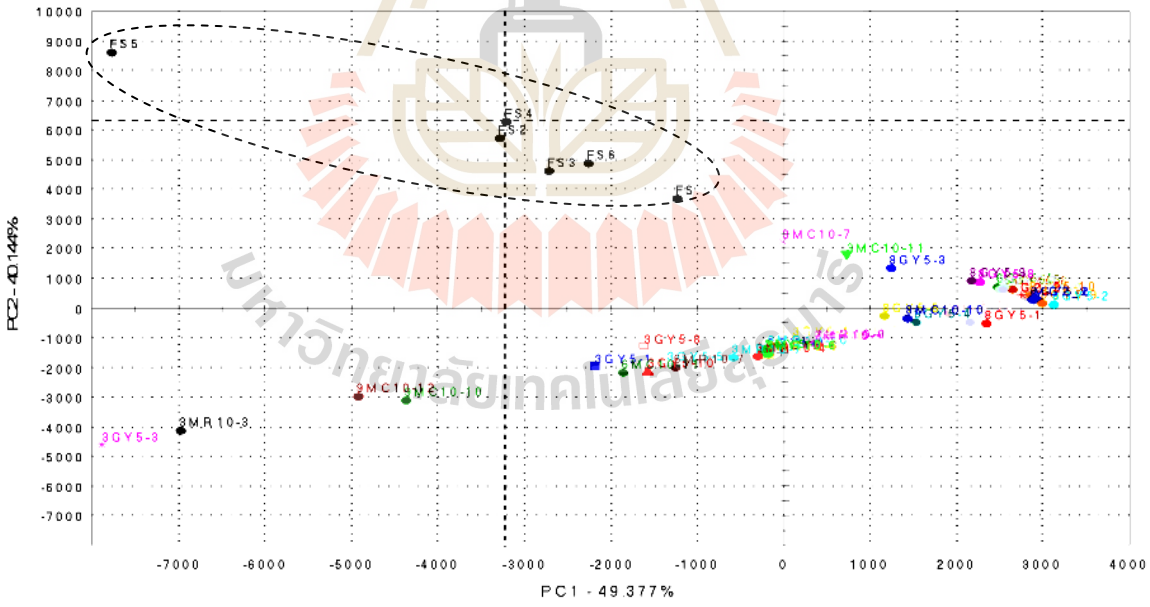

Figure 4.1 Principal component analysis of flavor compounds produced by 288 isolates of lactic acid bacteria compared to commercial fish sauces (FS, FS2, FS3, FS4, FS5, and FS6) by the electronic nose system.

Table 4.3 Morphological and physiological characteristics of 17 selected lactic acid bacterial isolates.

Characteristics	Selected lactic acid bacterial isolate								
	3MC10-7	3MC10-11	3MR10-3	3GY5-1	3GY5-8	6MC10-11	6MR10-7	8GY5-3	9MC10-10
Colony	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge
Colony diameter (mm)	0.5-1	0.5-1	0.5-1	0.5-1	0.5-1	0.5-1.5	0.5-1.5	0.25-1.25	0.5-1
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads
Cell size (µm)	0.69-1.04	0.43-0.65	0.71-1.09	0.52-0.75	0.66-0.81	0.64-0.90	0.69-0.82	0.65-0.75	0.47-0.71
Catalase test	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-
CO_2 production	-	-	-	-	-	-	-	-	-
Gram strain	+	+	+	+	+	+	+	+	+
Fish sauce production plant	1	1	1	1	1	1	1	1	1
Fermentation period (month)	3	3	3	3	3	6	6	8	9

Table 4.3 (Continued) Morphological and physiological characteristics of 17 selected lactic acid bacterial isolates.

Characteristics	Selected lactic acid bacterial isolate							
	9MC10-12	PM-10-5	PMC4-10-1	PMC4-10-2	PMC4-10-6	PMC4-10-7	PMC-11-5	PM-11-12
Colony	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge
Colony diameter (mm)	0.5-1	0.5-1	1-3	0.5-3	1-3	1-3	0.25-1	0.5-1
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads
Cell size (µm)	0.77-0.90	0.75-0.84	0.78-1.01	0.68-0.89	0.69-0.84	0.58-0.74	0.55-0.68	0.64-0.73
Catalase test	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-
CO_2 production	-	-	-	-	-	-	-	-
Gram strain	+	+	+	+	+	+	+	+
Fish sauce production plant	1	2	2	2	2	2	2	2
Fermentation period (month)	9	10	10	10	10	10	11	11

+: positive; -: negative.

4.2.2 Growth in hight salt concentration (25% NaCl)

Seventeen isolates of the selected lactic acid bacteria were tested for growth in fish broth containing 25% NaCl (FB25) with observing 3 cycles of cultivation. This ability is useful for bacteria applied as a starter culture for fish sauce fermentation. LAB able to grow in FB25 were likely to survive and/or proliferate during fish sauce fermentation (Udomsil, 2008). It was found that thirteen lactic acid bacterial isolates could survive in FB25 within 7 days at the 1st cycle of cultivation then slightly decreased at the 2nd and 3rd cycles. At the 3rd cylcle of cultivation, some isolates can survive in FB25 but did not increase cell counts. Isolates that grew in FB25 with an increase of cell counts were selected. Isolate PMC-11-5 showed the highest amount of cell counts approximately 2.26 Log CFU/ml. Udomsil (2008) reported that some lactic acid bacterial isolates from fish sauce can grow in FB25 and increased cell counts to approximately 1-2 Log CFU/ml. Therefore, 4 out of 17 isolates, namely 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5, were selected.

4.2.3 Biogenic amines formation

Four selected isolates (results of the section 4.2.2) were tested for their biogenic amines formation capabilities using modified glucose yeast extract peptone (mGYP) medium added with 5% NaCl and 0.25% of various amino acids (L-lysine, L-histidine, L-ornithine, and L-tyrosine) as precursors (Appendix A1.10). Cell counts using MRS agar containing supplemented with 0.5% $CaCO_3$ of all isolates were 6-7 Log CFU/ml. Cadaverine, histamine, putrescine, spermine, tryptamine, and tyramine were presented in samples at concentrations of 0.03, 0.09-0.45, 0.11-0.20, 0.53, 1.09, and 0.01-0.14 mg/100 ml, respectively, while spermidine was not detected (Table 4.4). Major biogenic amines found in fish sauce and various fermented fish products

were histamine, cadaverine, and tyramine while spermidine and spermine were minor biogenic amines (Yongsawatdigul *et al*., 2007). All four LAB isolates produced histamine and putrescine in mGYP containing 5% NaCl in the range of 0.09-0.45 and 0.11-0.20 mg/100 ml, respectively. It should be noted that histamine amount were lower than those previously reported in mGYP containing 5% NaCl (6.62 to 22.55 mg/100ml; Udomsil, 2008). This result indicated that histamine produced from selected LAB was far below the allowable limit concentration of 20 mg/100 ml (CFIA, 2013). Formation of histamine during fish sauce fermentation could be partly contributed from halophilic LAB (Udomsil, 2008). Kimura, Konagaya, and Fujii (2001) reported that *T. muriaticus* produced histamine at the level of 4.2 mg/100 ml in mGYP containing 20% NaCl after 10-day incubation and increased to 120 mg/100 ml after 20 days. This was similar to the findings of Sitdhipol *et al*. (2013) who reported that *T. halophilus* and *T. muriaticus* strains isolated from Thai fermented food products produced negligible amounts of histamine in histidine broth at the level of 626 mg/l. Cadaverine, another major biogenic amine, was slightly produced from isolate PMC-11-5 (0.03 mg/100 ml). Putrescine and cadaverine have been reported as potentiators for histamine toxicity (Silla Santos, 1996). Spermine was found only in the culture medium inoculated with isolate 3MR10-3 at the level of 0.53 mg/100 ml while tryptamine was produced by isolate 3MC-10-11 at the concentration of 1.09 mg/100 ml. Spermine and spermidine have been reported that they have no adverse effect on human health by usual intake of dietary, but they may react with nitrite to form carcinogenic nitrosoamines by excessive amounts (Zaman, Bakar, Selamat, and Bakar, 2010). Tyramine was formed by isolates 3MC10-11 and 3MR10-3 at the level of 0.53 and 1.09 mg/100 ml respectively. Bover-Cid and Holzapfel (1999) reported

that tyramine was the main amine formed by *Lactobacilus brevis*, *Lactobacilus buchneri*, and *Lactobacilus curvatus* ranging from 2.0-500.0 mg/100 ml. In addition, *T. halophilus* strains produced this biogenic amine in the range of 12.90-16.03 mg/100 ml (Udomsil *et al*., 2011). These results indicated that these lactic acid bacterial isolates did not increase biogenic amines in the medium fortified by amino acids and did not regarded as biogenic amine producers.

Table 4.4 Biogenic amine forming ability of 4 selected lactic acid bacterial isolates in mGYP medium incubated at 35°C for 48 h under anaerobic condition.

Bacterial isolate	Biogenic amine content (mg/100 ml)							Growth (CFU/ml)
	Cadaverine	Histamine	Putrescine	Spermidine	Spermine	Tryptamine	Tyramine	
3MC10-11	nd	0.18 ± 0.10	0.20 ± 0.01	nd	nd	1.09 ± 0.54	0.01 ± 0.21	1.64×10^7
3MR10-3	nd	0.09 ± 0.02	0.15 ± 0.14	nd	0.53 ± 0.75	nd	0.14 ± 0.03	9.05×10^7
6MR10-7	nd	0.45 ± 0.25	0.18 ± 0.35	nd	nd	nd	nd	2.99×10^7
PMC-11-5	0.03 ± 0.02	0.12 ± 0.03	0.11 ± 0.01	nd	nd	nd	nd	6.30×10^6

nd: not determined.

4.3 Identification of the selected lactic acid bacterial isolates

4.3.1 Morphological and physiological characterization

Four lactic acid bacterial isolates, namely 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5, were selected for identification due to their good growth in fish broth containing 25% NaCl and ability to produce low concentration of biogenic amines. Their colonies on MRS agar containing 5% NaCl and 0.5% $CaCO_3$ (48-h cultivation) were 0.25-3.00 mm, low convex, circular, smooth, and white. Cells of these Gram-positive bacteria were cocci and tetrads arrangement with the approximate cell sizes of 0.43-1.09 μm (Figure 4.2 and Table 4.5). All selected strains showed catalase and oxidase negative. They were not able to produce amylase, lipase, and proteinase, but could produce L-lactic acid as the sole metabolic product from glucose. Gas was not produced from D-glucose. All selected strains were considered as homofermentative.

These 4 selected lactic acid bacterial isolates showed different characteristics to the type strains *T. muriaticus* JCM 10006^T, *T. halophilus* subsp. *halophilus* DSM 20339^T, *T. halophilus* subsp. *flandriensis* DSM 23766^T, *T. osmophilus* DSM 23765^T, *T. koreensis* DSM 16501^T, and *T. solitarius* DSM 5634^T. All isolates had their optimum growth temperature in the range of 25-40°C and pH 6.0-10.0. The optimal NaCl concentration for growth in MRS broth of isolates 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5, were about 10-15, 6.5-10, 6.5-10, and 15-15%, respectively. All of the strains were able to grow in the presence of wide ranges of NaCl concentration (3 to 25%) and no growth occurs in the absence of NaC1. Results of NaCl concentration required for growths of these selected strains were similar to those of type strain *Tetragenococcus muriaticus* JCM 10006^T.

The API-system was used for biochemical characterization. The selected isolates 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5, were closed to *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactobacillus curvatus* subsp. *curvatus*, and *Lactobacillus plantarum* with preciseness at 85.8, 88.8, 61.9, and 56.1% homology, respectively (API 50CH/CHL, Biomérieux), but showed different morphological and physiological characteristics from all *Lactobacillus* species (Table 4.5). Results of morphological and physiological characteristics were not sufficient for identifying these isolates. They were then characterized by the comparison of 16S ribosomal RNA gene sequences.

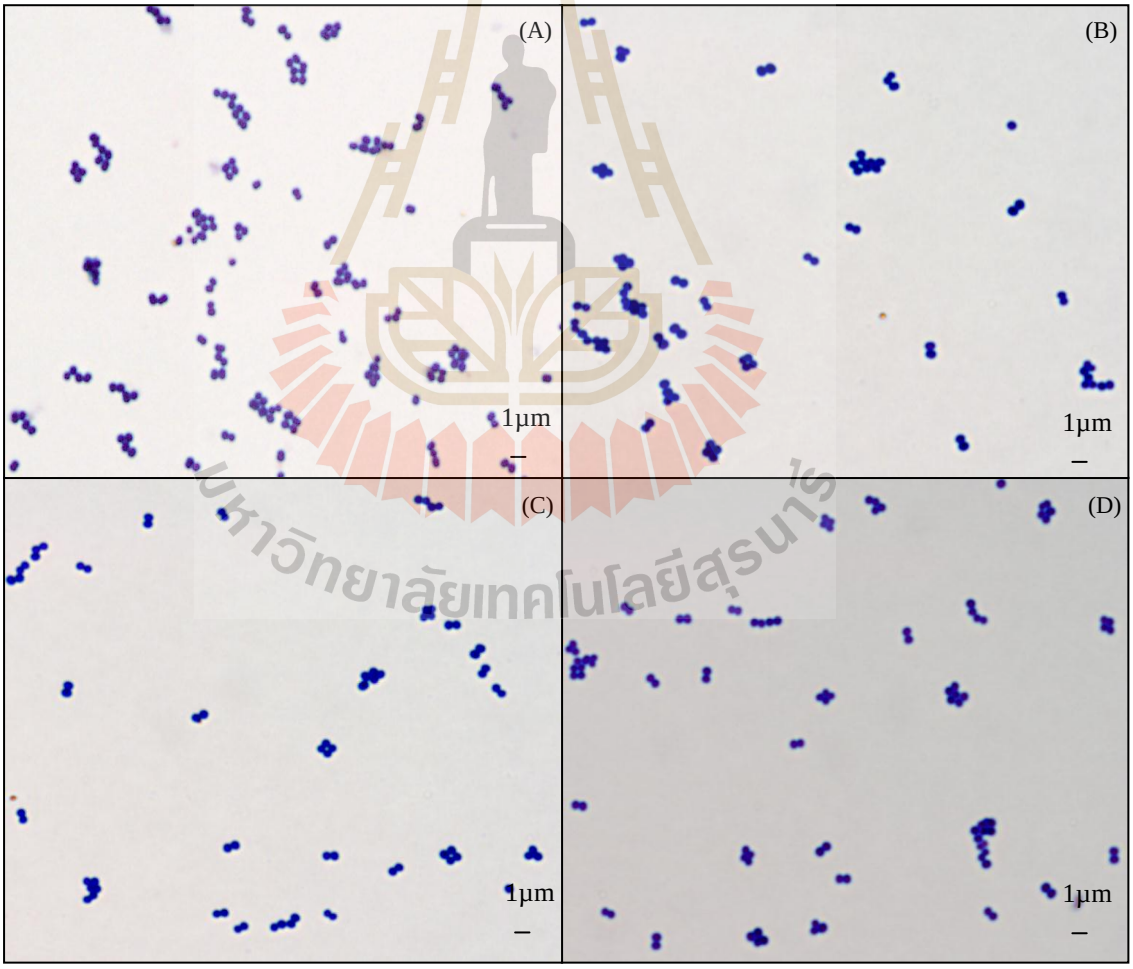

Figure 4.2 Cell morphology of the selected LAB isolates, namely 3MC10-11 (A), 3MR10-3 (B), 6MR10-7 (C), and PMC-11-5 (D).

Table 4.5 Comparison of physiological characteristics of 4 selected lactic acid bacteria compared to type strains of *Tetragenococcus*.

Characteristics	Selected bacterial isolate				Type culture strain[a]					
	3MC10-11	3MR10-3	6MR10-7	PMC-11-5	1	2	3	4	5	6
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads	Tetrads
Cell size (μm)	0.43-0.65	0.71-1.09	0.69-0.82	0.55-0.68	0.50-0.80	0.54-1.02	0.60-1.00	0.60-1.00	1.00	NA
Gram stain	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
Amylase	-	-	-	-	-	-	-	-	-	-
Lipase	-	-	-	-	-	-	-	-	-	-
Proteinase	-	-	-	-	-	-	-	-	-	-
Range of Temp. (°C)	25-40	25-40	25-40	25-40	15-40	15-40	15-37	15-30	15-37	15-40
Optimal Temp. (°C)	30	30	30	30	25-30	30-35	30	30	30	NA
Range of NaCl (%)	3-25	3-25	3-25	3-25	1-25	0-26	0-25	0-25	0-8	NA
Optimal NaCl (%)	10-15	6.5-10	6.5-10	15-18	7-10	5-10	5-10	NA	2-5	7-10
Range of pH	6-10	6-10	6-10	6-10	5.0-9.6	5.0-9.6	NA	NA	NA	NA
Optimal pH	9.0	9.0	9.0	9.0	7.5-8.0	7.0-8.0	7.0-9.0	8.0	9.0	7.5-8.0
Gas from D-glucose	-	-	-	-	-	-	-	-	-	-
Acid from:										
Glycerol	-	-	-	-	-	+	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	+	+	-	-	-
L-Arabinose	-	-	-	-	-	+	+	-	-	-
D-Ribose	+	+	+	+	+	+	+	-	+	-
D-Xylose	-	-	-	-	-	-	-	-	+	
L-Xylose	-	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-	-
Methyl-βD - xylopyranoside	-	-	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	+	+	-	+	+

Table 4.5 (Continued) Comparison of physiological characteristics of 4 selected lactic acid bacteria compared to type strains of *Tetragenococcus*.

Characteristics	Selected bacterial isolate				Type culture strain[a]					
	3MC10-11	3MR10-3	6MR10-7	PMC-11-5	1	2	3	4	5	6
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
D-Mannitol	+	+	+	+	+	-	-	+	+	+
D-Sorbitol	-	-	-	-	-	-	+			+
Methyl-αD-mannopyranoside	-	-	-	-	-	-	-	+	+	-
Methyl-αD-glucopyranoside	-	-	-	-	-	+	-	+	+	-
N-Acetylglucosamine	+	+	+	+	-	+	+	+	+	+
Amygdalin	-	-	-	-	-	+	+	-	+	+
Arbutin	+	+	+	-	-	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	-	+	-	-	-	+	+	+	-	+
D-Maltose	-	-	+	-	-	+	+	+	+	+
D-Lactose	-	-	-	-	-	-	+	-	+	+
D-Melibiose	-	-	+	-	-	-	+	-	-	+
D-Saccharose (Sucrose)	-	-	+	-	-	+	+	+	+	+
D-Trehalose	-	-	+	-	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	+	-	-	-	-	-	+	+
D-Rafinose	-	-	+	-	-	-	+	-	-	-
Amidon (Starch)	-	-	-	-	-	-	+	-	-	-

Table 4.5 (Continued) Comparison of physiological characteristics of 4 selected lactic acid bacteria compared to type strains of *Tetragenococcus*.

Characteristics	Selected bacterial isolate				Type culture strain[a]					
	3MC10-11	3MR10-3	6MR10-7	PMC-11-5	1	2	3	4	5	6
Glycogen	-	-	-	-	-	-	-	-	-	
Xylitol	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	-	-	+	+	+	+	+
D-Turanose	-	-	-	-	-	-	+	+	+	+
D-Lyxose	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	+	-	-	+
D-Fucose	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-
D-Arabitol	+	+	-	-	-	+	+	+	+	+
L-Arabitol	-	-	-	-	-	-	-	-	-	-
Potassium gluconate	-	-	-	-	NA	NA	NA	NA	NA	NA
Potassium 2-Ketogluconate	-	-	-	-	NA	NA	NA	NA	NA	NA
Potassium 5-Ketogluconate	+	-	-	+	NA	NA	NA	NA	NA	NA
Stereoisomer of lactic acid	L	L	L	L	L	L	L	L	L	L

+: positive; -: negative; NA: Not available; [a]1: *T. muriaticus* JCM 10006^T (data from Satomi *et al*., 1997); 2: *T. halophilus* subsp. *halophilus* DSM 20339^T (data from Collins, Williams, and Wallbanks, 1990); 3: *T. halophilus* subsp. *flandriensis* DSM 23766^T (data from Justé *et al*., 2012); 4: *T. osmophilus* DSM 23765^T (data from Justé *et al*., 2012; 5: *T. koreensis* DSM 16501^T (data from Lee *et al*., 2005); 6: *T. solitarius* DSM 5634^T (data from Ennahar and Cai, 2005).

4.3.2 16S ribosomal RNA gene sequence

The 16S rRNA sequence analysis was performed to assist the identification of these coccus bacteria. Genomic DNA of 4 selected bacterial strains was extracted for 16S DNA amplification by PCR using fD1/rP2 primers. The expected lengths of amplified fragments of the four isolates were similar in size being approximately 1,500 bp (Figure 4.3).

After sequencing, the amplified 16S rRNA gene fragments of the bacteria were aligned (Figure 4.4). The lengths of the 16S rDNA sequences from 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5 were 1477, 1468, 1471, and 1447 bp, respectively. The 16S rDNA sequences were compared to nucleotides database provided by NCBI using BLAST to ascertain their closest relatives. The similarity of 16S rDNA sequence of 4 lactic acid bacterial strains compared with NCBI blast of *Tetragenococcus* species were 95-99% (Tables 4.6 and 4.7). When phylogenetic tree analysis was performed, four isolates appeared to be closely related to the genus *Tetragenococcus* and fell in the same cluster of *T. muriaticus* JCM 10006^{T} (Figure 4.5).

At present, the genus comprises a limited number of species, including *T. halophilus* (Collins *et al.*, 1990), *T. muriaticus* (Satomi *et al*., 1997), *T. solitarius* (Ennahar and Cai, 2005), *T. koreensis* (Lee *et al.*, 2005), and *T. osmophilus* (Justé *et al.*, 2012). For the latter strain, new subspecies *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* have been proposed (Justé *et al.*, 2012).

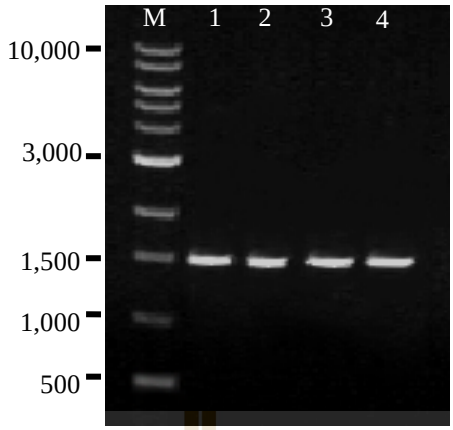

Figure 4.3 Agarose gel electrophoresis of PCR fragments obtained from the amplification of genomic DNA of selected isolates using primers fD1 and rP2. Lanes: M, 1 Kb DNA ladder (New England Biolabs) as a molecular weight marker; 1, bacterial isolates 3MC10-11; 2, 3MR10-3; 3, 6MR10-7; and 4, PMC-11-5.

All 6 type strains in the genus *Tetragenococcus* were used for comparison of physiological and biochemical characteristics with these 4 bacterial strains (Table 4.5). Although 16S rDNA sequence confirmed the taxonomic identity of the selected isolates as *T. muriaticus*, these isolates showed a wide variation in phenotypic characteristics among strains. *T. muriaticus* has been isolated from Thai fish sauce (Thongsanit *et al*., 2002; Sitdhipol *et al*., 2013). Typically, *T. muriaticus* is the only *Tetragenococcus* species that does not grow in the absence of NaCl. *Tetragenococcus muriaticus* prefers NaCl concentrations of 7 to 10 % and tolerates up to 26% NaCl. Isolates can grow up to 40°C on 10 % NaCl-GYP agar. The optimal pH for growth of tetragenococci is between 7.5 and 8.0. Determination of the fermentation pattern with API strips results in many negative reactions. This may be explained by its salt requirement for growth. Even at optimal salt concentrations, this species grows slower than the other *Tetragenococcus* species. Remarkably, *T.*

muriaticus fermented only a few of the carbon sources, including D-ribose, D-manitol, salicin and D-trehalose, depending on the strain (Justé *et al*., 2012), D-mannose and fructose (Satomi *et al*., 1997), D-glucose and arbutin (Kobayashi, Kimura, and Fuji, 2000). Based on phylogenetic tree (Figure 4.5), biochemical and physiological characteristics (Table 4.5), four bacterial strains appeared to be closely related to *T. muriaticus* JCM 10006^{T}. Therefore, four isolates of LAB isolated from fish sauce fermentation in this study are identified as *T. muriaticus*.

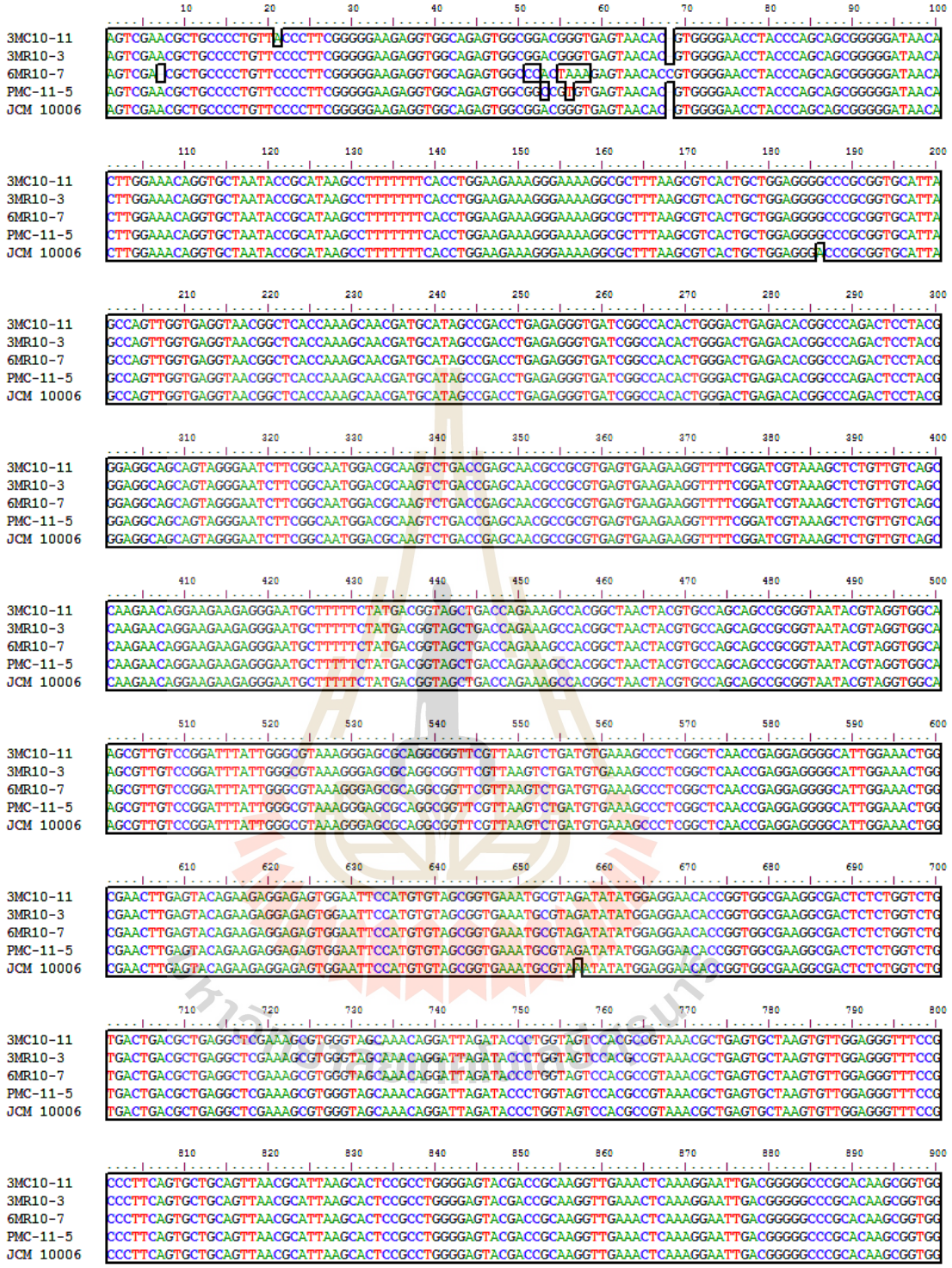

Figure 4.4 Sequence alignment of the partial 16S rDNA amplified by fD1 and rP2 primers, of lactic acid bacterial isolates 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5 compared to *Tetragenococcus muriaticus* JCM 10006^{T} using BioEdit program.

Figure 4.4 (Continued) Sequence alignment of the partial 16S rDNA amplified by fD1 and rP2 primers, of lactic acid bacterial isolates 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5 compared to *Tetragenococcus muriaticus* JCM 10006^{T} using BioEdit program.

Table 4.6 Similarity of 16S rRNA gene sequences of the bacterial strains 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5 compared to other closest relative bacteria from NCBI nucleotide sequence database (U.S.A.).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative	Length of sequence (nt) bp	Sequence homology (%)	GenBank accession number	Isolation source/ remark of the closest relative
3MC10-11	1477	*Tetragenococcus muriaticus* JCM 10006^T	1512	99.1	D88824	Fermented squid liver sauce
		Tetragenococcus osmophilus DSM 23765^T	1394	96.9	EU522083	Degraded sugar thick juice
3MR10-3	1468	*Tetragenococcus muriaticus* JCM 10006^T	1512	99.7	D88824	Fermented squid liver sauce
		Tetragenococcus osmophilus DSM 23765^T	1394	97.5	EU522083	Degraded sugar thick juice
6MR10-7	1471	*Tetragenococcus muriaticus* JCM 10006^T	1512	99.3	D88824	Fermented squid liver sauce
		Tetragenococcus osmophilus DSM 23765^T	1394	97.1	EU522083	Degraded sugar thick juice
PMC-11-5	1447	*Tetragenococcus muriaticus* JCM 10006^T	1512	99.7	D88824	Fermented squid liver sauce
		Tetragenococcus osmophilus DSM 23765^T	1394	97.5	EU522083	Degraded sugar thick juice

nt: nucleotide; bp: base pair.

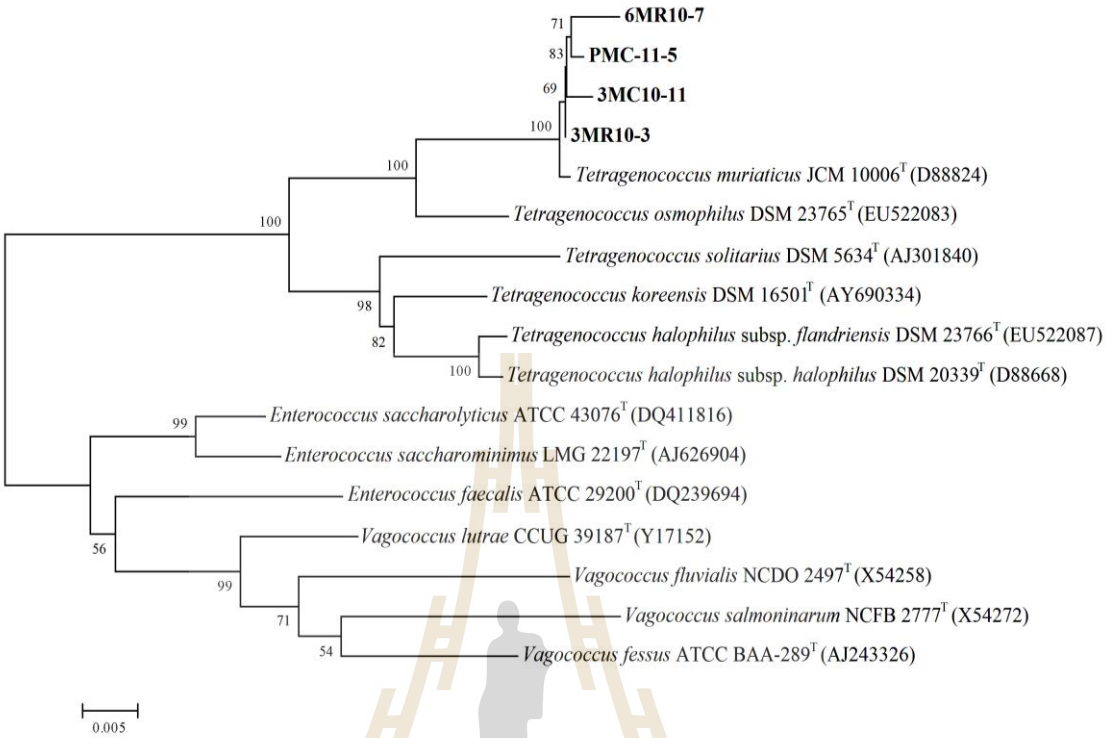

Figure 4.5 Phylogenetic tree of isolates 3MC10-11, 3MR10-3, 6MR10-7, PMC-11-5, and *Tetragenococcus* species, based on 16S rDNA sequences constructed by using the neighbor-joining method. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications.

Table 4.7 16S rRNA gene sequence similarity of the bacterial isolates 3MR10-3, 6MR10-7, PMC-11-5, and related species.

Bacterial strain	3MC10-11	3MR10-3	6MR10-7	PMC-11-5	1	2	3	4	5	6	7	8	9	10	11	12	13
3MC10-11	100																
3MR10-3	99.10	100															
6MR10-7	98.70	99.30	100														
PMC-11-5	99.10	99.70	99.40	100													
1	99.10	99.70	99.30	99.70	100												
2	96.90	97.50	97.10	97.50	97	100											
3	94.00	94.60	94.20	94.60	94	95	100										
4	94.40	94.90	94.60	94.90	94	97	96	100									
5	94.60	95.20	94.80	95.20	96	97	97	98	100								
6	94.50	95.10	94.70	95.10	94	96	96	98	99	100							
7	91.40	92.00	91.7	92.00	92	94	93	94	94	93	100						
8	91.60	92.20	91.9	92.20	92	94	92	93	94	93	98	100					
9	91.00	91.60	91.2	91.60	91	93	92	93	93	92	96	95	100				
10	91.10	91.70	91.3	91.70	92	93	92	92	93	93	95	95	96	100			
11	86.60	87.20	86.80	87.20	87	89	88	87	88	87	91	91	91	93	100		
12	86.00	86.50	86.10	86.50	88	89	87	88	88	88	91	90	91	94	91	100	
13	89.80	90.40	90.00	90.40	91	92	91	92	92	92	94	94	94	95	91	93	100

1: *T. muriaticus* JCM 10006^{T} (D88824); 2: *T. osmophilus* DSM 23765^{T} (EU522083); 3: *T. solitarius* DSM 5634^{T} (AJ301840); 4: *T. koreensis* DSM 16501^{T} (AY690334); 5: *T. halophilus* subsp. *flandriensis* DSM 23766^{T} (EU522087); 6: *T. halophilus* subsp. *halophilus* DSM 20339^{T} (D88668); 7: *Enterococcus saccharolyticus* ATCC 43076^{T} (DQ411816); 8: *E. saccharominimus* LMG 22197^{T} (AJ626904); 9: *E. faecalis* ATCC 29200^{T} (DQ239694); 10: *Vagococcus lutrae* CCUG 39187^{T} (Y17152); 11: *V. fluvialis* NCDO 2497^{T} (X54258); 12: *V. salmoninarum* NCFB 2777^{T} (X54272);13: *V. fessus* ATCC BAA-289^{T} (AJ243326).

4.3.3 Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rDNA

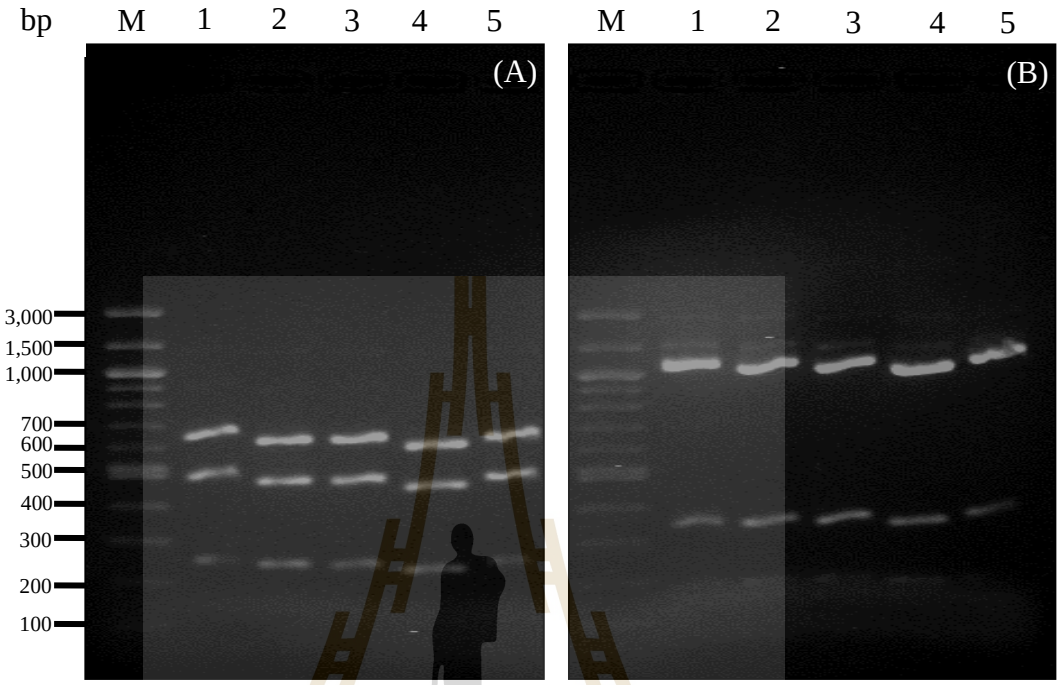

Figure 4.6 RFLP patterns of all LAB isolates and *T. muriaticus* JCM 10006^T obtained by digestion of 16S rDNA with restriction endonucleases *Alu*I (A) and *Mbo*I (B). Lanes M, Molecular weight marker (100-bp ladder); 1, 3MC10-11; 2, 3MR10-3; 3, 6MR10-7; 4, PMC-11-5; 5, *T. muriaticus* JCM 10006^T.

Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S rRNA genes was performed to confirm their species compared to *T. muriaticus* JCM 10006^T, a type strain. The patterns of fragments by digestion with *Alu*I and *Mbo*I of 4 LAB isolates were similar to those of the type strain of *T. muriaticus* JCM 10006^T (Figures 4.6A and 4.6B). Therefore, it can be confirmed that all isolates were *T. muriaticus*.

4.4 Development of flavor compounds by the selected lactic acid bacteria during fish sauce fermentation

4.4.1 Microbiological changes

The fermentation of fish sauce was performed in laboratory scale similar to traditional fermentation using fresh anchovy compared to enzyme-digested anchovy without and with inoculating 10% (v/w) of the selected bacterial starter cultures at approximate cell counts of 10^7 CFU/ml. Fish sauce products were obtained after 8 months of fermentation (Figures 4.7 and 4.8). At the initial stage of fermentation using fresh fish inoculated separately with 4 LAB strains, viable counts of LAB strains on MRS agar containing 5% NaCl added 0.5% $CaCO_3$ were 4-5 Log CFU/ml (Figure 4.9A). Between, 30 and 60 days of fermentation, bacterial counts (MRS agar containing 5% NaCl added 0.5% $CaCO_3$) increased up to 6-7 Log CFU/ml. Through 90 days, the number of counts gradually declined to 3-5 Log CFU/ml and was not detected at 240 days, while bacterial counts of control (without inoculation) were remained constant at 4 Log CFU/ml until 180 days of fermentation. These results implied that bacteria observed on MRS agar were likely to be the inoculated LAB. The halophilic bacterial counts on JCM 168 agar containing 20% NaCl and non-halophilic bacterial counts on PCA agar containing 10% NaCl showed similar trend as those of LAB counts (Figures 4.9B and 4.9C). However, bacterial counts on PCA agar containing 10% NaCl at 0 day were different at about only 1 Log CFU/ml. These results implied that the initial loads of bacteria were not the same which came from starter cultures and raw materials used for fish sauce fermentation. The bacterial counts of the control on JCM 168 agar could not be detected at the first day (Figure 4.9B). The halophilic bacteria may be inactivated by low salts.

For fermentation using enzyme-digested fish, the initial LAB counts on on MRS agar containing 5% NaCl added 0.5% $CaCO_3$ were 4-5 Log CFU/ml (Figure 4.10A). It sharply increased up to 6-7 Log CFU/ml and gradually declined to about 5-6 Log CFU/ml at 180 days. None of lactic acid bacteria was detected at 240 days of fermentation. In addition, the halophilic bacterial counts on JCM 168 containing 20% NaCl showed same profile as those of LAB counts (Figure 4.10B) but having some unsteady trend at time interval of fermentation.

However, bacterial counts on PCA agar containing 10% NaCl slightly decreased at 30 days and reached to 6-7 Log CFU/ml at 60 days (Figure 4.10C). It gradually declined and still remained in the system until 240 days. The bacterial counts of the control on MRS agar and JCM 168 agar could not be detected at the first day (Figure 4.10B). Some bacteria could be thermally inactivated for growth, since the samples were heated for 6 h at 50-60°C before fermentation. Interestingly, the bacterial counts of the control on PCA agar containing 10% NaCl were similar to samples inoculated with LAB strains. Although this agar medium used for detecting non-halophilic bacteria under aerobic condition, salt (10%) contained was likely to increase the growth of halophilic/halotolerant bacteria. Therefore, these results implied that bacteria counts detected on PCA agar containing 10% NaCl could be the microbial population of halophilic or halotolerant bacteria.

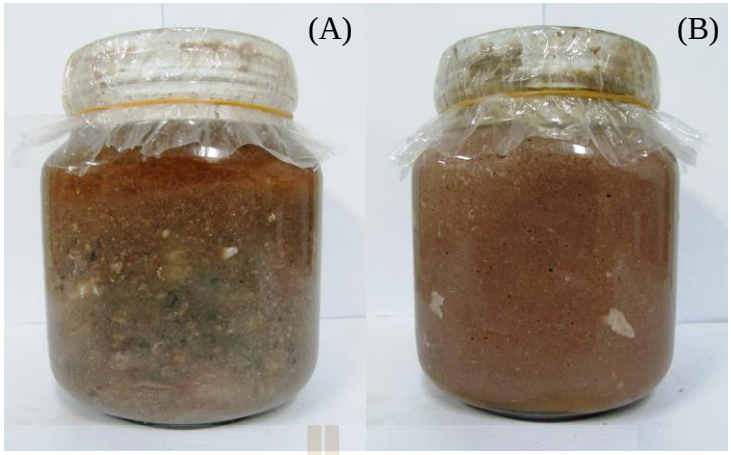

Figure 4.7 Fish sauce fermented for 8 months with the inoculation of *T. muriaticus* PMC-11-5 and contained in glass jar using fresh fish (1,000g, A) and enzyme-digested fish (B) for fermentation.

Figure 4.8 Fish sauce products inoculated with *T. muriaticus* PMC-11-5, using fresh fish (A) and enzyme-digested fish (C) compared to controls (B and D) in laboratory scale for 8 months of fermentation.

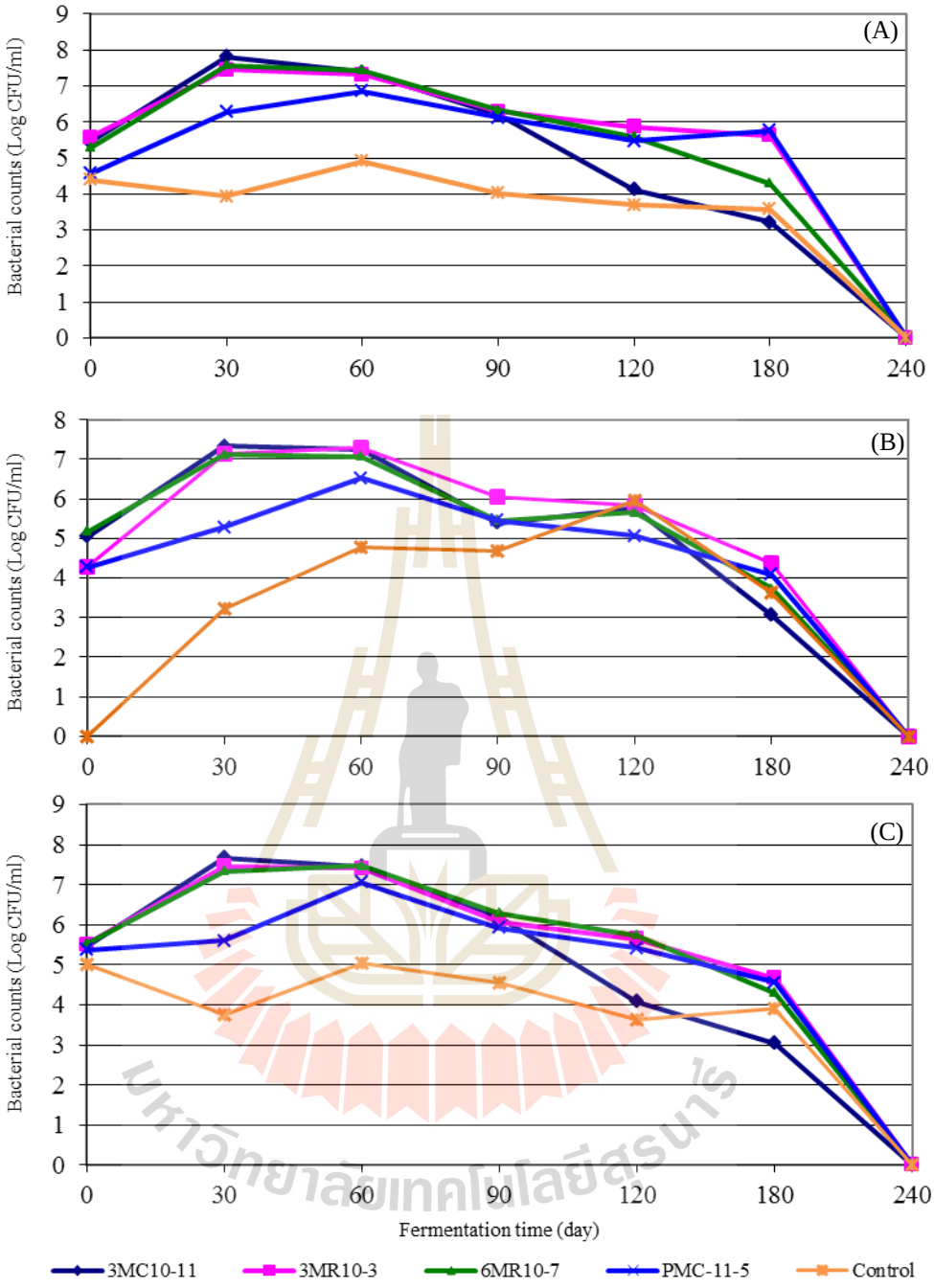

Figure 4.9 Changes in lactic acid bacterial counts on MRS agar containing 5% NaCl added 0.5% $CaCO_3$ (A), halophilic bacterial counts on JCM 168 agar containing 20% NaCl (B), and bacterial counts on PCA agar containing 10% NaCl (C) of fish sauce samples prepared from fresh fish incubated at room temperature for 240 days.

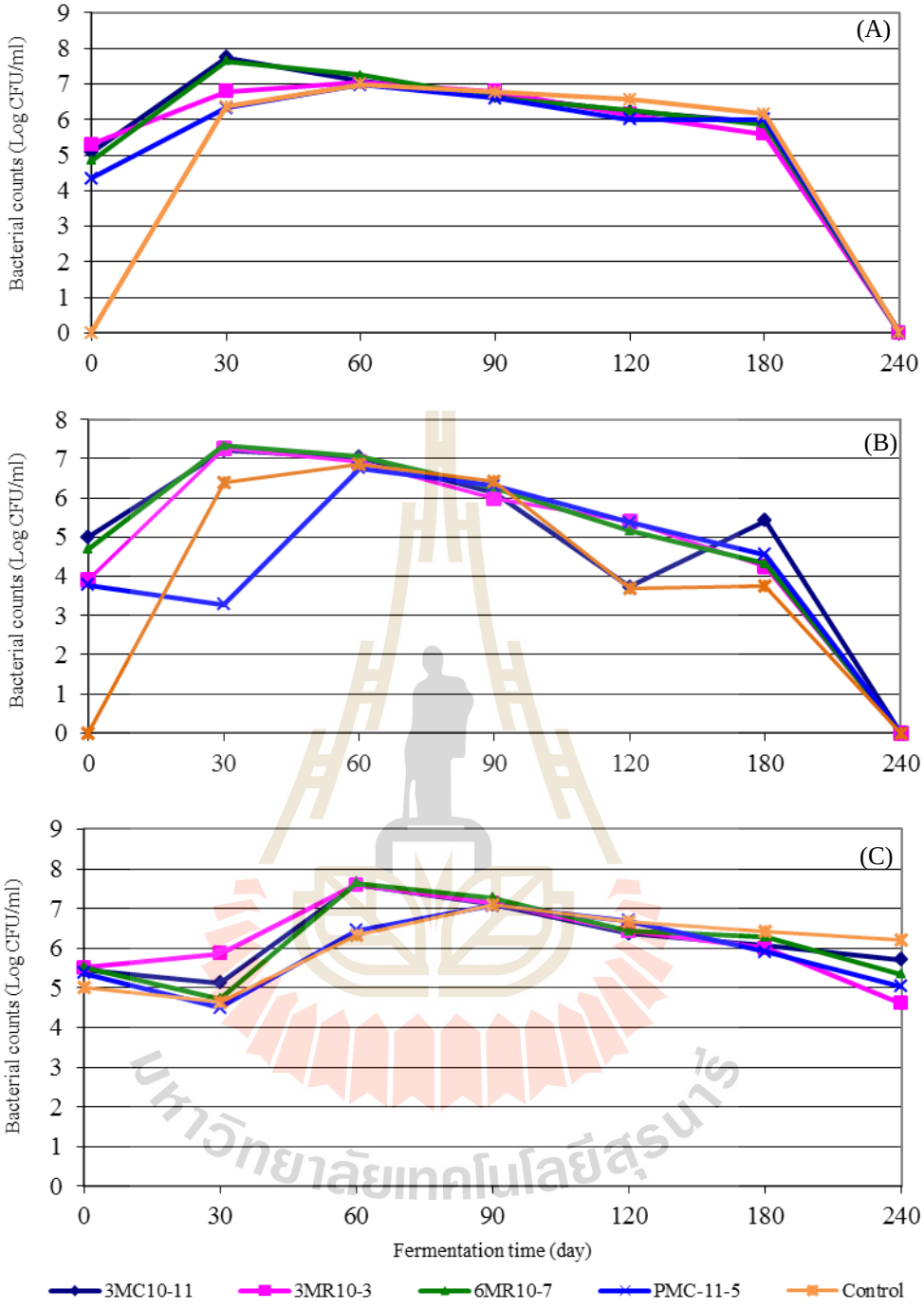

Figure 4.10 Changes in lactic acid bacterial counts on MRS agar containing 5% NaCl added 0.5% $CaCO_3$ (A), halophilic bacterial counts on JCM 168 agar containing 20% NaCl (B), and bacterial counts on PCA agar containing 10% NaCl (C) of fish sauce samples using enzyme-digested fish incubated at room temperature for 240 days.

4.4.2 Chemical analyses

4.4.2.1 α-Amino content

The initial α-amino contents of all samples from both fermentation methods were approximately 396-548 mM (Tables 4.8and 4.9) and higher than that of fermentation liquid at 3 days (less than 200 mM; Yongsawatdigul *et al*., 2004). The α-amino contents of both fermentation methods were the same at 0 day due to autolytic protein digestion in frozen fish used for fermentation. α-Amino contents increased up to 810-957 mM and higher than that of the controls (763-808 mM) at 8 months. An increase in α-amino content of the control samples due to proteolytic activity of microflora and fish digestive enzymes. Whereas, the α-amino contents increased in samples inoculated with 4 selected LAB were resulted from residual activity of fish endogenous (fermentation using fresh fish) and added exogenous proteinases (fermentation using enzyme-digested fish). It was observed that sample using fresh fish and inoculated *T. muriaticus* PMC-11-5 showed the highest α-amino contents at about 958 mM ($P < 0.05$), while the sample using enzyme-digested fish and inoculated *T. muriaticus* PMC-11-5 and *T. muriaticus* 6MR10-7 showed the highest content at 850 and 839 mM respectively ($P < 0.05$). This was concomitant with viable counts detected on MRS agar containing 5% NaCl added 0.5% $CaCO_3$, which remained in fermentation process until 180 days. Although LAB selected isolates, namely *T. muriaticus* 3MC10-11, *T. muriaticus* 3MR10-3, *T. muriaticus* 6MR10-7, and *T. muriaticus* PMC-11-5, did not show protein hydrolysis activity on skim milk agar, they appeared to hydrolyze fish protein during fish sauce fermentation, resulting in an increase of α-amino content.

Table 4.8 α-Amino content (mM) of fish sauce samples using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and incubated at room temperature for 8 months.

Fermentation time (day)	Fish sauce without and with inoculation of :				
	Control	3MC10-11	3MR10-3	6MR10-7	PMC-11-5
0	491.57 ± 10.46^{a}	396.15 ± 11.51^{b}	421.30 ± 53.35^{b}	449.41 ± 9.41^{ab}	498.96 ± 2.09^{a}
30	496.67 ± 1.05^{bc}	486.31 ± 45.01^{bc}	525.54 ± 12.56^{b}	446.34 ± 3.14^{c}	584.01 ± 7.33^{a}
60	530.73 ± 5.52^{bc}	510.45 ± 27.58^{bc}	580.66 ± 16.55^{ab}	481.59 ± 50.74^{c}	613.42 ± 9.93^{a}
90	541.56 ± 14.28^{a}	546.61 ± 7.14^{a}	543.00 ± 12.24^{a}	566.81 ± 15.31^{a}	496.83 ± 20.41^{b}
120	682.03 ± 6.45^{b}	691.16 ± 30.66^{ab}	711.12 ± 52.44^{ab}	757.90 ± 12.10^{a}	663.78 ± 8.067^{b}
180	670.75 ± 52.13^{c}	717.80 ± 27.73^{bc}	706.04 ± 24.40^{bc}	778.98 ± 32.17^{ab}	818.98 ± 6.66^{a}
240	807.92 ± 3.00^{c}	810.04 ± 12.00^{c}	820.65 ± 13.00^{ab}	852.48 ± 26.00^{b}	957.85 ± 9.00^{a}

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

Table 4.9 α-Amino content (mM) of fish sauce samples using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and incubated at room temperature for 8 months.

Fermentation time (day)	Fish sauce without and with inoculation of :				
	Control	3MC10-11	3MR10-3	6MR10-7	PMC-11-5
0	501.92 ± 12.55	508.58 ± 17.78	546.30 ± 41.84	496.75 ± 17.78	548.52 ± 24.06
30	274.61± 11.51^{d}	574.39 ± 18.84^{cd}	622.50 ± 5.23^{ab}	637.31 ± 21.98^{a}	595.85 ± 3.14^{bc}
60	550.23 ± 55.16^{bc}	917.63 ± 45.23^{a}	567.79 ± 22.27^{bc}	526.83 ± 4.41^{c}	627.20 ± 13.16^{b}
90	544.44 ± 4.08^{b}	649.78 ± 22.45^{a}	532.18 ± 60.20^{b}	516.31 ± 31.63^{b}	466.52 ± 2.04^{b}
120	644.95 ± 20.17^{b}	751.63 ± 43.56^{b}	631.26 ± 13.71^{b}	672.90 ± 12.91^{a}	668.91 ± 0.81^{b}
180	682.51 ± 42.15^{b}	754.67 ± 26.62^{b}	688.78 ± 26.62^{b}	922.51 ± 15.53^{a}	722.51 ± 25.51^{b}
240	763.37 ± 8.00^{b}	770.44 ± 8.00^{b}	762.66 ± 33.00^{b}	839.04 ± 5.00^{a}	849.65 ± 2.00^{a}

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

4.4.2.2 Oligopeptide content

Oligopeptide contents of samples inoculated with selected LAB strains gradually decreased throughout the course of fermentation, which were mainly target tyrosine (Tables 4.10 and 4.11). Typically, fish sauce contains about 20 g/l of nitrogen, of which 80% is in the form of amino acids (Je, Park, Jung, and Kim, 2005). This result implied that proteolytic activity was rapidly activated at the initial stage of fermentation, resulting in acceleration of oligopeptide hydrolysis. At initial stage of fermentation, oligopeptide contents of samples using enzyme-digested fish for fish sauce fermentation were higher than those of samples using fresh fish for fermentation. This could be because the added commercial proteinases contributed to protein hydrolysis, resulting of high content of oligopeptide. At 240 days of fermentation, oligopeptide contents of samples prepared from fresh fish and enzyme-digested fish were in the range of 16.07-20.71 and 18.00-31.29 mM, respectively. Samples inoculated with *T. muriaticus* 3MC10-11 showed the highest oligopeptide content at 20.04 and 31.29 mM of fish sauce fermentation using fresh fish and enzyme-digested fish respectively ($P < 0.05$). These results suggested that the addition of *T. muriaticus* 3MC10-11 increased protein hydrolysis of fish sauce fermentation.

Table 4.10 Oligopeptide content (mM) of fish sauce samples using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and incubated at room temperature for 8 months.

Fermentation time (day)	Control	3MC10-11	3MR10-3	6MR10-7	PMC-11-5
0	33.94 ± 0.63^{b}	28.69 ± 0.50^{c}	36.39 ± 1.57^{a}	35.95 ± 0.50^{ab}	35.12 ± 0.28^{ab}
30	24.56 ± 0.94^{a}	21.86 ± 1.16^{ab}	23.33 ± 0.28^{a}	23.04 ± 0.94^{a}	19.37 ± 1.67^{b}
60	24.41 ± 0.91	25.32± 0.03	26.32 ± 1.09	26.92 ± 1.53	26.82 ± 0.32
90	16.61 ± 0.03^{c}	20.48 ± 0.22^{ab}	21.99 ± 1.02^{a}	18.88 ± 0.89^{b}	19.08 ± 0.41^{b}
120	17.99 ± 0.13^{c}	19.36 ± 0.51^{b}	20.99 ± 0.06^{a}	18.06 ± 0.48^{c}	17.93 ± 0.54^{c}
180	18.32 ± 0.32^{bc}	17.28 ± 0.03^{c}	20.76 ± 0.26^{a}	18.15 ± 0.61^{bc}	18.92 ± 0.94^{b}
240	17.39 ± 0.58^{b}	20.04 ± 1.20^{a}	20.71 ± 0.49^{a}	17.42 ± 1.29^{b}	16.07 ± 0.43^{b}

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

4.4.2.3 Other physico-chemical properties

Total nitrogen content, salt content and pH were also analysed. Total nitrogen (TN) contents of all fish sauce samples inoculated with starter cultures were comparable to that of control (Table 4.12, P > 0.05). These compounds contributed to the specific aroma and flavor of fish sauce (Dougan and Howard, 1975). TN content is an index of the fish sauce product (Park *et al*., 2001). First grade fish sauce according to Thai Industry Standard must contained TN value greater than 2.0% (http://www.tisi.go.th). The increased nitrogen content suggested an increased hydrolysis of protein. The total nitrogen contents of all inoculated samples after 8 months fermentation were lower than that of first grade fish sauce. Salt content of fish sauce inoculated with starter cultures were in the range of 28-30% NaCl (Table 4.12). Changes of salt content during fermentation were minimal (Yongsawatdigul *et al*., 2007). The addition of starter cultures did not affect salt content of the sample.

The salt content of the finished product did not deviate from the standard of fish sauce which is set to be not less than 20% (Codex, 2011). The pH value of fish sauce samples were 6.06-6.36 (Table 4.12). Typically, organic acids, such as lactic acid and acetic acid, produced from lactic acid bacteria tended to lower pH of fish sauce during fermentation (Michihata, Sado, Yano, and Enomoto, 2000). However, pH did not drastically decrease. This was because no sugar (glucose in particular) in fish sauce fermentation, an important substrate, for lactic acid production of LAB.

Table 4.11 Oligopeptide content (mM) of fish sauce samples using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and incubated at room temperature for 8 months.

Fermentation time (day)	Control	3MC10-11	3MR10-3	6MR10-7	PMC-11-5
0	38.33 ± 0.53	32.92 ± 7.11	38.61 ± 1.57	37.72 ± 0.13	39.77 ± 0.57
30	20.84 ± 0.53^{b}	23.62 ± 0.57^{a}	24.69 ± 0.13^{a}	24.67 ± 0.66^{a}	20.42 ± 1.20^{b}
60	28.40 ± 2.37	26.95 ± 0.33	25.52 ± 0.91	28.45 ± 0.36	26.24 ± 1.66
90	25.50 ± 1.27^{a}	21.36 ± 0.00^{b}	22.01 ± 0.80^{b}	24.33 ± 1.27^{a}	21.45 ± 0.38^{b}
120	23.69 ± 0.57^{a}	19.75 ± 0.19^{c}	21.68 ± 0.19^{b}	24.47 ± 0.38^{a}	22.47 ± 0.03^{b}
180	24.53 ± 1.32	23.12 ± 1.26	21.90 ± 0.76	25.02 ± 0.09	22.98 ± 2.99
240	21.04 ± 0.89^{bc}	31.29 ± 0.12^{a}	18.85 ± 1.16^{cd}	21.86 ± 1.32^{b}	18.00 ± 1.20^{d}

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

Table 4.12 Physico-chemical properties of fish sauce samples inoculated with 4 selected lactic acid bacterial starter cultures and incubated at room temperature for 8 months.

Raw material for fermentation	Bacterial starter culture	Fish sauce product		
		Total nitrogen[a] (%)	Salt (%)	pH
Fresh fish	Control	1.62 ± 0.01[c]	29.17 ± 0.23	6.00 ± 0.01
	3MC10-11	1.58 ± 0.00[d]	30.48 ± 0.70	6.12 ± 0.02
	3MR10-3	1.65 ± 0.01[c]	28.34 ± 0.93	6.05 ± 0.01
	6MR10-7	1.62 ± 0.00[c]	28.84 ± 0.23	6.18 ± 0.03
	PMC-11-5	1.68 ± 0.01[b]	30.15 ± 0.23	6.23 ± 0.02
Enzyme-digested fish	Control	1.63 ± 0.01[c]	29.82 ± 0.23	6.13 ± 0.03
	3MC10-11	1.59 ± 0.01[d]	30.64 ± 0.46	6.13 ± 0.01
	3MR10-3	1.70 ± 0.01[ab]	30.81 ± 0.23	6.32 ± 0.01
	6MR10-7	1.69 ± 0.03[b]	30.32 ± 0.46	6.08 ± 0.01
	PMC-11-5	1.71 ± 0.01[a]	30.48 ± 0.23	6.14 ± 0.10

[a]: Different superscripts within a column indicate significant differences (P<0.05).

4.4.2.4 Volatile compound profiles

A total of 51 volatile compounds were detected and identified in inoculated fish sauces (using fresh fish and enzyme-digested fish) fermented for 2, 4 and 8 months (Tables 4.13 and 4.14; Appendix C). They were classified into 8 major groups: aldehydes; alcohols; ketones; sulfur-containing compounds; nitrogen-containing compounds; and acids. Aldehydes, alcohols, and acids were predominant compounds. Aldehydes, due to their low threshold values, are important aroma compounds in food products (Giri *et al*., 2010). The major parts of the aldehydes were contributed by 2-methyl-1-butanal, 3-methyl-1-butanal, and 2-methyl-1-propanal (Peralta *et al*., 1996; Fukami *et al*., 2002). Steinhaus and Schieberle (2007) reported

that 3-methyl-1-butanal and 2-methyl-1-butanal were produced by microbial action via amino-acid biosynthetic pathways of branched-chain amino acids, namely leucine, valine, and isoleucine. Although these aldehydes contributed to a meaty odor of fish sauce, they were not detected in all samples. These results implied that these 4 selected bacterial strains could not contribute to the formation of these distinctive aldehydes. However, fish sauce samples using enzyme-digested fish and added *T. muriaticus* 3MC10-11 and *T. muriaticus* PMC-11-5, had higher content of benzaldehyde than control ($P < 0.05$) at 8 months of fermentation. Benzaldehyde is an aromatic compound commonly found in cheese and dairy products. This compound predominantly plays an important role for flavor formation in cheese. Japanese fish sauce had high levels of benzaldehyde (Michihata *et al*., 2002). Interestingly, this compound was identified in the fish sauce inoculated with *T. halophilus* as a starter culture (Udomsil *et al*., 2011). Benzaldehyde was reported to responsible for sweet and cotton candy odor (Pham, Schilling, Yoon, Kamadia, and Marshall, 2008), and a wooden and bitter almond odor in fish sauce (Giri *et al*., 2010). Groot and Bont (1998) reported that *Lactobacillus. plantarum* could convert phenylalanine to phenylacetaldehyde via Strecker reaction, and it subsequently oxidized to benzaldehyde (McSweeney and Sousa, 2000). Other aldehydes such as, hexanal, 3-methyl-1-butenal, 3-methyl-2-butenal, heptanal, (*Z*)-2-hexanal, (*Z*)-4-hexanal, octanal, nanonal, (*E*)-2-octenal, (*EE*)-2,4-heptadienal, (*E*)-nonenal, (*EZ*)-2,6-nonadienal, 2-methl pentanal, and benzeneacetaldehyde were also found. These compounds were produced from chemical reaction and/or microbial action by degrading amino acids. The chemical reactions were lipid oxidation and other decomposition process during fermentation, resulting in branched/aromatic or short-

chain aldehydes (Giri *et al*., 2010). Branched, short chain aldehydes or aromatic aldehydes might have resulted from deamination of amino acid (McIver *et al*., 1982). In addition, aldehyde compounds derived from Strecker degradation (Maillard reaction).

Alcohols have been reported to contribute the major volatiles in fish sauce (Giri *et al*. 2010). The predominant alcohols found in all samples were 3-methyl-1-butanol, 1-pentanol, 1-hexanol, 1,5-hexadien-3-ol, and 3-(methylthio)-1-propanol. Interestingly, 2,3-butanediol was detected only in fish sauce using enzyme-digested fish for fermentation. It appeared to be responsible for fruity note. 3-methyl-1-butanol is converted from 3-methyl-1-butanal by branched-chain alcohol dehydrogenase activity of leucine transamination pathway (Smit *et al*., 2009). This results implied that this compound might be completely formed from 3-methyl-1-butanal which was produced in the fermentation. Thus, 3-methyl-1-butanal was not detected. 1- Propanol contributed to plastic, pungent, and musty odor. 3-(Methylthio)-1-propanol have been reported to be derived from 4-methylthio-2-oxobutyric acid, a transamination product of methionine, which yields 3-(methylthio)-1-propanal (Giri *et al*., 2010). Its presence in fish sauce might be desirable due to its meaty, nutty, and cooked potato-like notes (Wichaphon, Thongthai, Assavanig, and Lertsiri, 2012). However, most of alcohols have not been considered as being an influence role on overall acceptance in fish sauce due to the high odor threshold value (Michihata *et al*., 2002).

The major ketones were rarely detected in fish sauce samples (Tables 4.13 and 4.14; Appendix C). Michihata *et al*. (2002) reported that ketones contribute to cheesy note, but might not be responsible for fish sauce flavor due to

their high threshold. The amounts of 3-penten-2-one, 3-hydroxy-3-butanone, cyclohexanone, and (*EE*)-3,5-octadien-2-one were relatively low in fish sauce samples. Although, nitrogen-containing compounds are widely accepted as important aroma compounds in soy paste and contribute to prominent nutty and roasting odor, its contribution to overall odor of fish sauce was negligible (Yongsawatdigul *et al*., 2007; Peng *et al*., 2014). 2,5-Dimethyl pyrazine was predominant present in all fish sauce samples.

The major sulfur-containing compounds detected in fish sauce samples were dimethyl disulfide and dimethyl trisulfide. Dimethyl disulfide was not detected in fish sauce sample using fresh fish for fermentation added selected *T. muriaticus* strains. However, this compound was found in fish sauce sample using enzyme-digested fish but its content was lower that the control. The samples inoculated selected *T. muriaticus* strains showed lower content of dimethyl trisulfide than the control. Sulfur-containing compounds have been reported to originate from methionine, which are methanethiol, dimethyl disulfide, dimethyl trisulfide, methional, and methanethiol (Yvon and Rijnen, 2001). These compounds were converted by the catabolism of methionine via transamination pathway. Additionally, methanethiol resulted from methionine demethiolation is subsequently auto-oxidized to dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide (McSweeney and Sousa, 2000). Sulfur-containing compounds strongly contributed to the odor and flavor because of low their threshold values (Devos, Patte, Roualt, Laffort, and Gemert, 1995). Threshold values of, dimethyl disulfide and dimethyl trisulfide were 0.43 and 1.66 ppb in the vapor phase respectively, which are lower than that of dimethyl sulfide (2.24 ppb) (Devos *et al*., 1995). These compounds are contributed to

fecal note, an undesirable odor in fish sauce (Fukami *et al*., 2004). Udomsil *et al*. (2011) reported that ethyl acetate, an ester compound was detected in fish sauce samples added with *T. halophilus*. This compound has not been reported to play a significant role on fish sauce odor (Peralta *et al*., 1996), which is contributed to flowery and sweet odor (Wichaphon *et al*., 2012). The major acids found in fish sauce samples were acetic acid, butanoic acid, and 3-methylbutanoic acid. Acetic acid was abundant in Thai fish sauce (Yongsawatdigul *et al*., 2007). Butanoic acid appeared to be a predominant acid in patis (Peralta *et al*., 1997). 3-Methylbutanoic acid was the most abundant in shottsuru (Michihata *et al*., 2002) and was one of the principal contributors to the distinctive odor in nam-pla (Fukami *et al*., 2002). Shimoda *et al*. (1996) reported that volatile fatty acids, such as acetic acid, butanoic acid and 3-methylbutanoic acid, were responsible for cheesy odor of fish sauce. These results suggested that LAB strains isolated from fish sauce may play a significant role in flavor development in fish sauce. Adding these bacterial strains in fish sauce fermentation, they might help to enhance desirable odor of fish sauce.

Table 4.13 Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
2	1081	Hexanal	nd	nd	nd	0.477^{c}	nd	nd	0.751^{b}	nd	nd	1.176^{a}	nd	nd	0.326^{d}	nd	nd
8	1283	Octanal	0.190^{d}	nd	nd	0.231^{c}	nd	nd	0.276^{a}	nd	nd	0.257^{b}	nd	nd	0.189^{d}	nd	nd
14	1413	*(E)*-2-Octenal	0.191^{b}	nd	nd	0.183^{c}	nd	nd	0.308^{a}	nd	nd	0.073^{d}	nd	nd	0.186^{c}	nd	nd
16	1519	Benzaldehyde	0.549^{d}	nd	nd	0.305^{e}	0.073^{d}	0.033^{d}	0.778^{c}	0.193^{b}	0.514^{a}	1.251^{b}	0.144^{c}	0.294^{c}	2.066^{a}	0.319^{a}	0.338^{b}
		Alcohols															
4	1165	1-Butanol	nd	nd	nd	nd	nd	nd	0.661^{a}	0.175^{a}	0.357	0.035^{c}	nd	nd	0.231^{b}	nd	nd
5	1183	1-Penten-3-ol	nd	nd	nd	0.272^{b}	nd	nd	0.274^{b}	0.027^{a}	0.100	0.740^{a}	nd	nd	0.085^{c}	nd	nd
6	1230	3-Methyl-1-butanol	0.178^{c}	0.122^{a}	0.359	0.478^{b}	0.115^{c}	0.146	nd	nd	nd	0.697^{a}	0.120^{b}	0.199	0.070^{d}	0.102^{d}	0.288
7	1268	1-Pentanol	0.089^{c}	0.026^{a}	0.070^{a}	nd	nd	nd	nd	nd	nd	0.500^{a}	0.028	0.031^{c}	0.343^{b}	0.027	0.055^{b}
12	1362	1-Hexanol	nd	0.041^{a}	0.092^{a}	0.683^{a}	nd	nd	0.354^{c}	nd	nd	0.112^{d}	0.017^{b}	nd	0.428^{b}	0.015^{b}	0.094^{b}
18	1562	1-Octanol	0.079^{d}	0.018	0.064^{a}	0.232^{b}	nd	0.044^{b}	0.417^{a}	nd	nd	0.162^{c}	0.014	nd	nd	0.015	nd
23	1725	3-(Methylthio)-1-propanol	1.759^{b}	3.637^{a}	nd	0.194^{d}	0.725^{d}	0.954^{d}	0.932^{c}	1.925^{b}	1.140^{c}	0.093^{e}	nd	1.919^{b}	3.325^{a}	1.724^{c}	2.098^{a}
24	1922	Phenylethyl alcohol	0.653^{c}	0.699^{a}	0.385	nd	0.651^{b}	0.153	0.662^{b}	0.484^{d}	5.585	nd	nd	0.337	0.811^{a}	0.563^{c}	4.463
		Ketones															
3	1123	3-Penten-2-one	nd	0.172	nd	nd	nd	nd	nd	nd	nd	1.173	nd	nd	nd	nd	nd
9	1287	3-Hydroxy-2-butanone	nd	nd	nd	0.029	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 4.13 (Continued) Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Ketones															
19	1574	*(EE)*-3,5-Octadien-2-one	0.197^{c}	nd	nd	0.121^{d}	nd	nd	nd	nd	nd	0.275^{a}	nd	nd	0.232^{b}	nd	nd
		Sulfur-containing compounds															
1	1069	Dimethyl disulfide	nd	0.057	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
13	1371	Dimethyl trisulfide	nd	0.244^{a}	0.198^{a}	nd	nd	0.094^{b}	nd	0.106^{b}	0.096b^{a}	0.654	nd	0.022^{d}	nd	0.022^{c}	0.041^{c}
		Nitrogen-containing compounds															
10	1328	2,6-Dimethyl pyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.071	0.353
11	1334	2,5-Dimethyl pyrazine	nd	0.062^{c}	0.114^{a}	nd	3.015^{a}	0.064^{c}	nd	nd	nd	nd	0.248^{b}	0.094^{b}	nd	nd	nd
		Acids															
15	1453	Acetic acid	8.793^{a}	3.331^{b}	1.366^{e}	nd	1.771^{c}	3.785^{a}	2.578^{c}	4.818^{a}	2.282^{c}	2.956^{b}	1.262^{e}	2.013^{d}	1.263^{d}	1.375^{d}	2.820^{b}
17	1543	Propanoic acid	nd	0.920^{a}	0.116^{c}	nd	nd	0.266^{b}	0.665^{a}	0.064^{d}	0.112^{c}	0.567^{b}	0.077^{c}	0.292^{a}	nd	0.204^{b}	0.071^{d}
20	1576	2-Methylpropanoic acid	0.198^{c}	0.685^{a}	0.060^{e}	0.336^{b}	0.574^{b}	0.415^{d}	0.070^{d}	nd	0.473^{c}	0.412^{a}	0.085^{c}	0.559^{b}	nd	0.007^{d}	0.590^{a}
21	1627	Butanoic acid	34.671^{a}	29.352^{a}	nd	0.281^{e}	20.320^{b}	3.328^{a}	2.466^{d}	0.029^{e}	0.691^{c}	22.361^{c}	0.208^{c}	0.380^{d}	25.201^{b}	0.150^{d}	2.011^{b}
22	1673	3-Methylbutanoic acid	5.670^{d}	15.177^{a}	2.534^{e}	8.949^{b}	13.674^{b}	6.588^{d}	29.385^{a}	5.782^{d}	7.427^{c}	8.023^{c}	nd	9.342^{b}	nd	13.299^{c}	9.528^{a}

nd: Not detected; Different superscripts within a row indicate significant differences of each month (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).

Table 4.14 Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
2	1081	Hexanal	0.555^{d}	0.067^{b}	0.054^{a}	0.664^{b}	0.040^{d}	nd	0.585^{c}	nd	nd	0.405^{e}	0.136^{a}	0.025^{b}	1.009^{a}	0.047^{c}	nd
8	1283	Octanal	0.131^{b}	0.020^{b}	0.058^{a}	0.123^{c}	0.008^{c}	0.021^{b}	0.105^{d}	nd	nd	0.019^{e}	0.031^{a}	0.022^{b}	0.359^{a}	nd	nd
14	1413	*(E)*-2-Octenal	0.093^{c}	nd	nd	0.090^{c}	0.007^{d}	nd	0.121^{b}	0.011^{c}	0.282^{a}	0.082^{a}	0.020^{d}	nd	0.235^{a}	0.015^{b}	0.113^{b}
16	1519	Benzaldehyde	0.439^{c}	0.163^{b}	nd	0.373^{d}	0.117^{c}	0.469^{d}	0.278^{e}	0.095^{e}	4.234^{a}	0.472^{b}	0.304^{a}	0.684^{c}	0.572^{a}	0.109^{d}	1.053^{b}
		Alcohols															
4	1165	1-Butanol	nd	nd	0.037^{b}	nd	nd	nd	nd	nd	nd	nd	nd	0.113^{a}	0.104	nd	nd
5	1183	1-Penten-3-ol	nd	nd	0.027^{b}	0.272^{b}	nd	nd	0.234^{c}	0.024^{b}	0.026^{b}	0.306^{a}	0.057^{a}	nd	nd	nd	0.081^{a}
6	1230	3-Methyl-1-butanol	0.077^{e}	0.163^{b}	0.075^{c}	0.478^{a}	0.115^{c}	0.146	0.167^{d}	0.060^{e}	0.036^{c}	0.227^{c}	0.084^{d}	0.264^{b}	0.341^{b}	0.229^{a}	0.040^{a}
7	1268	1-Pentanol	0.108^{b}	0.054^{a}	0.025	nd	nd	nd	0.148^{a}	0.037^{b}	0.021	0.140^{a}	0.041^{b}	0.030	0.150^{a}	0.049^{a}	0.064
12	1362	1-Hexanol	0.056^{e}	0.059^{a}	0.034^{a}	0.683^{a}	nd	nd	0.092^{c}	0.019^{c}	0.014^{c}	0.078^{d}	0.036^{b}	0.032^{a}	0.104^{b}	0.034^{b}	0.025^{b}
18	1562	1-Octanol	0.054^{c}	0.016^{b}	nd	0.232^{a}	nd	0.044^{b}	0.020^{d}	0.008^{c}	nd	nd	0.028^{a}	nd	0.129^{b}	0.012b^{c}	0.127^{a}
23	1725	3-(Methylthio)-1-propanol	0.141^{c}	1.343^{a}	0.345^{c}	0.194^{b}	0.725^{b}	0.954^{a}	0.074^{d}	0.316^{e}	0.584^{b}	0.358^{a}	0.486^{c}	nd	0.360^{a}	0.364^{d}	0.300^{d}
24	1922	Phenylethyl alcohol	0.076^{d}	0.261^{e}	0.288^{a}	nd	0.651^{a}	0.153^{c}	0.187^{c}	0.324^{d}	nd	0.246^{b}	0.385^{c}	nd	0.379^{a}	0.414^{b}	0.191^{b}
		Ketones															
3	1123	3-Penten-2-one	nd	nd	0.070^{b}	nd	nd	nd	nd	nd	0.226^{a}	nd	nd	0.062^{c}	nd	nd	0.074^{b}
9	1287	3-Hydroxy-2-butanone	0.130^{a}	nd	0.029^{c}	0.466	0.012	0.090^{b}	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 4.14 (Continued) Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Ketones															
19	1574	*(EE)*-3,5-Octadien-2-one	0.086^{b}	0.018^{b}	0.066^{b}	0.088^{b}	nd	nd	0.069^{c}	nd	nd	nd	0.041^{a}	nd	0.152^{a}	nd	0.241^{a}
		Sulfur-containing compounds															
1	1069	Dimethyl disulfide	nd	nd	0.354^{a}	nd	nd	0.040^{e}	nd	nd	0.317^{b}	nd	nd	0.163^{c}	nd	0.006	0.049^{d}
13	1371	Dimethyl trisulfide	nd	nd	0.300^{a}	nd	nd	0.162^{c}	nd	0.019^{b}	0.055^{e}	nd	nd	0.221^{b}	nd	0.023^{a}	0.106^{d}
		Nitrogen-containing compounds															
10	1328	2,6-Dimethyl pyrazine	nd	nd	nd	nd	0.053^{a}	nd	nd	0.014^{b}	nd	nd	nd	nd	nd	nd	nd
11	1334	2,5-Dimethyl pyrazine	nd	nd	0.078^{d}	nd	0.159	0.137^{c}	nd	nd	0.302^{a}	nd	nd	0.138^{c}	nd	nd	0.153^{b}
		Acids															
15	1453	Acetic acid	nd	nd	0.641^{b}	0.264^{b}	0.165^{b}	1.699^{a}	1.256^{a}	0.109^{c}	nd	0.220^{c}	0.249^{a}	0.317^{c}	nd	nd	nd
17	1543	Propanoic acid	nd	nd	0.614^{b}	nd	nd	nd	0.124	nd	0.112^{d}	nd	nd	0.168^{c}	nd	0.082	0.621^{ac}
20	1576	2-Methylpropanoic acid	nd	nd	nd	nd	0.041	nd	0.160	nd	0.473	nd	0.115^{b}	nd	nd	0.199^{a}	nd
21	1627	Butanoic acid	nd	0.059^{b}	4.617^{c}	nd	nd	7.023^{a}	0.017^{b}	4.526^{a}	0.691^{d}	0.360^{a}	nd	0.005^{e}	nd	0.036^{c}	5.692^{b}
22	1673	3-Methylbutanoic acid	nd	0.055^{d}	0.374^{d}	0.691^{b}	1.059^{c}	0.413^{c}	nd	4.546^{a}	0.603^{a}	8.023^{a}	nd	0.100^{e}	0.308^{c}	4.014^{b}	0.567^{b}

nd: Not detected; Different superscripts within a row indicate significant differences of each month (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).

4.5 Draft genomes of the selected lactic acid bacterial strains involving unique flavor characteristics of Thai fish sauce

Two strains of lactic acid bacteria, 3MR10-3 and PMC-11-5, were selected for their draft genome analysis. The two bacterial strains were identified as different strains of *Tetragenococcus muriaticus* according to their physiological characteristics and 16S rDNA sequences. The genome of *T. muriaticus* 3MR10-3 and PMC-11-5 were sequenced by a whole-genome paired end strategy with a Roche 454 GS FLX Titanium sequencer. The quality trimming of paired-end reads produced from a 440-bp genomic library was performed. Two draft genomes were assembled using 454 Newbler GS de novo assembler software. The draft genome sequence of *T. muriaticus* 3MR10-3 consisted of 322 contigs for a total of 161,971 reads, counting up to 33,555,921 bases (a 13-fold coverage of the genome). About 98 percent of the reads (207 bp of an average read length) were assembled. The contig sizes ranged between 100 and 71,618 bp. The assembled sequence of *T. muriaticus* PMC-11-5 consisted of 376 contigs with sizes ranging between 101 and 89,653 bp. The assembly used 154,113 paired end 454 reads totaling 29,238,942 bases. 98 percent of the reads with an average read length of 190 bases was assembled.

The draft genome sizes of the bacterial strain 3MR10-3 (2,080,407 bp) and strain PMC-11-5 (2,103,938 bp) inferred from the total contig length was similar to the complete genome sizes of *T. muriaticus* DSM 15685^T (2,083,574 bp) and *T. halophilus* NBRC 12172^T (2,562,720 bp), which were ranked as the closest neighbor according to RAST genome analysis. The percent G+C contents in the total contigs of 3MR10-3 and PMC-11-5 were comparable to *T. muriaticus* DSM 15685^T and *T. halophilus* NBRC 12172^T, the reference strains, at 35.96, 36.03, 35.95, and 36.04%,

respectively (Table 4.15). By using the RAST server, a total numbers of predicted coding sequences (CDSs) of *T. muriaticus* 3MR10-3 and PMC-11-5 annotated, were 2,252 and 2,626 CDSs, respectively. Moreover, of the predicted 2,252 and 2,626 protein-coding genes, 47% and 47% were assigned subsystem categories, respectively. However, the draft genome of 3MR10-3 contained 2,348 CDSs, 1,414 of which were protein-coding genes with functional assignments and 934 of which are of unknown function. While 2,735 CDSs comprise of 1,288 and 1,447 functional and unknown functional genes, respectively, was found in the draft genome of PMC-11-5.

The 3MR10-3 draft genome contained five copies of rRNA operon which had only a very limited number of sequence polymorphisms. While, three rRNA copies of PMC-11-5 draft genome with no sequence polymorphism were found. These rRNA features were significantly different from *T. halophilus* NBRC 12172^{T} (Table 4.15). A total of 52 and 50 tRNA species of 3MR10-3 and PMC-11-5, respectively, were identified representing all 20 amino acids. Both draft genomes contained 13 genes encoding putative transposases (Tpases) and inactivated derivatives. However, there were 91 Tpases copies found in the reference strain, *T. halophilus* NBRC 12172^{T}. The draft genomes of two bacterial strains (3MR10-3 and PMC-11-5) contained more Tpases genes than those they were found, which may occur in the gap between contigs. The phage-related CDSs included phage structural protein, phage transcriptional protein, phage tail protein, phage portal protein, phage capsid protein phage shock protein, phage putative head morphogenesis protein, bacteriophage resistance protein, and recombinases (xerC, xerD, and DNA invertase Pin homolog).

Table 4.15 General features of draft genomes of *Tetragenococcus muriaticus* strains 3MR10-3 and PMC-11-5, in comparison to the reference strains *Tetragenococcus muriaticus* DSM 15685^{T} and *T. halophilus* NBRC 12172^{T}.

General features	3MR10-3	PMC-11-5	DSM 15685^{T}	NBRC 12172^{T}
Total number of bases, bp	2,080,407	2,103,938	2,083,574	2,562,720
DNA coding number of bases, %	85.50	84.61	85.98	87.22
DNA G+C contents, %	35.96	36.03	35.95	36.04
DNA scaffolds	322	376	110	1[a]
Total number of genes	2,431	2,812	2,184	2,633
Protein coding genes	2,348	2,735	2,079	2,555
with function prediction	1,414	1,288	1,775	1,711
without function prediction	934	1,447	304	844
RNA coding gene	83	77	105	78
rRNA genes	5	3	10	15
5S rRNA	3	1	3	5
16S rRNA	1	1	5	5
23S rRNA	1	1	2	5
tRNA genes	52	50	34	62
Insertion elements	34	39	45	104
Transposase genes	13	13	32	91
Phage-associated genes	16	20	6	5
Recombinases	5	6	7	8

[a]: assembled genome.

Halophilic bacteria respond to high external solute concentrations mostly by accumulating intracellular solutes to counteract the osmotic stress that might otherwise lead to loss of cellular turgor pressure, dehydration, and death. The

adaptation response is identified by two distinct strategies, including inorganic ion influx called “salt-in strategy” and organic compatible solute accumulation called “compatible-solute-in strategy”. Halophilic bacteria can grow optimally in the presence of extremely high salinities by either of salt and/or organic molecule accumulation (Oren, 2008). The $Na^+/K^+/H^+$ cycle plays a main role in the inorganic ion recycling. Organic compatible solutes, including proline, glycine betaine, choline, and carnitine, have been reported to be required not as growth factors but as osmotic and salt stabilizers in halophillic bacterial cells (Robert, Marrec, Blanco, and Jebbar, 2000). *Tetragenococcus muriaticus* 3MR10-3 and PMC-11-5 had their growth at 3 to 25% NaCl (optimum 6.5-10% and 15-18% respectively). Their genomes contained several osmoadaptation genes related to transporters which are responsible for inorganic ion and compatible solute uptake from hypersaline environments. The 3MR10-3 draft genome contained 2 genes related to K^+ transporters, compared to a total 3 genes in PMC-11-5. In contrast, these transporters were not found in the reference strain, *T. halophilus* NBRC 12172^T. These transporters could play a main role on initial rapid uptake of K^+. However, the incoming charge of K^+ is not compensated by the accumulation of Cl^- as it is. Instead, neutralization of K^+ is accomplished by the accumulation of a few organic anions. In addition, these bacterial draft genomes employed proline via ABC-type proline/glycine betaine transporter system, glycine betaine /choline transporter, and choline/carnitine/betaine transporter, which used for uptake compatible solute amino acid derivatives into bacterial cell. The last two transporters are a principal unique of halophillic bacteria (Takami, Takaki, and Uchiyama, 2002).

Lactic acid bacteria are fastidious microorganisms that require exogenous sources of peptides and amino acids as nitrogen sources through the activities of proteinases which are companied di- and tri-peptide and amino acid transport systems (Axelsson, 2004). Lactococci is the most extensively studied lactic acid bacteria for proteolytic system (Savijoki *et al*., 2006). Several metabolic routes can lead to the formation of flavor compounds when lactic acid bacteria are growing in milk (Liu, Nauta, Francke, and Siezen, 2008). Milk casein is hydrolyzed by a cell envelope-associated proteinase to form oligopeptides. These oligopeptides are then transported across the membrane by the oligopeptide transport system, di- and tri-peptide transporters and amino acid transporters. The intracellular oligopeptides are then hydrolyzed by cytoplasmic peptidase to form amino acids (Hutkins, 2006). Branched-chain amino acids (valine, leucine, and isoleucine), aromatic amino acids (tyrosine, tryptophan, and phenylalanine), and the sulfur-containing amino acids (methionine and cysteine) are important precursors of flavor compounds. Although amino acid catabolism by lactic acid bacteria has been well characterized, flavor-forming pathways of tetragenococci involved in fish sauce fermentation are not yet to be reported. Thus, the draft genomes of *T. muriaticus* 3MR10-3 and PMC-11-5 were analyzed for gene encoding enzymes of flavor compound forming pathway. There was no cell envelope-associated proteinase encoded in these two draft genomes, when compared to other casein utilizing lactic acid bacteria, including *Lactococcus lactis*, *Lactobacillus helveticus*, and *Lactobacillus bulgaricus* (Savijoki *et al*., 2006). However, transporters associated with free amino acid, di-/tri-peptide, and oligopeptide uptake were present and likely are responsible for the transport of the products of extracellular protein hydrolysis. The number of transport systems for

peptides and amino acids encoded in the draft genomes of 3MR10-3 and PMC-11-5 compared to the reference strains shown in Table 4.15. Draft genomes of 3MR10-3 and PMC-11-5 contained 4 and 11 genes, respectively, encoding free amino acid, di-/tri-peptide, and oligopeptide transporters. In contrast, only nine and six transport system proteins were identified in the complete genome of the reference strains *T. muriaticus* DSM 15685^{T} and *T. halophilus* NBRC 12172 respectively. However, the number of free amino acid transporters of two draft genomes was less than the reference strain. The different types of peptidases encoded in the studied draft genomes were also summarized in Table 4.16. These peptidases are most likely involved in the intracellular hydrolysis of transported peptides. Aminopeptidase and carboxypeptidase that hydrolyze a peptide bond at the amino-terminus and carboxy-terminal end of peptide, respectively, were encoded in both draft genomes. Di-/tri-peptides generated by endopeptidases, aminopeptidases, carboxypeptidases are next subjected to additional hydrolysis by tripeptidase, PepT, and dipeptidase, PepV (Savijoki *et al.*, 2006). These results suggested that these two bacterial strains containing genes involving nitrogen metabolism which may correlate to flavor formation in fish sauce.

Table 4.16 A total numbers of predicted peptide and amino acid transporters, and peptidases expressed in draft genomes of *Tetragenococcus muriaticus* 3MR10-3 and PMC-11-5 compared to *Tetragenococcus muriaticus* DSM 15685^T and *T. halophilus* NBRC 12172^T.

Protein	Number of gene			
	3MR10-3	PMC-11-5	DSM 15685^T	NBRC 12172^T
Peptide and amino acid transporters				
Di-/tri-peptide permease	1	1	2	1
Oligopeptide transport ATP-binding protein	1	1	-	1
Oligopeptide transport system permease protein	-	2	2	1
Periplasmic-binding component of an ABC superfamily oligopeptide transporter	3	5	4	2
ATP-binding component of an ABC superfamily oligopeptide transporter	-	2	1	1
ABC-type branched-chain amino acid transport systems, periplasmic component	3	1	1	-
Branched-chain amino acid uptake carrier	1	1	-	2
Arginine:ornithine antiporter, APA family	1	-	-	1
Glutamine transport system permease protein	4	5	-	6
Peptidases				
Endopeptidase, PepO	1	2	3	1
Oligoendopeptidase, PepF	3	3	1	1
Aminopeptidase, PepC	2	4	2	1
Aminopeptidase, PepS	1	1	3	1
Dipeptidase, PepV	-	-	3	1

Table 4.16 (Continued) A total numbers of predicted peptide and amino acid transporters, and peptidases expressed in draft genomes of *Tetragenococcus muriaticus* 3MR10-3 and PMC-11-5 compared to the reference strains *Tetragenococcus muriaticus* DSM 15685^T and *T. halophilus* NBRC 12172^T.

Protein	Number of gene			
	3MR10-3	PMC-11-5	DSM 15685	NBRC 12172
Peptidases				
Glutamyl aminopeptidase, PepA	1	1	-	1
Tripeptidase, PepT	-	1	1	1
Putative Xaa-Pro dipeptidase, PepQ	1	1	-	1
Carboxypeptidase	1	5	4	1
Histidine decarboxylase	-	-	1	-

-: Not detected.

The intracellular conversion of amino acids into volatile compounds proceeds at first by aminotranferases to their corresponding α-keto acids (Smit *et al*., 2005). In general, α-keto acids are generated from branched-chain amino acids, aromatic amino acids, and methionine by different aminotransferases via transamination pathway. From the draft genome of these two bacterial strains, branched-chain aminotransferase and aromatic aminotransferase, the essential enzymes for flavor formation were expressed, indicating that these bacterial strains have this pathway for the catabolism of amino acids. α-Keto acids, the central intermediates, are then further converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation (Kranenburg *et al*., 2002). The degradation of leucine, isoleucine, and phenylalanine via α-keto acids, including α-ketoisocaproate, α

-keto-3-methylpentanoate, and phenyl pyruvate, generates aldehydes and alcohols, including 3-methyl-1-butanal and 3-methyl-1-butanol from leucine, 2-methyl-1-butanal and 2-methyl-1-butanol from isoleucine, and benzaldehyde and phenylmethanol from phenylalanine, respectively (Giri *et al*., 2010). Branched-chain aldehydes, such as 3-methyl-1-butanal, 2-methyl-1-propanal, 2-methyl-1-butanal, were reported to be one of distinctive volatile compounds of nam-pla (Thai fish sauce) (Yongsawatdigul *et al*., 2007) and responsible for a meaty note (Fukami *et al*., 2002). Interestingly, two key enzymes, including 2-keto acid decarboxylase (*kdc*) and alcohol dehydrogenase (*adh*), play a role in decarboxylation and dehydrogenation, respectively (Atsumi, Hanai, and Liao, 2008). Excepting *adh*, the *kdc* was not identified in the draft genomes of 3MR10-3 and PMC-11-5 as well as *T. muriaticus* DSM 15685^{T} and *T. halophilus* NBRC 12172^{T} genomes. It suggests that the decarboxylase genes of these strains as well as the reference strains did not express. However, it has been shown that benzaldehyde converted from phenylalanine (Groot and Bont, 1998), was the predominant compound in fish sauce fermented by these strains. The 2-methylbutanol could be further converted to 2-methylbutanal by the action of alcohol dehydrogenase (Larroy, Fernandez, Gonzalez, Pares, and Biosca, 2002), which was present in the genome. Volatile acids were the most abundant group of volatile compounds in fish sauce (Saisithi *et al*., 1996). In addition, volatile fatty acids, such as butanoic acid, and 3-methylbutanolic acid, had been reported to contribute the flavor of fish sauce (Park *et al*., 2001, Fukami *et al*. 2002), which were accumulated significantly in the fermented fish sauce samples. These volatile acids are generated from dehydrogenation by aldehyde dehydrogenases, which were encoded in the draft genomes. Besides the transamination pathway, sulfuric aroma

compounds such as dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide, are derived from methionine and cysteine via elimination pathway by cystathionine lyases (Smit *et al*., 2005). These enzymes were also encoded in the draft genomes. Interestingly, genes encoding biogenic amines which accumulate during fish sauce fermentation were not detected. This is in agreement with our previous study that showed low amounts of biogenic amines in fish sauce sample inoculated with selected bacterial strains. However, it have been reported that *T. muriaticus* has been characterized in terms of its histamine production ability and histidine decarboxylase property and has previously received considerable attention as a histamine-producing bacterium (Kimura *et al*., 2001).

4.6 Detection of bacteria found in Thai fish sauce samples by ARISA and 16S rRNA gene sequencing

4.6.1 Microorganism changes during fish sauce fermentation

The initial total bacterial and lactic acid bacterial counts of fish sauce samples from two different vats were approximately 10^3 CFU/ml and slightly declined to about 10^2 CFU/ml at 4-5 months of fermentation process. However, the microbial cell numbers started to increase gradually between the middle stage to the final stage of the process and remained constant until 10 month (Figure 4.11). In addition, halophilic bacterial counts were also found about 10^3 CFU/ml at initial stage but were not detected at the middle stage. However, a number of halophilic bacteria increased dramatically at the final stage and presented constant thereafter. Our results also found the trend of cell counts in fish sauce fermentation of both vats was similar but cell counts of vat 1 was higher than those of vat 2. Our results implied that

microbial population observed at the initial stage of fish sauce fermentation process was likely microflora from fish and salt as well as fermentation vat. The results indicated microbial succession occurred during fish sauce fermentation process. The bacteria remained living in system throughout the fermentation process which was similar to those of the previous study (Udomsil, 2008). In addition, the majority of microorganisms growing in the process were most likely halophiles because they can grow and survive in hypersaline environment (Saisithi, 1994). Yongsawatdisul *et al.* (2007) also reported that halophilic bacteria were found during the fish sauce fermentation period of 3 to 12 months and played an important role in fish sauce fermentation. Bacterial population was vat-dependent because bacteria was loaded in the process differed in each vat.

4.6.2 ARISA assay

The ARISA method is based on the analysis of the intergenic 16S-23S spacer regions within the ribosomal operon. ARISA profiles of amplicons generated by PCR of bacterial ITS sequences of 20 fish sauce samples yielded a total of 232 amplicon lengths (ALs). Each AL likely represents a bacterial species. The number of ALs detected in the samples ranged from 13-56. A correspondence analysis of ARISA profile of fish sauce samples collected from different month of vat 1 of only one plant resulted in a different in fish sauce microbiota. It was found that 1 and 2 months of fish sauce samples were different from other samples. However, amplicons of 1, 3, 7 and 8 months of fish sauce of vat 2 indicated a difference in bacterial community composition. These data were comparable with bacterial counts of fish sauce samples from vats 1 and 2 (Figure 4.12), which were cultured bacteria.

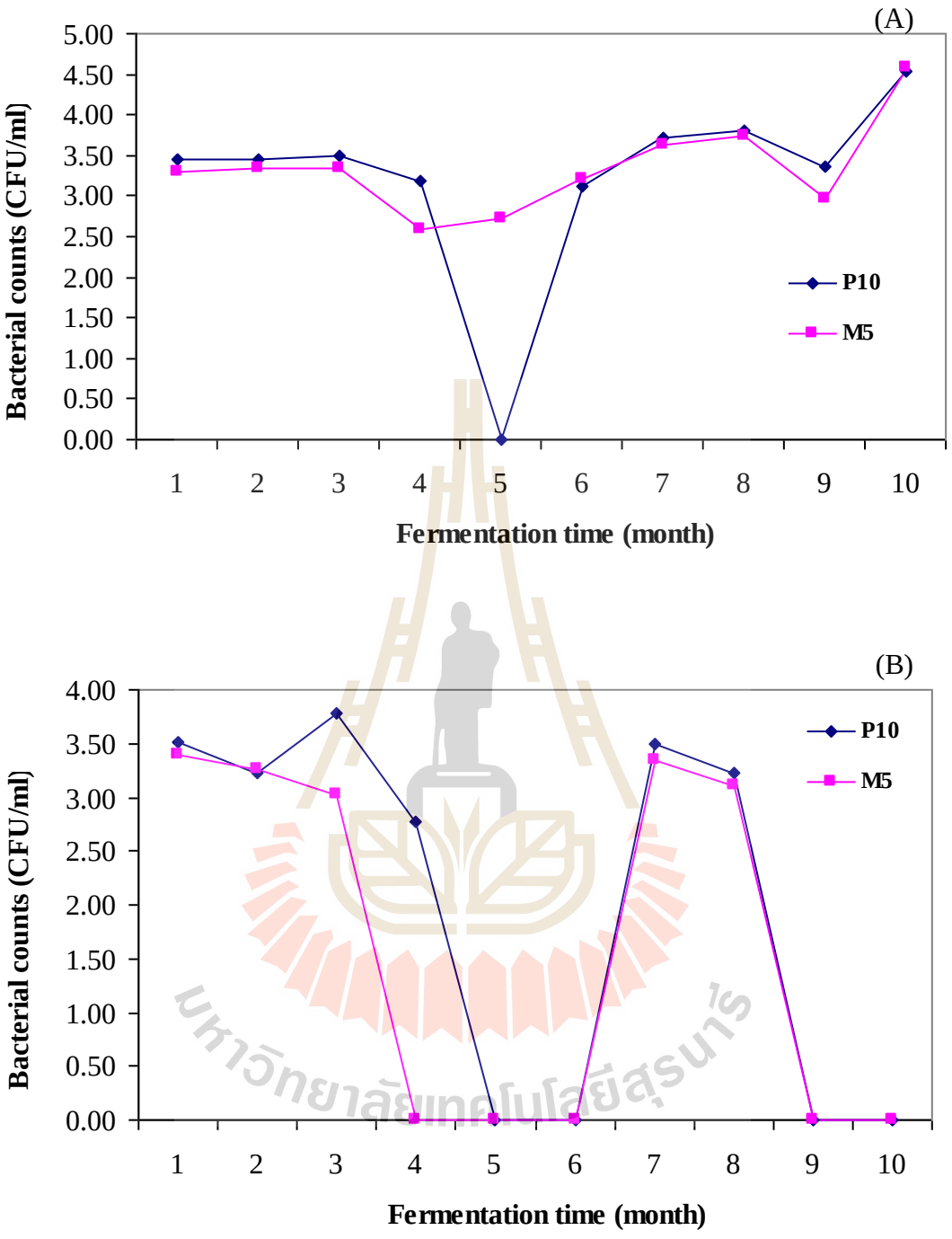

Figure 4.11 Changes in total bacterial and lactic acid bacterial counts of fish sauce samples (1-10 months) of vats 1 (A) and 2 (B) enumerated using PCA containing 10% NaCl (P10), and MRS agar containing 5% NaCl and 0.5% $CaCO_3$ (M5).

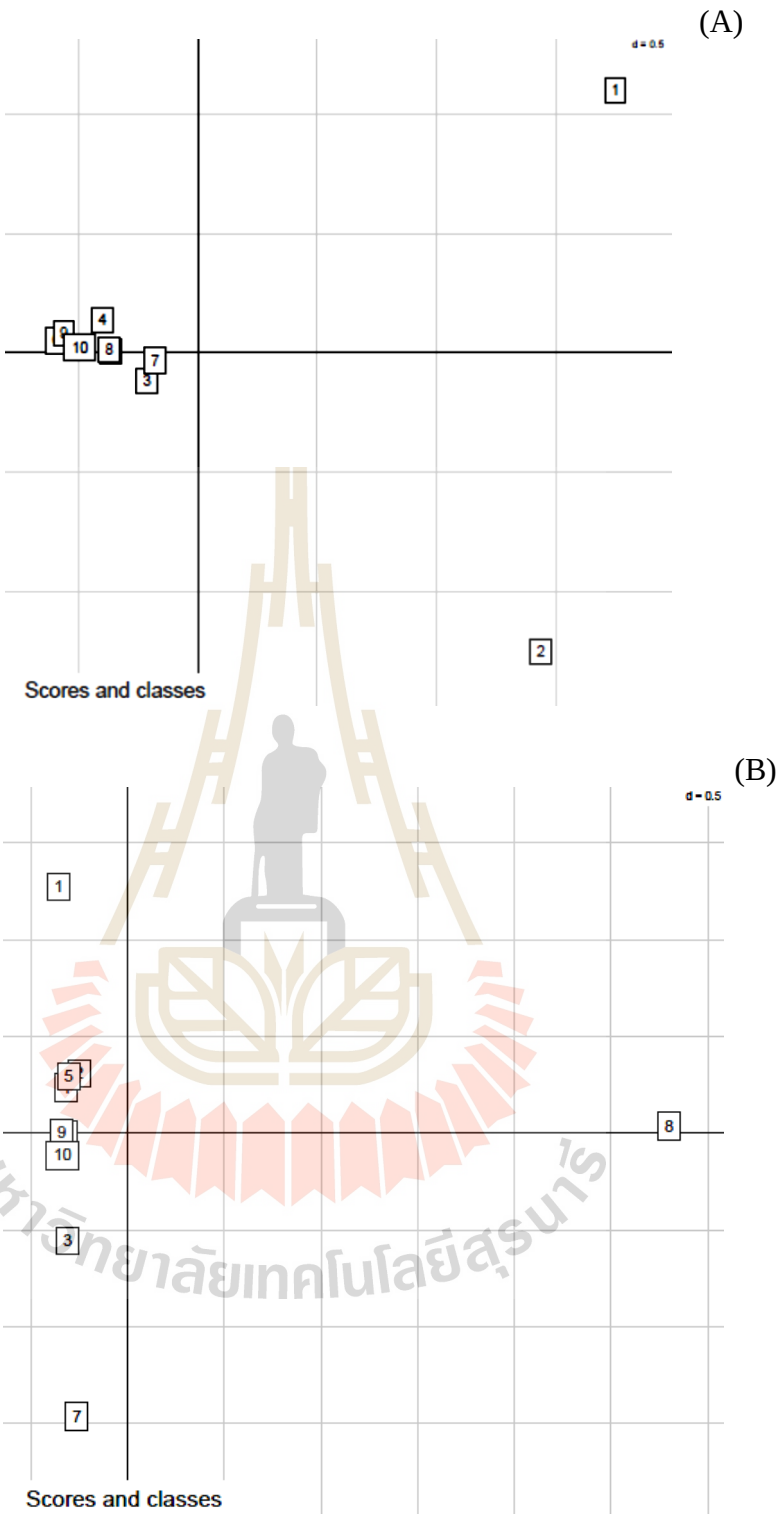

Figure 4.12 Between-group analysis of ARISA profiles of fish sauce samples (1-10 months) of vats 1 (A) and 2 (B), and represent the collective variance for each fermentation period.

The ARISA data obtained reveals that a total of 232 different ALs were detected across all 20 fish sauce samples at 1-10 months of fermentation, and 13-56 ALs detected per sample. Each peak represented fragments from at least one bacterial species since 20% of different genera had the same spacer size. Therefore, the study showed that there was at least 1 bacterial species detected in each sample. Between-group analysis indicated that bacterial community composition differed between fermentation months suggesting the shift of bacterial group (e.g. from aerobe to anaerobe or from protein degrader to ammonia utilizer/methanogen) and between vats suggesting the variation among replicates as well. This difference is due to the diversity of bacterial community that plays a significant role during fish sauce fermentation. A number of bacteria have been reported to involve in fish sauce fermentation, such as *Halobacterium salinarium* (Thongthai and Suntinanalert, 1991), *Streptococcus* sp., *Micrococcus varians*, *Staphylococcus saprophyticus*, *Pediococcus halophilus* (*Tetragenococcus halophilus*) (Beddows *et al*., 1980; Owen and Mendoza, 1985; Saisithi, 1994), *Halococcus* sp., *Halobacillus* sp. SR5-3, *Lentibacillus salicampi*, *Lentibacillus halophilus* sp. nov., *L. juripiscarius* sp. nov., *Pseudomonas* sp. (Hiraga *et al*., 2005; Namwong *et al*., 2005, 2006; Tanasupawat *et al*., 2006), *Staphylococcus xylosus* (Fukami *et al*., 2004), *Bacillus subtilis* (Uchida *et al*., 2004), *Filobacillus* sp. (Hiraga *et al*., 2005), *Virgibacillus* sp. (Sinsuwan *et al*., 2007 and 2008), *Tetragenococcus muriaticus* (Satomi *et al*., 1997), and *T. halophilus* (Thongsanit *et al*., 2002). These are microflora from fish, solar salt, and fermentation tank. The important roles of these bacteria found in fish sauce are protein degradation and flavor aroma improvement (Udomsil, 2008).

4.6.3 Bacterial community analysis by 16S rRNA gene sequencing

The composition of bacterial community and dynamic in fish sauce samples at 1-12 months of fermentation was investigated by 16S rRNA gene sequencing. A total of 1,864,163 filtered reads were obtained from the 30 samples; the number of reads varied from 23,642 to 87,579 per sample, with an average of 62,139 reads per sample. This sequencing depth allowed for identification of components of the bacteria at the level of genus.

After the taxonomic status of each read was assigned by QIIME, 8 and 8 phyla were detected from fish sauce in vat 1 and 2 respectively (Figures 4.13 and 4.14). The predominant genera in rank order of the bacteria in vat 1 of fish sauce samples at 1-12 months of fermentation were *Halanaerobium*, *Staphylococcus*, *Tetragenococcus*, *Salinivibrio*, *Lactobacillus*, *Bacillus*, *Salinicoccus*, *Pseudomonas*, *Flavobacterium*, *Virgibacillus*, and *Lentibacillus* (Figure 14.13), while the predominant genera in rank order of the bacteria in vat 2 of fish sauce samples were *Staphylococcus*, *Tetragenococcus*, *Lactobacillus*, *Halanaerobium*, *Salinicoccus*, *Pseudomonas*, *Flavobacterium*, *Salinivibrio*, *Bacillus*, *Virgibacillus*, and *Lentibacillus* (Figure 14.14).

Predominant bacterial species could be found in fish sauce, when considered to their isolation habitat. They are classified as halotolerants and extreme halophiles. These are microflora from fish, solar salt and fermentation tank (Lopetcharat *et al.*, 2001). Comparison of the dominant components of the microbiotas in vat 1 of fish sauce samples at 1-12 months of fermentation by correspondence analysis (Figure 14.13), revealed an extensive occurrence of *Tetragenococcus* throughout fermentation process. In addition, an increase in the

percentages of *Tetragenococcus* during the fermentation period of 8-12 months was indicated, which was comparable to that of vat 2 (Figure 14.14). *T. halophilus* and *T. muriaticus* strains are widely found in many batches of fish sauce fermentation in Thailand (Thongsanit *et al*., 2002) and dominated at 1-7 months of fish sauce fermentation. However, the present study indicated this bacterial genus was detected from 1-12 months of the fermentation. Udomsil *et al*. (2011) reported that *T. halophilus* could play an important role in aroma formation of fish sauce during fermentation process. *Staphylococcus* was another dominant genus found in fish sauce samples. *S. xylosus* had been isolated from fish-sauce mush (moromi) made from frigate mackerel and played an important role in unfavorable note reduction of fish sauce (Fukami *et al*., 2004). The results showed that the genus *Haloanaerobium*, widely distributed in surface saline ecosystems such as hypersaline lakes and subsurface ecosystems, was found in the middle stage of fish sauce fermentation. It was classified as halophilic bacteria that grew only anaerobically in the manufacturing process of Japanese seafood, puffer fish ovaries fermented with rice-bran or *fugunoko nukaduke* (Kobayashi *et al*., 2000). This will be the first report of this genus found in fish sauce fermentation process. Moreover, *Bacillus* and *Pseudomonas* species, which apparently originated mainly from marine fish, were presumed to be halophilic or halotolerant (Vihelmsson *et al*., 1996; Noguchi *et al*., 2004). *Salinicoccus*, *Salinivibrio*, *Flavobacterium*, and *Lactobacillus* are considered as halotolerants which were found at the first time in fish sauce fermentation (Chamroensaksri *et al*., 2009; Dobson, Colwell, Mcmeekin, and Franzmann, 1993; Tanasupawat, Shida, Okada, and Komagata, 2000). It can be assumed that these bacterial species may survive at the first stage of fish sauce fermentation process and thereafter these bacteria will die,

however their DNA still remained in the process. *Lentibacillus* and *Virgibacillus*, the moderately halophilic bacteria, are widely distributed in environments containing high NaCl concentrations, such as saline lakes and fish sauce. Tanasupawat *et al*. (2006) reported that *Lentibacillus halophilus* was isolated from fish sauce (nam-pla) collected in Thailand at various stages of fish sauce fermentation process. The use of proteinase producing bacteria, *Virgibacillus* sp., as a starter culture was reported to reduce the fermentation time to 4 months and increased the desirable volatile compounds of fish sauce (Sinsuwan *et al*., 2007). However, from the 16S sequencing results, *Lentibacillus* and *Virgibacillus* were likely found less than other halophiles in fish sauce fermentation process. Fish sauce sampling and DNA extraction steps could be the main reason why a number of these bacterial genera were found lower than the previous study data.

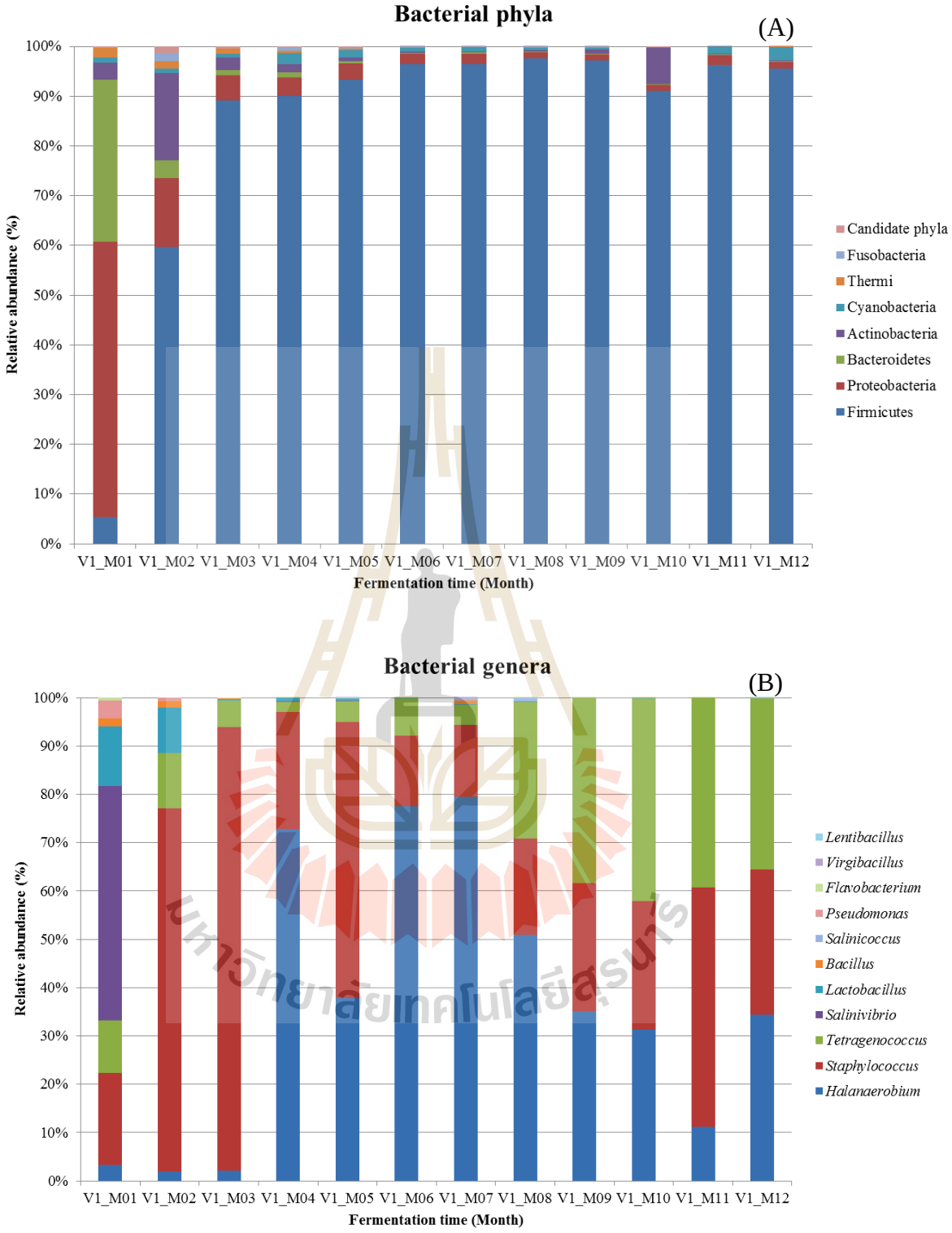

Figure 4.13 The microbiota composition of fish sauce samples during fermentation period at 1-12 months, at the (A) phylum or (B) genus level, from vat 1.

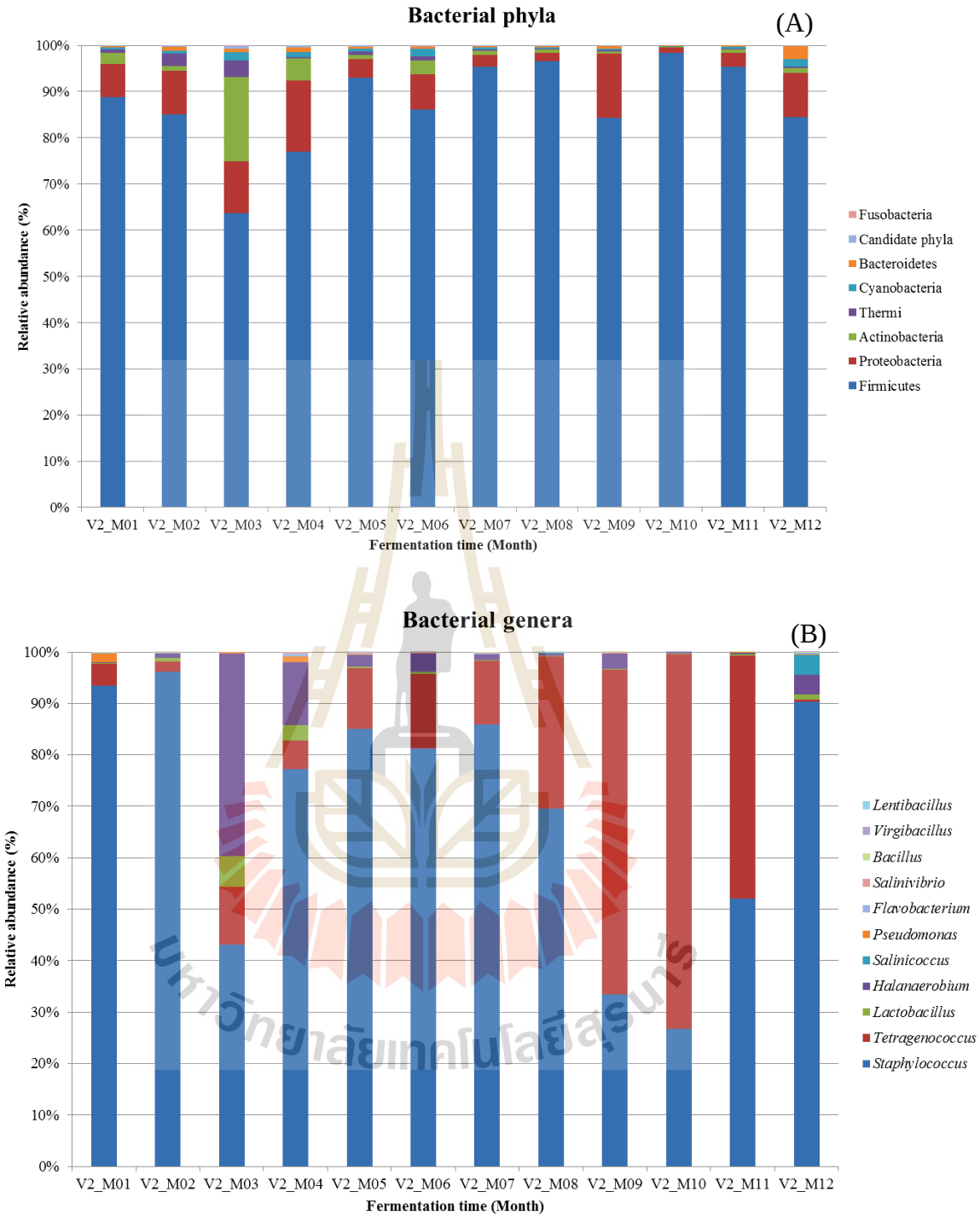

Figure 4.14 The microbiota composition of fish sauce samples during fermentation period at 1-12 months, at the (A) phylum or (B) genus level, from vat 2.

CHAPTER V

CONCLUSION

A total of 288 lactic acid bacterial isolates from Thai fish sauce samples were collected and screened for volatile compound production. Thirty eight isolates from different period of fish sauce fermentation process (1-12 months) were selected based on their flavor production abilities using modified suitable medium. Four isolates, namely 3MC10-11, 3MR10-3, 6MR10-7, and PMC-11-5), could grow well in high salt concentration which was fish broth containing 25% NaCl (FB25). Four isolates produced low concentration of biogenic amines in mGYP medium fortified by amino acids. All isolates were identified as *T. muriaticus* according to their morphological and physiological characteristics, and 16S rRNA gene sequence. *Tetragenococcus muriaticus* 3MC10-11, *T. muriaticus* 3MR10-3, *T. muriaticus* 6MR10-7, and *T. muriaticus* PMC-11-5 were selected for study flavor compound development during fish sauce fermentation. The fermentation of fish sauce was performed in laboratory scale similar to traditional fermentation using fresh anchovy compared to enzyme-digested anchovy without and with inoculating 10% (v/w) of the selected bacterial starter cultures at approximate cell counts of 10^7 CFU/ml. The population of the inoculated LAB detected at the initial stage of fermentation was 4-6 Log CFU/ml. At 60 days of fermentation, bacterial counts increased up to 6-7 Log CFU/ml, gradually declined to 3-6 Log CFU/ml after 180 days of fermentation, and was not detected at 240 days of fermentation. Total α-amino content of all inoculated samples ranged

from 810 to 957 mM after 240 days of both fermentations, which were higher than the control (763-808 mM). Fish sauce samples added *T. muriaticus* 6MR10-7 and *T. muriaticus* PMC-11-5 showed higher α -amino content than the control ($P < 0.05$). Oligopeptide contents of fish sauce samples fermented with fresh and enzyme-digested fish were 16.07-20.71 and 18.00-31.29 mM respectively. Total nitrogen content of fish sauce samples was comparable to the controls. The fish sauce samples using enzyme-digested fish added *T. muriaticus* PMC-11-5 showed higher oligopeptide content than the control ($P < 0.05$). Other physico-chemical characteristics were also comparable to those of controls. A total of 51 volatile compounds were identified in fish sauces added LAB at 2, 4, and 8 months of fermentation including aldehydes, alcohols, ketones, sulfur-containing compounds, and nitrogen-containing compound. Fish sauce samples using enzyme-digested fish and added *T. muriaticus* 3MC10-11 and *T. muriaticus* PMC-11-5, had higher content of benzaldehyde than control ($P < 0.05$) at 240 days of fermentation. Benzaldehyde was responsible for bitter almond odor in fish sauce. All fish sauce sample added starter cultures showed lower sulfur-containing compounds, which contributed to undesirable odor than the control. Moreover, dimethyl sulfide was not detected in all fish sauce samples. Butanoic acids and 3-methylbutanoic acids were the major acids in the fish sauce samples, which contributed to cheesy note in fish sauce. These four bacterial isolates could, thus, be potentially used as starter cultures for fish sauce fermentation.

Automated ribosomal intergenic spacer analysis (ARISA) profiles of fish sauce samples using polymerase chain reaction (PCR), yielded a total of 232 amplicon lengths (ALs). The number of ALs, which represents a bacterial species, detected in

the fish sauce samples ranged from 13-56. From the analysis of bacterial community in fish sauce samples at 1-12 months of fermentation by 16S rRNA gene sequencing, a total of 1,864,163 reads were obtained in a ranging of 23,642 to 87,579 per sample. Some predominant genera of bacteria found in fish sauce samples during fermentation at 1-12 months could be concluded as follows: *Halanaerobium*, *Staphylococcus*, *Tetragenococcus*, *Salinivibrio*, *Lactobacillus*, *Bacillus*, *Salinicoccus*, *Pseudomonas*, *Flavobacterium*, *Virgibacillus*, and *Lentibacillus*. Interestingly, *Tetragenococcus* was found throughout fish sauce fermentation process (1-12 months of fermentation). ARISA and 16S sequencing techniques were successfully introduced to investigate bacterial community in fish sauce fermentation, which will provide important data for the development of the fermentation process.

Tetragenococcus muriaticus 3MR10-3 and *T. muriaticus* PMC-11-5 were selected for draft genome study. According to comparative genomic analysis, draft genome sequences of two bacterial strains reveal a number of protein coding sequences (CDSs) involving regulation and uptake of inorganic ions and osmoprotectants for adaptation to high saline environments. The essential enzymes for flavor formation were encoded in these bacterial strains. Interestingly, genes ending biogenic amines which are normally presented in *T. muriaticus* were not detected. These draft genome sequences of the two *T. muriaticus* strains are useful for explanation of their growth and survival in saline protein-rich environments, and flavor formation in fish sauce. Further study is needed to get the completed genome sequences for the insightful understating of the existed information of *T. muriaticus*.

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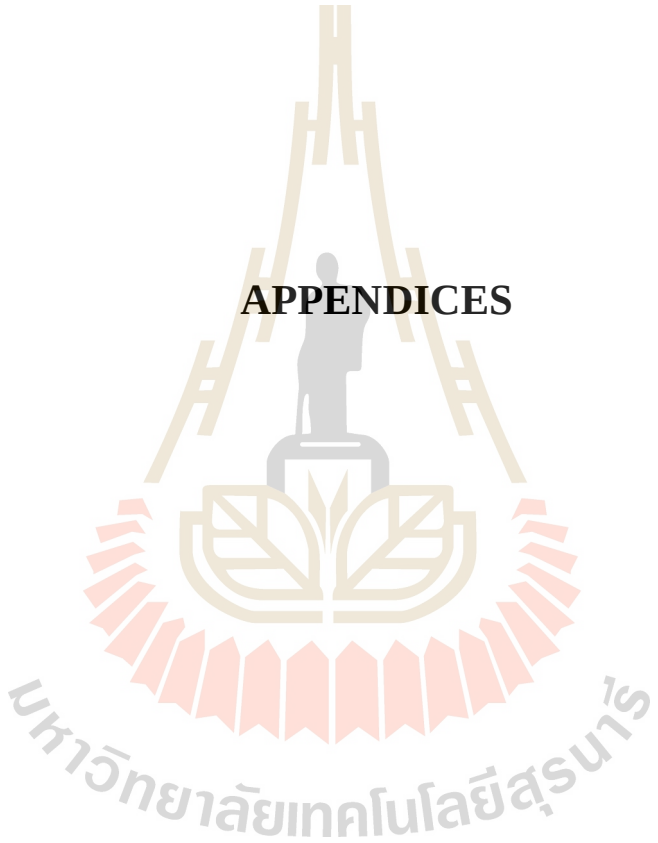
APPENDICES



APPENDIX A

CULTURE MEDIA AND REAGENT PREPARATION

A.1 Culture media

A.1.1 De Man, Rogosa and Sharpe agar (MRS agar) containing 5 or 10% NaCl

Glucose	20.00	g
Proteose peptone	10.00	g
Beef extract	10.00	g
Yeast extract	5.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Tween-80	1.00	ml
K_2HPO_4	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Sodium chloride	50.00 or 100.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 115°C for 10 min.

A.1.2 MRS agar containing 5% or 10% NaCl and 0.5% $CaCO_3$

Glucose	20.00	g
Proteose peptone	10.00	g
Beef extract	10.00	g
Yeast extract	5.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Tween-80	1.00	ml
K_2HPO_4	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Sodium chloride	50.00 or 100.00	g
$CaCO_3$	5.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 115°C for 10 min.

A.1.3 M17 agar containing 5% or 10% NaCl

Phytone peptone	5.00	g
Polypeptone	5.00	g
Yeast extract	5.00	g
Beef extract	2.50	g
Lactose	5.00	g
Ascorbic acid	0.50	g
β-Disodium glycerophosphate	19.00	g
1.0 M $MgSO_4.7H_2O$	1.00	ml
Sodium chloride	50.00 or 100.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 121°C for 15 min.

A.1.4 Glucose yeast extract peptone (GYP) agar containing 5% or 10% NaCl

Glucose	10.00	g
Peptone	10.00	g
Yeast extract	10.00	g
Sodium acetate	10.00	g
$FeSO_4.7H_2O$	10.00	mg
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	10.00	mg
Sodium chloride	50.00 or 100.00	g
Agar	15.0	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 121°C for 15 min.

A.1.5 Modified Glucose Yeast Peptone medium (mGYP medium) (Bover-Cid and Holzapful, 1999)

Glucose	10.00	g
Yeast extract	10.00	g
Peptone	5.00	g
Tween 80	5.00	g
Amino acid (L-lysine, L-histidine, L-tyrosine and L-ornithine) of each concentration	2.50	g
Sodium acetate trihydrate	2.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.7H_2O$	0.01	g
$FeSO_4.7H_2O$	0.01	g
Sodium chloride	50.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 121°C for 15 min.

A.1.6 Plate count agar (PCA) containing 10% NaCl

Tryptone	5.00	g
Yeast extract	2.50	g
Dextrose	1.00	g
Sodium chloride	100.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 121°C for 15 min.

A.1.7 JCM medium no.168

Casamino acids	5.00	g
Yeast extract	5.00	g
Sodium glutamate	1.00	g
tri-Sodium citrate	3.00	g
Potassium chloride	2.00	g
$MgSO_4.7H_2O$	20.00	g
$FeCl_2.4H_2O$	36.00	mg
$MnCl_2.4H_2O$	0.36	mg
Sodium chloride	200.00	g
Agar	20.00	g
Add distilled water to bring volume up to	1,000.00	ml

The medium was autoclaved at 121°C for 15 min. pH 7.0

A.1.8 MRS agar added 1% soluble starch and 5% NaCl (modified from MRS medium; Atlas and Parks, 1997)

Soluble starch	10.00	g
Proteose peptone	10.00	g
Beef extract	10.00	g
Yeast extract	5.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Tween-80	1.00	ml
K_2HPO_4	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Sodium chloride	50.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 115°C for 10 min.

A.1.9 MRS agar added 1% Tween 80 and 5% NaCl (modified from MRS medium; Atlas and Parks, 1997)

Tween 80	10.00	ml
Proteose peptone	10.00	g
Beef extract	10.00	g
Yeast extract	5.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Tween-80	1.00	ml
K_2HPO_4	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Sodium chloride	50.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 115°C for 10 min.

A.1.10 MRS agar added 1% skim milk and 5% NaCl (modified from MRS medium; Atlas and Parks, 1997)

Skim milk	10.00	g
Proteose peptone	10.00	g
Beef extract	10.00	g
Yeast extract	5.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Tween-80	1.00	ml
K_2HPO_4	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Sodium chloride	50.00	g
Agar	15.00	g
Add distilled water to bring volume up to	1,000.00	ml

pH 7.0

The medium was autoclaved at 115°C for 10 min.

A.2 Reagent

A.2.1 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol (95%)	20.00	ml
Mixed thoroughly		
Ammonium oxalate (1% Aqueous solution)	80.00	ml

A.2.2 Hydrogen peroxide (3% solution)

Hydrogen peroxide	3.00	g
Distilled water	100.00	ml

A.2.3 Iodine solution (Gram's iodine)

Iodine	1.00	g
Potassium iodide	2.00	g
Added distilled water and brought volume up to	300.00	ml

A.2.4 Dansyl chloride solution (10 mg/ml)

Dansyl chloride	0.50	g
Added acetone and brought volume up to	50.00	ml

A.2.5 Tetramethyl-p-phenylenediamine dihydrochloride (1% solution)

Tetramethyl-p-phenylenediamine dihydrochloride	1.00	g
Added distilled water and brought volume up to	100.00	ml

A.2.6 Saline-EDTA (0.15 M NaCl + 0.1 M EDTA, pH 8.0)

Sodium chloride	8.76	g
EDTA (di-Sodium salt)	37.22	ml
Added distilled water and brought volume up to	1000.00	ml

The reagent was autoclaved at 121°C for 15 min.

A.2.7 Lysozyme (0.75 mg/ml)

Lysozyme	7.50	mg
Added distilled water and brought volume up to	10.00	ml

A.2.8 Tris-HCl buffer (0.1M, pH 9.0)

Tris	1.21	g

Adjust to pH 9.0 using 1N HCl

Added distilled water and brought volume up to	100.00	ml

A.2.9 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100.00	mg
Added distilled water and brought volume up to	1000.00	ml

A.2.10 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.

A.2.11 RNAase (10 mg/ml)

RNAase	10.00	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20°C.

A.2.12 Proteinase K

Proteinase K	4.00	mg
50 mM Tris-HCl (pH 7.5)	1.00	ml

Use freshly prepared solution.

A.2.13 Saline-sodium citrate (20X)

NaCl	175.30	g
Sodium citrate	88.20	g
Add distilled water and bring volume up to	1,000.00	ml

A.2.14 Phosphate buffer (0.2125 M, pH 8.2)

di-Sodium hydrogen phosphate (Na_2HPO_4)	30.1665	g
Add distilled water and bring volume up to	1,000.00	ml

A.2.15 TNBS (Picrylsulfonic acid solution) (0.05% v/v)

TNBS (5% v/v)	1.00	ml
Add distilled water and bring volume up to	100.00	ml

A.2.16 HCl (0.1 N)

HCl (5% v/v)	9.00	ml
Add distilled water and bring volume up to	1,000.00	ml

A.2.17 Folin phenol reagent (Reagent C, 1 N)

HCl (5% v/v)	9.00	ml
Add distilled water and bring volume up to	1,000.00	ml

A.2.18 Reagent D (Reagent A + Reagent B) for Lowry method

Prepare Reagent D by mixing Reagent A containing sodium carbonate in 0.1 N NaOH with Reagent B containing 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate

APPENDIX B

CHROMATOGRAMS OF VOLATILE COMPOUNDS

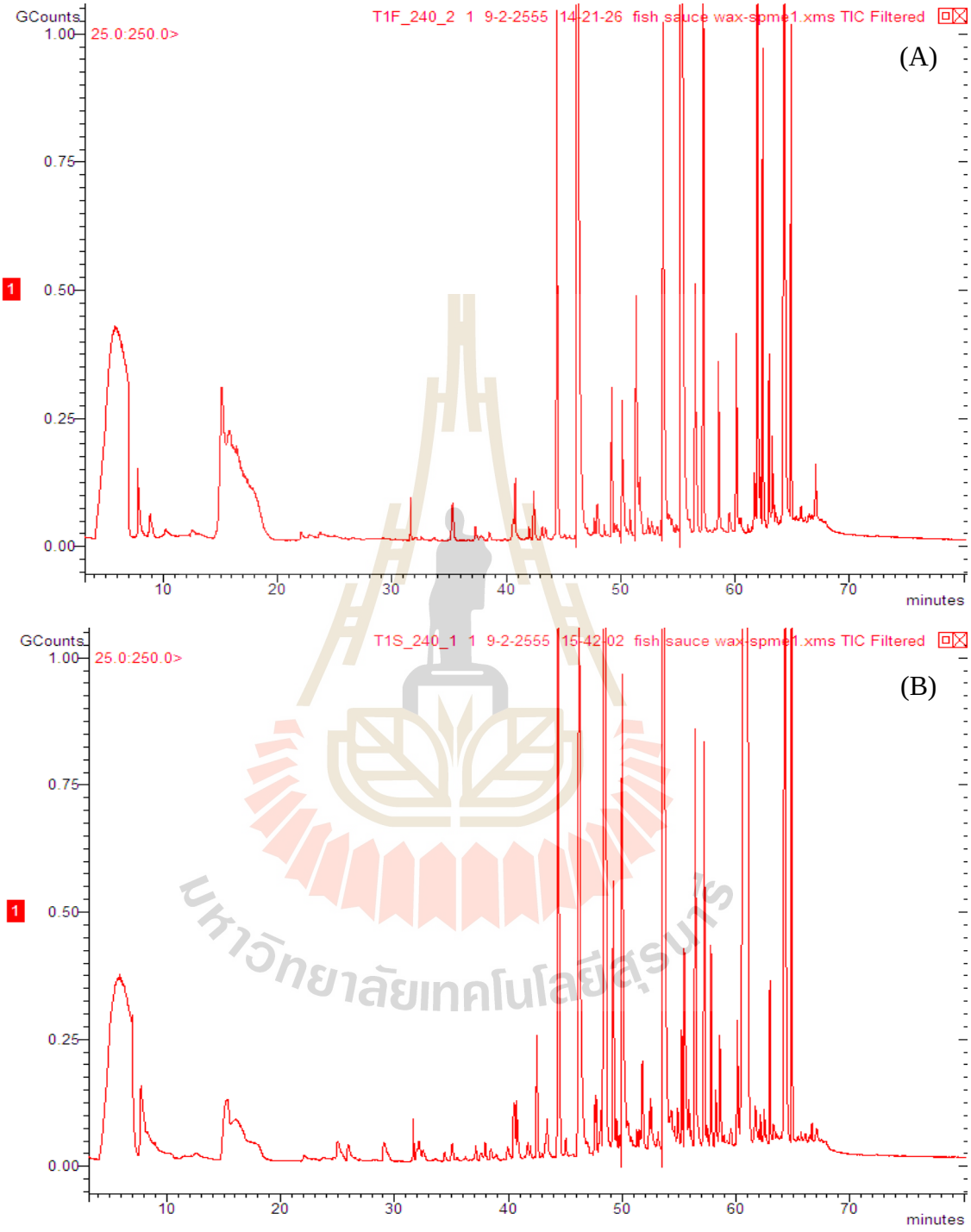


Figure 1C Chromatograms of volatile compounds of fish sauce samples using fresh fish (A) and enzyme-digested fish (B)inoculated with *T. muriaticus* 3MC10-11 fermented for 8 months.

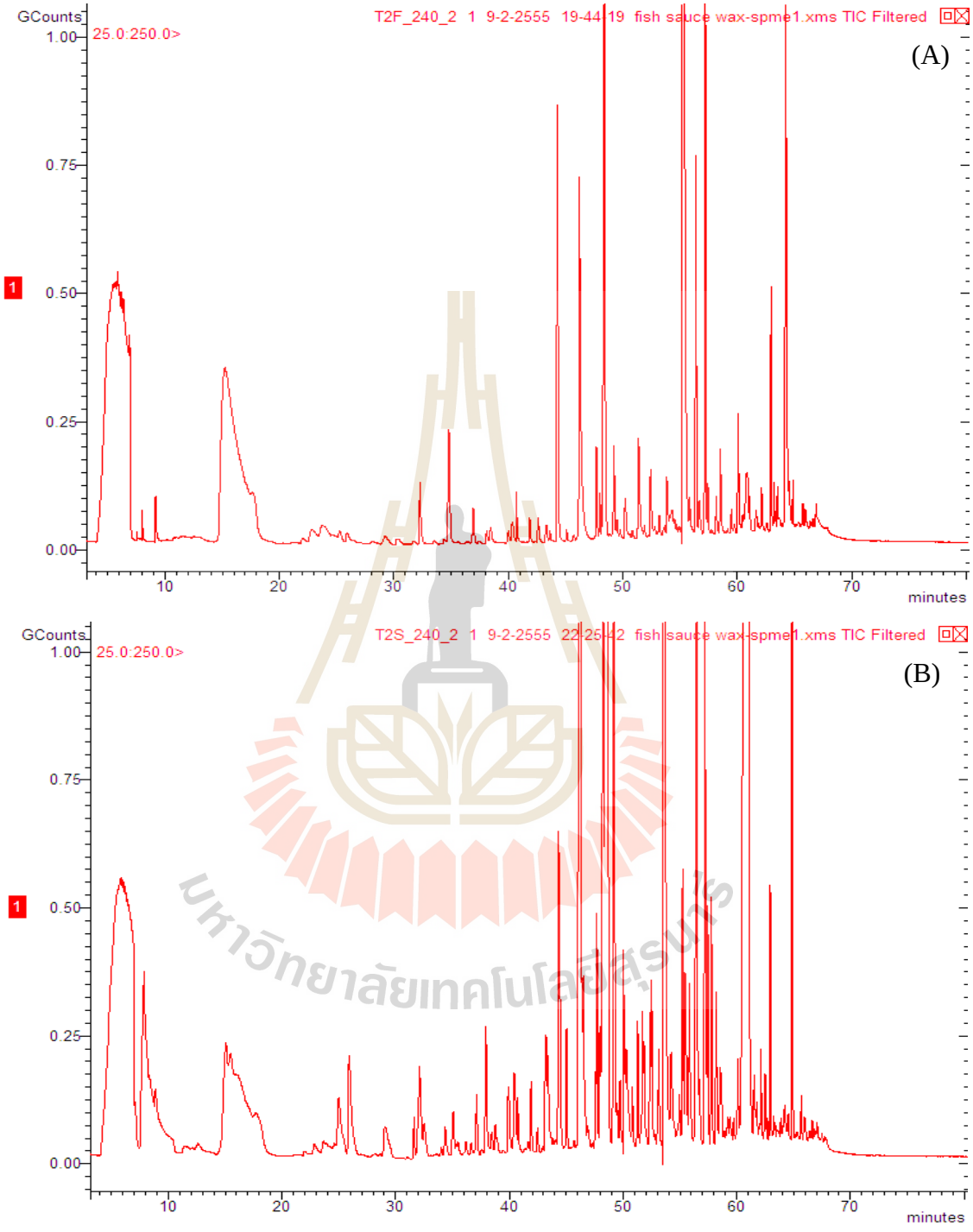


Figure 2C Chromatograms of volatile compounds of fish sauce samples using fresh fish (A) and enzyme-digested fish (B) inoculated with *T. muriaticus* 3MR10-3 fermented for 8 months.

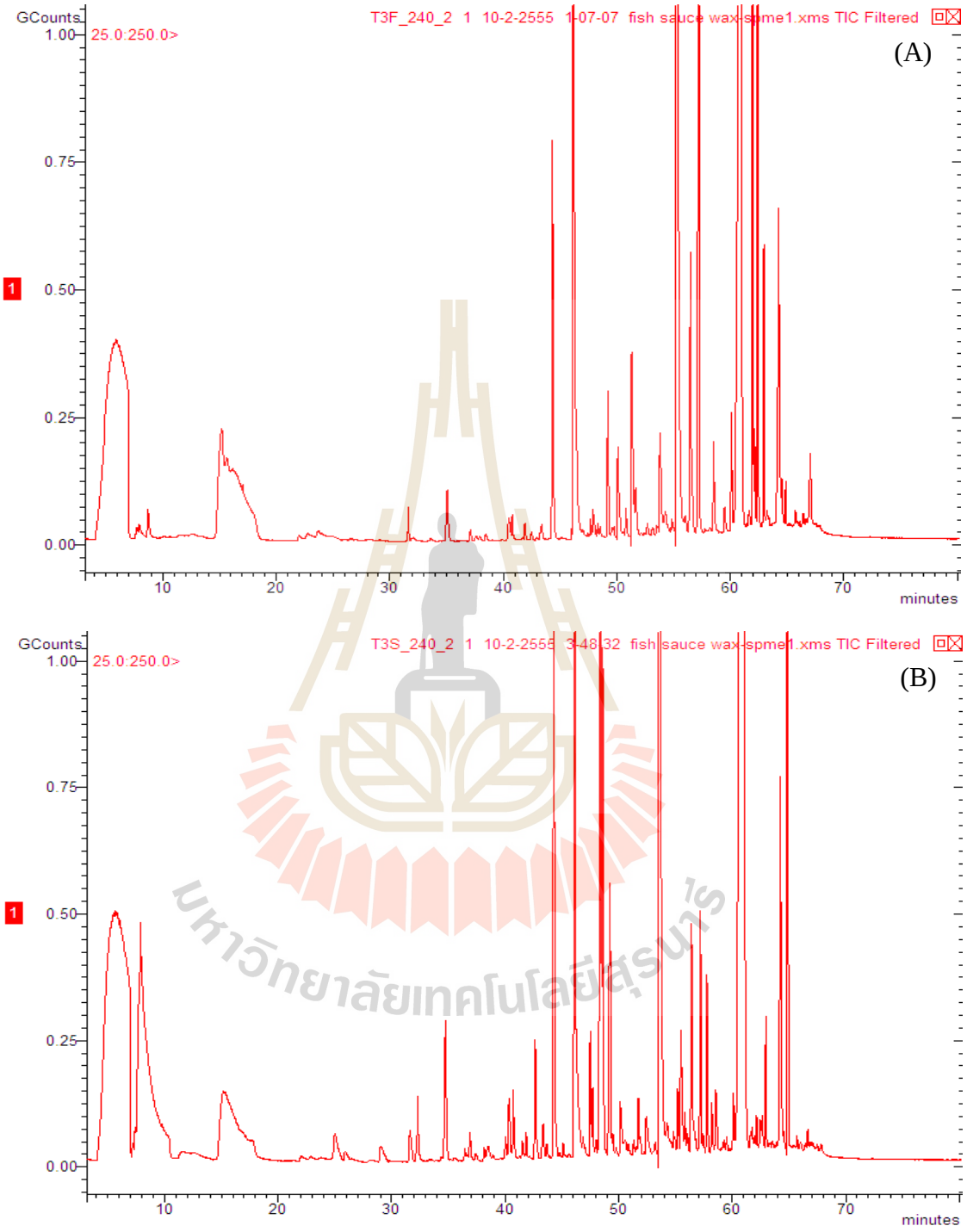


Figure 3C Chromatograms of volatile compounds of fish sauce samples using fresh fish (A) and enzyme-digested fish (B) inoculated with *T. muriaticus* 6MR10-7 fermented for 8 months.

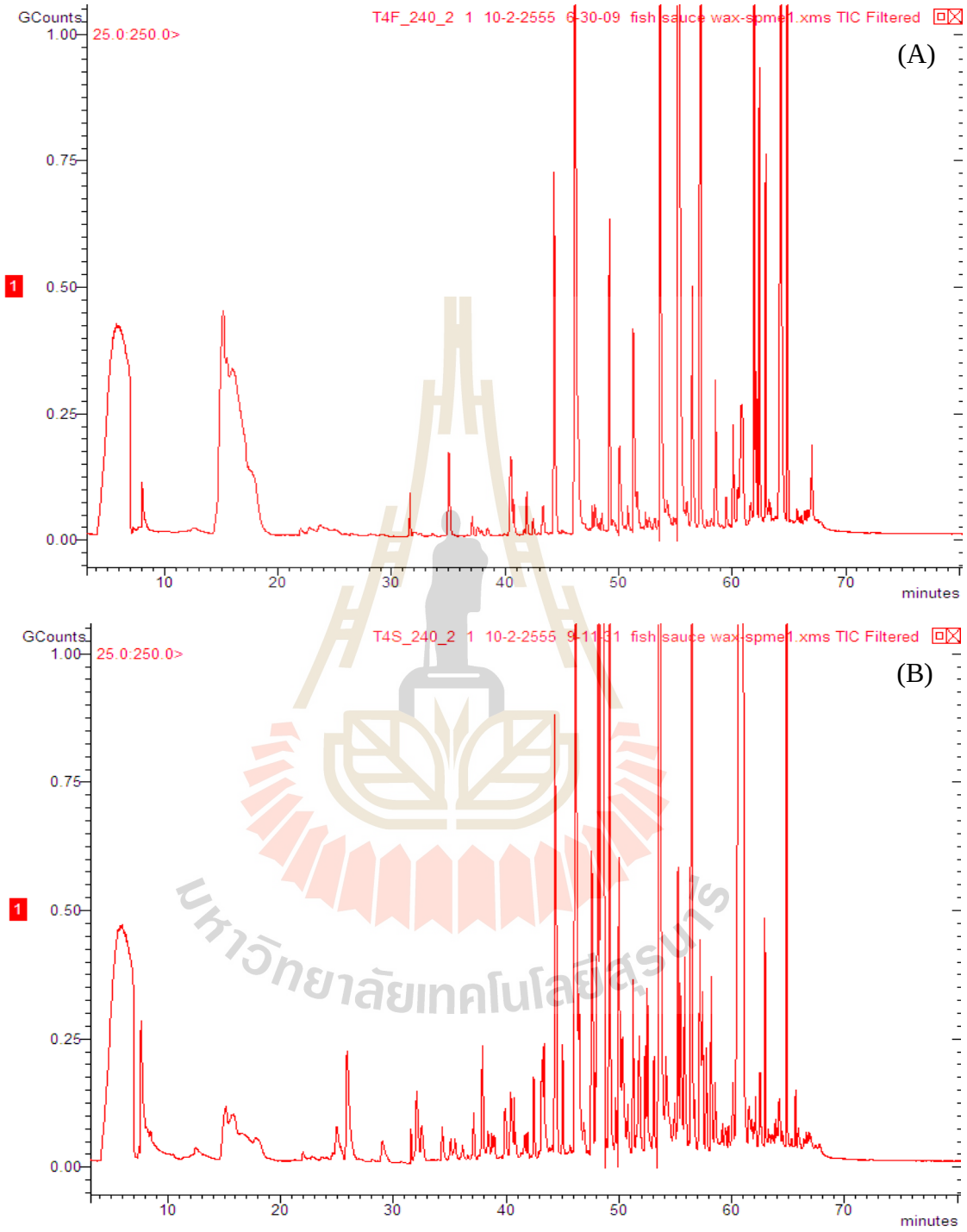


Figure 4C Chromatograms of volatile compounds of fish sauce samples using fresh fish (A) and enzyme-digested fish (B) inoculated with *T. muriaticus* PMC-11-5 fermented for 8 months.

APPENDIX C

VOLATILE COMPOUNDS FOUND DURING FISH SAUCE FERMENTATION

Table 1D Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
3	1081	Hexanal	nd	nd	nd	0.477	nd	nd	0.751	nd	nd	1.176	nd	nd	0.326	nd	nd
5	1128	3-Methyl-1-butenal	nd	nd	nd	0.084	nd	nd	0.165	nd	nd	nd	nd	nd	nd	nd	nd
7	1166	2-Methyl-2-butenal	nd	0.028	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	1173	Heptanal	nd	nd	nd	0.329	nd	nd	1.410	nd	nd	nd	nd	nd	nd	nd	nd
10	1214	*(Z)*-2-Hexanal	nd	nd	nd	0.142	nd	nd	0.271	nd	nd	nd	0.024	nd	nd	nd	nd
12	1237	*(Z)*-4-Hexanal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15	1283	Octanal	0.190	nd	nd	0.231	nd	nd	0.276	nd	nd	0.257	nd	nd	0.189	nd	nd
25	1384	Nanonal	0.178	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
26	1413	*(E)*-2-Octenal	0.191	nd	nd	0.183	nd	nd	0.308	nd	nd	0.073	nd	nd	0.186	nd	nd
31	1472	*(EE)*-2,4-Heptadienal	0.563	nd	nd	0.536	nd	nd	0.994	nd	nd	nd	nd	nd	0.264	nd	nd
34	1519	Benzaldehyde	0.549	nd	nd	0.305	0.073	0.033	0.778	0.193	0.514	1.251	0.144	0.294	2.066	0.319	0.338
35	1533	*(E)*-2-Nonenal	0.134	nd	nd	nd	nd	nd	nd	nd	nd	0.032	nd	nd	0.082	nd	nd
41	1588	*(EZ)*-2,6-Nonadienal	0.216	nd	nd	nd	nd	nd	0.449	nd	nd	0.272	nd	nd	0.260	nd	nd
42	1603	2-Methyl pentanal	nd	nd	nd	0.256	nd	nd	0.272	nd	nd	0.324	nd	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 1D (Continued) Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
45	1649	Benzeneacetaldehyde	nd	0.370	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.105	nd
		Alcohols															
1	948	Ethanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.019	nd	nd
6	1165	1-Butanol	nd	nd	nd	nd	nd	nd	0.661	0.175	0.357	0.035	nd	nd	0.231	nd	nd
9	1183	1-Penten-3-ol	nd	nd	nd	0.272	nd	nd	0.274	0.027	0.100	0.740	nd	nd	0.085	nd	nd
11	1230	3-Methyl-1-butanol	0.178	0.122	0.359	0.478	0.115	0.146	nd	nd	nd	0.697	0.120	0.199	0.070	0.102	0.288
13	1268	1-Pentanol	0.089	0.026	0.070	nd	nd	nd	nd	nd	nd	0.500	0.028	0.031	0.343	0.027	0.055
20	1332	*(E)*-2-Penten-1-ol	nd	nd	nd	nd	nd	nd	0.159	nd	nd	nd	nd	nd	0.189	nd	nd
23	1362	1-Hexanol	nd	0.041	0.092	0.683	nd	nd	0.354	nd	nd	0.112	0.017	nd	0.428	0.015	0.094
29	1454	1-Octen-3-ol	nd	nd	nd	0.834	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
30	1461	1-Heptanol	nd	nd	nd	0.577	nd	nd	0.747	nd	nd	nd	nd	nd	nd	nd	nd
33	1487	1,5-Hexadien-3-ol	0.049	0.067	1.832	0.059	0.037	0.038	0.729	0.043	0.018	0.948	0.063	nd	0.895	0.043	nd
37	1562	1-Octanol	0.079	0.018	0.064	0.232	nd	0.044	0.417	nd	nd	0.162	0.014	nd	nd	0.015	nd
40	1583	2,3-Butanediol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 1D (Continued) Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Alcohols															
43	1621	*(E)*-2-Octen-1-ol	0.199	nd	nd	0.220	nd	nd	0.331	nd	nd	0.626	nd	nd	nd	nd	nd
46	1663	2-Furanmethanol	nd	0.085	nd	nd	0.076	nd	nd	nd	nd	nd	0.049	nd	nd	2.521	nd
49	1725	3-(Methylthio)-1-propanol	1.759	3.637	nd	0.194	0.725	0.954	0.932	1.925	1.140	0.093	nd	1.919	3.325	1.724	2.098
51	1922	Phenylethyl alcohol	0.653	0.699	0.385	nd	0.651	0.153	0.662	0.484	5.585	nd	nd	0.337	0.811	0.563	4.463
		Ketones															
4	1123	3-Penten-2-one	nd	0.172	nd	nd	nd	nd	nd	nd	nd	1.173	nd	nd	nd	nd	nd
16	1287	3-Hydroxy-2-butanone	nd	nd	nd	0.029	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17	1290	Cyclohexanone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
18	1318	2,3-Octanedione	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
38	1574	*(EE)*-3,5-Octadien-2-one	0.197	nd	nd	0.121	nd	nd	nd	nd	nd	0.275	nd	nd	0.232	nd	nd
50	1732	*(E)*-3-Octen-2-one	nd	nd	nd	0.366	nd	nd	0.694	nd	nd	0.933	nd	nd	0.791	nd	nd
		Sulfur-containing compounds															
2	1069	Dimethyl disulfide	nd	0.057	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
24	1371	Dimethyl trisulfide	nd	0.244	0.198	nd	nd	0.094	nd	0.106	0.096	0.654	nd	0.022	nd	0.022	0.041

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 1D (Continued) Changes of volatile compounds during fish sauce fermentation using fresh fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Nitrogen-containing compounds															
14	1275	2-Methyl pyrimidine	0.122	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
19	1328	2,6-Dimethyl pyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.071	0.353
21	1334	2,5-Dimethyl pyrazine	nd	0.062	0.114	nd	3.015	0.064	nd	nd	nd	nd	0.248	0.094	nd	nd	nd
22	1353	2,3-Dimethyl pyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
27	1441	2,5-Diethyl pyrazine	nd	0.083	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
32	1483	Tetramethyl pyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Acids															
28	1453	Acetic acid	8.793	3.331	1.366	nd	1.771	3.785	2.578	4.818	2.282	2.956	1.262	2.013	1.263	1.375	2.820
36	1543	Propanoic acid	nd	0.920	0.116	nd	nd	0.266	0.665	0.064	0.112	0.567	0.077	0.292	nd	0.204	0.071
39	1576	2-Methylpropanoic acid	0.198	0.685	0.060	0.336	0.574	0.415	0.070	nd	0.473	0.412	0.085	0.559	nd	0.007	0.590
44	1627	Butanoic acid	34.671	29.352	nd	0.281	20.320	3.328	2.466	0.029	0.691	22.361	0.208	0.380	25.201	0.150	2.011
47	1673	3-Methylbutanoic acid	5.670	15.177	2.534	8.949	13.674	6.588	29.385	5.782	7.427	8.023	nd	9.342	nd	13.299	9.528
48	1688	3-Methylpropanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.222	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 2D Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
3	1081	Hexanal	0.555	0.067	0.054	0.664	0.040	nd	0.585	nd	nd	0.405	0.136	0.025	1.009	0.047	nd
5	1128	3-Methyl-1-butenal	0.036	nd	nd	nd	nd	nd	nd	nd	nd	0.081	nd	nd	nd	nd	nd
7	1166	2-Methyl-2-butenal	nd	nd	nd	0.071	nd	nd	nd	nd	nd	nd	nd	nd	0.080	nd	nd
8	1173	Heptanal	0.122	nd	nd	0.215	nd	0.064	0.186	nd	nd	0.112	0.061	nd	0.275	nd	nd
10	1214	*(Z)*-2-Hexanal	0.123	0.010	nd	0.117	nd	nd	0.125	nd	nd	0.010	0.011	nd	0.196	nd	nd
12	1237	*(Z)*-4-Hexanal	0.018	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.072	nd	nd
15	1283	Octanal	0.131	0.020	0.058	0.123	0.008	0.021	0.105	nd	nd	0.019	0.031	0.022	0.359	nd	nd
25	1384	Nanonal	0.134	0.019	nd	0.121	nd	nd	0.091	0.011	nd	0.116	0.032	nd	0.372	nd	nd
26	1413	*(E)*-2-Octenal	0.093	nd	nd	0.090	0.007	nd	0.121	0.011	0.282	0.082	0.020	nd	0.235	0.015	0.113
31	1472	*(EE)*-2,4-Heptadienal	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.074	nd	nd	nd	nd	nd
34	1519	Benzaldehyde	0.439	0.163	0.469	0.373	0.117	0.469	0.278	0.095	4.234	0.472	0.304	0.684	0.572	0.109	1.053
35	1533	*(E)*-2-Nonenal	0.057	0.008	nd	0.068	0.004	nd	0.095	nd	nd	0.051	nd	nd	nd	nd	0.074
41	1588	*(EZ)*-2,6-Nonadienal	0.087	0.028	nd	nd	nd	nd	nd	nd	0.294	nd	nd	nd	nd	0.012	0.210
42	1603	2-Methyl pentanal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 2D (Continued) Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Aldehydes															
45	1649	Benzeneacetaldehyde	nd	0.214	nd	nd	0.066	nd	nd	0.025	nd	nd	nd	nd	nd	0.184	nd
		Alcohols															
1	948	Ethanol	nd	nd	nd	nd	nd	nd	0.286	nd	nd	nd	nd	nd	nd	nd	nd
6	1165	1-Butanol	nd	nd	0.037	nd	nd	nd	nd	nd	nd	nd	nd	0.113	0.104	nd	nd
9	1183	1-Penten-3-ol	nd	nd	0.027	0.272	nd	nd	0.234	0.024	0.026	0.306	0.057	nd	nd	nd	0.081
11	1230	3-Methyl-1-butanol	0.077	0.163	0.075	0.478	0.115	0.146	0.167	0.060	0.036	0.227	0.084	0.264	0.341	0.229	0.040
13	1268	1-Pentanol	0.108	0.054	0.025	nd	nd	nd	0.148	0.037	0.021	0.140	0.041	0.030	0.150	0.049	0.064
20	1332	*(E)*-2-Penten-1-ol	0.147	nd	nd	nd	nd	nd	0.256	nd	nd	nd	nd	nd	0.171	nd	nd
23	1362	1-Hexanol	0.056	0.059	0.034	0.683	nd	nd	0.092	0.019	0.014	0.078	0.036	0.032	0.104	0.034	0.025
29	1454	1-Octen-3-ol	nd	nd	nd	0.834	nd	nd	nd	nd	nd	0.372	0.152	nd	nd	0.158	nd
30	1461	1-Heptanol	nd	nd	nd	0.577	nd	nd	nd	nd	nd	nd	nd	nd	0.198	nd	nd
33	1487	1,5-Hexadien-3-ol	0.259	0.067	nd	0.059	0.037	0.038	0.328	0.079	0.004	0.279	0.095	nd	0.385	0.084	nd
37	1562	1-Octanol	0.054	0.016	nd	0.232	nd	0.044	0.020	0.008	nd	nd	0.028	nd	0.129	0.012	0.127
40	1583	2,3-Butanediol	0.036	0.166	0.142	nd	nd	nd	0.598	0.171	0.223	0.049	0.239	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 2D (Continued) Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Alcohols															
43	1621	*(E)*-2-Octen-1-ol	0.108	nd	nd	0.220	nd	nd	nd	nd	nd	nd	nd	nd	0.172	nd	nd
46	1663	2-Furanmethanol	0.076	0.053	nd	nd	0.076	nd	0.090	0.042	0.125	0.077	0.042	nd	nd	0.064	nd
49	1725	3-(Methylthio)-1-propanol	0.141	1.343	0.345	0.194	0.725	0.954	0.074	0.316	0.584	0.358	0.486	nd	0.360	0.364	0.300
51	1922	Phenylethyl alcohol	0.076	0.261	0.288	nd	0.651	0.153	0.187	0.324	nd	0.246	0.385	nd	0.379	0.414	0.191
		Ketones															
4	1123	3-Penten-2-one	nd	nd	0.070	nd	nd	nd	nd	nd	0.226	nd	nd	0.062	nd	nd	0.074
16	1287	3-Hydroxy-2-butanone	0.130	nd	nd	0.029	0.466	0.012	0.090	nd	nd	nd	nd	nd	nd	nd	nd
17	1290	Cyclohexanone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.025	0.281	nd	nd	nd
18	1318	2,3-Octanedione	nd	0.048	nd	0.038	nd	0.066	0.035	0.033	0.240	nd	0.042	0.038	nd	0.042	0.106
38	1574	*(EE)*-3,5-Octadien-2-one	0.086	0.018	0.066	0.088	nd	nd	0.069	nd	nd	nd	0.041	nd	0.152	nd	0.241
50	1732	*(E)*-3-Octen-2-one	nd	0.010	nd	0.158	0.018	0.015	0.208	0.036	0.061	nd	0.063	nd	0.546	0.064	nd
		Sulfur-containing compounds															
2	1069	Dimethyl disulfide	nd	nd	0.317	nd	nd	0.040	nd	nd	0.354	nd	nd	0.163	nd	0.006	0.049
24	1371	Dimethyl trisulfide	nd	nd	0.300	nd	nd	0.162	nd	0.019	0.055	nd	nd	0.221	nd	0.023	0.106

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

Table 2D (Continued) Changes of volatile compounds during fish sauce fermentation using enzyme-digested fish inoculated with 4 selected lactic acid bacterial starter cultures and fermented at room temperature for 8 months.

No.	RI[a]	Volatile compound	Relative peak area[b]													
			Control			3MC-10-11			3MR10-3			6MR10-7			PMC-11-5		
			2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo	2mo	4mo	8mo
		Nitrogen-containing compounds															
14	1275	2-Methyl pyrimidine	nd	nd	nd	nd	0.008	0.018	nd	nd	nd	nd	nd	nd	nd	nd	nd
19	1328	2,6-Dimethyl pyrazine	nd	nd	nd	nd	0.053	nd	nd	0.014	nd	nd	nd	nd	nd	nd	nd
21	1334	2,5-Dimethyl pyrazine	nd	nd	0.078	nd	0.159	0.137	nd	nd	0.302	nd	nd	0.138	nd	nd	0.153
22	1353	2,3-Dimethyl pyrazine	nd	0.011	0.031	nd	nd	nd	nd	nd	nd	nd	nd	0.005	nd	nd	0.039
27	1441	2,5-Diethyl pyrazine	nd	nd	nd	nd	nd	nd	nd	nd	6.478	nd	nd	1.375	nd	nd	nd
32	1483	Tetramethyl pyrazine	nd	0.021	0.209	nd	0.066	nd	nd	0.018	0.148	nd	nd	0.199	nd	nd	0.338
		Acids															
28	1453	Acetic acid	nd	nd	0.641	0.264	0.165	1.699	1.256	0.109	nd	0.220	0.249	0.317	nd	nd	nd
36	1543	Propanoic acid	nd	nd	0.614	nd	nd	nd	0.124	nd	0.112	nd	nd	0.168	nd	0.082	0.621
39	1576	2-Methylpropanoic acid	nd	nd	nd	nd	0.041	nd	0.160	nd	0.473	nd	0.115	nd	nd	0.199	nd
44	1627	Butanoic acid	nd	0.059	4.617	nd	nd	7.023	0.017	4.526	0.691	0.360	nd	0.005	nd	0.036	5.692
47	1673	3-Methylbutanoic acid	nd	0.055	0.374	0.691	1.059	0.413	nd	4.546	0.603	8.023	nd	0.100	0.308	4.014	0.567
48	1688	3-Methylpropanoic acid	nd	nd	nd	0.446	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Note: nd, Not detected; [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); 2 mo, 2 months of fermentation; 4 mo, 4 months of fermentation; 8 mo, 8 months of fermentation.

APPENDIX D

DEPOSITION OF BACTERIAL RIBOSOMAL SEQUENCES IN GENBANK (U.S.A.)

Table 1E 16S rRNA genes (partial sequences) of *Tetragenococcus muriaticus* deposited in GenBank (U.S.A.).

Isolation source	Bacterial strain	Nucleotide sequence	
		Length	NCBI accession no.
Sample from fish sauce fermentation process at 3rd month	3MC10-11	1477	KM042031
Sample from fish sauce fermentation process at 3rd month	3MR10-3	1468	KM042032
Sample from fish sauce fermentation process at 6th month	6MR10-7	1471	KM042033
Sample from fish sauce fermentation process at 11th month	PMC-11-5	1447	KM042034

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