ROLE OF LACTIC ACID BACTERIA ON CHEMICAL COMPOSITIONS OF FISH SAUCE

Natteewan Udomsil

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Food Technology

Suranaree University of Technology

Academic Year 2008

บทบาทของแบคทีเรียกรดแล็กติกต่อองค์ประกอบทางเคมีของน้ำปลา

นางสาวนัทธีวรรณ อุดมศิลป์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2551

ROLE OF LACTIC ACID BACTERIA ON CHEMICAL COMPOSITIONS OF FISH SAUCE

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

Thesis Examining Committee

_____

(Asst. Prof. Dr. Piyawan Gasaluck)

Chairperson

_____

(Assoc. Prof. Dr. Jirawat Yongsawatdigul)

Member (Thesis Advisor)

_____

(Asst. Prof. Dr. Sureelak Rodtong)

Member

_____

(Assoc. Prof. Dr. Somboon Tanasupawat)

Member

_____

(Prof. Dr. Pairote Sattayatham)

Vice Rector for Academic Affairs

_____

(Asst. Prof. Dr. Suwayd Ningsanond)

Dean of Institute of Agricultural Technology

นัทธีวรรณ อุดมศิลป์ : บทบาทของแบคทีเรียกรดแล็กติกต่อองค์ประกอบทางเคมีของน้ำปลา (ROLE OF LACTIC ACID BACTERIA ON CHEMICAL COMPOSITIONS OF FISH SAUCE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 141 หน้า.

วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาบทบาทของแบคทีเรียกรดแล็กติกที่ชอบเจริญในที่เค็มที่คัดแยกจากกระบวนการหมักน้ำปลาต่อการเกิดกลิ่นรสและองค์ประกอบทางเคมีของน้ำปลา จากการคัดแยกแบคทีเรียกรดแล็กติกจากบ่อหมักน้ำปลา ช่วงเดือนที่ 1-12 จำนวน 64 ไอโซเลท คัดเลือกได้ 18 ไอโซเลท ที่มีความสามารถในการเจริญในอาหารเหลวที่เตรียมจากสารสกัดจากปลากะตัก (Fish broth) ที่เติมเกลือโซเดียมคลอไรด์ 25% (FB25) และสามารถย่อยสลายโปรตีนปลา จากนั้นได้คัดเลือกต่อ ได้แบคทีเรียจำนวน 7 ไอโซเลท คือ M11 MS33 MRC10-1-3 MRC5-5-2 MCD10-5-10 MCD10-5-15 และ MRC10-7-8 จากความสามารถในการเจริญในอาหารเหลว MRS ที่เติมเกลือโซเดียมคลอไรด์ ร่วมกับความสามารถในการย่อยสลายโปรตีนปลา เมื่อศึกษากิจกรรมของเอนไซม์อะมิโนเพปทิเดสภายในเซลล์ (Intracellular aminopeptidase) พบว่าแบคทีเรียทั้ง 7 ไอโซเลทแสดงกิจกรรมสูงต่อสารตั้งต้นอะลานีน (Alanine) (2.85-3.67 หน่วย/มิลลิลิตร) และยังพบว่ามีกิจกรรมปานกลางต่อสารตั้งต้นเมทไธโอนีน(Methionine) ลิวซีน (Leucine) อาร์จินีน (Arginine) และกลูทามิค (Glutamic) แบคทีเรียทั้ง 7 ไอโซเลทสร้างฮีสตามีนในอาหารเลี้ยงเชื้อเหลว mGYP ที่มีความเข้มข้นเกลือโซเดียมคลอไรด์ 5% และ 25% ในระดับ 6.62-22.55 และ 13.14-20.39 มิลลิกรัม/100มิลลิลิตร ตามลำดับ สารระเหยเด่นที่พบใน FB25 ที่เติมแบคทีเรียกรดแล็กติกทั้ง 7 ไอโซเลท คือสารระเหย 1-โพรพานอล (1-Propanol) 2-เมททิลโพรพาแนล (2-Methylpropanal) และ เบนซัลดีไฮด์ (Benzaldehyde) เมื่อระบุชนิดของแบคทีเรียกรดแล็กติกที่คัดเลือกได้โดยอาศัยลักษณะสัณฐานวิทยาและสรีรวิทยาพบว่า เป็นแบคทีเรียแกรมบวก มีรูปร่างเซลล์กลม มีการจัดเรียงตัวทั้งเป็นคู่ (Pairs) และแบบสี่เซลล์ (Tetrads) ผลการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene ของแบคทีเรีย 7 ไอโซเลท มีความเหมือนของลำดับนิวคลีโอไทด์กับ *Tetragenococcus halophilus* ATCC 33315 ในระดับ 99% และแบบแผนของนิวคลีโอไทด์ที่วิเคราะห์ด้วยเทคนิค Restriction fragment length polymorphism (RFLP) ของทั้ง 7 ไอโซเลท มีความเหมือนเช่นเดียวกับ *T. halophilus* ATCC 33315 จึงระบุได้ว่าคือ *T. halophilus* เมื่อศึกษาลักษณะทางสรีรวิทยาและสมบัติทางชีวเคมีของแบคทีเรียทั้ง 7 ไอโซเลท พบว่ามีความแตกต่างกันและแตกต่างจากแบคทีเรียคู่เทียบ *T. halophilus* ATCC 33315

เตรียมกล้าเชื้อแบคทีเรียกรดแล็กติก 4 ไอโซเลท คือ *T. halophilus* MS33 *T. halophilus* MRC10-1-3 *T. halophilus* MCD10-5-10 และ *T. halophilus* MRC10-7-8 ในอาหาร FB25 โดยมีจำนวนแบคทีเรียเริ่มต้น 10^6 CFU/มิลลิลิตร เพื่อใช้ในการหมักน้ำปลา พบว่าจำนวนแบคทีเรียกรดแล็กติกลดลงจากวันเริ่มต้นประมาณ 3-4 Log CFU/มิลลิลิตร ที่ระยะเวลาการหมัก 60 วัน และไม่พบแบคทีเรียกรดแล็กติกที่เวลาการหมักมากกว่า 90 วัน ตัวอย่างที่เติมแบคทีเรียกรดแล็กติกหมักเป็นระยะเวลา 6 เดือนมีปริมาณแอลฟาอะมิโน 780-784 มิลลิโมลาร์ ซึ่งสูงกว่าตัวอย่างควบคุม ($P < 0.05$) ตัวอย่างที่เติม *T. halophilus* MRC10-1-3 และ *T. halophilus* MCD10-5-10 มีปริมาณฮีสตามีนต่ำกว่าตัวอย่างควบคุม ($P < 0.05$) คุณลักษณะทางเคมีกายภาพของน้ำปลาที่เติมกล้าเชื้อเทียบเท่ากับน้ำปลาที่หมักด้วยวิธีแบบดั้งเดิม และน้ำปลาที่เติมกล้าเชื้อมีปริมาณของกรดอะมิโนอิสระโพรลีน (Proline) อะลานีน (Alanine) วาลีน (Valine) และไลซีน (Lysine) เพิ่มขึ้น และน้ำปลาที่เติม *T. halophilus* MS33 มีปริมาณกลูทามิค (Glutamic) ทั้งหมดสูงที่สุด ($P < 0.05$) เมื่อวิเคราะห์สารระเหยในตัวอย่างน้ำปลาพบว่าสารระเหยเด่นที่พบคือ 2-เมธิลโพรพาแนล (2-Methylpropanal) 2-เมธิลบิวทาแนล (2-Methylbutanal) 3-เมธิลบิวทาแนล (3-Methylbutanal) และเบนซัลดีไฮด์ (Benzaldehyde) และไม่พบสารระเหยในกลุ่มไดซัลไฟด์ซึ่งเป็นสาเหตุของกลิ่นอุจจาระ(Fecal note) ในตัวอย่างการหมักน้ำปลาที่เติม *T. halophilus* MCD10-5-10 จากการประเมินคุณภาพทางประสาทสัมผัสของน้ำปลาที่เติมแบคทีเรียกรดแล็กติกพบว่า การยอมรับโดยรวม (Overall acceptance) ไม่มีความแตกต่างจากน้ำปลาทางการค้าที่หมัก 12 เดือน ดังนั้นการใช้แบคทีเรียกรดแล็กติกในการหมักน้ำปลาสามารถปรับปรุงลักษณะทางเคมีและองค์ประกอบสารให้กลิ่นของน้ำปลาได้

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2551

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NATTEEWAN UDOMSIL : ROLE OF LACTIC ACID BACTERIA ON CHEMICAL COMPOSITIONS OF FISH SAUCE. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 141 PP.

FISH SAUCE/VOLATILE COMPOUNDS/*TETRAGENOCOCCUS HALOPHILUS*/ /INTRACELLULAR AMINOPEPTIDASE/BIOGENIC AMINES

The objectives of this study were to investigate the role of halophilic lactic acid bacteria isolated from fish sauce fermentation on flavor formation and chemical composition of fish sauce. Lactic acid bacteria (LAB) were isolated from fish sauce mash fermented at various times ranging from 1 to 12 months. Sixty-four isolates were obtained and 18 of them were selected, based on the ability to grow in fish broth containing 25% NaCl (FB25) and to hydrolyze fish protein. Subsequently, seven isolates were chosen, namely M11, MS33, MRC10-1-3, MRC5-5-2, MCD10-5-10, MCD10-5-15, and MRC10-7-8, based on the growth in MRS containing NaCl and the ability to hydrolyze fish protein. Intracellular aminopeptidase activities of 7 isolates were high toward Ala-*p*-nitroanilide (Ala-pNA) (2.85-3.67 U/ml of crude enzyme) and moderate activity toward Met-pNA, Leu-pNA, Arg-pNA and Glu-pNA. They produced histamine in mGYP broth containing 5 and 25% NaCl ranging from 6.62-22.55 and 13.14-20.39 mg/100ml, respectively. Predominant volatile compounds of FB25 added LAB 7 isolates were 1-propanol, 2-methylpropanal, and benzaldehyde. Bacterial identification which was based on morphological and physiological characteristics revealed that these isolates were Gram-positive cocci with pairs/tetrads. The result of 16S rRNA gene sequences showed homology to

Tetragenococcus halophilus ATCC 33315 at 99%. The restriction fragment length polymorphism (RFLP) patterns of all isolates were also similar to *T. halophilus* ATCC 33315. Thus, these isolates were identified as *T. halophilus*. Physiological and biochemical characteristics were varied among isolates and differed from T. *halophilus* ATCC 33315.

Four treatments of fish sauce fermentation were prepared by adding *T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8 cultured in FB25 with approximate cell count of 10^6 CFU/ml. LAB counts of inoculated samples decreased 3-4 Log CFU/ml at 60 days of fermentation and were not detected after 90 days. α-Amino content of 6-mo-old fish sauce samples were 780-784 mM and higher than the control ($P < 0.05$). Addition of *T. halophilus* MRC10-1-3 and *T. halophilus* MCD10-5-10 resulted in lower histamine content than the control ($P < 0.05$). Physico-chemical characteristics of fish sauce added starter cultures were comparable to those of fish sauce fermented conventionally. Fish sauce inoculated with starter cultures showed an increase in free proline, alanine, valine, and lysine. Fish sauce inoculated *T. halophilus* MS33 showed the highest total glutamic acid content ($P < 0.05$). The major volatile compounds identified in the fish sauce inoculated with LAB starter culture were 2-methylpropanal, 2-methylbutanal, 3-methylbutanal and benzaldehyde. Dimethyl disulfide, a compound attributing to fecal note, was not detected in the sample added *T. halophilus* MCD10-5-10. Overall acceptance based on sensory evaluation of all fish sauce samples inoculated with starter cultures was comparable to the commercial fish sauce fermented for 12 months. The use of LAB

for fish sauce fermentation appeared to improve chemical characteristics and volatile composition of a fish sauce product.

School of Food Technology

Academic Year 2008

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

ACKNOWLEDGEMENTS

I am deeply grateful to my thesis advisor, Assoc. Prof. Dr. Jirawat Yongsawatdigul, for his support, understanding, and kindness during my graduate program. He always spent a lot of his time to help me solve my problem, not only me but to all his students.

I am very grateful to Asst. Prof. Dr. Sureelak Rodtong for her guidance, valuable advices, encouragement and other supports throughout my work. My deepest gratitude to the thesis committee, Assoc. Prof. Dr. Somboon Tanasupawat, for providing *Tetragenococcus halophilus*^{T} and *Tetragenococcus muriaticus*^{T} and his valuable comments and advices on my work.

Thanks to all members of Food technology who have made my time in laboratory enjoyable and memorable. My sincere thanks are to the entire members of F2105 and Biotechnology laboratory for their friendships, suggestion, and support of chemical reagents and instruments. I wish to thank P'Jiw for her kind suggestion and being my best sister. I wish to thank Ton for his understanding and helping me whenever I needed.

This work was supported by a grant from the National Center for Genetic Engineering and Biotechnology (BIOTEC) under the grant No. BT-B-01-FT-19-5014.

Lastly, I would like to express my deepest gratitude to my parents who have always believed in me and give me the great opportunity.

Natteewan Udomsil

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

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

LIST OF ABBREVIATIONS (Continued)

%	=	Percentage
PCR	=	Polymerase chain reaction
RFLP	=	Restriction fragment length polymorphism
rpm	=	Revolution per minute
s	=	Second
SAS	=	Statistical analysis system
sp.	=	Species
subsp.	=	Subspecies

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce is a seasoning commonly used in Asian countries and it is produced by mixing fish with salt at a ratio of 3:1 and fermenting for 12-18 months (Shimoda, Peralta, and Osajima, 1996). Fish proteins are hydrolyzed during fermentation by both bacterial and fish proteinases (Fukami, Funatsu, Kawasaki, and Watabe, 2004). The resulting product is a clear brown liquid with salty taste and distinctive odor. Fermentation of fish sauce is considered to be a traditional process with long fermentation time. In the past, the reduction of salt concentration and/or the addition of enzymes (Beddow and Ardeshir, 1979; Fu, You, and Kim, 2001; Gildberg, Hermes, and Orejana, 1984), addition of acid and alkaline (Gildberg and Shi, 1994; Thongthai and Suntinanalert, 1991) and raising temperature (Lopetcharat and Park, 2002) were used to accelerate fish sauce fermentation. However, these methods have some limitations because of their cost, harsh condition, high energy expenditure and negative sensory characteristics in the finished product. Therefore, they have not been applied commercially. Recently, Yongsawatdigul, Rodtong, and Raksakulthai (2007) reported that proteinase-producing bacteria, namely *Virgibacillus* sp., could accelerate fish sauce fermentation. α-Amino content of 4-month-old fish sauce fermentation added *Virgibacillus* sp. were higher than the control (without *Virgibacillus* sp.). Similarly, Aquerreta, Astiasarán, and Bello (2001) reported that salt-tolerant

proteinases produced by *Bacillus subtilis* could increase α-amino content during 40 days of fish sauce fermentation. These studies indicated that the use of proteinase-producing bacteria as a starter culture could reduce fish sauce fermentation time. One concern of using bacterial starter culture is biogenic amine formation of such a starter culture. Histamine is important biogenic amines having vasoactive effects in human. Histamine intoxication causes an increase in blood pressure, severe headache, hypertension, vomiting, and renal intoxication (Lehane and Olley, 2000). Tyramine has been related to hypertension in patients under antidepressive treatment with mono-amine oxidase inhibitor (MAOI). Other biogenic amines, such as putrescine and cadaverine, may boost the toxicity of histamine. Histamine was regulated in fish sauce at the maximum level of 20 mg/100ml by Canada and 50 mg/100ml by the U.S.A. (Brillantes, Pakon, and Totakien, 2002). Generally, commercial fish sauce products contain a wide range of histamine of 10 to 100 mg/100ml (Brillantes et al., 2002). Fish sauce containing low histamine would be more desirable and safer to consumers. Thus, an ideal starter culture for fish sauce fermentation should not produce histamine.

Many bacteria species belonging to *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Pediococcus*, extremely halophilic red archaea, and halophilic lactic acid bacteria, namely *Tetragenococcus*, were found in fish sauce fermentation (Fukami et al., 2004; Thongthai, McGenity, Suntinanalert, and Grant, 1992). These bacteria produced proteinases that can hydrolyze fish protein. Lactic acid bacteria (LAB) were one of the most important bacteria in fermented food, such as soy sauce, kimchi, fermented fish, fermented sausage and fermented ham (Lee, Kim, and Kunz, 2006). They were used as a starter culture for those fermented foods. The use of

starter culture could reduce fermentation time and improve desirable odor and taste (Omafuvbe, Abiose, and Shonukan, 2002). *Lactococcus lactis* used as a starter culture for cheese production shortened cheese ripening and improved odor. It could produce ketone and volatile fatty acids compounds that were contributed to cheesy aroma (Ishikawa et al., 2006). However, the use of LAB as a starter culture for fish sauce fermentation has not been reported.

Halophilic LAB are described as bacteria which require sodium chloride (NaCl) for growth and was tolerant of high NaCl concentration (>18%) (Holt, Krieg, Sneath, Staley, and Williams, 1994). *Tetragenococcus* were halophilic LAB found in fish sauce and salt fermented food. Satomi, Kimura, Mizoi, Sato, and Fuji (1997) showed that *T. muriaticus* was a new moderately halophilic LAB isolated from squid liver sauce. Thongsanit, Tanasupawat, Keeratipibul, and Jitikavanich (2002) isolated *T. halophilus* and *T. muriaticus* from fish sauce (nam-pla). Moreover, *T. halophilus* and *T. muriaticus* were found in Indonesian soy mash (Roling, and Verseveld, 1996), Japanese soy sauce (Nakagawa and Kitahara, 1959), salted anchovies (Villar, Holgado, Sanchez, Trucco, and Oliver, 1985), Japanese-fermented puffer fish ovaries (Kobayashi, Kimura, and Fuji, 2000), fermented black bean and fermented mustard (Chen, Yanagida, and Hsu, 2006), and Indonesian ‘terasi’ shrimp paste (Kobayashi et al., 2003). Despite of its prevalence, the role of *Tetragenococcus* on chemical characteristics of fermented food has not been systematically investigated.

Halophilic LAB 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, Shimoda, and Osajima, 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. Because intracellular aminopeptidases converted peptides and/or oligopeptides to amino acids (Smit et al., 2005). Amino acids which were converted to flavor compound by LAB can be separated into three groups; (i) aromatic amino acids (tyrosine, tryptophan, and phenylalanine), (ii) branched chain amino acids (leucine, isoleucine, and valine), and (iii) sulfur-containing amino acid (methionine). Amino acids of each group produced different flavor compounds (Marilley and Casey, 2004). *L. lactis* contributed to flavor formation during cheese ripening (Marilley and Casey, 2004). The role of *Tetragenococcus* on flavor characteristics of fish sauce has not been reported despite of its prevalence during fermentation. Therefore, LAB found in fish sauce fermentation might have ability to produce and/or improve flavor characteristics of fish sauce products.

Three distinctive notes: ammoniacal; cheesy; meaty, are found in fish sauce. These compounds derived from the reaction of protein hydrolysis and lipid oxidation during microbial fermentation (Beddows, Ardeshir, and Daud, 1980). The ammonical notes were produced by ammonia, amine and nitrogen-containing compounds, such as trimetylamine, pyrazine, 2-methylpyrazine and 2-ethylpyridine. The ketone volatile compounds were 2-butanone, acetone, 3-methyl-2-butanone, 2-hexanone and 2-pentanone which contributed to cheesy note. The aldehyde compounds including 2-methylpropanal, 2-methylbutanal, 3-methylbutanal and benzaldehyde contributed to meaty note of fish sauce. Dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide

were volatile compounds responsible for undesirable fecal note (Shimoda et at., 1996). *Staphylococcus xylosus* added to fish sauce mash (moromi) improved fish sauce odor (Fukami et al., 2004). It reduced dimethyl trisulfide in finished product. The addition of *Tetragenococcus* to fish sauce fermentation might also reduce undesirable odor of fish sauce.

LAB can be identified on the basis of morphological characteristics. However, this approach is not reliable for all groups of bacteria which possess limited morphological differentiation (Entis et al., 2001). Many techniques for bacterial identification rely on the use of physiological tests or commercially available kits, such as API system. However, phenotypic characteristics still have limitation for species identification. Phylogenetic approaches for identifying and classifying LAB have become a major part of taxonomic studies. The 16S ribosomal RNA (rRNA) gene sequences have been used for identification of LAB (Johansson et al., 1995).

The purposes of this research were to identify halophilic LAB isolated from fish sauce fermentation. In addition, to investigate changes of flavor compounds and chemical properties of fish sauce inoculated the selected LAB isolates.

1.2 Research objectives

The objectives of this research were:

1) To isolate and select halophilic lactic acid bacteria from fish sauce fermentation,
2) To identify the selected isolates of halophilic lactic acid bacteria using morphological, physiological, and biochemical characteristics as well as genetic analyses,

3) To investigate the role of selected isolates on changes of volatile compounds and chemical characteristics of fish sauce.

1.3 Research hypotheses

Specific strain of halophilic LAB isolates could improve flavor and chemical characteristics of fish sauce. These halophilic LAB would have potential for use as a starter culture in fish sauce fermentation.

1.4 Scope of the study

Halophilic lactic acid bacteria were isolated from fish sauce fermentation at 1, 3, 5, 7, 9 and 12 months. These bacteria could produce intracellular aminopeptidases and should not produce biogenic amines, especially histamine. The identification of halophilic lactic acid bacteria were carried out according to Holt, Krieg, Sneath, Staley, and Williams (1994) and Thongsanit et al. (2002). Phenotypic and genomic analyses were achieved using biochemical tests and/or the gene sequence analysis amplified by polymerase chain reaction (PCR) technique and cloning of 16S rRNA gene in vector according to Weisburg, Barns, Pelletier, and Lane (1991). In addition, restriction fragment length polymorphism (RFLP) technique was used for bacterial identification (Sato, Yanagida, Shinohara, Yokotsuka, 2000). Changes of volatile compounds and chemical characteristics of fish sauce inoculated LAB isolates were also studied.

CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce fermentation

Fish sauce is a clear brown liquid with salty taste and mild fishy odor. Fish sauce has been wildly used in Southeast Asian countries as a seasoning (Lopetcharat, Choi, Park, and Daeschel, 2001). Fish sauce comes with different names according to the country of origin, such as budu in Malaysia, yu lu in China, patis in Philippines, shotturu in Japan, nuoc mam in Vietnam, ketjapikan or bakasang in Indonesia, ngapi in Myanmar, and nam-pla in Thailand (Sanceda, Suzuki, and Kurata, 2003). Generally, fish sauce is produced by a conventional process. Fish and salt are mixed in the ratio of 3:1 and placed in layers in a loosely covered container. Brine will be reaching to the top of the fish flesh within the first week of fermentation. After 12-18 months of fermentation, the supernatant is firstly transferred from the fermentation tank to the ripening tank. After 2-12 weeks of ripening, first grade fish sauce (nam-pla) is obtained (Lopetcharat et al., 2001; Sanceda et al., 2003). Chemical composition of the first grade fish sauce contains about 20 g/L of total nitrogen of which 16 g/L is in the form of amino acids, 25-28% of salt, 0.2-0.7% of ammonium, and pH 5.1-5.7 (Park et al., 2001). There are separations of fish sauce quality into A and B grade as shown in Table 2.1.

Table 2.1 Standard parameters for fish sauce in Thailand.

Parameters	Grade A	Grade B
Total nitrogen (g/ L)	> 20	14-16
Protein (%)	> 12.5	> 9.38
Sodium chloride (%)	25-28	25-27.9
pH	5.1-5.7	5.1-5.7
Relative density at 25 °C	> 1.20	> 1.20
Amino acid (g/ dL)	1.0	0.75
Glutamic acid (g/ total nitrogen)	0.4-0.65	0.4-0.60

Source: http://www.tisi.go.th

Fish sauce is resulted from natural hydrolysis by endogenous and microbial enzymes. The major change during the fermentation period is the conversion of proteins to small peptides and free amino acids (Lopetcharat et al., 2001). Generally, most of polypeptide decreases during the fermentation period and the amino acid content increases. Saisithi (1994) reported that amino acids found in fish sauce are taurine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine and citrulline during fermentation, pH value drops due to the release of free amino acids. In addition, fatty acid composition does not change greatly during fermentation, but total lipids decrease (Kim, Koizumi, Jeong, and Jo, 1994). In addition to the chemical composition of fish, microorganisms in fish are also important to the quality of fish sauce. Fish microflora varies depending upon season, place, transportation, species, storage, and catching methods. Microorganisms found in fish include *Escherichia coli*, *Pseudomonas* sp., *Serratia* sp., *Enterococcus*, *Enterobactor*, *Lactobacillus*, and *Aeromonas*, etc. (Jay, 1986). However, microorganisms including some pathogenic

microbes are inactivated during fermentation by high salt content. Moreover, the concentration of salt affects the function of various endogenous enzymes that play an important role in protein degradation during fermentation (Orejana and Liston, 1982).

Fish species also affects the type of proteins that serve as nutrient for microorganism and substrates for enzymes, both of which hydrolyze proteins into small peptides and amino acids. Most proteins in fish except connective tissue and other stroma proteins are hydrolyzed into small peptides and amino acids. The small peptides, free amino acids, ammonium, and trimethylamine (TMA), contribute to the specific aroma and flavor of fish sauce (Dougan and Howard, 1975). The aroma of fish sauce is primarily resulted from aerobic and anaerobic bacteria in the fermentation tank (Beddows and Ardeshir, 1980). Halophilic aerobic spore formers are predominant microorganism in fish sauce. *Bacillus* was found to be dominant in nam-pla and they produced a measurable amount of volatile acids. *Staphylococcus* was isolated and produced twice as much volatile acid as *Bacillus*. *Micrococcus* also played a major role in aroma production in nam-pla. Additionally, *Streptococcus* spp. produced a measurable amount of volatile acids (Saisithi et al., 1966). At present, *Tetragenococcus* was found in fish sauce fermentation. The role of *Tetragenococcus* on flavor formation of fish sauce has not been reported.

Biogenic amines are one of important quality indices of fish sauce. They are organic basic compounds which occur in different kinds of food, such as fishery products and fermented foods. Biogenic amines are generated by decarboxylation of amino acids through substrate specific enzymes of bacteria (Brink, Damink, Joosten, Huis, and Veld, 1990). The formation of biogenic amines has been found in many groups of bacteria, such as *Pseudomonas* sp., *Staphylococcus* sp., and some lactic acid

bacteria. Food allergy is caused by histamine, the accumulation of which in food is a result of bacterial histidine decarboxylase. Histidine in fish muscle is the primary source for histamine formation by bacterial histidine decarboxylase. Histamine content in fish sauce is very important for safety. The maximum allowable histamine content of fish sauce based on Canadian Food Inspection Agency (CFIA) is 20 mg/100ml (CFIA 2003) and 50 mg/100ml by U.S.A. (Brillantes et al., 2002). Other biogenic amines, such as cadaverine and putrescine, have been reported to enhance toxicity of histamine because they inhibit diamine oxidase and histamine N-metyltransferase (Stratton, Hutkins, and Taylor, 1991). Moreover, tyramine is a particular concern as it is the most common cause of monoamine intoxication (MAI). MAI is characterized by an increase in blood pressure, hypertension, and prostration. Brillantes et al. (2002) concluded that histamine formation in fish sauce can be controlled by lowering the storage temperature of fish used as a raw material and implementing hygienic practices. Thus, the quality of raw material is a key factor in controlling histamine in fish sauce products.

2.2 Flavor compounds of fish sauce

Fish sauce has a characteristic flavor which often serves as a quality indicator (Sanceda et al., 2001). Three major notes in fish sauce are ammoniacal, cheesy, and meaty note (Dougan and Howard, 1975; Beddows et al., 1976). These compounds are derived from protein hydrolysis, lipid oxidation products by autolytic and microbial fermentation (Beddows et al., 1980). Saisithi et al. (1966) reported that the activity of bacteria isolated from 9 mo-old fish sauce were capable of producing volatile acids and other products, such as ketone and aldehyde which contributed to flavor and

aroma of fish sauce. Ammonia, amines, and other nitrogen compounds were responsible for the ammonical note. Low molecular weight volatile fatty acids (VFA) were responsible for cheesy note. Aldehydes were responsible for the meaty odor (Dougan and Howard, 1975; Fukami et al., 2002; Sanceda, Kurata, Suzuki, and Arakawa, 1992). Peralta, Shimoda, and Osajima (1996) identified 124-155 volatile compounds of fish sauce including acids, nitrogen-containing compounds, sulfur-containing compounds, aldehydes, ketones and aromatic hydrocarbons. Fukami et al. (2004) reported that 2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio) propanal, and 3-methylbutanoic acid contributed to fishy note. The sweaty note was attributed from 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide. 2-Ethylpyridine, 2-methylpropanal and 2-methylbutanal contributed to the meaty note. In addition, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide were volatile compounds responsible for fecal note which was undesirable in fish sauce (Shimoda et at., 1996). These studies indicated that flavor compounds in fish sauce were formed in a complex fashion.

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). Butanoic acid, 3-methylbutanoic acid, pentanoic acid, and 4-methylpentanoic acid, are also considered to be associated with the cheesy note in fish sauce, and they show low odor threshold value (Devos, Patte, Roualt, Laffort, and Gemert, 1995; Peralta et al., 1996; Shimoda et al., 1996). Meaty odor was produced by the oxidation of lipid. Glutamic acid also contributes to the meaty flavor in fish sauce (Devos et al., 1995).

Michihata, Yano, and Enomoto. (2002) reported that the major volatile compounds in Japanese fish sauce, *Ishiru*, were aldehydes, nitrogen-containing compounds, sulfur-containing compounds, and ketones. Aldehydes derived from lipid oxidation during fermentation, and branched/aromatic or short-chain aldehydes might occur from deamination of amino acids. Cha and Cadwallader (1998) reported that aldehyde compounds positively affected overall flavor of tuna sauce which were produced from chemical reaction or microbial degradation of amino acids.

Accumulation of amino acids and peptides during fermentation has a considerable effect on flavor of fish sauce (Peralta et al., 1996). Microbial proteinases, especially, intracellular aminopeptidases, were the key enzymes that were important for flavor formation. Because intracellular aminopeptidases converted peptides and/or oligopeptides to amino acids (Smit, Smit, and Engels, 2005). Amino acids which were converted to flavor compound by lactic acid bacteria (LAB) can be separated into three groups; (i) aromatic amino acids (tyrosine, tryptophan and phenylalanine), (ii) branched-chain amino acids (leucine, isoleucine and valine) and (iii) sulfur-containing amino acid (methionine) (Marilley and Casey, 2004). Smit et al. (2005) reported that leucine was converted to 2-methylpropanoic acid and methionine was converted to methional which contributed to cheesy and boiled potato-like, respectively. Marilley and Casey (2004) reported that valine and methionine were converted to 3-metylbutanol and dimethyl disulfide, respectively. Therefore, amino acids are important precursor for flavor compounds.

Aminopeptidases were important enzymes that were responsible for flavor formation. Aminopeptidases are parts of the proteolytic system of LAB. In addition to their role in LAB growth, they contribute to development of flavor by releasing single

amino acid residues from peptides (Magboul and McSweeney, 1999). Aminopeptidases produced from LAB, such as, *Lactobacillus sake*, *Lb. plantarum* and *Lb. curvatus*, contributed to flavor formation in fermented foods. Leucyl aminopeptidase from *Lb. curvatus* was characterized and showed high activity toward leucine (Magboul and McSweeney, 1999). Similarly, aminopeptidase from *Lb. sake* exhibited high activity toward leucine and alanine substrates (Sanz and Toldra, 1997). The catabolic products of leucine were 2-methylbutanal, 3-methylbutanal and 3-methylbutanol (Masson, Hinrichsen, Talon, and Montel, 1999). These results indicated that they contributed to flavor development during cheese ripening. Moreover, release of positively charged amino acid residues by aminopeptidase from *Lb. plantarum* suggested that it may have an impact on cheese debittering (Macedo, Tavares, and Malcata, 2003). However, aminopeptidase activity of *Tetragenococcus* has not yet been studied.

2.3 Microorganisms in fish sauce fermentation

Fish sauce contains high salt concentration of 25-30%, thus microorganisms found during fish sauce production are generally classified as halophiles (Thongthai et al., 1992). Halophilic bacteria found in fish sauce can be categorized as halotolelant, slightly halophilic, moderately halophilic, and extremely halophilic bacteria (Lopetcharat et al., 2001). These are microflora from fish, solar salt, and fermentation tank. The important role of these bacteria found in fish sauce are protein degradation and flavor aroma improvement.

Halobacterium sp. and *Halococcus* sp. have been reported to be found during fish sauce fermentation (Norberg and Hofsten, 1968). The optimal NaCl concentration

for *Halobacterium* sp. growth is 20-30% and *Halococcus* sp. requires more than 15% of NaCl concentration for growth. Therefore, *Halobacterium* sp. and *Halococcus* sp. are classified as extremely halophilic bacteria. *Halobacterium* sp. showed ability in gelatin and casein hydrolysis, while *Halococcus* sp. hydrolyzed gelatin. Chaiyanan, Maugel, Huq, Robb, and Colwell (1999) isolated *Halobacillus thailandensis* sp. nov. from fish sauce which produced extracellular proteolytic enzymes. *Halobacterium salinarium* and *Halobacterium cutirubrum* produced extracellular proteinase at 25% NaCl (Ihara, Wanatabe, and Tamura, 1997; Thongthai et al., 1992; Thongthai and Suntinanalert, 1991). Therefore, these halophilic bacteria contributed to hydrolysis of fish protein during fish sauce fermentation. Halophilic bacterial enzymes are active at high concentration of NaCl or KCl and required divalent cations for activity and stability (Demirijan, Moris, and Cassidy, 2001). Moreover, *Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Coryneform* bacteria (Thongthai and Suntinanalert, 1991), *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* is proteinase-producing halophilic bacteria found in fish sauce (Satomi et al., 1997; Thongsanit et. al., 2002). Similarly, 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, *Bacillus subtilis* was found in Vietnamese and Laotain fish sauce (Tran and Nagano, 2000).

The important roles of proteinase-producing bacteria are their ability to produce extracellular and/or cell-bound proteinases to hydrolyze fish protein to

peptides and amino acids. Some amino acids can be used as a substrate for LAB. *Tetragenococcus* were halophilic LAB found in fish sauce. Satomi et al. (1997) showed that *T. muriaticus* was a new moderately halophilic LAB isolated from squid liver sauce. Thongsanit et al. (2002) isolated *T. halophilus* and *T. muriaticus* from fish sauce (nam- pla). According to Saisithi (1966), *Halobacterium* and total halophilic bacteria were 2-4 Log CFU/ml at the initial stage of fish sauce fermentation (between 1-5 months) and decreased to 2-3 Log CFU/ml at the final stage of fermentation, whereas halophilic LAB were dominant at the final stage. Final stage of fermentation at 7^{th} -12^{th} month was a period when color, aroma and flavor are fully developed (Saisithi, 1994). Therefore, halophilic LAB found in fish sauce could play a significant role in aroma and flavor development.

2.4 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are defined as Gram-positive, non-sporulating, catalase-negative, aerotolerant or microaerophile, acid tolerant, nutritionally fastidious, strictly fermentative organisms that lack cytochromes and produce lactic acid as the major end product of carbohydrate metabolism (Christensen, Dudley, Pederson, and Steele, 1999). At least 21 genera of *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Enterococcus*, *Lactococcus*, *Vagococcus*, *Carnobacterium*, *Weissella*, *Oenococcus*, *Alloiococcus*, *Dolosicoccus*, *Dolosigranulum*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, and *Lactosphaera* (Axelsson, 2004). LAB are classified into different genera based on their cell morphology, growth at different temperature, ability to

grow at high salt concentration, acid or alkaline tolerance, glucose fermentation, and configuration of lactic acid produced (Carr, Chill, and Maida, 2002).

LAB has been characterized primarily by their ability to form various isomers of lactic acid from the fermentation of glucose. Ratio of L- to D- lactic acid produced by LAB has been employed for the classification of LAB (Hammes, Weiss, and Halzapfel, 1992). The stereoisomer of lactic acid produced by LAB can be classified into 3 types (L, DL, and D). The L-type means a content of L-lactic acid 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). In addition, all genera are classified as either homofermentater or heterofermentater based on the end product of their fermentation. The homofermentaters produce lactic acid as the major product of glucose fermentation. The heterofermentaters produce various products including lactic acid, carbon dioxide, acetic acid, and ethanol from glucose fermentation (Jay, 1986; Singleton and Sainsbury; 1987). Two main sugar fermentation pathways can be distinguished among LAB. Glycolysis pathway (Embden-Meyerhof-Parnas pathway) results exclusively in lactic acid as the end product under standard condition, and the metabolism is referred to as homofermentation. Glucose is fermented via glycolysis pathway by the enzyme aldolases directly to lactic acid. In case of heterofermentation, bacteria use the alternate pentose monophosphate pathway, converting six carbon sugars to five carbon sugars (pentoses) by phosphoketolase enzymes. Moreover, the resulting products of heterofermentation are diacetyls and aldehydes which are aroma- and flavor-enhancing substances (Frosbisher, Hinsdill, Crabtree, and Goodheart, 1974; Sharpe and Fryer, 1965).

The conversion of peptides to free amino acids and the subsequent utilization of these amino acids is a central metabolic activity of LAB. The peptidase system is involved in the hydrolysis of exogenous peptides to obtain essential amino acids for growth. The amino acids formed by peptidase system can be utilized for various processes, such as protein synthesis, generation of metabolic energy, and recycling of reduced cofactor (Christensen, Dudley, Pedersen, and Steele, 1999). Amino acid catabolism by anaerobic/fermentative microorganisms is also believed to have an important role in their ability to obtain energy in nutrient-limited environment. For example, arginine and histidine are known to provide energy via substrate level phosphorylation.

At least 16 peptidases responsible for the conversion of the released peptides into free amino acids have been characterized in LAB (Christensen et al., 1999). The routes for the breakdown of the released amino acids may involve deamination, decarboxylation and desulfuration reactions. It is now recognized that aroma compounds formation of LAB resulted from amino acid degradation via catabolic pathway (Yvon and Rijnen, 2001). Therefore, catabolic activities of amino acid have the potential to flavor compounds formation.

LAB are known for their use as starter cultures in several fermented food, such as acidophilus milk, yogurt, buttermilk, and cheese including fermented meat, fermented vegetable, fermented sausage, cured ham, wine, pickles, and saukerkraut (Schillinger and Lücke, 1987). They can improve the flavor and texture of food and inhibit the growth of spoilage bacteria. However, the use LAB as a starter culture in fish sauce fermentation has not been investigated.

2.5 Characteristic and identification of *Tetragenococcus* species.

Pediococcus halophilus was proposed to be reclassified in a new genus as *Tetragenococcus* since 1990. The bacteria described as a halophilic microorganism requires sodium chloride (NaCl) for growth and is tolerant to NaCl concentration more than 18% (Collins, Williams, and Wallbanks, 1990; Hozapfel et al., 2006). Collins, Williams, and Wallbanks (1990) reported that *P. halophilus* is phylogenetically more closely related to enterococci and carnobacteria than to pediococci and lactobacilli on the basis of 16S rRNA sequence data. The different characteristics between pediococci and tetragenococci are shown in Table 2.2. The genus *Tetragenococcus* comprised only the single recognized species *Tetragenococcus halophilus* (Anonymous, 1994). Subsequently, Satomi et al. (1997) found a new species, *Tetragenococcus muriaticus* that was moderately halophilic bacteria isolated from squid liver sauce. Recently, Lee et al. (2005) reported that *Tetragenococcus koreensis* sp. nov. was a new species isolated from kimchi. Ennahar and Cai (2005) reported that phylogenetic analysis of 16S rRNA gene sequences revealed that *Enterococcus solitarius* is not a member of the genus *Enterococcus*, but is related to species of the genus *Tetragenococcus*. Therefore, *E. solitarius* was also transferred to the genus *Tetragenococcus* and reclassified as *Tetragenococcus solitarius* comb. nov. At present, the genus *Tetragenococcus* comprises of four species: *T. halophilus*, *T. muriaticus*, *T. koreensis*, and *T. solitarius*.

Table 2.2 The different characteristics between the genera *Tetragenococcus* and *Pediococcus*.

Characteristics	*Pediococcus*	*Tetragenococcus*
Growth tolerance to 18% NaCl	-	+
Growth at pH 5.0	+	-
pH 9.0	-	+
Facultative aerobic	+	+
Gas from glucose fermentation	-	-
Configulation of lactic acid from glucose	DL/L(+)	L(+)
Starch fermentation	-	-
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp

Source: Holzapeel, Franz, Ludwig, Back, and Dicks (2006)

Tetragenococcus are Gram positive cocci (0.5-1.0 μm in diameter) and cell form is tetrads or pairs. Colonies are circular, white, low convex, smooth, and non-pigmented. Catalase test is always negative. There are several *Tetragenococcus* strains that have been found in a variety of fermented food, including fish sauce (Thongsanit et al., 2002), soy sauce (Stiles and Holzapfel, 1997), Indonesian soy mash (Kecap) (Roling et al., 1996), kimchi and fermented ham (Lee et al., 2005), and salted anchovies (Villar et al., 1985). Four species of *Tetragenococcus* can be described follows:

Tetragenococcus halophilus was found in salted fermented food, such as fish sauce and soy sauce. It is spherical with 0.5-1.0 μm in diameter. Cells are non-motile. Colonies on MRS agar plate are circular, low convex with entire margin, and non-

pigmented (Thongsanit et al., 2002). They are homofermentative and microaerophile. It cannot grow at 45 °C. Optimum NaCl concentration for growth was between 5-10 % and can grow between pH 5.0-9.0. *T. halophilus* does not reduce nitrate. Some strains retain their ability to hydrolyze arginine (Lee et al, 2005; Thongsanit et al., 2002). Most strains are capable of producing acid as shown in Table 2.3.

Kobayashi, Kimura, and Fuji (2000) isolated *T. halophilus* from Indonesian terasi shrimp paste. It can ferment L-arabinose, D-mannitol, maltose, D-melizitose, maltotriose, glycerol, and sucrose. Therefore, *T. halophilus* was suggested to play a role in carbohydrate decomposition of fermented food products. The optimum NaCl obtained for growth of *T. halophilus* isolated from terasi was 3 to 7%. It grows well at pH 7.5 and 30 °C. Moreover, *T. halophilus* was isolated from several sources. Roling and Verseveld (1996) isolated *T. halophilus* from soy mash fermentation. Uchida (1982) showed *T. halophilus* population of a single batch of Japanese-type soy sauce fermentation was very heterogeneous regarding substrate utilization and also *T. halophilus* isolated from Chinese soy mash showed different sugar utilization (Abe and Uchida, 1989). The different composition of soy sauce fermentation has an effect on sugar utilization of *T. halophilus*.

Tetragenococcus muriaticus was firstly isolated from fermented squid liver sauce by Satomi et al. (1997). Cells are cocci (0.5-0.8 μm in diameter) and non-motile. Colonies are white, convex, smooth, and up to 1.5 mm in diameter. It grows at 1-25% NaCl with optimum concentration of 7-10%. No growth is found in the absence of NaCl. The optimum growth temperature is 25 to 30 °C and can grow at 15-40 °C but not at 45 °C. Growth is found at pH 5.0-9.6 with the optimal of 7.5-8.0.

Table 2.3 Important characteristics of species in the genus *Tetragenococcus* sp.

Characteristics	*T. halophilus*[a] ATCC 33315	*T. muriaticus*[b] JCM 10006	*T. koreensis*[c] DSM 16501	*T. solitarius*[d] JCM 8736
Optimum temperature (°C)	30	25-30	15-30	NA
Growth at 40 °C	-	+	-	+
45 °C	-	-	-	+
Growth range of pH	5.0-9.0	5.0-9.6	NA	NA
Optimum pH	NA	7.5-8.0	9.0	6.5
Range of NaCl (%)	0-25	1-25	0-8	NA
Optimum NaCl (%)	5-10	7-10	2-5	NA
Gas from D-glucose	-	-	-	NA
Acid from:				
Amygdalin	+	-	+	+
L-Arabinose	-	-	-	-
D-Cellobiose	+	-	-	+
D-Galactose	+	-	-	+
Glucose	+	+	+	NA
Glycerol	+	-	NA	-
D-Lactose	-	-	-	-
D-Maltose	+	-	+	+
D-Raffinose	-	-	NA	-
L-Rhamnose	-	-	NA	-
D-Mannitol	-	+	+	+
D-Mannose	+	+	+	+
D-Melibiose	-	-	NA	-
Sucrose	+	-	+	+
D-Melezitose	+	-	+	+
α-Methyl-D-glucoside	+	-	-	+
D-Ribose	+	+	+	-
D-Sorbitol	-	-	NA	-
Sorbose	+	-	-	-
D-Trehalose	+	-	+	+
D-Xylose	+	-	-	-
Xylitol	NA	-	+	-
Turanose	NA	-	+	+
D-Tagatose	NA	-	-	+
D-Arabitol	NA	-	+	+
Gluconate	NA	-	+	+

NA = Not available, [a]Thongsanit et al. (2002), [b]Satomi et al. (1997), [c]Lee et al. (2005), [d]Ennahar and Cai (2005).

It is facultatively anaerobic. Arginine is not metabolized. Nitrate is not reduced to nitrite by this species. It produces histamine (44 mg/100 g) (Thongsanit et al., 2002). Acids can be produced from several sugars (Table 2.3). The cell wall peptides contain Lys-D-Asp. The G+C content in DNA was 36.5 mol% (Satomi et al., 1997).

Phenotypic characteristics used to differentiate between *T. muriaticus* and *T. halophilus* is the growth temperature. *T. muriaticus* grows at 40 °C but *T. halophilus* does not. Moreover, *T. halophilus* can grow in the absence of NaCl but *T. nuriaticus* required at least 1% NaCl for growth (Table 2.3). In addition, *T. muriaticus* can be distinguished from *T. halophilus* by the lack of fermentation of L-arabinose, sucrose, and lactose and the ability to produce histamine (Satomi et al., 1997). However, these phenotypic differences are not sufficient to absolutely differentiate 2 species. Some variations in sugar utilization have previously been reported within population of *T. halophilus* (Abe and Uchida, 1989; Roling and Verseveld, 1996). A more reliable method for determining species affiliations is 16S rRNA gene sequence analysis, which clearly differentiates *T. muriaticus* from *T. halophilus*. In adition, DNA-DNA relatedness between the 2 species based on DNA hybridization was less than 50% (Satomi et al., 1997).

Tetragenococcus koreensis was another species isolated from kimchi (Lee et al., 2005). Similarity of this species based on 16S rRNA gene sequence as compared to *T. halophilus* and *T. muriaticus* is 97.8% and 95.9%, respectively. The level of DNA-DNA relatedness is only 9.7% similarity to *T. halophilus* and 39.0% to *T. muriaticus*. *T. koreensis* can produce acid as shown in Table 2.3. The characteristics of *T. koreensis* were described by Lee et al. (2005). Cells are cocci (1 μm in diameter), non-motile, non-spore forming. Catalase and oxidase are negative. Growth is detected

in trypticase soy agar at 30 °C under either aerobic or anaerobic condition. Growth is also found at 15-30 °C but not at 4 °C or above 37 °C. Optimum growth temperature is 30 °C and optimal pH is 9.0. Growth occurs at 0-8 % NaCl with the optimum at 2-5 %. It demonstrates homofermentative production. Nitrate is not reduced to nitrite or nitrogen gas by this speies. Moreover, the important characteristic of *T. koreensis* is the ability to produce rhamnolipid biosurfactant.

Enterococcus solitarius was transferred to the genus *Tetragenococcus* and reclassified as *Tetragenococcus solitarius* (Ke et al., 1999; Monstein et al., 2001). *E. solitarius* was firstly isolated from human sample (ear exudate) (Colline et al., 1989). This strain was identified on the basis of conventional physiological tests. However, these tests often led to misidentification of this particular species (Bunchelman et al., 1993; Ruoff et al., 1990; Tsakris et al., 1998). Results of 16S rRNA gene sequence were different from those of *Enterococcus* species (Patel et al., 1998; William, Rodrigues, and Collins, 1991). Similarity sequence of *E. solitarius* with *T. halophilus* and *T. muriaticus* were 93.8% and 93.7%, respectively (Ennahar and Cai, 2005). The results of carbohydrate utilization are shown in Table 2.3. *E. solitarius* does not ferment lactose, which is similar to *Tetragenococcus*. While other *Enterococcus* species can produce acid from lactose (Ennahar and Cai, 2005). In addition, *T. solitarius* displayed the similar pattern of sugar fermentation to *T. halophilus*. Both species can produce acids from several sugars including galactose, cellobiose, sucrose, and maltose (Table 2.3). The level of 16S rRNA gene sequence similarity of *E. solitarius* to *T. halophilus* and *T. muriaticus* were 93.8% and 93.7%, respectively. In addition, similarity compared with other *Enterococcus* species was only 80.0 -

90.0%. *E. solitarius* showed only 25.7 - 28.6% DNA-DNA relatedness to *T. halophilus* and 25.4 - 27.2% to *T. muriaticus* (Ennahar and Cai, 2005).

Halophilic LAB was identified based on their phenotypic and biochemical tests. Their characteristics have been performed using traditional test method or API kit (Schleifer et al., 1995). Ijong et al. (1996) identified LAB isolated from bakasang (Indonesian fermented fish sauce) based on physiological and biochemical characteristics. *Micrococcus* sp., *Streptococcus* sp., and *Pediococcus* sp. were identified during 40 days of bakasang fermentation. Kuda, Okamoto, and Yano (2002) isolated halophilic bacteria from 7 salted fish samples and identified using physiological characteristics and biochemical tests. The characteristics of isolated lactococci were similar with the genus *Tetragenococcus*. Nair and Surendran (2005) isolated 650 isolates from various samples of fresh and frozen fish and prawns. All isolates were identified based on phenotypic and biochemical characteristics. There were 433 isolates identified as *Lactobacillus* and separated into 13 species. There were 217 cultures belonging to the genus *Lactobacillus* but could not be assigned to species by these characteristics. Thus, phenotypic and biochemical characteristics still have limitation for species identification.

Molecular characteristics have become important taxonomic tools, such as 16S rRNA gene sequence analysis and DNA-DNA hybridization. Moreover, restriction fragment length polymorphism (RFLP) is a molecular method of genetic analysis that allows microorganism to be identified based on unique patterns of the restriction enzyme cutting in specific regions of DNA (Johansson et al., 1995). Satomi et al. (1997) isolated *Tetragenococcus* strain X-1 from squid liver sauce. The 16S rRNA sequence was different from that of *T. halophilus*. The level of DNA-DNA

relatedness with *T. halophilus* was less than 50%. Moreover, the result of RFLP patterns of *Tetragenococcus* X-1 strain confirmed that *Tetragenococcus* strain X-1 was different from *T. halphilus*, and identified as a new species of *T. muriaticus.* Kobayashi et al. (2003) isolated *Tetragenococcus* from terasi shrimp paste. They used RFLP analysis and 16S rRNA sequence for bacterial identification. They classified isolated microorganism into two groups. The first group showed the highest level similarity of 16S rRNA sequence with *T. halophilus* (99%) and *T. muriaticus* revealed high similarity with the second group. The RFLP patterns of the first and second groups were the same as *T. halophilus* and *T. muriaticus*, respectively. Hanagata et al. (2003) reported that 102 isolates obtained from shoyu mash were identified as *T. halophilus* on the basis of 16S rRNA sequence. Thongsanit et al. (2002) used DNA-DNA hybridization and cellular fatty acid composition analysis to identify *T. halophilus* and *T. muriaticus* from fish sauce. Forty strains showed high similarity with *T. halophilus* (70.0% to 109.1%) and 38 strains showed high similarity with *T. muriaticus* (70.9% to 105.6%). Cellular fatty acid composition of 40 strains and 38 strains showed similarity with *T. halophilus* and *T. muriaticus*, respectively.

Therefore, 16S rRNA gene sequence, RFLP, and DNA-DNA hybridization are useful molecular techniques for halophilic LAB identification. However, phenotypic characterization was also important and should be used in combination with the molecular techniques.

2.6 Salt tolerance of halophilic lactic acid bacteria

LAB requiring NaCl for growth are defined as halophilic LAB. The genus *Tetragenococcus* are the only LAB known to thrive at high NaCl concentration (>

18%) (Roling and Verseeld, 1997). Strains of *T. halophilus* are able to grow at a salt concentration > 18% NaCl and are involved in lactic fermentation of fermented food. A second species, *T. muriaticus* isolated from Japanese fermented squid liver sauce was described by Satomi et al. (1997). High salt and pH (around 9.0) tolerance of *Tetragenococcus* are two paramenters used to selectively detect and isolate this genus from food products. *T. halophilus* and *T. muriaticus* grow between salt concentration ranging from 12-26% in fermented salt product. *T. halophilus* is also associated with other food under reduced water activities, such as cured anchovies, where it becomes the dominant bacterium at the end of the curing process. This organism can grow under both aerobic and anaerobic condition (Villar et al., 1985). Habitats of halophilic LAB rich in salt and protein. Typical habitats of *Tetragenococcus* are salted anchovies (Villar et al., 1985), fermented fish (Tanasupawat and Daengsubha, 1983), the Japanese moromi (Sakaguchi and Mori, 1969), the Indonesian soy sauce (Roling and Van Versefeld, 1996), squid liver sauce (Satomi et al., 1997), and terasi shrimp paste (Kobayashi et al., 2003). These strains are used for inoculation of mashes for moromi fermentation in soy sauce production (Simpson and Taguchi, 1995). *T. halophilus* is also found in pickling brines (Back, 1978).

The *Tetragenococcus* genus like other genera of gram-positive moderately halophilic bacteria, such as *Halobacillus*, *Marinococcus*, *Salinicoccus*, and *Nesterenkonia*, requires 0.5 to 30% NaCl and optimum NaCl of 10% (Ventosa, Nieto, and Oren, 1998). Halophilic LAB have evolved mechanisms that enable them to adapt to high salinity while maintain an osmotic equilibrium between inside and outside of the cells. They can achieve osmotic balance by the accumulation of salts and/or organic molecules. They uptake or synthesize a compound called compatible solutes

(Csonka and Epstein, 1996; Kempf and Bremer, 1998). The compatible solutes also called osmolytes, including sugars and polyols, such as trehalose and glycerol, amino acids, such as glutamate and proline, and amino acid derivatives, such as betaines, and ectoines (Csonka and Epstein, 1996; Jebbar, Talibart, Gloux, Bernard, and Blanco, 1992; Kempf and Bremer, 1998). The term compatible solutes are generally used for low molecular weight organic compounds that accumulate to high intracellular levels under osmotic stress and are compatible with the metabolism of the cell. The accumulation of compatible solutes helps to maintain turgor pressure, cell volume, and concentration of electrolytes, all important elements for cell proliferation (Robert, 2005). Moreover, osmolytes are found in membrane integrity, protein folding and stability (Bremer and Kramar, 2000).

Organic osmolytes separated into three groups; (i) zwitterionic solutes (glycine betaine, carnitine, β-glutamine, and ectoine) (ii) nonchanged solutes (trehalose, sucrose, α-glucosylglycerol, and N-acetylglutaminylglutamine amide), and (iii) anionic solutes (β-glutamte, hydroxybutyrate, sulfotrehalose, and α-glucosylglycerate) (Robert, 2005). Glycine betaine is the major effective osmoprotectant for LAB at high and low salinities concentration (Robert, Marrec, Blanco, and Jebbar, 2000). In LAB, all transport systems involved in glycine betaine uptake are not induced but occur to be activated by exposure of the cells to high osmotic pressure (Baliarda et al., 2003). *T. halophilus* is the only known LAB which has a choline-glycine pathway, allowing accumulation of glycine betaine through the conversion of its precursor choline. Moreover, *T. halophilus* is the first LAB which can convert choline into glycine betaine under aerobic growth condition and accumulates glycine betaine during salt stress (Robert et al., 2000). MRS medium

consists of yeast extract and beef extract which contain significant amounts of glycine betaine, choline, and carnitine. Thus, *T. halophilus* is able to uptake some of those solutes from the medium, rendering its growth in a wide range of salt concentration (Robert et al., 2000). In addition, *T. halophilus* accumulates compatible solutes in the cell by uptaking from environment when the osmolarity is raised. *T. halophilus* has two transport systems: the first, carnitine transport system taking up carnitine, glycine betaine, and choline. The second is glycine betaine transport system taking up glycine betaine only. Certain halophilic bacteria can use glycine betaine not only as an osmotic stabilizer but also as a carbon and energy source (Ventosa et al., 1998). *Halomonas* sp. utilizes glycine betaine as the carbon and energy source by oxidation. However, the use of glycine betaine as carbon/energy source is inhibited in the presence of another carbon, such as glucose. Thus, glycine betaine was served as an osmotic solute for maintaining osmotic balance only (Cummings, Williamson, and Gilmour, 1993).

Compatible solutes are not the only efficient compounds for the adaptation of bacteria to hyperosmotic condition. Potassium ion (K^+) is also known to be a key ion for osmotic adaptation in many bacteria. For example, *Halobacterium* required K^+ for growth under high salinity. K^+ ion was pumped from the environment into the cell. The concentration of K^+ inside the cell was comparable to the concentration of sodium ion (Na^+) outside the cell. Therefore, *Halobacterium* used K^+ as a compatible solute and maintained the concentration of the cell balance and/or internal pH (Moral, Severin, Ramos-Cormenzana, Truper, and Galinski. 1994). In case of *T. halophilus*, K^+ is not essential in the response to osmotic changes. Since K^+ concentration in the cell does not increase in correlation with increasing external NaCl concentration. The

accumulation of glycine betaine does not affect the intracellular concentration of K^+. The relative small role of K^+ in controlling the of osmotic balance has also been observed in other LAB and most moderately halophilic bacteria (Ventosa et al., 1998).

CHAPTER III

MATERIALS AND METHODS

1. Isolation of lactic acid bacteria from fish sauce fermentation

Lactic acid bacteria (LAB) were isolated from fish sauce samples fermented at 1, 3, 5, 7, 9 and 12 months, using De Man, Rogosa, and Sharpe (MRS) agar containing 5 and 10% NaCl and MRS agar containing 5 and 10% NaCl added 0.5% $CaCO_3$ in previous study (Yongsawatdigul, 2006). Population of LAB were shown in Table 3.1. Spread plate technique was used for the isolation of LAB. The plates were incubated at 30 °C for 3-5 days under anaerobic condition using an anaerobic chamber (SHEL LAB, Sheldon Manufacturing Inc., IA., U.S.A.). After incubation, 64 colonies with different morphological characteristics were randomly selected for further identification.

2. Screening of proteinase-producing halophilic lactic acid bacteria

The criteria for selection of LAB were the ability to grow in fish broth containing high salt concentration (25% NaCl) and high proteolytic activities. All isolates were tested for the following characteristics:

Table 3.1 Lactic acid bacteria counts (CFU/ml) of various samples collected from two different plants.

Fish sauce production plant	Fermentation time (month)	Total viable counts (CFU/ml)			
		MRS agar		MRS agar (containing 0.5% $CaCO_3$)	
		5% NaCl	10% NaCl	5% NaCl	10% NaCl
A	1	0	0	$5.80x10^3$	0
	3	0	0	$1.40x10^2$	0
	5	0	0	$3.08x10^2$	0
	7	<30(25)	0	$4.75x10^2$	0
	9	55	0	$1.30x10^2$	0
	12	0	0	$3.65x10^2$	0
B	1	<30(21)	0	$7.60\ x10^3$	0
	3	0	0	$1.20\ x10^4$	$2.30\ x10^4$
	5	0	0	$5.00\ x10^3$	$1.30\ x10^4$
	7	0	0	$1.80\ x10^4$	$3.50\ x10^3$
	9	0	0	$1.80\ x10^3$	$2.70\ x10^4$
	12	$8.80\ x10^2$	0	$1.80\ x10^3$	$2.60\ x10^4$

2.1 Growth in fish broth

Sixty-four isolates were tested for growth in fish broth containing 25% NaCl (FB25). Fish broth was prepared by boiling 1 part of anchovy (*Stolephorus indicus*) with 2 parts of distilled water for 20 min (Yongsawatdigul, Rodtong, and Raksakulthai, 2007). The fish slurry was filtered through cheesecloth and supernatant was collected. pH was adjusted to 7.0 and the media was autoclaved at 121 °C for 15 min. The purified isolates were cultured in 5 ml of MRS broth containing 10% NaCl. The inoculum size of 2% (approximate 10^5-10^6 CFU/ml) was added to 5 ml FB25 and incubated at 30 °C for 7 days under anaerobic condition using an anaerobic chamber. Growth was enumerated using MRS agar containing 5 and 10% NaCl added 0.5%

$CaCO_3$ and incubated at 30 °C for 3-5 days under anaerobic condition. The pH was measured using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland).

2.2 Proteolytic activity of lactic acid bacteria

When the incubation time was attained, remaining protein content and trichloroacetic acid (TCA)-soluble oligopeptides content in fish broth were determined as an indicator of proteolytic activity. Cell cultures in fish broth were centrifuged at 10,000×g for 10 min at 4 °C, and supernatants were collected. Protein content of supernatant was monitored by Bradford protein assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard. In addition, 20% TCA was added to supernatant at a ratio 1:1 and left at 4 °C overnight. Subsequently, it was centrifuged at 10,000×g for 10 min at 4 °C and supernatant was collected for oligopeptides content by Lowry method using tyrosine as a standard. The residual protein and oligopeptide content relative to the respective original values were calculated. These isolates showing high TCA-soluble oligopeptide content were selected and evaluated for their growth in MRS broth containing various NaCl concentration.

2.3 Growth in MRS broth

Based on the ability to grow in FB25 and proteolytic ability, eighteen isolates were selected. Inoculum was prepared by culturing all isolates in 5 ml of MRS broth containing 10% NaCl added 0.5% $CaCO_3$ and incubated at 30°C for 3 days under anaerobic condition. The inoculum size of 2% was added to 5 ml of MRS broth containing 0, 10, and 18% NaCl added 0.5% $CaCO_3$ and incubated at 30 °C for 3-5 days under anaerobic condition. Growth at all conditions was enumerated using MRS agar containing 10% NaCl added 0.5% $CaCO_3$ and incubated at 30 °C for 3-5 days under anaerobic condition.

3. Characteristics of halophilic lactic acid bacteria for the use as starter cultures for fish sauce fermentation

Seven selected isolates from 2.3 were tested for aminopeptidases activity, biogenic amine formation, and volatile compound formation in fish broth containing 25% NaCl.

3.1 Aminopeptidase activities and volatile formation of halophilic LAB

3.1.1 Intracellular aminopeptidase activities assay

3.1.1.1 Cell-free extract preparation

Seven selected LAB were cultured in MRS broth containing either 5 or 10% NaCl, incubated at 30°C for 3-5 days under anaerobic condition. The cell-free extract was prepared according to Magboul and McSweeney (1999) with slight modifications. Cells were harvested by centrifuging at 10,000×g for 15 min at 4 °C. The cells were added 250 µl of 5 mg/ml lysozyme and incubated at 37 °C for 1 h. Subsequently, the cells were washed twice with 0.2 M phosphate buffer (pH 7.5) and 2 ml of the same buffer was added. The cells were disrupted using a sonicator (Sonics and Materials Inc., Newton, U.S.A) at 100 Hz, 0 °C for 30 min. Cell debris was removed by centrifugation at 10,000×g for 15 min at 4 °C. The supernatant was used to determine intracellular aminopeptidases activity (Magboul and McSweeney, 1999; Sanz et al., 1997).

3.1.1.2 Aminopeptidase assay

The aminopeptidase activities were determined using several amino *p*-nitroanilide derivatives as described by Magboul and McSweeney (1999). One ml of reaction mixture consisting of 0.1 ml of crude enzyme, 0.1 ml of substrates (Glu-

pNA, Arg-pNA, Leu-pNA, Ala-pNA, Met-pNA and Lys-pNA at concentration of 20 mM) and 0.8 ml of 0.2 M phosphate buffer (pH 7.5). The reaction mixture was incubated at 60 °C for 2 h and 80% acetic acid was added to stop reaction. The release of *p*-nitroanilide was measured using a spectrophotometer (GBC UV/VIS 916; GBC Scientific Equipment PTY, Ltd., Australia) at 405 nm. One unit of enzyme activity (U) was defined as the release of 1 nmole of *p*-nitroanilide per minute (Magboul and McSweeney, 1999; Sanz et al., 1997).

3.1.2 Formation of volatile compounds in fish broth

The seven selected isolates were cultured on MRS agar containing 10 % NaCl added 0.5% $CaCO_3$ and incubated at 30 °C for 48 h under anaerobic condition. One loopful of pure culture was inoculated to 50 ml of fish broth containing 25% NaCl and incubated at 30 °C for 14 days under anaerobic condition. After incubation, bacteria were enumerated using MRS agar containing 10% NaCl added 0.5% $CaCO_3$ and cell 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 XPT^{TM}, Teledyne Tekmar, Mason, OH., U.S.A.). Ten milliliter of 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 60 °C at a flow rate of 40 ml/min. The volatile compounds were trapped by Tenax TA column and were desorbed at 225 °C for 4 min. Separation of the desorbed volatile compounds were achieved by gas chromatography-mass spectrometry (Varian Inc., Walnut Creek, CA., U.S.A.) connected to a capillary column (DB-WAX, 60 m x 0.25 mm x 0.25 μm Agilent Technologies, Redwood, CA., U.S.A.). The oven temperature was increased from 25 to 200 °C at 15 °C/min. The volatile compounds were

identified using a quadrupole mass detector (Mass spectrometer 1200L quadrupole, Varian Inc., Walnut Creek, CA., U.S.A.). The mass spectra of volatile compounds in fish sauce were obtained by electron ionization (EI) at 70 eV. The data of mass spectra and retention times were reported. The content of volatile compounds was calculated from peak areas relative to the internal standard (cyclohexanol). Kovats Index of each compound was calculated and mass spectral data were compared with authentic data (National Institute of Standards; NIST data) (Shimoda et al., 1996).

3.2 Biogenic amines forming ability of selected LAB isolates

Seven selected LAB were tested for biogenic amines forming ability using modified Glucose Yeast Peptone (mGYP) broth added 5 and 25% NaCl and fortified with 0.25% of each amino acid (L-lysine, L-histidine, L-ornithine and L-tyrosine) (Bover-Cid and Holzapfel, 1999). Seven selected LAB were crossed streak on MRS agar containing 10% NaCl added 0.5% $CaCO_3$ to obtain a single colony. One loopful of pure culture was transferred into 10 ml of mGYP broth and incubated at 30°C for 5-7 days under anaerobic condition. After incubation, LAB population was enumerated using MRS agar containing 10% NaCl added 0.5% $CaCO_3$. Cells were removed by centrifuging at 10,000×g for 15 min at 4 °C. Supernatants were collected to determine biogenic amines using high performance liquid chromatography (HPLC). One ml of supernatant was added with 100 µl of 1 µg/ml diaminoheptane as an internal standard, 200 µl of 0.2 N NaOH, and 300 µl of saturated sodium bicarbonate. Two milliliters of 10 mg/ml dansyl chloride was added. The mixture solution was incubated at 40 °C for 45 min. Subsequently, they were added 300 µl of 30% ammonia solution to eliminate dansyl chloride residue. The mixture solution was adjusted to 5 ml using acetonitrile and centrifuged at 2,500×g for 5 min. The

supernatant was collected and filtered through a 0.45-μm membrane filter (Agilent Technologies Inc., Palo Alto, CA., U.S.A.). Dansyl-derivatized biogenic amines were separated using a mobile phase consisting of the mixture of acetonitrile and 0.02 M acetic acid (1:9) as a solvent A. The mixture of solvent B was prepared from 0.02 M acetic acid, acetonitrile and methanol (1:4.5:4.5) at a flow rate of 1 ml/min with a Hypersil BDS column C_{18} (3 μm, 100 × 4 mm, Agilent Technologies Inc., Palo Alto, CA., U.S.A.) equipped with HPLC (HP 1100, Agilent Technologies Inc., Palo Alto, CA., U.S.A.). Fifty percent of solvent B was run isocratically for 5 min. Subsequently, the gradient elution was started and ended at 90% solvent B in 25 min. The column was equilibrated with 50% solvent B for 10 min before the next injection. Diode array detector was set at 254 nm and 550 nm as a reference wavelength (Yongsawatdigul, Choi, and Udomporn, 2004).

4. Identification of lactic acid bacteria

4.1 Morphological and biochemical characteristics

Seven selected isolates were cultured on MRS agar containg 10% NaCl and 0.5% $CaCO_3$, and incubated at 30°C for 48 h under anaerobic condition. Cell morphology and cell arrangement were observed by Gram staining. Catalase activity and gas production from glucose were conducted according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Other biochemical tests were carried out using API 50 CH/CHL kits (BIO-Merieux, Marcy-I, Etoile, France).

The production of proteinase, amylase, and lipase was tested using MRS agar containing 5 or 10% NaCl and 1% skim milk, 1% starch, 1% Tween80, respectively. Each LAB isolate was point inoculated onto these media and incubated at 30 °C for 5

days under anaerobic condition. A positive reaction of the proteolytic test was indicated by clear zone around colony. Starch hydrolysis was visualized by adding iodine solution to the plate. A clear zone around colony indicated the positive result. A positive reaction of lipase was a white precipitate of fatty acid around colony.

4.2 Physiological characteristics

4.2.1 Optimal growth condition

The seven isolates were tested for growth at different NaCl concentrations, temperature, and pHs as follows:

The inoculum size of 2% (approximate 10^6 CFU/ml) was added into 5 ml of MRS broth containing 0, 3, 6.5, 10, 15, 18, 20 and 25% NaCl, pH of all media was adjusted to 7.0. The inoculated cultures were incubated at 30 °C for 1-7 days under anaerobic condition.

For optimum temperature, the inoculum size of 2% was added into 5 ml of MRS broth containing 5% NaCl, pH 7.0 and incubated at various temperatures, 10, 15, 20, 25, 30, 35, 40 and 45 °C under anaerobic condition at 30 °C for 3-5 days.

For optimum pH, the inoculum size of 2% was added into 5 ml of MRS broth containing 5% NaCl and adjusted to different pHs of 4.5, 5.0, 6.0, 7.0, 8.0, and 9.0. The culture was incubated at 30 °C for 3-5 days under anaerobic condition.

Growth of LAB at each condition was enumerated using MRS agar containing 5% NaCl added 0.5% $CaCO_3$.

4.2.2 Stereoisomer of lactic acid produced

Seven isolates were grown in 10 ml MRS broth containing 10% NaCl incubated at 30 °C for 3-5 days under anaerobic condition. The cells were removed by centrifuging at 10,000×g for 10 min at 4 °C. The supernatant was filtered through a

0.45-μm membrane filter and was diluted 100 times with deionized water. HPLC (Water2487, waters Inc., Midford, MA., U.S.A.) equipped with a Chiral Astec CLC-L column (5 μm, 4.6 mm × 15 cm, Sigma Chemical Co., St. Louis, MO., U.S.A.) was used to separate D/L lactic acid. Mobile phase (0.005 M $CuSO_4$) was run isocratically at a flow rate of 0.8 ml/min. Injection volume was 100 μl. Lactic acid was detected by UV detector at 254 nm (Manome et al., 1998).

4.3 Ribosomal RNA gene analysis

4.3.1 Genomic DNA extraction

Seven isolates were grown in 10 ml MRS broth containing 10% NaCl incubated at 30 °C for 3 days under anaerobic condition. The cell pellets were collected by centrifuging at 10,000×g for 5 min at 4 °C. Cells were washed twice using 50 mM Tris-ethylenediamine buffer (TE buffer) pH 8.0 and resuspended in 480 μl lysis buffer (50 mM sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0), then 60 μl of 10 mg/ml lysozyme was added. The mixture was incubated at 37 °C for 1 h, and 60 μl of 20% SDS was added and gently mixed. Subsequently, the mixture was incubated at 65 °C for 10 min and 4 μl of 2 mg/ml RNase solution was added and incubated at 37 °C for 30 min. After incubation, 600 μl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed. The reaction mixture was centrifuged at 12,500 rpm for 5 min at 4 °C. Supernatant was collected and 450 μl of isopropanol was added. DNA was allowed to precipitate at -80 °C for 30 min. Then, the mixture was centrifuged at 12,500 rpm for 5 min at 4 °C and added 600 μl of 70% ethanol. Ethanol was removed by centrifugation. Subsequently, DNA was dried by incubating at 37 °C for 30 min. Finally, 25 μl of TE buffer was added and incubated

at 65 °C for 15 min (Vassu et al., 2002). Purity of DNA was investigated by electrophoresis using 0.9% agarose gel (Low EEO Agarose, BIO 101, Inc., La Jolla, CA., U.S.A.). The purified DNA was kept at -20 °C until use.

4.3.2 PCR amplification of 16S rRNA gene

Fifty µl of the mixture contained 4 µl of 25 mM $MgCl_2$, 5 µl of 2 µM of each nucleoside triphosphate (dATP, dCTP, dGTP, dTTP; Invitrogen™ Life Technologies, Foster, CA., U.S.A.), 5 µl of 10X PCR buffer (200 mM Tris-HCl, pH 8.0, 500 mM KCl; Invitrogen™ Life Technologies, Foster, CA., U.S.A.), 2 µl of 10 pmole of each primer (fD1 and rP2, Sigma Proligo, Helios, Singapore) (Table 3.2), 0.5 µl of 5 U Taq polymerase (Invitrogen™ life technologies, Foster, CA., U.S.A.) and 10-150 ng of 4 µl Genomic DNA. PCR product size was 1,500 bp. Thermo cycle program was set as follows: 95 °C for 2 min; 35 cycles of 95 min for 45 s; 55 °C for 45 s and 72 °C for 2 min; and final extension step at 72 °C for 10 min (Thermo electron corporation Px2 Thermal Cycler, San Francisco, CA., U.S.A.). PCR products were separated on 1% agarose gel electrophoresis and visualized by ethidium bromide.

4.3.3 Cloning of 16S rRNA gene in vector

4.3.3.1 Competent cell preparation

Preparation of competent cells were conducted according to Molecular Cloning laboratory manual (Sambrook and Russell, 2001). *E. coli* DH5α was cultured in 3 ml of Luria-Bertani broth (LB broth) and incubated at 37 °C for 14-16 h at 200 rpm. One hundred µl cell suspension were transferred into 50 ml LB broth and incubated at 37 °C for 3 h with a shaking speed of 200 rpm ($OD._{600}$ = 0.4). Cells

Table 3.2 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene.

Primer	Primer sequence (5′ to 3′)	Target region	Reference
16S rRNA gene amplification			
fD1	5′-AGAGTTTGATCCTGGCTCAG-3′	8-27	Weisburg et al. (1991)
rP2	5′-ACGGCTACCTTGTTACGACTT-3′	1490-1511	Weisburg et al. (1991)
Nucleotide sequencing			
T7	5′-TAATACGACTCACTATAGGG -3′	53-72	Hans et al. (2002)
SP6	5′-TAATACGAC TCACTATAGGG -3′	2896-2916	Hans et al. (2002)
Forward	5′-TAACTACGTGCCAGCAGCC-3′	515-533	Design from nucleotide sequencing results of this study
Reverse	5′-CGACAACCATGCACCACCTG-3′	1008-1027	Design from nucleotide sequencing results of this study

were harvested by centrifuging at 4,000 rpm for 5 min at 4 °C. Supernatant was decanted and the cell pellets were resuspend in 30 ml of ice-cold deionized water and centrifuged at 4,000 rpm for 5 min at 4 °C. Subsequently, 30 ml of ice-cold 10% glycerol was added. Cells were collected by centrifuging at 4,000 rpm for 5 min at 4 °C. Finally, one ml of ice-cold Glycerol Yeast Tryptone (GYT) broth was added into cell pellet. One hundred µl of competent cell was aliquoted and immersed into liquid nitrogen and kept at -70 °C throughout the study.

4.3.3.2 Plasmid transformation to *E. coli* DH5α

The bacterial 16S rRNA gene from PCR amplification were purified with Wizard Gel/PCR product kit (Promega corporation, Madison, WI., U.S.A.) and

ligated into pGEM-T easy vector (Promega corporation, Madison, WI., U.S.A.) following the company's instruction. The recombinant vector was transformed into *E. coli* DH5α by an electroporator (Electroporator 2510, Eppendorf AG, Hamburg, Germany) set at 1800 V. The transformed *E. coli* DH5α was selected by blue-white selection method (Sambrook and Russell, 2001). The plasmid vector was purified using Wizard DNA purified kit (Promega corporation, Madison, WI., U.S.A.) and the vector was cut using *Eco*RI (Invitrogen^{TM} life technologies, Foster, CA., U.S.A.) and incubated at 37 °C for 6 h to verify DNA insertion. DNA fragments were detected on 2% agarose gel electrophoresis.

4.3.4 DNA sequence analysis

The pGEM plasmid was used for sequence analysis of cloned 16S rRNA fragments. T7/SP6 primer was used to sequencing (Table 3.2). Nucleotide sequence data were obtained from DNA sequencing software of ABI 3730xl DNA analyzer (Model 373, Forster, CA., U.S.A.). The sequences were compared to local alignment search of GenBank database using the BLAST version 2.2.9 program of the National Center for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment was performed using CLUSTAL_X (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins, 1997). Phylogenetic tree was constructed by the Maximum Pasimony method with software MEGA version 4.0 (Kumar, Tamura, Jakobsen, and Nei, 2004). The robustness of relationships was evaluated by a bootstrap analysis through 1,000 bootstrap replications.

4.3.5 Restriction fragment length polymorphism (RFLP) analysis

The PCR product of 16S rDNA was digested with two restriction endonucleases. Approximately 500 ng of 16S rDNA was added with 1 μl of the 10×

reaction buffer and 4 U of *Alu*I and *Mbo*I restriction endonucleases (New England Biolabs, Beverly, MA., U.S.A.), then diluted to a final volume of 10 μl with MillQ water. The reaction mixture was incubated at 37 °C for 3 h. The digested DNA fragments were separated by 2% agarose gel elecrophoresis. DNA fragment patterns of 7 isolates were compared to those of *Tetagenococcus halophilus* ATCC 33315 (Sato, Yanagida, Shinohara, Yokotsuka, 2000).

5. The use of lactic acid bacteria as starter cultures for fish sauce Fermentation

5.1 Preparation of starter culture

Four selected LAB namely MS33, MRC10-1-3, MCD10-5-10, and MRC 10-7-8, were cultured in 100 ml of fish broth containing 25% NaCl, pH 7.0 at 30 °C for 7-10 days under anaerobic condition to obtain the cells approximately of 10^5-10^6 CFU/ml. Fish broth was prepared by boiling 1 part of anchovy (*Stolephorus* sp.) with 2 parts of distilled water for 20 min. Then, fish extract was filtered and NaCl was added to contain 25%. pH was adjusted to 7.0. The extract was autoclaved at 121 °C for 15 min (Yongsawatdigul, Rodtong, and Ruksakulthai, 2007).

5.2 Fish sauce fermentation

One kg of thawed anchovy contained in a glass jar was incubated in a 65 °C water bath until temperature at the center of the sample reached 65 °C. Subsequently, 0.25% of Alcalase 2.4L was added and the samples were incubated for 2 h. The samples were then cooled to 50 °C and added 0.5% of Flavouzyme 500L, and further incubated at 50 °C for 4 h (Yongsawatdigul et al., 2007). The 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 added 10% of fish broth without LAB. All treatments were incubated in a 35 °C incubator for 6 months. Microbiological changes, pH and α- amino content were monitored at 0, 14, 30, 60, 90, 120, 150 and 180 days of fermentation. Volatile compounds of the sample were analyzed at 30, 120 and 180 days. Other physico-chemical properties, such as salt content, color, ammonical nitrogen and total nitrogen content, were determined at 180 days of fermentation.

5.2.1 Microbiological changes

Fish sauce mash (10 g) was aseptically taken from the glass jar at various fermentation times (0, 14, 30, 60, 90, 120, 150 and 180 days) and enumerated for LAB population by spread plate method using MRS agar containing 18% NaCl and 0.5% $CaCO_3$ (MRS18%+0.5% $CaCO_3$) and incubated at 30 °C for 5-7 days under anaerobic condition. Population of haloplilic bacteria were enumerated using JCM168 medium containing 18% NaCl and incubated at 35 °C for 5-7 days under aerobic condition.

5.2.2 Chemical analyses

5.2.2.1 α–Amino acid content

Fish sauce samples fermented at 0, 14, 30, 60, 90, 120, 150 and 180 days were measured for α–amino content using trinitrobenzenesulfonic acid (TNBS) method with L-leucine as a standard (Field, 1979). The reaction mixture consisted of 0.05% (w/v) TNBS in deionized water, 1% (w/v) sodium dodecyl sulfate (SDS), 0.1 N HCl, and 0.2125 M phosphate (pH 8.2). One hundred μl of fish sauce was added 1 ml of 0.2125 M phosphate (pH 8.2). Subsequently, one ml of 0.05% TNBS was added

and the mixture was incubated at 50 °C for 1 h. After incubation, the reaction was stopped by addition of 0.1 N HCl 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).

5.2.2.2 Biogenic amines content

Biogenic amines content of fish sauce samples fermented for 6 months were determined as described in 3.2.

5.2.2.3 Physico-chemical properties of fish sauce

Fish sauce samples (6 months) were analyzed for ammonical nitrogen, total nitrogen and salt content (AOAC, 1995). The color of fish sauce samples were measured spectrophotometrically. The sample was diluted with deionized water at a ratio 1:4 and absorbance was determined at 450 nm. The pH was measured using pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland).

5.2.2.4 Amino acid profiles analysis

Six-month-old fish sauce samples and commercial fish sauce (fermented for 12 months) were analyzed for total and free amino acid profiles. To determine total amino acids, samples were diluted to distilled water with a ratio 1:10. Two ml of diluted samples was added 2 ml of 12 N HCl containing 1% phenol and hydrolyzed at 110 °C for 24 h using an autoclave. Hydrolyzed samples were dried by rotary evaporation at below 50 °C. Total amino acids were determined by directly diluting all samples with 20 ml of distilled water and filtered through a 0.22-μm membrane filter. Amino acids quantification was performed using amino acid analyzer (Model Biochrom 30, Pharmacia-Biotech, UK). Sodium citrate buffers at pH 3.2 to 4.9 were used as a mobile phase. In addition, to determine free amino acids, 2

ml of diluted samples were added 100 mg of 5'-sulfosalicylic acid (SSA) and left at 1 h for protein precipitation. Precipitate protein was removed by centrifuging at 1,500×g for 10 min. Supernatant was collected for free amino acid analysis. Standard amino acids were analyzed in the same condition to identify retention time. The amounts of amino acids were expressed as μmol of amino acid per 100 ml of fish sauce.

5.2.2.5 Analysis of volatile compounds in fish sauce

Samples fermented for 30, 120 and 180 days were analyzed for volatile compounds using purge and trap as described in 3.3.

6. Sensory evaluation

Fish sauce samples were evaluated by trained panelists. The panel consisted of 10 individuals who deal with fish sauce tasting on the regular basis as part of quality control task. The panelists were asked to give acceptance scores for 4 attributes: color, odor, flavor and overall acceptance, using the 7-point hedonic scale. Six fish sauce samples (four fish sauce samples inoculated with LAB, control and traditionally fermented sample from fish sauce manufacturer) were randomly separated into 2 groups. The first group containing 3 samples was presented to each panelist in a random order in the first day. The second group was evaluated on the following day. Samples were not added sugar or any other additives. Ten ml of samples contained in a 15-ml glass cup with approximately 2-cm headspace was served to each panelist. Before sensory evaluation, the sample cups were covered with lids and left at room temperature (approximately 28 °C) for 30 min. The panelists compared odor by opening the lid of the cup and sniffing. Flavor preference were assesses by tasting

approximately 0.5 ml of fish sauce samples using a plastic spoon. The panelists were asked to use drinking water and plain cracker for rinsing their mouth before tasting the next sample.

7. Statistical analyses

All chemical experiments were analyzed in duplicates. Statistical analysis was evaluated in Completely Randomized Design (CRD). Statistical analysis of sensory evaluation experiment was evaluated in Randomized Complete Block Design (RCBD) with Statistical Analysis System (SAS) (SAS Inst. Inc., Cary, NC., U.S.A.). The day of sensory evaluation was assigned as a block. Two-way analysis of variance (ANOVA) was used to determine significant differences between samples and days. Means comparison by Duncan's Multiple Range Test (DMRT) were used to determine differences between mean at $P < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of lactic acid bacteria from fish sauce fermentation

Lactic acid bacteria (LAB) in the commercial fish sauce samples fermented for 1, 3, 5, 7, 9, and 12 months were isolated in previous study (Yongsawatdigul, 2006). LAB counts of samples from factory A were approximately 10^2-10^3 CFU/ml on MRS agar containing 5% NaCl added 0.5% $CaCO_3$. LAB counts of samples from factory B were 10^3-10^4 CFU/ml on MRS agar containing 5 and 10% NaCl added 0.5% $CaCO_3$. LAB counts from factory B were generally higher than those of factory A. This could be due to variation of raw material and fermentation process among plants. It should be noted that the medium added $CaCO_3$ showed higher LAB counts than medium without $CaCO_3$. pH of medium without $CaCO_3$ gradually decreased due to the accumulation of lactic acid produced by LAB. This could limit the growth of LAB, rendering the lower count. Addition of $CaCO_3$ in the medium neutralized lactic acid and maintained the optimum pH (Guoqiang, Kaul, and Mattiasson, 1991). Furthermore, calcium ion could be used for substrate transport of lactococci (Teuber, 1995). Therefore, addition of $CaCO_3$ in MRS promoted growth of lactococci. For this reason, the use of MRS added $CaCO_3$ promoted the growth of several LAB and showed higher LAB counts. LAB were not detected in MRS agar containing 10% NaCl from both factories. Therefore, MRS containing 5% and 10% NaCl added 0.5% $CaCO_3$ appeared to be in appropriated for the selection of LAB in fish sauce mash

sample. Kilinc, Cakli, Tolasa, and Dincer (2006) reported that LAB counts during 1 to 57 days of fish sauce fermentation in 6 samples were 10^4-10^7 CFU/ml. The number of LAB count in all fish sauce samples increased corresponding to a decrease in pH and LAB counts continued to decrease after 15 days of fermentation. Ijong and Ohta (1996) found a variety of LAB during bakasang (Indonesian fish sauce) fermentation, namely *Lactobacillus*, *Streptococcus* and *Pediococcus*. Taira, Funatsu, Satomi, Takano, and Abe (2007) found *Tetragenococcus* sp. dominated after 60 days of nuoc mam (Vietnam fish sauce) fermentation. Saisithi (1994) reported that halophilic LAB were dominant at 7^{th} -12^{th} month of fish sauce fermentation, corresponding to a period when color, aroma and flavor were fully developed. However, LAB were found throughout 12 months of fermentation in this study. Sixty four isolates of LAB were randomly selected based on different colonies appearance.

4.2 Screening of proteinase-producing halophilic lactic acid bacteria

A total of 64 isolates from fish sauce mash samples were tested for growth in high salt environment. Fish broth containing 25% NaCl (FB25) was used for this purpose. Fish broth contained mainly fish extract from anchovy, the main raw material used for fish sauce fermentation. The chemical composition of fish broth would be comparable to that of fish sauce fermentation. Since the selected LAB were intended to be used 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.

LAB that grew in FB25 with an increase of cell count approximately 1-2 Log CFU/ml within 7 days were selected (Table 4.1). It should be noted that pH of FB25 slightly decreased. A carbon source, such as glucose, is typically required for lactic

acid production of LAB. FB25 was likely to contain less amount of glucose and other sugars, resulting in low acid production. Oligopeptide content indicating hydrolysis of fish protein was also used as one of selection criteria. Some isolates, namely MCD5-1-1, MCD10-1-1, M11, MS33, MRC5-5-2 and MRC10-7-8, resulted in an increase of oligopeptide content, indicating their proteolytic activity (Table 4.1). Some isolates showed a decrease in oligopeptide content, suggesting that oligopeptides were utilized for bacterial growth (Table 4.1). Proteolysis is essential for liberating amino acids for flavor development (Smit, Engels, and Smit, 2009). Probably, these isolates might be able to produce flavor compounds in fish sauce. Eighteen isolates were selected for further study based on the ability to grow in FB25 and/or to hydrolyze fish protein (Table 4.1). Although, oligopeptide contents of some isolates (e.g. MRC10-1-3, MRC10-1-4, MCD10-5-10, MCD10-5-11, MCD10-5-12, and MCD10-5-15) were lower than that of original FB25, they were selected. Because they could show ability to utilize oligopeptide leading to amino acids, which served as a substrate for flavor formation. Therefore, these LAB might be useful for improving fish sauce flavor (Smit et al., 2005).

Eighteen selected isolates were tested for cell morphology and ability to hydrolyze skim milk protein at 5% NaCl (Table 4.2). There were 5 isolates showing of skim milk protein. However, some isolates did not hydrolyze skim milk protein but showed ability to hydrolyze fish protein in FB25 (Table 4.1). It should be noted that proteinases produced by LAB could hydrolyze fish protein at high NaCl concentration. These results indicated that isolated LAB might produce salt-stable proteinases which could hydrolyze fish protein during fish sauce fermentation.

Table 4.1 Changes of bacterial growth, protein, oligopeptide content and pH value of fish broth containing 25% NaCl inoculated 64 isolates.

Fermentation period (month)	Bacterial[a] isolate code	Δ Growth[b] (Log CFU/ml)	Δ Protein content[c] (%)	Δ Oligopeptide[c] (%)	pH[d]
1	**MCD5-1-1**	**1.75**	**-15.56**	**3.15**	**6.22**
	MRS5-1-1	1.61	6.60	-1.05	6.13
	MRS5-1-2	0.89	8.67	-16.53	6.25
	MRS5-1-3	3.89	67.66	58.04	6.04
	MRS5-1-4	-0.61	-27.20	5.54	6.16
	MCD5-1-4	0.58	12.15	10.13	6.38
	MRS5-1-5	1.85	13.67	20.44	6.13
	MCD5-1-5	1.10	-12.73	4.23	6.22
	MCD5-1-6	1.20	-29.83	2.36	6.05
	MRS5-1-6	1.35	19.21	3.25	6.26
	MCD5-1-7	-0.31	-23.91	-0.83	6.33
	MCD10-1-1	**1.35**	**17.04**	**4.11**	**5.97**
	MRC10-1-1	-0.69	-23.58	-12.98	5.92
	MRC10-1-2	-0.02	-25.65	-6.01	5.93
	MRC10-1-3	**1.68**	**-20.89**	**-9.35**	**5.81**
	MRC10-1-4	**1.39**	**-27.98**	**-14.89**	**6.21**
	MRC10-1-5	-0.71	-31.00	4.79	6.43
	MRC10-1-6	1.54	-12.59	-13.55	6.22
	MRC10-1-7	1.69	-16.77	-3.33	6.18
	MRC10-1-8	-0.41	-21.75	4.82	6.19
	MRC10-1-9	0.77	-41.70	-45.35	6.39
	MRC10-1-10	1.37	-15.02	-5.69	6.08
	MRC10-1-11	-0.07	-31.37	-15.49	6.44

Table 4.1 (continued)

Fermentation period (month)	Bacterial[a] isolate code	Δ Growth[b] (Log CFU/ml)	Δ Protein content[c] (%)	Δ Oligopeptide[c] (%)	pH[d]
1	M7	1.29	17.26	-11.66	6.36
	M9	**0.87**	**16.93**	**17.86**	**6.28**
	M11	**2.1**	**11.82**	**8.98**	**6.30**
	M20	-0.69	7.58	10.70	6.29
	MS33	**1.25**	**6.60**	**9.27**	**6.33**
3	MRC5-3-1	1.08	23.82	18.79	6.74
	MRC10-3-1	**0.83**	**-30.37**	**-0.14**	**6.27**
	MRC10-3-3	-0.63	-19.46	-6.77	6.18
	MRC10-3-4	0.11	-38.18	-11.55	6.37
	MRC10-3-5	-0.99	-9.63	-1.66	5.98
	MRC10-3-6	**1.04**	**-25.65**	**-9.73**	**5.82**
	MRC10-3-7	0.62	3.37	-24.28	6.24
	MRC10-3-8	**0.82**	**-13.81**	**-3.04**	**6.20**
5	MRC5-5-1	1.02	-24.06	-13.74	5.93
	MRC5-5-2	**1.56**	**-36.57**	**1.41**	**6.55**
	MRC5-5-3	0.03	-32.98	-23.94	6.39
	MCD10-5-10	**1.51**	**-0.59**	**-4.01**	**5.86**
	MCD10-5-11	**1.28**	**-16.50**	**-2.65**	**6.14**
	MCD10-5-12	**0.21**	**-53.65**	**-19.08**	**6.42**
	MCD10-5-13	-0.92	-11.92	-3.24	6.2
	MCD10-5-14	**1.09**	**-4.26**	**2.36**	**6.21**
	MCD10-5-15	**1.23**	**-33.60**	**-9.26**	**5.86**
	MCD10-5-16	1.10	-8.69	-9.03	6.17
7	M5-7-1	-0.44	-14.61	-8.34	6.23
	M5-7-2	-2.42	-6.78	-20.33	6.36

Table 4.1 (continued)

Fermentation period (month)	Bacterial[a] isolate code	Δ Growth[b] (Log CFU/ml)	Δ Protein content[c] (%)	Δ Oligopeptide[c] (%)	pH[d]
7	M5-7-4	-0.06	0.18	1.38	6.2
	M5-7-5	-2.81	-11.85	-8.05	6.36
	MRC5-7-5	0.63	-22.87	-9.45	6.54
	M5-7-6	-0.74	-4.75	-14.14	6.36
	MRC10-7-8	**2.63**	**-4.14**	**28.46**	**6.2**
	MRC5-7-11	0.19	10.41	37.85	6.71
	MRC10-7-15	1.65	-10.49	-7.73	6.17
	MRC10-7-16	**1.60**	**9.21**	**-17.96**	**6.22**
9	M5-9-1	-3.13	-7.50	-16.59	6.34
	M5-9-3	-2.67	-5.76	49.12	6.60
	M5-9-4	-0.77	-6.63	-16.10	6.35
	M5-9-5	-3.43	-22.87	-18.46	6.33
	M5-9-6	-3.31	-17.94	-2.55	6.31
	M5-9-7	-0.33	-13.01	-0.58	6.30
	M5-9-8	-2.98	-26.64	-1.96	6.31
	M5-9-9	-1.30	49.01	7.24	6.56
	M5-9-10	-1.01	-22.00	-13.35	6.31

[a]Bold indicates the selected isolate.

[b]difference in bacterial count compared to initial count of 10^5-10^6 cell/ml.

[c]difference of protein and oligopeptide content compared to initial content of fish broth.

[d]initial pH of FB25 is 6.54.

Cell morphology of all selected LAB were cocci and pairs and/or tetrads arrangement (Table 4.2). LAB having pairs and/or tetrads and being able to grow ranging from 0-10% NaCl are *Pediococcus* while those tolerant to NaCl concentration

Table 4.2 Growth of the selected 18 isolates in MRS containing various NaCl concentrations.

Fermentation period (Month)	Bacterial[a] isolate code	Cell shape/ arrangement	Proteinase (Casien hydrolysis)	Growth[b] (Log CFU/ml)		
				MRS[c]	MRS10[d]	MRS18[e]
1	MCD5-1-1	Cocci/Tetrads	+	5.33	8.35	8.26
	MCD 10-1-1	Cocci/Pairs, Tetrads	-	3.91	10.42	9.05
	MRC10-1-3	Cocci/Pairs, Tetrads	-	6.02	**10.05**	9.95
	MRC10-1-4	Cocci/Tetrads	-	7.64	10.11	8.03
	M9	Cocci/Pairs, Tetrads	+	7.80	10.07	9.70
	M11	Cocci/Pairs, Tetrads	+	7.80	**10.32**	9.88
	MS33	Cocci/Pairs	+	6.47	**8.12**	**8.69**
3	MRC10-3-1	Cocci/Pairs, Tetrads	-	6.38	8.34	8.66
	MRC10-3-6	Cocci/Pairs, Tetrads	-	6.35	8.59	9.22
	MRC10-3-8	Cocci/Pairs, Tetrads	-	5.59	9.42	9.36
5	**MRC5-5-2**	Cocci/Tetrads	+	6.30	**10.13**	9.54
	MCD10-5-10	Cocci/Pairs, Tetrads	-	6.34	8.24	**10.16**
	MCD10-5-11	Cocci/Pairs, Tetrads	-	6.04	8.24	8.06
	MCD10-5-12	Cocci/Pairs, Tetrads	-	5.67	9.28	8.59
	MCD10-5-14	Cocci/Pairs, Tetrads	-	6.16	8.70	7.43
	MCD10-5-15	Cocci/Pairs, Tetrads	-	6.36	**9.28**	**9.69**
7	**MRC10-7-8**	Cocci/Pairs, Tetrads	-	5.95	9.60	**11.79**
	MRC10-7-16	Pairs, Tetrads	-	6.06	8.11	8.78

[a]Bold indicates the selected isolate.

[b]Initial cell count were 10^5-10^6 cells/ml.

[c]MRS broth.

[d]MRS containing 10% NaCl.

[e]MRS containing 18% NaCl.

more than 18% are *Tetragenococcus* (Hozapfel et al., 2006). To differentiate between 2 groups, all 18 isolates were tested for growth in MRS containing either 10 or 18% NaCl. These results indicated that LAB isolated from fish sauce fermentation might belong to the genus *Tetragenococcus* since all isolates grew in MRS containing 18% NaCl (Table 4.2). In addition, all 18 isolates were also tested for growth in MRS broth that was not added NaCl in order to primarily differentiate between *T. halophilus* and *T. muriaticus*. *T. halophilus* is known that it can grow in the absence of NaCl but *T. muriaticus* required at least 0.5% NaCl for growth (Satomi et al., 1997) All isolates grew in MRS not added NaCl (MRS). Isolates from the 1st month samples grew in MRS containing 10 (MRS10) and 18% NaCl (MRS18) better growth than MRS alone (Table 4.2). The isolates from the 3rd, 5th, and 7th month also grew best at 10 and 18% NaCl (Table 4.2). The results suggested that selected LAB were halophilic LAB with growth characteristics similar to *T. halophilus*.

From Table 4.2, the selected LAB from the 1st month can be separated into 2 groups: The first group including MCD 10-1-1, MRC10-1-3, MRC10-1-4, M9, and M11, showed the highest growth at MRS10 (Table 4.2). The second group, MCD5-1-1 and MS33, grew well at MRS10 and MRS18 (Table 4.2). Thus, MRC10-1-3 and M11 were chosen from the first group while isolates MS33 from the second group were selected for further study. The isolate MS33 was selected due to its higher oligopeptide production than MCD5-1-1. Four isolates from 5th month and 7th were chosen, namely MRC5-5-2, MCD10-5-10, MCD10-5-15, and MRC10-7-8. The isolates MRC5-5-2 and MCD10-5-10 were selected based on their growth in MRS10 and MRS18, respectively (Table 4.2). In addition, MRC5-5-2 could increase

oligopeptide content in FB25 (Table 4.1). The isolate MCD10-5-10 was selected because of its high growth in MRS10 and ability to growth in FB25. The isolate MCD10-5-15 was selected due to its high growth in MRS10 and MRS18. It should be noted that MCD10-5-10 and MCD10-5-15 showed a decrease in oligopeptide content in FB25. This result indicated that these isolates utilized oligopeptide for growth and might produce flavor compounds from oligopeptides. MRC10-7-8 was selected since it showed high growth in MRS18 and FB25. Moreover, it showed the highest oligopeptide content in FB25. Therefore, 7 out of 18 isolates, namely MRC10-1-3, M11, MS33, MRC5-5-2, MRC10-5-10, MRC10-5-15, and MRC10-7-8, were selected.

4.3 Characteristics of halophilic lactic acid bacteria for the use as starter cultures for fish sauce fermentation

4.3.1 Intracellular aminopeptidase activities

Seven selected isolates were assayed for intracellular aminopeptidase activities. All isolates showed high activity toward alanine with activity of 2.85-3.67 U/ml followed by leucine with activity of 1.90-2.37 U/ml (Figure 4.1). Activities toward other substrates, such as methionine, lysine, ariginine, and glutamic, were also detected. Intracellular aminopeptidase activity of the selected isolates showed broad substrate specificity. Amino acid resulted from aminopeptidase activity could be important precursor for volatile compounds formation. Leucine is normally converted to carboxylic acids, such as 2-methylpropanoic acid and 3-methylpropanoic acid

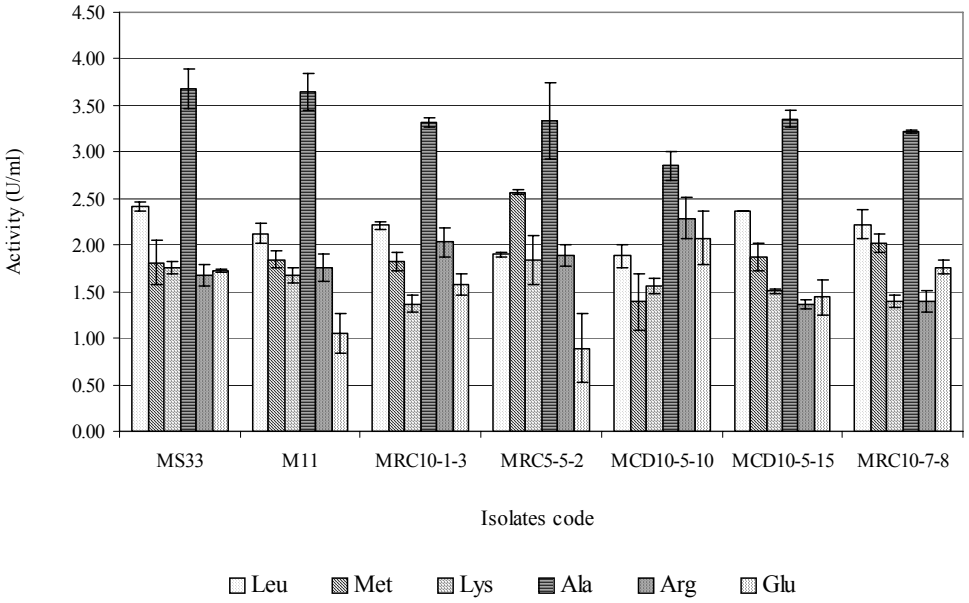

Figure 4.1 Activity of intracellular aminopeptidases of selected LAB on various substrates (Leu = leucine-pNA; Met = methionine-pNA; Lys = lysine-pNA; Ala = alanine-pNA; Arg = arginine-pNA; Glu = glutamic-pNA). Bar indicates the standard deviation.

which contributed to cheesy note, while methionine was converted to methional and methanethiol responsible for boiled potato-like and cooked cabbage flavor, respectively (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001). In addition, leucine catabolism activity may result in 3-methylbutanal, subsequently, converted to 3-methylbutanol and 3-methylbutanoic acid via reduction and oxidation, respectively (Smit, Engels, and Smit, 2009; Yvon and Rijnen, 2001). Intracellular aminopeptidase activities toward glutamic acid could be useful for flavor improvement since glutamic contributed to meaty flavor of fish sauce (Devos et al., 1995; Masson, Hinrichsen,

Talon, and Montel, 1999; Smit et al., 2005). Macedo et al. (2003) reported that aminopeptidase of *Lb. plantarum* was the most active on leucine, followed by arginine, lysine, valine, alanine, and methionine and activities toward glutamic and glycine were not detected. Sanz and Toldra (1997) also reported that aminopeptidase from *Lb. sake* exhibited high activity toward leucine and alanine, resulting in increase free amino acids and small peptides contributing to flavor (Sanz and Toldra, 1997). Therefore, high aminopeptidases activity of the selected LAB might be an important characteristic which contribute to a precursor of aldehydes and other volatile compounds of fish sauce.

4.3.2 Formation of volatile compounds in fish broth

Seven isolates were tested for production of volatile compounds in fish broth containing 25% NaCl (FB25). The role of LAB on volatile compounds formation in fish sauce or high salt products has not been reported. A total of 22 volatile compounds were identified (Table 4.3). They were separated into 4 groups: alcohol; aldehyde; ester; ketone. Alcohols compounds detected were isopropyl alcohol, 1-propanol, 1-butanol, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol, and 1-hexanol. FB25 inoculated MS33, MRC5-5-2, MCD10-5-15, and MRC10-7-8 showed higher isopropyl alcohol content than the control (Table 4.3, $P < 0.05$). Moreover, 1-butanol, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol, and 1-hexanol were only found in samples added MRC10-1-3 (Table 4.3, $P < 0.05$). These results indicated that halophilic LAB played an important role in alcohol formation in fish broth containing high salt (25%). However, most thresholds of alcohol compounds are high (Michihata et al., 2002). Marilley and Casey (2004) reported that LAB could produce alcohol

Table 4.3 Volatile compounds of fish broth containing 25% NaCl inoculated with selected LAB and incubated at 30 °C for 14 days under anaerobic condition.

No.	RI[b]	Compounds	Relative peak area[a]							
			Control	M11	MS33	MRC 10-1-3	MRC 5-5-2	MCD 10-5-10	MCD 10-5-15	MRC 10-7-8
		Alcohols								
8	1011	Isopropyl alcohol	0.063[b]	ND	0.501[a]	ND	0.560[a]	ND	0.354[a]	0.453[a]
10	1041	2-Butanol	ND	ND	0.022	ND	0.003	ND	ND	0.004
11	1049	1-Propanol	0.026[c]	0.001[d]	0.063[b]	0.082[b]	0.104[a]	ND	ND	0.063[b]
13	1110	2-Methyl-1-propanol	ND	ND	0.024	ND	0.019	ND	ND	0.021
14	1185	1-Butanol	ND	0.023[b]	ND	2.638[a]	ND	ND	ND	ND
15	1193	1-Penten-3-ol	ND	0.110[b]	ND	1.64[a]	ND	ND	ND	ND
16	1205	3-Methyl-1-butanol	ND	0.019[b]	ND	0.513[a]	ND	ND	ND	ND
17	1255	1-Pentanol	ND	0.019[c]	0.033[b]	0.119[a]	0.036[b]	ND	ND	0.031[b]
19	1327	(*E*)-2-Penten-1-ol	0.064	ND	0.018	ND	0.018	0.004	0.016	0.017
20	1336	(*Z*)-2-Penten-1-ol	0.018	ND	ND	0.145	ND	ND	ND	ND
21	1360	1-Hexanol	ND	0.072[b]	0.024[c]	0.464[a]	0.020[c]	0.014[c]	ND	0.017[c]
		Aldehydes								
1	784	Propanal	0.030	ND	0.004	ND	0.002	0.001	ND	ND
2	800	2-Methylpropanal	ND	ND	0.372[a]	ND	0.301[a]	0.126[b]	0.325[a]	0.006[c]
6	909	2-Methylbutanal	0.107	ND	0.038	ND	ND	0.028	ND	ND
7	911	3-Methylbutanal	0.091	0.006	0.034	ND	0.018	ND	ND	0.034
12	1097	Hexanal	0.024	ND	ND	ND	0.018	ND	ND	0.016
22	1459	Benzaldehyde	0.004[c]	0.061[b]	0.039[b]	0.755[a]	0.034[b]	0.045[b]	0.033[b]	0.034[b]

Table 4.3 (continued)

No.	RI[b]	Compounds	Relative peak area[a]							
			Control	M11	MS33	MRC 10-1-3	MRC 5-5-2	MCD 10-5-10	MCD 10-5-15	MRC 10-7-8
		Ester								
4	863	Ethyl acetate	0.101	0.013	0.045	0.119	0.044	0.005	0.045	0.051
		Ketones								
3	809	Acetone	0.202	0.292	0.391	0.513	0.313	0.054	ND	0.360
5	888	2-Butanone	ND	0.018	0.020	0.296	ND	ND	ND	0.021
9	1028	2,3-Butanedione	ND	0.007	0.011	0.111	0.008	ND	ND	0.003
18	1315	Cyclohexanone	ND	ND	0.086	ND	0.080	ND	ND	0.084

Note: Bacterial cell count of all isolates were 10^7-10^8 cell/ml; ND= not detected.
Different superscripts within a row indicate significant differences ($P < 0.05$).
[a]The values represent the ratio of the peak area of any compound to that of internal standard (cyclohexanol).
[b]Retention indices calculated for DB-WAX column using n-alkanes as standards.

from proteins, lipids, and lactose via proteolysis, lipolysis, and glycolysis pathway, respectively. Helinck, Bars, Moreau, and Yvon (2004) reported that *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus* and *Streptococcus thermophilus* produced large amount of 3-methylbutanol from phenylalanine, valine, and leucine. Amino acids were converted to α-keto isocaproic acid by a transamination reaction, which requires the presence of an α-ketoglutarate as the amino group acceptor (Smit, Smit, and Engels, 2005). α-Keto isocaproic acid was further converted to 2-methylbutanal and 3-methylbutanal by decarboxylase activity, followed by alcohol dehydrogenase reduction to 2-methylbutanol and 3-methylbutanol, respectively (Smit, Engels, and Smit, 2009).

Major aldehydes compounds were identified as 2-methylpropanal and benzaldehyde. FB25 samples added MS33, MRC5-5-2, and MCD10-5-15 showed higher amount of 2-methylpropanal than the control (Table 4.3, $P < 0.05$). Moreover, FB25 samples added MRC10-1-3 contained the highest amount of benzaldehyde ($P < 0.05$). Addition of the selected LAB did not increase 2-methylbutanal and 3-methylbutanal when compared to the control (Table 4.3). Branched-chain aldehydes, such as 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal are potent flavor compounds.

In many food products, aldehydes are key flavor compounds. They are generally perceived as malty and meaty note that is desirable odor (Marilley and Casey, 2004). An important process leading to the formation of compounds like 3-methylbutanal is the non-enzymic, heat-induced, Strecker degradation of amino groups with reducing sugar moieties (Fukami et al., 2002; Sanceda et al., 1992).

Benzaldehyde derived from aromatic amino acids (phenylalanine) and contributed to bitter almond (Curioni and Bosset, 2002). Smit, Engels, and Smit (2009) reported that 2-methylpropanal, 2-methylbutanal and 3-methylbutanal were produced from amino acid catabolism. Initial step involves transamination of leucine, valine, and isoleucine, and subsequently aldehyde decarboxylation. Besides enzymatic conversion, chemical oxidation of α-keto isocaproic acid is catalyzed by Mn^{2+} and results in 2-methylpropanal. The activity can be modulated by the Mn^{2+} and oxygen concentration (Smit and Engels, 2004).

Less amount of ethyl acetate was found in all samples (Table 4.3). Aldehydes was reduced to alcohols and oxidized to carboxylic acid. Alcohols and carboxylic acid are substrates for esterase leading to ester (Marilley and Casey, 2004). Acetone, 2-butanone, 2,3-butanedione, and cyclohexanone were found in varied amount (Table 4.3). Therefore, some isolates (MS33, MRC5-5-2, and MCD10-5-15) of LAB showed potential to increase meaty odor at high salted fermented products. Sulfur-containing compounds, such as dimethyl sulfide and dimethyl disulfide which contributed to undesirable fecal note in fish sauce (Fukami et al., 2004) were not detected in FB25 inoculated with selected LAB. This result indicated that all selected LAB are unlikely to cause undesirable odor in fish sauce product. From these results, 7 selected LAB showed different profile of volatile compounds production in FB25 and demonstrated the potential of flavor production in high salt environment.

4.3.3 Biogenic amines-forming ability of selected LAB

Seven isolates were also tested for biogenic amines production using mGYP medium containing 5 and 25% NaCl fortified with amino acids. Cell counts of all

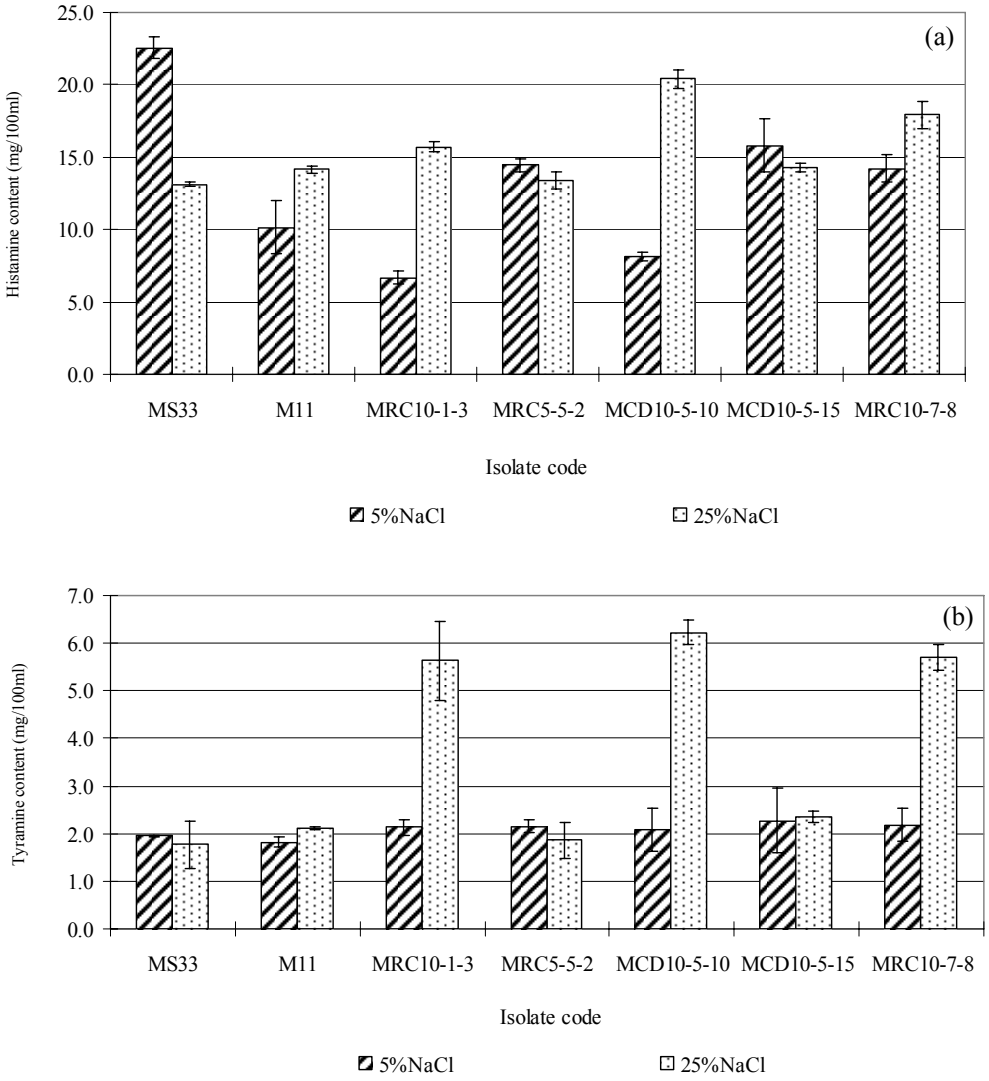

Figure 4.2 Histamine (a) and tyramine content (b) of the selected isolates in mGYP medium containing 5 and 25% NaCl and incubated at 30 °C for 5-7 days under anaerobic condition.

isolates were 7-8 Log CFU/ml. All isolates produced histamine in mGYP containing 5 and 25% NaCl in the range of 6.62-22.55 and 13.14-20.39 mg/100ml, respectively (Figure 4.2a). It was evident that formation of histamine could not be prevented even

at high salt concentration (25% NaCl). Formation of histamine during fish sauce fermentation could be partly contributed from halophilic LAB. According to Karnop (1988), *T. halophilu*s was the main histamine-producing bacteria in semi-preserved anchovies. Thongsanit et al. (2002) reported that *T. halophilus* and *T. muriaticus* isolated from fish sauce produced histamine in histidine-containing medium at 10% NaCl in the level of 4.46 and 52.29 mg/100ml, respectively. Kimura et al. (2001) reported that *T. muriaticus* isolated from squid liver sauce was classified as histamine-producing bacteria (Satomi et al., 1997). It was able to produce histamine at NaCl concentration ranging from 3 to 20%. The optimal NaCl concentration for histamine formation of *T. muriaticus* was 5-7%.

It also produced 120 mg/100ml histamine at 20% NaCl in mGYP medium (Kimura et al., 2001). Thus, both *T. muriaticus* and *T. halophilus* are prevalent in salted food products and might play a role in histamine formation of such products.

Other important biogenic amine produced by selected LAB was tyramine with lower amount than histamine (Figure 4.2b). Tyramine contents in mGYP containing 5% NaCl of all 7 isolates were comparable (1.70-2.20 mg/100ml). Some isolates (MRC10-1-3, MCD10-5-10, and MRC10-7-8) showed higher tyramine level at 25% NaCl (5.60-6.20 mg/100ml) than at 5% NaCl (1.90-2.20 mg/100ml) (Figure 4.2b). Bover-Cid and Holzapfel (1999) reported that tyramine was the main amine formed by *Lb. curvatus*, *Lb. brevis*, and *Lb. buchneri*. These LAB produced tyramine ranging from 2.0-500.0 mg/100ml. Pons-Sánchez-Cascado et al. (2005) reported that *Lactococcus lactis* and *Pediococcus pentosaceous* isolated from ice-preserved anchovies showed a great tyrosine decarboxylase activity.

Histamine and tyramine are the most important biogenic amines due to their toxicological effect (Bover-Cid and Holzapfel, 1999). Other biogenic amines were not detected in all selected isolates. It should be mentioned that the medium used in this study was fortified by amino acids, served as substrates for amino decarboxylase. Such a condition is likely to be different from fish sauce fermentation. Substrates of amino decarboxylase in the fermentation tank are fish protein that might not be totally available for the enzyme as compared to amino acids directly added in mGYP medium. Therefore, the ability of these selected LAB to produce biogenic amines in the fermentation tank could be lower than in mGYP medium.

4.4 Identification of halophilic lactic acid bacteria

4.4.1 Phenotypic and biochemical characteristics of bacterial isolates

The selected LAB were Gram positive cocci with cell arrangement as pairs/tetrads form (Figure 4.3). Colonies on MRS agar were 1-2 mm, white, circular, low convex, and smooth. These isolates were initially identified using API system. All selected isolates were biochemically identified as *Carnobacterium piscicola* and *Lactobacillus plantarum* (Table 4.4). However, the results from API identification were not logical because all 7 isolates were cocci while *C. piscicola* and *Lb. plantarum* are rod shape.

Based on cell morphology, cell arrangement, and ability to growth at 18% NaCl of the selected LAB were similar to *T. halophilus* (Table 4.5). Therefore, physiological and biochemical characteristics of the selected LAB were compared with *T. halophilus*^{T}.

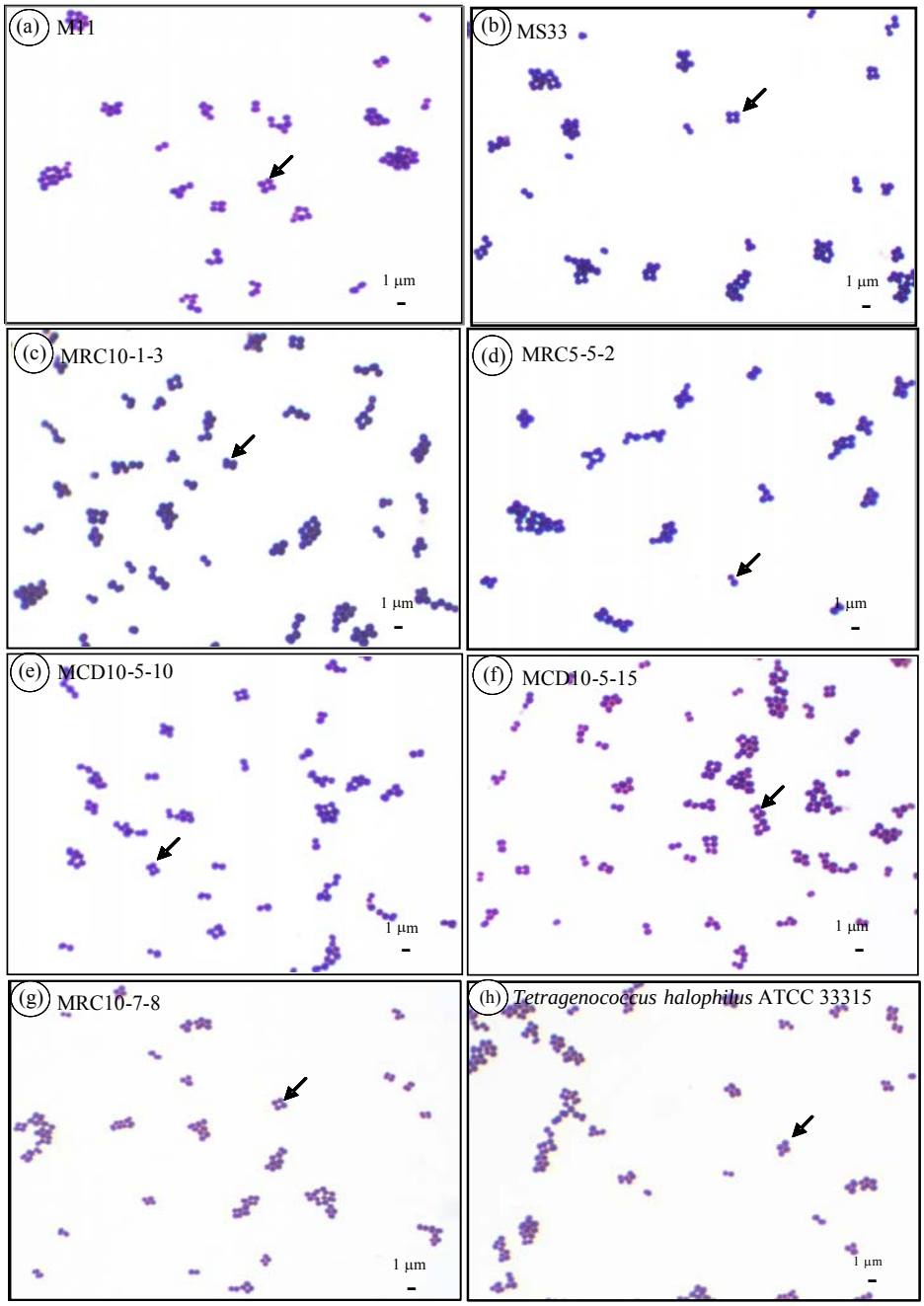

Figure 4.3 Gram stain and cell arrangement of selected LAB isolated from 1st month (a-c), 5th (d-f), 7th (g) and *T. halophilus* ATCC 33315 (h).

Table 4.4 Comparison of physiological characteristics of 7 selected isolates and identification results according to biochemical characteristics.

Month of fermentation	Bacterial Isolate code	Gram stain	Cell shape/ Arrangement/ Size (μm)	Oxidase	Catalase	Proteinase[a]	Lipase[b]	Amylase[c]	Fermentive metabolism		Identification result according to biochemical characteristics[d]	
									Homo-	Hetero-	Closest relative	Identity (%)
1	MRC 10-1-3	+	Cocci/Pairs, Tetrads/0.88-1.00	-	-	-	-	-	+	-	*Carnobacterium piscicola*	NR
	M11	+	Cocci/Pairs, Tetrads/0.69-0.89	-	-	+	+	-	+	-	*Lactobacillus plantarum*	NR
	MS33	+	Cocci/Pairs/ 0.68-0.88	-	-	+	+	-	+	-	*Lactobacillus plantarum*	NR
5	MRC 5-5-2	+	Cocci/Tetrads/ 0.68-0.98	-	-	-	-	-	+	-	*Carnobacterium piscicola*	NR
	MCD 10-5-10	+	Cocci/Pairs, Tetrads/0.59-0.78	-	-	-	-	-	+	-	*Lactobacillus plantarum*	NR
	MCD 10-5-15	+	Cocci/Pairs, Tetrads/0.59-0.88	-	-	-	-	-	+	-	*Lactobacillus plantarum*	61.9
7	MRC 10-7-8	+	Cocci/Pairs, Tetrads/0.78-0.89	-	-	-	-	-	+	-	*Lactobacillus plantarum*	NR

[a]Casein hydrolysis; [b]Tween80 hydrolysis; [c]Starch hydrolysis; [d]API system (API 50CH/L for lactic acid bacteria; bioMérieux).

NR = Not reported.

Table 4.5 Comparison of phenotypic characteristics of the selected lactic acid bacteria and *Tetragenococcus halophilus*.

Characteristics	Type culture strain		Bacterial isolate from fish sauce sample[c]						
	T. halophilus[a] ATCC 33315	*T. halophilus*[b] JCM 5888	M11	MS33	MRC10-1-3	MRC5-5-2	MCD10-5-10	MCD10-5-15	MRC10-7-8
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Tetrads	Tetrads	Pairs, Tetrads	Pairs, Tetrads	Pairs, Tetrads	Pairs, Tetrads	Pairs, Tetrads	Pairs, Tetrads	Pairs, Tetrads
Cell size (μm)	0.5-1.0	0.5-1.0	0.69-0.89	0.68-0.88	0.88-1.0	0.68-0.98	0.59-0.78	0.59-0.88	0.78-0.88
Catalase	-	-	-	-	-	-	-	-	-
Growth at 40 °C	-	+	+	+	+	+	+	+	+
45 °C	-	NA	-	-	+	+	+	-	+
Growth at pH 4.5	+	NA	+	+	+	+	+	+	+
5.0	+	NA	+	+	+	+	+	+	+
6.5	+	NA	+	+	+	+	+	+	+
7.0	+	NA	+	+	+	+	+	+	+
8.0	+	NA	+	+	+	+	+	+	+
9.0	+	NA	+	+	+	+	+	+	+
Growth at 0% NaCl	+	NA	+	+	+	+	+	+	+
15%	+	NA	+	+	+	+	+	+	+
20%	+	NA	+	+	+	+	+	+	+
25%	+	NA	+	+	+	+	+	+	+
Optimum NaCl (%)	5-10	NA	5-10	5-10	5-10	5-10	5-10	5-10	5-10
Gas from D-glucose	-	-	-	-	-	-	-	-	-
Acid from:									
Amygdalin	+	+	+	+	+	+	+	+	-

Table 4.5 (continued)

Characteristics	Type culture strain		Bacterial isolate from fish sauce sample[c]						
	T. halophilus[a] ATCC 33315	*T. halophilus*[b] JCM 5888	M11	MS33	MRC10-1-3	MRC5-5-2	MCD10-5-10	MCD10-5-15	MRC10-7-8
L-Arabinose	+	+	-	-	-	-	-	-	-
D-Arabinose	+	NA	-	-	-	-	-	-	-
D-Cellobiose	+	+	+	+	+	+	+	+	+
Dextrin	-	NA	NA	NA	NA	NA	NA	NA	NA
Esculin	+	NA	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	-	+	+	+	+	-
D-Lactose	-	-	-	-	-	-	-	-	-
D-Maltose	+	+	+	+	+	+	+	+	-
D-Raffinose	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	NA	-	-	-	-	-	-	-
D-Mannitol	-	-	+	+	-	+	+	+	+
D-Mannose	+	NA	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-	-	-
D-Saccharose	+	+	+	-	+	+	+	+	+
D-Melezitose	+	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	+	NA	NA	NA	NA	NA	NA	NA	NA
D-Ribose	+	+	+	+	+	+	+	+	+
Salicin	+	NA	+	+	+	+	+	+	-
D-Sorbitol	-	-	+	+	-	-	+	+	+
L-Sorbose	-	NA	NA	NA	NA	NA	NA	NA	NA

Table 4.5 (continued)

Characteristics	Type culture strain		Bacterial isolate from fish sauce sample[c]						
	T. halophilus[a] ATCC 33315	*T. halophilus*[b] JCM 5888	M11	MS33	MRC10-1-3	MRC5-5-2	MCD10-5-10	MCD10-5-15	MRC10-7-8
D-Trehalose	+	-	+	-	+	+	+	+	-
D-Xylose	-	+	-	-	-	-	-	-	-
L-Xylose	-	NA	-	-	-	-	-	-	-
Xylitol	-	NA	-	-	-	-	-	-	-
Gentiobiose	+	NA	+	+	+	+	+	+	-
D-Turanose	+	+	+	-	+	+	+	+	+
D-Lyxose	-	NA	-	-	-	-	-	-	-
D-Tagatose	+	-	-	-	+	-	-	-	-
D-Fucose	-	NA	-	-	-	-	-	-	-
L-Fucose	-	NA	-	-	-	-	-	-	-
D-Arabitol	+	+	+	+	+	+	+	+	-
L-Arabitol	-	NA	-	-	-	-	-	-	-
Configuration of lactic acid	L	L	L	L	L	L	L	L	L

NA = Not available.

[a]Phenotypic characteristics were tested of this study.

[b]Ennahar and Cai (2005)

[c]M11, MS33, and MRC10-1-3 isolated from the 1st month fermentation; MRC5-5-2, MCD10-5-10, and MCD10-5-15 isolated from the 5th month fermentation; MRC10-7-8 isolated from the 7th month fermentation.

These 7 isolates showed different characteristics to the type strain (Table 4.5). Seven isolates grew at 0-25% NaCl, pH 4.5-9.0 and produced acid from D-cellobiose, esculin, D-galactose, D-mannose and D-ribose but not L-arabinose, lactose, D-melibiose, D-melezitose, raffinose, and rhamnose (Table 4.5).

Isolate MRC10-7-8 did not ferment amygdalin, maltose and salicin. In this study, 4 isolates (MRC10-1-3, MRC5-5-2, MCD10-5-10 and MRC10-7-8) grew at 40 and 45 °C, while the others (M11 and MS33) grew at temperature up to 40°C. Only L-lactic acid was produced by 7 isolates (Table 4.5).

4.4.2 Genotypic characterization

The genomic DNA was extracted from 7 isolates and used for 16S rDNA amplification. The size of amplified DNA fragments was about 1,500 bp using fD1/rP2 primer (Figure 4.4).

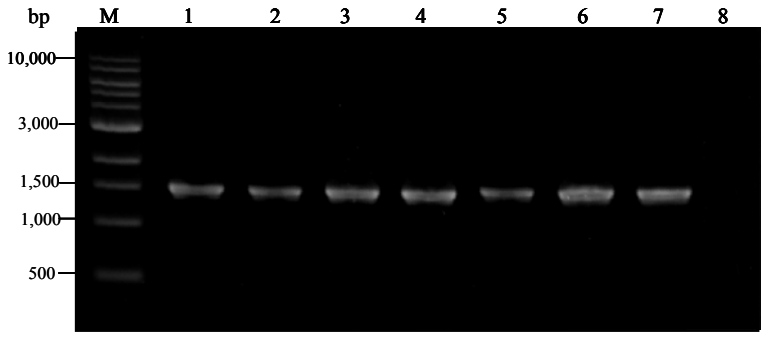

Figure 4.4 Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rDNA using primer fD1/rP2. Lanes M, Molecular weight marker (1-kb ladder, Fermentas Life Sciences); 1, M11; 2, MS33; 3, MRC10-1-3; 4, MRC5-5-2; 5, MCD10-5-10; 6, MCD10-5-15; 7, MRC10-7-8; and 8, negative control.

The similarity of 16S rDNA sequence of 7 isolates compared with NCBI blast of *T. halophilus* were 99% (Table 4.6).

Table 4.6 Similarity of 16S rRNA gene sequence of lactic acid bacteria isolates compared with other bacteria from NCBI Nucleotide sequence database (U.S.A.).

Bacterial isolate code	Length of sequence (bp)	Nucleotide sequence comparison, identification results and details				
		Closest relative	Length of sequence (bp)	Sequence homology (%)	NCBI Accession no.	Isolation source/ remark of closest relative
M11	1520	*Tetragenococcus halophilus* ATCC 33315	1558	99.0	D88668	Traditional Chinese fermented soybean paste
		Tetragenococcus koreensis strain DSM 16501	1492	97.0	AY690334	Traditional Korean food (Kimchi)
MS33	1522	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				
MRC 10-1-3	1522	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				
MRC 5-5-2	1521	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				
MCD 10-5-10	1520	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				
MCD 10-5-15	1520	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				
MRC 10-7-8	1522	*T. halophilus* ATCC 33315 and *T. koreensis* DSM 16501 with the same sequence homology as isolate M11				

Similarity of 7 isolates when compared to a type strain, *T. halophilus* ATCC 33315, *T. koreensis* DSM 16501, *T. solitarius* DSM 5634, and *T. muriaticus* JCM 1006 were 97-98, 95, 92-93 and 92%, respectively (Table 4.7). Sequence similarity with type strains of *Enterococcus* sp. and *Vagococcus* sp. were 89-90% and 83-91%, respectively (Table 4.7). These results demonstrated that 7 isolates appeared to be closely related to the genus *Tetragenococcus*.

When phylogenetic tree analysis was performed, all seven isolates fell in the same cluster of *T. halophilus* ATCC 33315 (Figure 4.5). Therefore, seven isolates of LAB isolated from fish sauce fermentation in this study are identified as *T. halophilus*. *Tetragenococcus* has been found in fish sauce fermentation (nam-pla) by Thongsanit et al. (2002). Forty strains were identified as *T. halophilus* and 38 strains were identified as *T. muriaticus*. In this study, only *T. halophilus* was found throughout the fermentation period of 1^{st}-7^{th} month. *T. halophilus* might be the prevalent LAB in fish sauce fermentation. The distinct phenotypic difference between these 2 species is that *T. halophilus* can grow in the absence of NaCl but *T. muriaticus* required NaCl concentration at least 0.5% for growth (Satomi et al., 1997). *T. halophilus* found in this study also grew in the absence of NaCl. *T. koreensis* was another species isolated from kimchi (Lee et al., 2005). It grows at 0-8% NaCl with the optimum at 2-5% NaCl. Therefore, *T. koreensis* was unlikely to be found in fish sauce fermentation with high salt content.

Although 16S rDNA sequence confirmed the taxonomic identity of 7 isolates as *T. halophilus*, these isolates showed a wide variation in phenotypic characteristics among strains (Table 4.5).

Table 4.7 Similarity of 16S rRNA gene sequence of all isolated LAB and related species.

	Bacterial isolates from fish sauce samples							Type culture strains[a]										
Bacterial	MS33	M11	MCD 10-5-10	MRC 10-7-8	MRC 5-5-2	MRC 10-1-3	MCD 10-5-15	1	2	3	4	5	6	7	8	9	10	11
MS33	100																	
M11	99	100																
MCD10-5-10	99	98	100															
MRC10-7-8	99	99	99	100														
MRC5-5-2	99	99	99	99	100													
MRC10-1-3	99	99	99	99	99	100												
MCD10-5-15	99	99	99	99	99	99	100											
1	97	97	97	98	98	98	98	100										
2	95	95	95	95	95	95	95	95	100									
3	95	95	95	95	95	95	95	95	93	100								
4	92	92	92	93	92	92	92	94	92	92	100							
5	90	90	90	90	90	90	90	92	90	90	91	100						
6	89	89	89	90	90	90	90	90	92	89	89	96	100					
7	83	83	83	84	84	84	84	83	84	82	81	85	88	100				
8	83	83	83	84	84	83	83	82	84	82	82	86	87	90	100			
9	87	87	87	88	87	87	87	88	90	86	87	91	93	89	90	100		
10	91	91	91	91	91	91	91	90	90	91	89	93	93	87	87	91	100	
11	85	85	85	86	86	86	86	86	88	84	86	89	91	88	88	91	88	100

Note; [a] 1: *Tetragenococcus halophilus* ATCC 33315 (D88668), 2: *Tetragenococcus koreensis* DSM 16501 (AY690334), 3: *Tetragenococcus solitarius* DSM 5634 (AJ301840), 4: *Tetragenococcus muriaticus* JCM 10006 (D88824), 5: *Enterococcus saccharominimus* LMG 22197 (AJ626904), 6: *Enterococcus saccharolyticus* ATCC 43076 (DQ411816), 7: *Vagococcus salmoninarum* NCFB 2777 (X54272), 8: *Vagococcus fluvialis* NCDO 2497 (X54258), 9: *Vagococcus lutrae* CCUG 39187 (Y17152), 10: *Enterococcus faecalis* ATCC 29200 (DQ239694), 11: *Vagococcus fessus* ATCC BAA-289 (AJ243326).

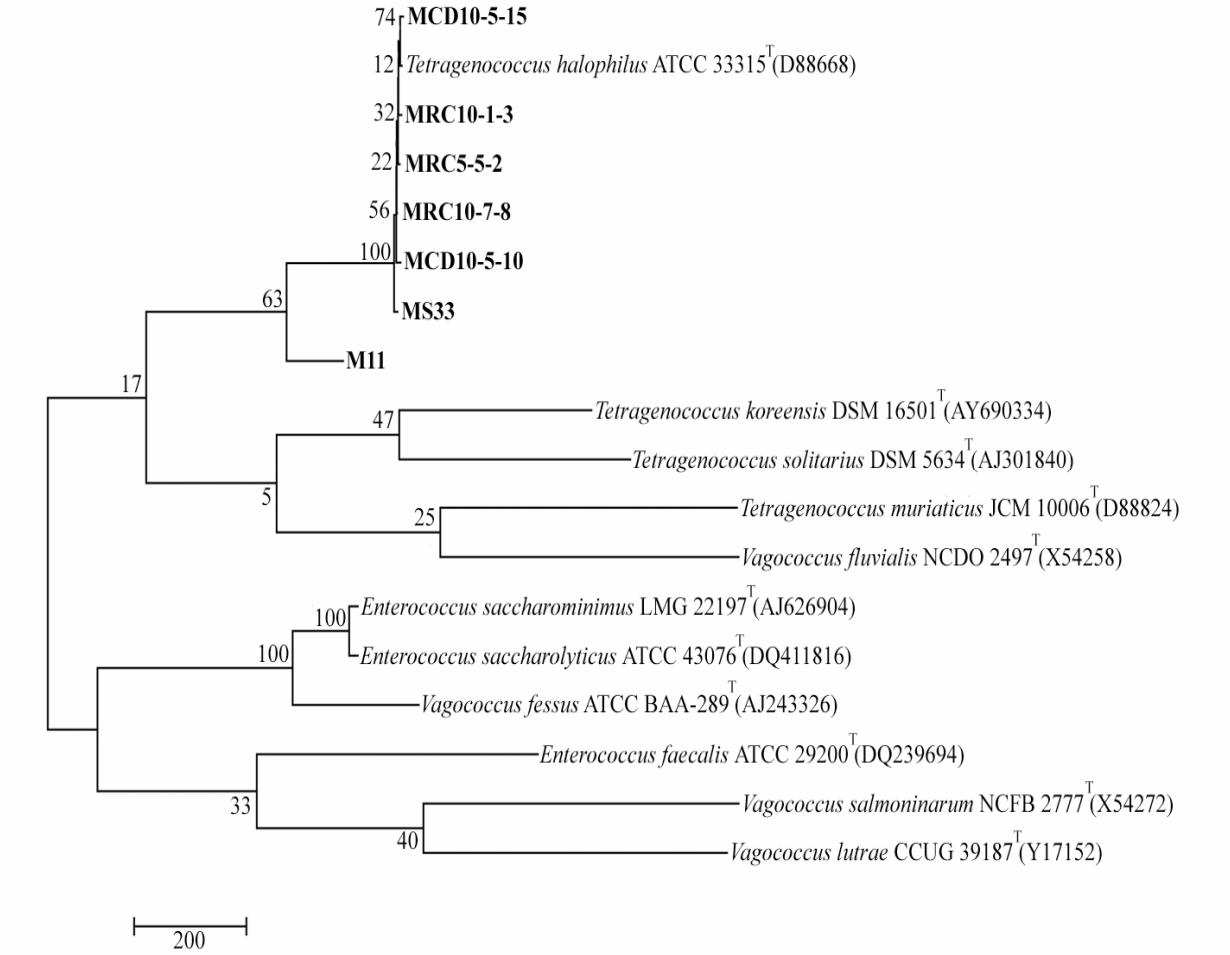

Figure 4.5 Phylogenetic tree of lactic acid bacteria isolated from fish sauce fermentation, based on 16S rRNA gene sequence data. Bar indicates 200 substitutions per nucleotide position.

Variations in phenotypic characteristics, such as sugar fermentation and growth temperature, among *T. halophilus* have been reported (Kobayashi et al., 2003; Thongsanit et al., 2002). *T. halophilus* IAM 1676 grew at 40 °C and produced acid from L-arabinose (Kobayashi et al., 2003), while *T. halophilus* ATCC 33315 did not grow at 40 °C and did not produce acid from L-arabinose (Thongsanit et al., 2002).

Chen et al. (2005) obtained 29 isolates from fermented mustard and identified as *T. halophilus*, which could grow at 45 °C. Kobayashi et al. (2003) and Holzapfel et al. (2006) reported that genus *Tetragenococcus* produced L-lactic. Collin et al. (1990) reported that L-lactic is the major end product of glucose metabolism of *Tetragenococcus*, while D-lactic is typically produced in trace amount. However, to confirm these results, restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene was performed. The patterns of fragments obtained from *Alu*I and *Mbo*I of 7 isolates were similar to those of the type strain of *T. halophilus* ATCC 33315 (Figure 4.6a, b). Therefore, it can be confirmed that all isolates were *T. halophilus*. Previously, Kobayashi et al. (2003) used 16S rDNA sequence and RFLP analysis to identify LAB that were isolated from shrimp paste. They were identified as *T. halophilus*. Similarly, Chen et al. (2006) isolated LAB from fermented mustard and were identified as *T. halophilus* based on RFLP and 16S rDNA sequence.

However, *T. halophilus* found in this study showed different characteristics from those previously reported. They showed ability to hydrolyze fish protein, milk protein (casein), lipid (Tween80), and production of volatile compounds in fish broth containing 25% NaCl. *Tetragenococcus* is the only genus of LAB that was halophilic. *T. halophilus* was usually found in food containing high salt concentration. For example, *T. halophilus* was isolated from soy sauce (Hanagata et al., 2003; Tanasupawat, Thongsanit, Okada, and Komagata, 2003), salted anchovies (Villar et al., 1985), fermented fish (Tanasupawat and Daengsubha, 1983), Japanese moromi(Sakaguchi and Mori, 1969), Indonesian baceman type (Roling and Van Versefeld, 1996), and squid liver sauce (Satomi et al., 1997).

The salt and sugar tolerance of *T. halophilus* was enhanced by uptaking of

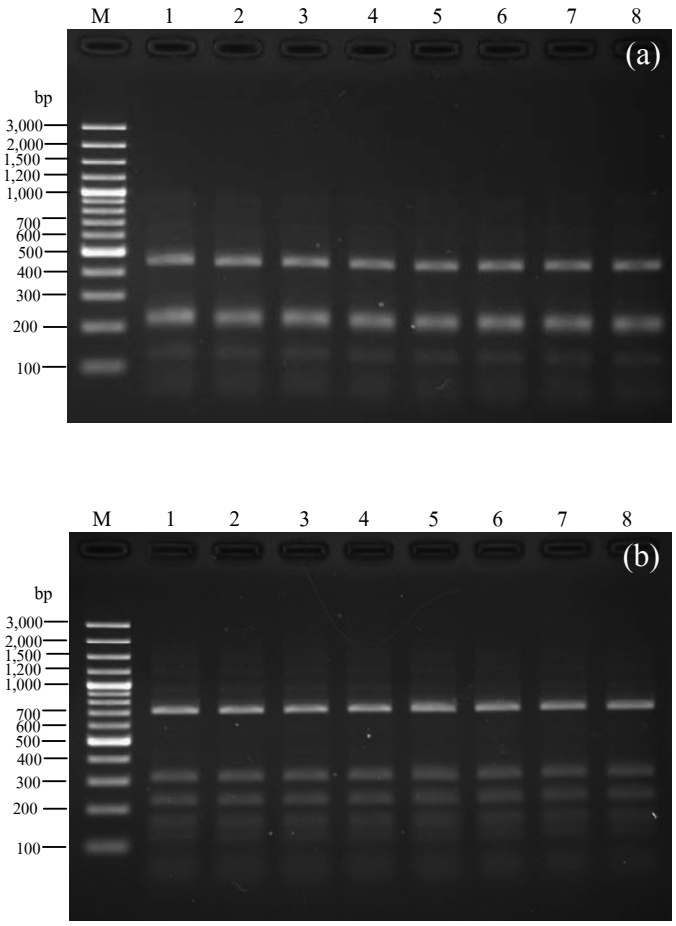

Figure 4.6 RFLP patterns of all LAB isolates and *T. halophilus* ATCC 33315 obtained by digestion of 16S rDNA with restriction endonucleases *Alu*I (a) and *Mbo*I (b). Lane M, Molecular weight marker (100-bp ladder); 1, M11; 2, MS33; 3, MRC10-1-3; 4, MRC5-5-2; 5, MCD10-5-10; 6, MCD10-5-15; 7, MCD10-7-8; 8, *T. halophilus* ATCC 33315.

glycine betaine for osmotic balance between inside and outside of the cells (Csonka and Epstein, 1996; Kempf and Bremer, 1998). Therefore, *T. halophilus* can survive in high salt and sugar concentration. Recently, *T. halophilus* also appeared as the dominant microflora during storage of sugar thick juice (Justé et al., 2008).

4.5 The use of lactic acid bacteria as starter cultures for fish sauce Fermentation

Four selected isolates namely, *T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10, and *T. halophilus* MRC10-7-8, were selected from 4.2.3 based on their volatile compounds formation. *T. halophilus* MS33 and *T. halophilus* MRC10-7-8 also produced many volatile compounds in FB25 and showed distinctive odor. *T. halophilus* MRC10-1-3 produced the highest alcohol content in FB25 and MCD10-5-10 appeared to produce different odor from others. Starter cultures from these 4 isolates were prepared for fish sauce fermentation.

4.5.1 Microbiological changes

The initial LAB counts of inoculated fish sauce mash on MRS agar containing 18% NaCl added 0.5% $CaCO_3$ (MRS18%+0.5% $CaCO_3$) were 5 Log CFU/ml (Figure 4.7a). After 60 days of fermentation, LAB count decreased to 3-4 Log CFU/ml (Figure 4.7a) and was not detected at 90 days, whereas LAB in the control could not be enumerated. Therefore, bacteria found on MRS agar were likely to be the inoculated LAB. The bacterial count of the control on JCM 168 medium could not be detected at the first day (Figure 4.7b). Since the samples were heated for 6 h at 50-60 °C, halophilic/halotolerant bacteria could be thermally inactivated. Bacterial counts in JCM 168 media showed the same profile to MRS18%+0.5% $CaCO_3$.

When colonies grown in JCM 168 medium were randomly selected and re-streaked on MRS18%+0.5% $CaCO_3$, they grew under anaerobic condition (Figure 4.8a, b). In addition, they were catalase negative, Gram-positive cocci with pairs/tetrads cell arrangement.

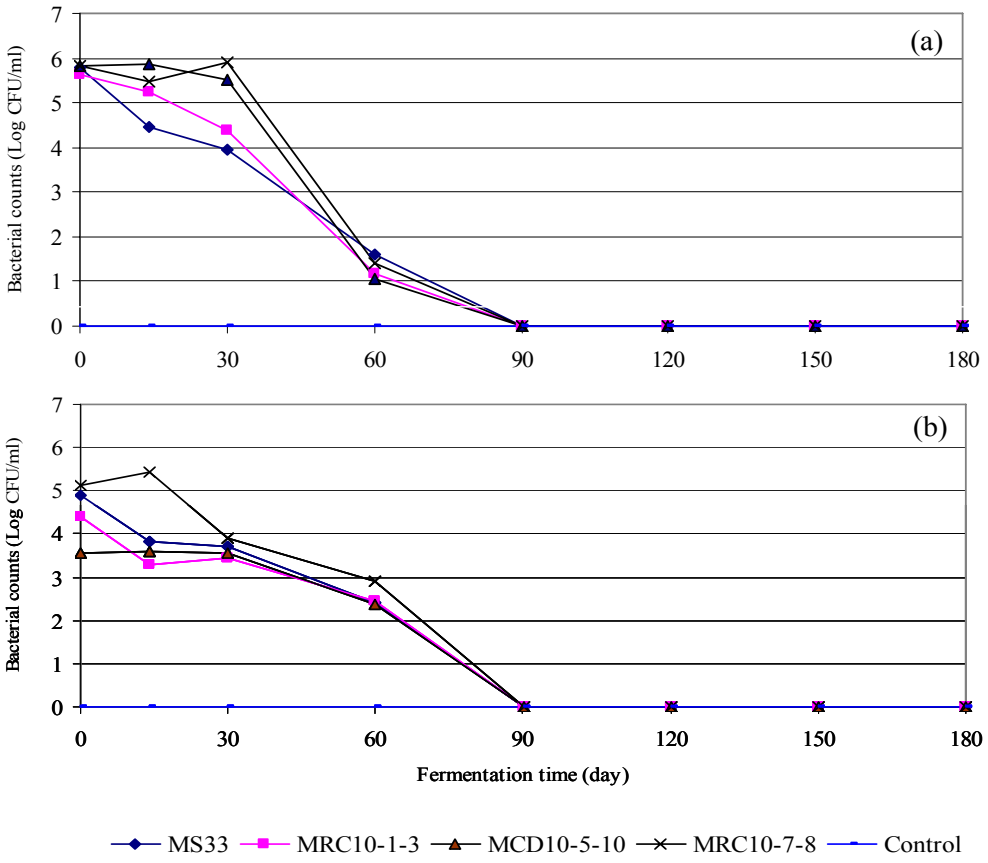

Figure 4.7 Changes in lactic acid bacteria counts on MRS agar containing 18% NaCl added 0.5% $CaCO_3$ incubated at 30 °C under anaerobic condition for 3-5 days (a) and halophilic bacteria counts on JCM 168 containing 18% NaCl incubated at 35 °C under aerobic condition for 3-5 days (b) of fish sauce samples inoculated with bacterial starter cultures and incubated at 35 °C for 6 months (control = sample without addition of starter culture).

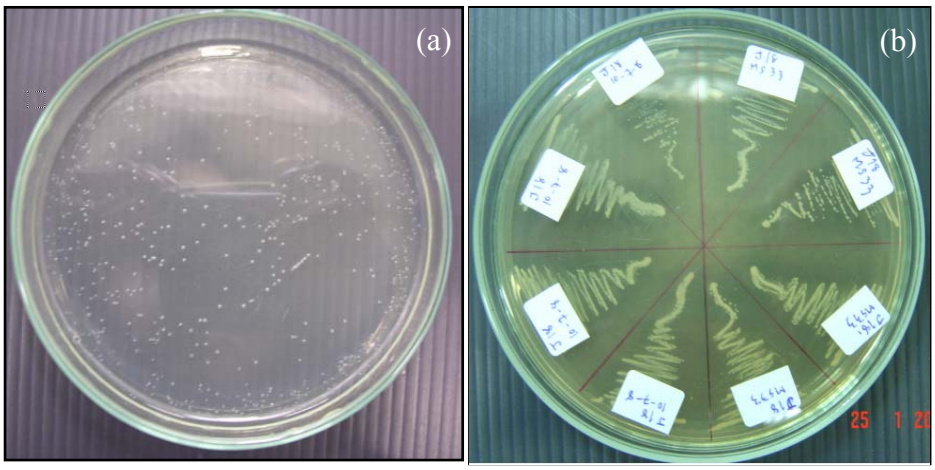

Figure 4.8 Bacteria counts from fish sauce fermentation inoculated with LAB on JCM 168 containing 18% NaCl incubated at 35 °C under aerobic condition for 5-7 days (a) and colonies selected from JCM168 on MRS agar containing 18% NaCl added 0.5% $CaCO_3$ incubated at 30 °C under anaerobic condition for 5-7 days (b).

These results suggested that colonies grew in JCM 168 medium were likely to be the inoculated LAB. Leisner et al. (2001) reported that bacterial counts on plat count agar (PCA) in acid-fermented condiment (tempoyak) were LAB. Colonies of LAB on PCA were small, grey or white, catalase negative, and able to grow under aerobic condition (Leisner et al., 2001).

4.5.2 α–Amino acid content

α–Amino acid content of fish sauce samples added LAB starter cultures were 779-784 mM and higher than that of the control at 6 months ($P < 0.05$, Table 4.8). Although population of all starter cultures added into fish sauce fermentation did not

Table 4.8 α–Amino acid content (mM) of fish sauce samples inoculated with LAB bacterial starter cultures and incubated at 35 °C for 6 months.

Fermentation time (day)	control	MS33	MRC10-1-3	MCD10-5-10	MRC10-7-8
0	436.49±24.11	445.87±38.58	462.92±4.82	458.65±42.20	487.64±4.82
14	584.08±28.09^{b}	636.12±32.76^{ab}	643.48±20.26^{ab}	691.74±51.14^{a}	657.00±38.17^{ab}
30	644.89±40.45	684.14±23.51	648.08±52.08	676.95±27.80	687.65±24.58
60	611.60±85.11	679.52±69.84	672.03±80.43	711.51±27.00	701.15±87.47
90	676.13±15.55	726.92±40.20	672.91±67.14	744.95±38.79	730.49±52.01
120	658.64±1.57	704.50±8.52	727.01±32.38	740.35±79.42	744.61±69.28
150	691.46±12.75	724.17±7.94	760.58±15.03	786.15±45.81	796.73±78.26
180	707.46±3.58^{b}	783.96±19.18^{a}	783.38±13.28^{a}	779.65±32.58^{a}	782.41±10.92^{a}

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

increase during fermentation, α-amino content increased in all samples when compared to the control (Table 4.8). Although *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10, and *T. halophilus* MRC10-7-8 did not hydrolyzed skim milk agar, they appeared to hydrolyze fish protein during fish sauce fermentation, resulting in an increase of α–amino acid content.

An increase of α-amino content indicated an increase of oligopeptides and/or free amino acids of these samples. LAB could use free amino acids resulted from protein hydrolysis to produce flavor compounds (Law and Haandrinkman, 1995; Magboul and McSweeney, 1999). These results indicated that all selected LAB strains (*T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8) accelerated the protein hydrolysis during fish sauce

fermentation. This is the first report demonstrating ability to hydrolyze fish protein of *T. halophilus*.

4.5.3 Biogenic amines content in fish sauce

Tryptamine, putrescine, cadaverine and histamine were biogenic amines found in 6 month-old fish sauce, while tyramine, spermine, and spermidine were not detected in all samples (Table 4.9). Biogenic amines were resulted from amino decarboxylase acitivity produced by bacteria. Several LAB such as *Lb. curvatus*, *Lb. buchneri*, *Lb. brevis*. *Leuconostoc* spp., and *Pediococcus* spp. are known to produce decarboxylase enzymes, resulting in the formation of histamine, tryptamine and tyramine (Bover-Cid and Holzapfel, 1999; Christensen et al., 1999; McSweeney and Sousa, 2000).

Table 4.9 Biogenic amines contents (mg/100ml) of fish sauce samples inoculated with LAB starter cultures and incubated at 35 °C for 6 months.

Fish sauce sample	Tryptamine	Putrescine	Cadaverine	Histamine
Control	16.03 ± 1.06	ND	2.40 ± 0.50^{b}	10.24 ± 3.12^{a}
MS33	14.39 ± 1.15	ND	ND	6.06 ± 0.01^{ab}
MRC10-1-3	15.66 ± 1.80	ND	2.48 ± 0.74^{b}	4.50 ± 1.73^{b}
MCD10-5-10	12.90 ± 1.64	2.33 ± 1.76	6.12 ± 2.70^{a}	4.50 ± 1.94^{b}
MRC10-7-8	13.70 ± 1.62	ND	2.10 ± 0.01^{b}	7.34 ± 3.33^{ab}

Different superscripts within a column indicate significant differences ($P < 0.05$).
ND = Not detected.

Tryptamine was dominant biogenic amine while putrescine was negligible. Fish sauce fermentation carried out in the laboratory was prepared from fresh anchovy, resulting in relatively low putrescine content. Yongsawatdigul et al. (2004) reported that putrescine was the major biogenic amine found in spoiled fish. Histamine contents of 4 fish sauce samples (*T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8) were 4.50-7.34 mg/100ml which were lower than the control ($P < 0.05$, Table 4.9).

Biogenic amines of samples added *T. halophilus* MS33 and *T. halophilus* MRC 10-7-8 were not different from the control (Table 4.9). It should be noted that addition of starter culture did not increase histamine in fish sauce product. Biogenic amines are generated by decarboxylation of amino acids through substrate specific enzymes produced by bacteria presenting in fish sauce fermentation.

The maximum allowable histamine content of fish sauce based on Canadian Food Inspection Agency (CFIA) is 20 mg/100ml (CFIA 2003). Histamine content of fish sauce samples in this study was in the limit of CFIA standard. *T. halophilus* MRC10-1-3 and *T. halophilus* MCD10-5-10 showed the ability to decrease histamine level in fish sauce. It should be noted that these LAB produced relatively high level of histamine (9-27 mg/100ml) in the culture broth (mGYP broth) (Figure 4.3). This was because mGYP broth was added amino acids allowing more available substrates for bacterial amino decarboxylases the real fermentation system. Based on this study, these isolates not only increased α- amino content but also reduced histamine.

Leuschner, Heidel, and Hammes (1998) reported that diamine oxidases located in cytoplasm of *Micrococcus varians*, *Brevibacterium linens*, and coryneform bacteria were responsible for degradation of histamine and tyramine. Similarly, Enes-

Dapkevicius, Robert-Nout, Rombouts, Houben, and Wymenga (2000) reported that some strains of *Lb. sake* isolated from fish paste degraded histamine. *T. halophilus* is typically reported to produce histamine, particularly in a culture medium (Satomi et al., 1997; Thongsanit et al., 2002). This is the first evidence suggesting the histamine reduction of *T. halophilus* in the fermentation system. *T. halophilus* might have enzymes that could degrade biogenic amines. Further study is need to clarify biogenic amine reduction ability of *T. halophilus*.

4.5.4 Physico-chemical properties of fish sauce

Total nitrogen (TN) contents of all fish sauce samples inoculated with starter cultures were comparable to that of control (Table 4.10, $P > 0.05$). TN included the amount of protein nitrogen and non-protein nitrogen compounds, such as ammonia, free amino acids, nucleotide, peptides, urea, and trimethylamine (TMA) (Shahidi, Sikorski, and Pan, 1994). These compounds contribute to the specific aroma and flavor of fish sauce (Dougan and Howard, 1975). Dougan and Howard (1975) reported that about 80% of TN in fish sauce remained in the form of amino acids. Moreover, TN content in fish sauce depended on fish species and chemical composition of fish (Tungkawachara, Park, and Choi, 2003). TN content is an index used to classify the quality of fish sauce (Park et al., 2001). TN value of the first grade fish sauce according to Thai Industry Standard must be greater than 2.0%. Samples inoculated with starter cultures demonstrated the first grade quality after 6 months fermentation (Table 4.10).

Ammonical nitrogen (AN) content of all samples inoculated with starter cultures and control were 0.1% (Table 4.10). An increasing of AN content is related to the degradation of polypeptide (Tungkawachara, Park, and Choi, 2003).

Table 4.10 Physico-chemical properties of fish sauce samples were inoculated with bacterial starter culture and incubated at 35 °C for 6 months.

Samples	Total nitrogen (%)	Ammonical nitrogen (%)	Browning index	Salt (%)	pH
Control	2.03 ± 0.08	0.11 ± 0.01	0.455 ± 0.01	25.98 ± 0.41	5.46 ± 0.00[a]
MS33	2.10 ± 0.00	0.11 ± 0.01	0.446 ± 0.01	26.71 ± 0.41	5.42 ± 0.01[b]
MRC 10-1-3	2.07 ± 0.03	0.10 ± 0.00	0.453 ± 0.01	25.91 ± 0.88	5.42 ± 0.00[b]
MCD 10-5-10	2.05 ± 0.57	0.11 ± 0.00	0.449 ± 0.00	26.20 ± 0.88	5.42 ± 0.01[b]
MRC 10-7-8	2.16 ± 0.12	0.11 ± 0.00	0.449 ± 0.03	25.83 ± 0.41	5.39 ± 0.00[c]

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

Furthermore, AN content indicated that primary nitrogenous compounds contributed to ammonical note in fish sauce (Dougan and Howard, 1975; Beddows et al., 1976; McIver, Brooks, and Reineccius, 1982). However, the influence of AN on consumer acceptance has not been reported (Lopetcharat and Park, 2002).

The browning index was comparable among samples (Table 4.1, $P > 0.05$). Maillard reaction is responsible for the brown color of fish sauce during ripening step (Lopetcharat et al., 2001). Lee, Homma, and Aida (1997) reported that fish and soy sauce became darker with melanoidine produced by Maillard reaction during storage.

Salt content of fish sauce inoculated with starter cultures were 25-26% NaCl which complied with TISI standard (Table 4.10). Addition of starter cultures did not affect salt content of the sample. The pH value of fish sauce samples were 5.39-5.46 (Table 4.10). Fish sauce inoculated with starter cultures, namely *T. halophilus* MS33,

T. halophilus MRC10-1-3, *T. halophilus* MCD10-5-10, and *T. halophilus* MRC10-7-8, showed lower pH than the control ($P < 0.05$). Michihata, Sado, Yano, and Enomoto (2000) reported that organic acids, such as lactic acid and acetic acid, also tended to lower pH of fish sauce during fermentation. Although LAB were added, they did not drastically decrease pH. This was because fish sauce fermentation did not contain sugar (glucose in particular), an important substrate for lactic acid production of LAB.

4.5.5 Amino acid profiles

Free amino acid contents of samples inoculated with starter culture were higher than the commercial fish sauce (Table 4.11). The commercial sample used in this study was the blended sample containing 2.07% TN, 28.31% Salt, pH 5.36, and 11.78 mg/100ml histamine. Free glutamic acid of fish sauce added starter cultures were comparable to the control and commercial fish sauce. Glutamic acid contributes to umami flavor of fish sauce. The typical flavor of glutamic acid is meaty (Jaing et al., 2007; Sanceda et al., 1990). Free histidine of fish sauce inoculated with starter cultures showed higher than commercial fish sauce (Table 4.11, $P < 0.05$) that corresponded with low histamine content of fish sauce added starter cultures (Table 4.9). Moreover, free proline, alanine, valine, and lysine increased in all fish sauce added starter culture (Table 4.11). Kranenberg et al. (2002) and Macedo et al. (2000) reported that peptidases of LAB separated into 4 groups were endopeptidases, aminopeptidases, di-/tri-peptidases, and proline-specific peptidases. Therefore, *T. halophilus* could exhibit proline specific peptidase activity, resulting in high content of free proline. Park et al. (2001) reported that proline and alanine contributed to sweetness and were responsible for the flavor characteristic of sea urchin roe. The results suggested that addition of starter culture also increased free amino acids

Table 4.11 Free amino acid contents (μmol/100ml) of fish sauce sample inoculated with starter cultures fermented for 6 months and fish sauce fermented conventionally.

Amino acid	Free amino acid					
	Control	MS33	MRC 10-1-3	MCD 10-5-10	MRC 10-7-8	Com[a]
Asp	414.5	427.8	411.5	427.9	429.3	425.1
Thr	443.5	461.6	447.2	455.4	462.4	416.1
Ser	398.4[a]	277.8[b]	364.2[a]	261.1[b]	286.0[b]	355.3[a]
Glu	617.2	646.4	620.7	639.6	646.0	681.5
Pro	733.7[a]	857.5[a]	839.6[a]	802.2[a]	850.8[a]	457.2[b]
Gly	283.2[b]	303.4[b]	279.1[b]	304.1[b]	305.3[b]	415.1[a]
Ala	734.4	777.1	734.8	767.5	783.3	759.0
Cys	25.6[ab]	24.1[ab]	30.5[a]	33.2[a]	30.0[a]	15.3[b]
Val	599.0	621.8	610.1	612.4	623.5	533.5
Met	171.3	171.9	185.2	174.9	174.2	165.9
Ile	309.1	319.7	317.6	306.7	312.0	277.2
Leu	365.1	376.8	377.5	353.9	358.1	358.7
Tyr	49.3	51.3	51.9	46.1	50.8	43.5
Phe	241.5	250.1	243.4	244.3	247.1	207.1
His	233.4[a]	239.8[a]	240.5[a]	240.4[a]	245.9[a]	196.6[b]
Lys	623.7	651.4	630.1	641.4	653.0	638.9
Arg	400.0[a]	224.6[b]	332.8[a]	233.5[b]	223.6[b]	34.8[c]
Total	7189.1	7579.6	7375.9	7423.4	7566.8	7009.5

[a]Commercial fish sauce fermented for 12 months.
Different superscripts within a row indicate significant differences ($P < 0.05$).

contributing to flavor characteristic of fish sauce. Hydrolysis of oligopeptides to free amino acids could be resulted from aminopeptidases activity of LAB starter cultures since these LAB showed broad substrate aminopeptidases activity. It should be noted that glutamic, proline and alanine were predominant amino acids in all fish sauce samples (Table 4.11). These results coincidentally corresponded with high intracellular aminopeptidase activitiy toward alanine and glutamic of these LAB

(Figure 4.2). Therefore, increasing of free amino acids of fish sauce was mainly resulted from aminopeptidases activity of LAB starter cultures. Fish sauce added starter culture showed higher arginine content than the commercial fish sauce ($P < 0.05$). Typically, arginine was converted to ornithine via Krebs-Hanseleit urea cycle, which was further converted to putrescine by a bacterial decarboxylase (Mathews and van Holde, 1990; Silla Santos, 1996). Therefore, low level of arginine in commercial fish sauce indicated low freshness quality of raw material used. Fish used in the laboratory scale fermentation had high freshness quality than these in the fish sauce plant. For this reason, arginine of fresh fish was greater than poor fish.

Total amino acid content of fish sauce added *T. halophilus* MS33 was comparable to free amino acids and showed higher content than others (Table 4.13, $P < 0.05$). These results indicated that amino acids in fish sauce added *T. halophilus* MS33 were presented in the form of free amino acids. Fish sauce added *T. halophilus* MS33 showed higher total amino acid content than other isolates and control (Table 4.12, $P < 0.05$). It should be noted that fish sauce added *T. halophilus* MS33 showed higher total glutamine acid content than others and was comparable to the commercial sample (Table 4.12, $P < 0.05$). These results suggested that *T. halophilus* MS33 could increase total glutamic acid, contributing to desirable meaty flavor of fish sauce. In addition, total amino acid content of commercial fish sauce was higher than free amino acids, suggesting that amino acids in commercial fish sauce existed in the form of peptides rather than free amino acids (Table 4.11, 4.12). These result suggested that the addition of LAB in fish sauce fermentation increased oligopeptide degradation to free amino acid in fish sauce sample.

Table 4.12 Total amino acid contents (μmol/100ml) of fish sauce sample inoculated with starter cultures fermented for 6 months and fish sauce fermented conventionally.

Amino acid	Total amino acid					
	Control	MS33	MRC 10-1-3	MCD 10-5-10	MRC 10-7-8	Com[a]
Asp	503.2	522.9	474.8	517.3	506.5	489.0
Thr	372.3	375.9	361.3	374.5	368.6	444.9
Ser	371.4	336.8	351.9	301.3	310.1	449.0
Glu	630.5[b]	918.9[a]	678.7[b]	671.1[b]	665.7[b]	846.7[a]
Pro	495.7	634.6	498.2	500.8	490.8	444.0
Gly	508.1	495.5	488.9	511.7	505.5	490.6
Ala	547.3	546.1	532.5	555.0	550.5	555.5
Cys	41.6[b]	59.7[b]	49.3[b]	46.2[b]	46.7[b]	111.2[a]
Val	379.4[b]	418.2[b]	380.0[b]	383.4[b]	382.4[b]	551.2[a]
Met	205.5	222.6	202.4	207.6	206.4	278.3
Ile	323.3	343.0	324.0	322.9	322.8	428.2
Leu	351.7[b]	399.7[b]	359.1[b]	347.0[b]	345.8[b]	578.4[a]
Tyr	53.6[b]	93.6[b]	61.5[b]	50.4[b]	53.1[b]	231.0[a]
Phe	260.6[b]	347.2[ab]	278.6[b]	278.9[b]	269.4[b]	494.4[a]
His	297.3	311.6	300.9	299.7	299.0	367.1
Lys	415.8	515.2	454.6	505.6	506.8	533.0
Arg	370.7	262.7	334.1	238.2	221.1	306.6
Total	6848.8[c]	7675.3[b]	6898.2[c]	6913.6[c]	6855.2[c]	8709.8[a]

[a]Commercial fish sauce fermented for 12 months.
Different superscripts within a row indicate significant differences ($P < 0.05$).

4.5.6 Volatile compounds in fish sauce

A total of 29 volatile compounds were identified in the fish sauce fermented for 1, 4 and 6 months are shown in Table 4.13. They were separated into 8 groups: alcohols; aldehydes; ketone; ester; sulfur-containing compounds; nitrogen-containing compound; aromatic hydrocarbon (Table 4.13). Shimoda et al. (1996) reported that volatile fatty acids, such as acetic acid, propanoic acid, 2-methylpropanoic acid,

butanoic acid and 3-methylbutanoic acid, contributed to cheesy odor of fish sauce. Volatile fatty acids were not detected in this study. The major alcohols found in the samples were 1-butanol, 1-penten-3-ol, 1-pentanol, and 1-propanol. 1-Butanol content in fish sauce inoculated LAB was comparable to that of the control. 1- Propanol content of commercial fish sauce was higher than others (Table 4.13). It should be noted that alcohols decreased during fermentation. The role of alcohol on overall acceptance in fish sauce has not been reported since the odor threshold was high (Michihata et al., 2002). Michihata et al. (2002) found a large number of alcohols in *Ishiru* (Japanese fish sauce) that might be resulted from LAB fermentation.

Fish sauce samples added *T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8, showed high content of 2-methylbutanal and 3-methylbutanal (Table 4.13). Those isolates also produced the same compounds in FB25 with lower amount than in fish sauce fermentation (Table 4.3). Fukami et al. (2002) and Peralta et al. (1996) reported that 2-methylpropanal and 2-methylbutanal contributed to meaty odor of fish sauce samples. Commercial fish sauce showed higher content of 2-methylbutanal than others (P <0.05). 3-Methylpropanal content of fish sauce added *T. halophilus* MS33 and *T. halophilus* MRC10-1-3 were comparable to commercial fish sauce. Fish sauce added starter cultures appeared to have higher content of 2-methylpropanal than control ($P < 0.05$). *T. halophilus* MRC10-1-3 produced the highest content of 2-methylpropanal in fish sauce ($P < 0.05$). These results suggested that these 4 selected bacteria could contribute to the formation of important volatile compounds in fish sauce. Other aldehydes such as, propanal, 2-methyl-2-butenal, and 3-(methylthio) propanal, were also found (Table 4.13). 3-(Methylthio) propanal was also found in tuna sauce and

occurred from degradation of methionine (Cha and Cadwallader, 1998; Forss, 1979). Cha and Cadwallader (1998) reported that 3-methylbutanal content in tuna sauce was high, which may positively affect the overall flavor. Sheldon and Lindsay (1971) reported that threshold values of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal were 0.10, 0.13, and 0.06 mg/l, respectively. Therefore, aldehydes compounds contributed to the overall flavor due to their low odor threshold value (Shimoda et al., 1996). These compounds were generated from chemical reaction and/or microbial degradation of amino acids. Chemical reactions responsible for aldehyde formation were lipid oxidation and deamination of amino acids during fermentation, resulting in branched/aromatic or short-chain aldehydes (Michihata et al., 2002). In addition, aldehyhe compounds occurred from Strecker degradation (Maillard reaction). The maillard reaction is very important for the formation of brown color and flavor. Steinhaus and Schieberle (2007) reported that 3-Methylbutanal and 2-methylbutanal are among the most important odorants in soy sauce and are thought to be essentially produced by microbial action via amino-acid biosynthetic pathways of branched-chain amino acids leucine, valine and isoleucine. Benzaldehyde which is an aromatic compound commonly found in cheese and dairy products were detected in all samples. Groot and Bont (1998) reported that *Lb. plantarum* could convert phenylalanine to phenylacetaldehyde via Strecker reaction, and phenylacetaldehyde further oxidized to benzaldehyde (McSweeney and Sousa, 2000). This compound is important for flavor development in cheese. These results indicated that, LAB isolated from fish sauce may play a significant role in desirable flavor and/or aroma formation in fish sauce.

Table 4.13 Changes of volatile compounds during fish sauce fermentation of samples added LAB and fish sauce fermented conventionally.

No.	RI	Peak Name[b]	Relative peak area[a]														
			Com[c]	Control			MS33			MRC10-1-3			MCD10-5-10			MRC10-7-8		
				1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo
		Alcohols																
10	1022	Ethanol	0.065[a]	0.561	0.371	0.040[a]	3.263	0.136	0.072[a]	0.309	0.169	0.007[b]	2.970	0.007	ND	2.032	0.087	0.018[b]
11	1041	2-Butanol	0.063	ND	0.003	0.001	ND	0.002	0.001	ND	0.003	ND	ND	ND	0.001	ND	0.002	0.002
12	1049	1-Propanol	1.094	ND	0.004	0.011	ND	0.004	0.006	0.097	ND	ND	ND	0.017	ND	0.026	ND	0.001
15	1110	2-Methyl-1-propanol	0.062	ND	0.003	0.002	ND	0.001	0.004	0.026	0.004	0.001	ND	0.004	ND	ND	0.002	0.003
17	1172	3-Pentanol	0.035	ND	ND	ND	ND	0.000	ND	ND	0.001	ND	0.040	ND	0.002	ND	ND	ND
18	1188	2-Pentanol	0.005	ND	ND	ND	ND	0.001	ND	ND	0.001	ND	ND	ND	ND	ND	ND	ND
19	1185	1-Butanol	0.128[a]	0.165	0.065	0.026[b]	0.183	0.071	0.027[b]	0.035	0.062	0.029[b]	0.239	0.023	0.027[b]	0.216	0.041	0.028[b]
20	1193	1-Penten-3-ol	0.214[a]	2.288	0.345	0.061[b]	3.061	0.238	0.045[b]	2.073	0.379	0.043[b]	2.861	0.098	ND	2.097	0.136	0.058[b]
21	1205	3-Methyl-1-butanol	0.024	0.049	0.022	0.001	0.362	0.026	0.010	0.250	ND	ND	0.489	0.006	ND	0.497	0.024	0.002
22	1255	1-Pentanol	0.014	0.210	0.046	0.007	0.212	0.029	0.004	0.098	0.025	0.005	0.074	0.016	0.003	0.097	0.020	0.007
24	1327	(*E*)-2-Penten-1-ol	0.010	0.107	0.002	0.004	0.042	0.014	ND	0.081	0.007	0.001	0.064	0.008	0.008	ND	0.007	0.003
25	1336	(*Z*)-2-Penten-1-ol	0.027	0.329	0.086	0.003	0.369	0.079	0.005	0.341	0.075	0.008	0.424	0.012	0.009	0.354	0.033	0.013
27	1444	2-Ethyl-1-hexanol	0.001	ND	0.040	0.003	ND	0.038	0.002	ND	0.014	0.002	ND	ND	0.003	ND	0.019	0.004
		Aldehydes																
1	784	Propanal	0.511	0.570	ND	ND	0.608	0.029	ND	0.174	0.032	ND	ND	ND	ND	0.278	0.011	ND
3	830	2-Methylpropanal	0.017[c]	0.687	ND	ND	0.149	0.372	0.065[b]	0.250	0.351	0.252[a]	ND	0.157	0.076[b]	0.157	0.217	0.053[b]
6	906	2-Methylbutanal	0.591[a]	0.964	0.606	0.220[b]	0.209	0.545	0.245[b]	0.265	0.464	0.247[b]	ND	0.237	0.227[b]	0.254	0.351	0.234[b]
7	911	3-Methylbutanal	0.292[a]	0.964	0.552	0.232[a]	1.491	0.586	0.255[a]	1.186	0.766	0.282[a]	1.476	0.197	0.073[c]	1.319	0.408	0.188[b]
14	1104	2-Methyl-2-butenal	0.179	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.004	ND

Table 4.13 (continued)

No.	RI	Peak Name[b]	Relative peak area[a]														
			Com[c]	Control			MS33			MRC10-1-3			MCD10-5-10			MRC10-7-8		
				1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo	1mo	4mo	6mo
29	1615	3-(Methylthio) propanal	0.051	ND	0.103	0.015	ND	0.080	0.019	ND	0.017	0.026	ND	0.023	0.016	ND	0.025	0.018
28	1459	Benzaldehyde	0.025	0.429	0.251	0.014	0.175	0.177	0.023	0.208	0.196	0.024	0.162	0.106	0.028	0.258	0.102	0.030
		Ketones																
5	888	2-Butanone	0.894[a]	1.139	0.489	0.312[b]	1.075	0.460	0.270[b]	1.249	0.531	0.241[b]	1.513	0.127	0.009[c]	1.187	0.329	0.282[b]
8	929	3-Methyl-2-butanone	0.075	ND	0.004	0.020	0.000	0.002	0.008	ND	0.002	0.012	ND	ND	ND	ND	ND	0.008
9	980	2-Pentanone	0.017	0.275	0.049	ND	0.092	0.020	ND	ND	0.002	ND	ND	ND	ND	ND	0.010	ND
13	1050	2,3-Butanedione	0.006	ND	0.053	0.008	ND	ND	0.003	ND	0.003	0.003	ND	0.003	ND	ND	0.006	0.007
23	1315	Cyclohexanone	0.117	ND	0.023	0.046	ND	0.025	0.043	ND	0.025	0.042	ND	0.026	0.046	ND	0.028	0.048
		Esters																
4	863	Ethyl acetate	0.146[a]	ND	0.007	0.006[b]	0.092	ND	ND	ND	0.158	0.013[b]	ND	0.126	0.121[a]	0.030	0.003	0.134[a]
		Sulfur-contaning compounds																
2	818	Dimethyl sulfide	0.002	0.106	ND	ND	0.216	0.023	ND	0.035	ND	ND	0.155	ND	ND	0.039	0.011	ND
16	1120	Dimethyl disulfide	0.075[a]	0.058	0.030	0.038[b]	0.044	0.020	0.013[c]	0.030	0.023	0.010[c]	0.027	0.037	ND	0.014	0.070	0.028[b]
		Nitrogen-containing compounds																
18	1176	Methylpyrazine	0.009	0.006	0.003	0.005	ND	0.023	0.003	ND	0.027	0.003	0.010	0.010	0.005	ND	0.010	0.005
26	1377	2,6-Dimethyl pyrazine	0.008	ND	ND	0.001	ND	0.009	0.002	ND	0.001	0.002	ND	ND	ND	ND	0.005	0.004

Note: ND= Not detected; Different superscripts within a row indicate significant differences ($P < 0.05$).

[a]The values represent the ratio of the peak area of any compound to that of internal standard (cyclohexanol).

[b]Retention indices calculated for DB-WAX column using n-alkanes as standards.

[c]Commercial fish sauce fermented conventionally.

The major ketones found in this study were 2-butanone and 2-methyl-2-butanone (Table 4.13). Ketones seem to be responsible for the cheesy note (Michihata et al., 2002; Peralta et al., 1996). The amounts of these compounds were comparable to the control. The amount of 2-pentanone, 2,3-butanedione, and cyclohexanone were relatively low. However, these compounds were unlikely to affect fish sauce odor because of their high odor threshold in the vapor phase of 1.55 to 7.76 mg/l (Devos et al., 1995; Michihata et al., 2002).

Ester compound detected in fish sauce samples was ethyl acetate (Table 4.13). Fish sauce added *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8 showed ethyl acetate content comparable to commercial fish sauce. Esters are usually found in fermented seafoods which were derived from esterification of alcohols with carboxylic acids that are formed by microbial and enzymatic decomposition of lipids. These compounds have not been reported to play a significant role on fish sauce odor (Cha and Cadwallader, 1995; Devos et al., 1995; Peralta et al., 1996).

Methylpyrazine and 2,6-dimethyl pyrazine were negligible in all fish sauce samples and commercial fish sauce (Table 4.12). Sancedra, Kurata, and Arakawa (1986) reported that pyrazines contributed to burnt and sweet odors of noucmam (Vietnamese fish sauce). 2,6-Dimethyl pyrazine and 2-ethylpyridine were contributed to sweaty note (Fukami et al., 2004). Fukami et al. (2004) found that fish sauce sample treated with *Staphylococcus* strain R4Nu could reduce undesirable note of 2-ethylpyridine in fish sauce.

Sulfur-containing compounds identified in fish sauce samples were dimethyl sulfide and dimethyl disulfide (Table 4.13). The fish sauce sample added *T. halophilus* MS33 showed lower content of dimethyl sulfide and dimethyl disulfide

than the control and commercial fish sauce ($P < 0.05$). Dimethyl disulfide was not detected in fish sauce sample added *T. halophilus* MCD10-5-10. Sulfur compounds originating from methionine are methanethiol, dimethyl disulfide, dimethyl trisulfide, methional and methanethiol (Yvon and Rijnen, 2001). The catabolism of methionine can occur via transamination pathway, resulting in sulfur compounds. In addition, demethiolation of methionine resulting in methanethiol, subsequently, auto-oxidized to dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide (Bonnarme, Psoni, and Spinnler, 2000; McSweeney and Sousa, 2000). Dimethyl disulfide content of commercial fish sauce was higher than others ($P < 0.05$). Sulfur-containing compounds strongly contributed to the odor and flavor because of low their threshold values (Devos et al., 1995). These compounds are responsible for fecal note that is undesirable odor in fish sauce (Fukami et al., 2004). Fish sauce consumption has been limited in Japan, Europe, and U.S.A., which is partly due to a strong odor characteristic (Fukami et al., 2004). Therefore, the 4 selected starter cultures added in fish sauce fermentation might help to eliminate undesirable odor and/or promote desirable odor characteristic of fish sauce.

4.5.7 Sensory evaluation of fish sauce

Sensory evaluation based on color, odor, and overall acceptance of all fish sauce was shown in Table 4.14. The color and odor of fish sauce samples inoculated with starter cultures were comparable to the control and the commercial fish sauce ($P > 0.05$), although the profile of volatile compounds by GC-MS analysis were different.

Table 4.14 Hedonic score of color, odor, flavor, and overall acceptance of fish sauce samples inoculated with LAB starter cultures and commercial sample.

Samples	Attributes			
	Color	Odor	Flavor	Overall acceptance
Commercial	5.45 ± 1.04	4.73 ± 1.74	4.73 ± 1.35[a]	4.91 ± 1.38
Control	4.82 ± 0.40	4.27 ± 1.01	3.55 ± 0.82[b]	4.09 ± 0.94
MS33	4.82 ± 0.60	4.00 ± 1.18	3.64 ± 1.36[ab]	4.27 ± 1.10
MRC10-1-3	5.09 ± 0.83	4.45 ± 0.82	3.73 ± 1.49[ab]	4.09 ± 1.58
MCD10-5-10	4.82 ± 0.60	4.18 ± 1.08	3.91 ± 1.22[ab]	4.36 ± 1.12
MRC10-7-8	4.91 ± 0.83	4.09 ± 0.70	3.73 ± 1.27[ab]	3.91 ± 1.30

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

Flavor preferences of fish sauce inoculated with starter cultures were comparable to the commercial fish sauce ($P > 0.05$). It should be noted that odor and flavor quality of 6-mo-old fish sauce samples added starter culture were equivalent to the sample conventionally fermented for 12 months. Most of panelists who were familiar with fish sauce tasting revealed that fish sauce inoculated with starter culture exhibited less strong odor than the commercial fish sauce. Addition of LAB could be a promising means to produce fish sauce with less strong odor which could be more widely acceptable to consumers worldwide.

CHAPTER V

CONCLUSIONS

Most lactic acid bacteria were found in fish sauce samples collected from factories during 1-7 months fermentation. Seven isolates could grow in fish broth containing 25% NaCl (FB25) and hydrolyzed fish protein in FB25 but did not hydrolyze skim milk protein. Seven isolates showed high intracellular aminopeptidase activity toward Ala-pNA. In addition, they produced volatile compounds: alcohol; aldehyde; ester; ketone, in FB25. Some isolates (M11, MS33, MRC10-1-3, MRC5-5-2, MCD10-5-10, MCD10-5-15, and MRC10-7-8) produced histamine in mGYP medium containing 5 and 25% NaCl at the level of 6.62-22.55 and 13.14-20.39 mg/100ml, respectively. All isolates were identified as *T. halophilus* based on the phenotypic and genotypic characteristics. All isolates showed variation in physiological and biochemical characteristics.

Tetragenococcus halophilus MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MCD10-5-10, and *T. halophilus* MRC10-7-8 were selected for use as a starter culture for fish sauce fermentation. The population of the inoculated LAB in the anchovy hydrolysate remained 1-2 Log CFU/ml at 60 days and was not detected at 90 days of fermentation. Total α-amino content of fish sauce inoculated with starter cultures ranged from 774 to 784 mM after 6 months of fermentation. Fish sauce samples added starter cultures showed higher α-amino content than the control ($P < 0.05$) but comparable to fish sauce fermented conventionally ($P > 0.05$). Biogenic amines found

in fish sauce added starter cultures were tryptamine, cadaverine, and histamine. Histamine content of fish sauce inoculated with *T. halophilus* MRC10-1-3 and *T. halophilus* MCD10-5-10 was lower than the control ($P < 0.05$). Total nitrogen content of fish sauce added starter culture was comparable to the commercial fish sauce. Physico-chemical characteristics were also comparable to those of commercial fish sauce. LAB starter culture increased free amino acids (proline, alanine, valine, and lysine). Total amino acids, especially glutamic acid, was increased in fish sauce added *T. halophilus* MS33. A total of 29 volatile compounds were identified in fish sauce added LAB including alcohols, aldehydes, ketone, ester, sulfur-containing compounds, and nitrogen-containing compound. 2-Methylpropanal showed high content in all samples added LAB starter culture. 2-Metylbutanal and 3-methylbutanal was also found in all samples. These compounds contributed to desirable meaty note in fish sauce. All fish sauce sample added starter cultures showed lower sulfur-containing compounds than the control and commercial fish sauce. Moreover, dimethyl disulfide which contributed to fecal note was not detected in fish sauce inoculated *T. halophilus* MCD10-5-10. All fish sauce samples inoculated with starter cultures exhibited similar overall acceptance to the commercial fish sauce fermented 12-mo-old. Therefore, the selected *T. halophilus* accelerated fish sauce fermentation and improved chemical characteristics of fish sauce. Therefore, LAB could be potential strains to be used as a starter culture in fish sauce fermentation.

REFERENCES

REFERENCES

AOAC. (1995). Salt (chlorine as sodium chloride) in seafood: volumetric method, Section 35.1.18, Method 937.09. **In Official Methods of Analysis of AOAC International.** (pp. 16-17). AOAC International, Gaithersburg, MA, USA.

Abe, K., and Uchida, K. (1989). Correlation between depression of catabolite control of xylose metabolism and a defect in the phosphoenolpyruvate: mannose phosphotransferase system in *Pediococcus halophilus*. **J. Bacteriol**. 171: 1793-1800.

Aquerreta, Y., Astiasarán, I., and Bello, J. (2001). Use exogenous enzymes to elaborate the roman fish sauce 'garum'. **J. Sci. Food Agric**. 82: 107-112.

Anonymous. (1994). Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List no. 49. **Int. J. Syst. Bacteriol**. 44: 370-371.

Axelsson, L. (2004). Lactic acid bacteria: Classification and Physiology. In S. Salminen, A. V. Writght, and A. Ouwehand (eds.). **Lactic acid bacteria; Microbiology and Functional Aspects.** (3rd ed., pp.1-100). New York: Marcel Dekker., Inc.

Back, W. (1978). Elevation of *Pediococcus cerevisiae* subsp. *dextrinicus* Coster and White to species status *Pediococcus dextrinicus* (Coster and White) comb. nov. **Int. J. Syst. Bacteriol** 28: 523-527.

Baliardaa, A., Roberta, H., Jebbarc, M., Blancoc, C., Deschampsa, A., and Marrec, C. L. (2003). Potential osmoprotectants for the lactic acid bacteria *Pediococcus pentosaceus* and *Tetragenococcus halophilus*. **Int. J. Food Microbiol**. 84: 13-20.

Beddows, C. G., Ardeshir, A. G., and Daud, W. J. (1980). Development and origin of volatile fatty acid in Budu. **J. Sci. Food Agric**. 31: 86-92.

Berdague, J. L., Monteil, P., and Talon, R. (1993). Effects of starter cultures on the formation of flavor compounds in dry sausage. **Meat Sci**. 35: 275-287.

Bonnarme, P., Psoni, L., and Spinnler, H. E. (2000). Diversity of L-methionine catabolism pathways in cheese-ripening bacteria. **Appl. Environ. Microbiol**. 66: 5514-5517.

Bover-Cid, S., and Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. **Int. J. Food Microbiol**. 53: 33-41.

Bradford, M. M. (1976). Method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. **Anal. Biochem**. 72: 248-254.

Bremer, E., and Kramer, R. (2000). Coping with osmotic challenges. In Storz, G., and Hengge-Aronis, R. (eds.). **Through accumulation and release of compatible solutes in bacteria.** (pp. 79-97). Bacterial Stress Responses. ASM Press, Washington, DC.

Brillantes, S., Pakon, S., and Totakien, A. (2002). Histamine formation in fish sauce production. **J. Food Chem. Toxicol.** 67: 2090-2094.

Brink, B. T., Damink, C., Joosten, H. J., Huis, T., and Veld, J. (1990). Occurrence and formation of biologically active amines in foods. **Int. J. Food Microbiol.** 11: 73-84.

Buschelman, B. J., Bale, M. J., and Jones, R. N. (1993). Species identification and determination of high-level aminoglycoside resistance among enterococci. Comparison study of sterile body fluid isolates, 1985–1991. **Diagn. Microbiol. Infect Dis.** 16: 119-122.

[CFIA] Canadian Food Inspection Agency. (2003). Fish Inspection. Act. Ottawa, Canada: Dept. of Justice.

Carr, F. J., Chill D., and Maida N. (2002). The lactic acid bacteria: A Literature Survey. **Crit. Rev. Microbiol**. 28(4): 281-370.

Cha, Y. J., and Cadwallader, K. R. (1995). Volatile components in salt-fermented fish and shrimp pastes **J. Food Sci.** 60: 19-24.

Cha, Y. J., and Cadwallader, K. R. (1998). Aroma active compounds in skipjack tuna sauce. **J. Agri. Food Chem.** 46: 1123-1128.

Chaiyanan, S., Maugel, T., Huq, A., Robb, F. T. and Colwell, R. R. (1999). Polyphasic taxonomy of a novel *Halobacillus thailandensis* sp. nov. isolated from fish sauce. **J. Appl. Microbiol.** 22: 360-365.

Chen, Y. S., Yanagida, F., and Hsu, J. S. (2006). Isolation and characterization of lactic acid bacteria from *dochi* (fermented black beans), a traditional fermented food in Taiwan. **J. Gen. Appl. Microbiol**. 43: 229-235.

Christensen, J. E., Dudley, E. G., Pedersen, J. A., and Steele, J. L. (1999). **Peptidases and amino acid catabolism in lactic acid bacteria**. In W. N. Konings, O. P.

Kuipers, and J. H. J. Huis Veld (eds.), Lactic acid bacteria: Genetics, metabolism and applications (pp. 217-246). Dordrecht: Kluwer Academic.

Collins, M. D., Williams, A. M., and Wallbanks, S. (1990). The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. **FEMS Microbiol. Lett**. 70**:** 255-262.

Csonka, L. N., and Epstein, W. (1996). Osmoregulation. In Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (eds.). ***Escherichia coli* and *Salmonella*: cellular and molecular biology** (pp. 1210-1223.). ASM Press, Washington, D.C.

Cummings, S. P., Williamson, M. P., and Gilmour, D. J. (1993). Tugor regulation in a novel *Halomonas* species. **Arch. Microbiol**. 160: 319-323.

Curioni, P. M. G., and Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography–olfactometry. **Int. Dairy J.** 12: 959-984.

Demirijan, D. C., Moris, F. V., and Cassidy, C. S. (2001). Enzymes from extremophlies. **Chemical Biology**. 5: 144-151.

Devos, M., Patte, F., Roualt, J., Laffort, P., and Gemert, L. J. (1995). Standardized human olfactory thresholds. **J. Odor Res. Eng**. 26: 27-47.

Dougan, J., and Harward, G. E. (1975). Some flavoring constituents of fermented fish sauces. **J. Sci. Food Agric**. 26: 887.

Enes-Dapkevicius, M. L. N., Robert-Nout, M. J., Rombouts, F. M., Houben, J. H., and Wymenga, W. (2000). Biogenic amine formation and degradation by

potential fish silage starter microorganisms. **Int. J. Food Microbiol**. 57: 107-114.

Ennahar, S., and Cai, Y. (2005). Biochemical and genetic evidence for the transfer of *Enterococcus solitarius* Collins et al. 1989 to the genus *Tetragenococcus* as *Tetragenococcus solitarius* comb. nov. **Int. J. Syst. Evol. Microbiol**. 55: 589-592.

Entis, P., Fung, D. F. C., Griffiths, M. W., McIntyre, L., Russel, S., Sharpe, A. N., and Tortorello, M. L. (2001). **Compendium of Methods for the Microbiological Examination of Food** (4th ed.). Rapid Methods for Detection, Identification, and Enumeration. American Public Health Association. U.S.A.

Fields, R. (1979). The measurement of amino groups in proteins and peptides. **Biochem**. 124: 571-590.

Frosbisher, M., Hinsdill, R. D., Crabtree, K. T. and Goodheart, C. R. (1974). **Fundamentals of Microbiology**, (9th ed., pp. 561-575). Co., Toronto.

Forss, D. A. (1979). Review of the progress of dairy science: mechanisms of formation of aroma compounds in milk and milk products. **J. Dairy Res**. 46: 691-706.

Fukami, K., Funatsu, Y., Kawasaki, K., and Watabe, S. (2004). Improvement of fish sauce odor by treatment with bacteria isolated from the fish-sauce mush (moromi) made from frigate mackerel. **J. Food Sci**. 69(2): 45-49.

Fukami, K., Ishiyama, S., Yaouramaki, H., Masuzawa, T., Nabeta, Y., Endo, K., and Shimoda, M. (2002). Identification of distinctive volatile compounds in fish sauce. **J. Agric. Food Chem.** 50: 5412-5416.

Fu, X. T., You, S. G., and Kim, S. M. (2008). Characterization of a salt-tolerant acid protease produced by *Bacillus megaterium* KLP-98 and its protential as a fermentation starter for the manufacture of fish sauce. **J. Food Biochem.** 32: 279-298.

Garvie, E. I. (1986). Genus *Pediococcus* Claussen 1903, 68AL, p. 1075-1079. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology, vol. 2**. The Williams & Wilkins Co., Baltimore, Md.

Geisen, R., Lucke, F. K., and Krockel, L. (1992). Starter and prospective cultures for meat and meat products. **Fleischwirtschaft**. 72: 894-898.

Gildberg, A., Hermes, J. E., and Orejana, F. M. (1984). Acceleratation of autolysis during fish sauce fermentation by adding acid and reducing the salt content. **J. Sci. Food Agric**. 35: 1363-1369.

Gibbs, P. A. (1987). Novel use for lactic acid fermentation in food preservation. **J. Appl. Bacteriol.** 51-58.

Gildberg, A., and Shi, X. Q. (1994). Recovery of tryptic enzymes from fish sauce. **Process Biochem**. 29: 151-155.

Guoqiang, D., Kaul, R., and Mattiasson, M. (1991). Evaluation of alginate - immobilized *Lactobacillus casei* for lactate production. **Appl. Microbiol. Biotechnol.** 36: 309-314.

Hammes, W. P., Weiss, N., and Halzapfel, W. (1992). The genera *Lactobacillus* and *Carnobacterium*. **The Prokayrotes**, (2nd ed., pp. 1535–1594), Vol. II, Springer-Verlag, New York.

Hanagata, H., Shida, O., and Takagi, H. (2003). Toxonomic homogeneity of a salt tolerant lactic acid bacteria isolated from shoyu mash. **J. Gen. Appl. Microbiol**. 49: 95-100.

Hans, G. H. J., Erwin, G. Z., Elaine E. V., Marteau, P., Akkermans, A. D. L., and Willem, M. V. (2002). Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. **Appl. Environ. Microbiol**. 68(1): 114-123.

Helinck, S., Bars, D. L., Moreau, D., and Yvon, M. (2004). Ability of thermophilic lactic acid bacteria to produce aroma compounds from amino acids. **Appl. Environ. Microbiol**. 3855-3861.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994). **Bergey's Manual of Determinative Bacteriology**. (9^{th} ed.). Wilkins, Baltimore.

Hozapfel, W. H., Franz, M. A. P., Ludwig, W., Back, W., and Dicks, M. T. (2006). The genera *Pediococcus* and *Tetragenococcus*. **Prokaryotes**. 4: 229-266.

Ihara, K., Watanabe, S., and Tamura, T. (1997). *Haloarcula argentinensis* sp. nov. and *Haloarcula* mukohátáei sp. nov., two new extremely halophilic archaea collected in Agentina. **Int. J. Syst. Bacteriol**. 47: 73-77.

Ijong, F. C. and Ohta, Y. J. (1996). Physiochemical and microbiological changes associated with Bakasang processing: A traditional Indonesian fermented fish sauce. **J. Sci. Food Agric**. 71: 69-74.

Ishikawa, M., Kodama, K., Yasuda, H., Okamot-Kainuma, A., Koizumi, K., andYamasato, K. (2006). Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses. **Lett. Appl. Microbiol**. 44: 308-313.

Jaing, J. J., Zeng, Q. X., Zhu, Z. W., and Zhang, L. Y. (2007). Chemical and sensory changes associated Yu-lu fermentation process- a traditional Chinese fish sauce. **Food chem**. 104: 1629-1634.

Jay, M. J. (1986). Fermented foods and related products of fermentation. **Modern Food Microbiology.** (3rd ed.). Van Nostrand Reinhold Company, New York.

Jebbar, M., Talibart, R. Gloux, K. Bernard, T. and Blanco, C. (1992). Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. **J. Bacteriol.** 174: 5027-5035.

Johansson, M. L., Quednau, M., Ahrne, S., and Molin, G. (1995). Classification of *Lactobacillus plantarum* by restriction endonuclease analysis of total chromosomal DNA using conventional agarose gel electrophoresis. **Int. J. Syst. Bacteriol**. 45: 670-675.

Justé, A., Lievens, B., Frans, I., Marsh, T., Klingeberg, M., Michiels, C. W., and Willems, K. A. (2008). Genetic and physiological diversity of *Tetragenococcus halophilus* strains isolated from sugar- and salt-rich environments. **Microbiol**. 154: 2600-2610.

Karnop, V.G. (1988). Histamine in salzardellen. **Arc Lebensmittelhyg**. 39: 57-84.

Ke, D., Picard, F. J., Martineau, F., Nard, C., Roy, P. H., Ouellette, M., and Bergeron, M. G. (1999). Development of a PCR assay for rapid detection of enterococci. **J. Clin. Microbiol.** 37: 3497-3503.

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. **Arch. Microbiol.** 170: 319-330.

Kilinc, B., Cakli, S., Tolasa, S., and Dincer, T. (2006). Chemical, microbiological and sensory changes associated with fish sauce processing. **Eur. Food Res. Technol**. 222: 604-613.

Kimura, B., Konagaya, Y., and Fujii, T. (2001). Histamine formation by *Tetragenococcus muriaticus*, a holophilic lactic acid bacterium isolated from fish sauce. **Int. J. Food Microbiol**. 70: 71-77.

Kim, D. S., Koizumi, C., Jeong, B. Y., and Jo, K. S. (1994). Studies on the lipid content and fatty acid composition of anchovy sauce. **J. Korean Fish Soc.** 27: 469-475.

Kobayashi, T., Kajiwara, M., Wahyuni, M., Kitakado, T., Hamada-Sato, N., Imada, C., and Watanabe, E. (2003). Isolation and characterization of halophilic lactic acid bacteria isolated from terasi shrimp paste: A traditional fermented seafood product in Indinesia. **J. Gen. Appl. Microbiol**. 49: 279-286.

Kobayashi, T., Kimura, B., and Fuji, T. (2000). Differentiation of *Tetragenococcus* populations occurring in products and manufacturing process of puffer fish ovaries fermented with rice-bran. **Int. J. Food Microbiol**. 56: 211-218.

Kumar, S., Tamura, K., Jakobsen, I. B., and Nei, M. (2004). MEGA4: molecular evolutionary genetics analysis software. **Bioinformatics**. 17: 1244-1245.

Lee, G. D., Chun, S. S., Kho, Y. H., and Chun, H. K. (1998). Purification and properties of an extracellular leucine aminopeptidase from the *Bacillus* sp. N2. **J. Appl. Microbiol.** 84: 561-566.

Lee, J. Y., Kim, C. J., and Kunz, B. (2006). Identification of lactic acid bacteria isolated from Kimchi and studies on their suitability for application as starter culture in the production of fermented sausages. **J. Meat Sci**. 72: 437-445.

Lee, M., Kim, M. K., Vancanneyt, M., Swings, J., Kim, S. H., Kang. M.S., and Lee, S. T. (2005). *Tetragenococcus koreensis* sp. nov., a novel rhamnolipid-producing bacterium. **Int. J. Syst. Evol. Microbiol**. 55: 1409-1413.

Lee, Y. S., Homma, S., Aida, K. (1997). Characterization of melanoidine in soy sauce and fish sauce by electrofocusing and high performance gel permeation chromatography. **Nippon Shokuhin Kogyo Gakkaishi**. 34: 313-319.

Lehane, I., and Olley, J. (1999). Histamine (*Scombroid*) Fish poisoning: review. **National office of animal and plant health**. Canberra, Australia.

Leisner, J. J., Vancanneyt, M., Rusul, G., Pot, B., Lefebvre, K., Fresi, A., and Tee, L. K. (2001). Identification of lactic acid bacteria constituting the predominating microflora in an acid-fermented condiment (tempoyak) popular in Malaysia. **Int. J. Food Microbiol**. 63: 149-157.

Leuschner, R. G., Heidel, M., and Hammes, W. P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. **Int. J. Food Microbiol**. 39: 1-10.

Lopetcharat, K., Choi, Y. J., Park, J. W., and Daeschel, M. A. (2001). Fish sauce products and manufacturing : a review. **Food. Rev. Int.** 17(1): 65-88.

Lopetcharat, K., and Park, J. W. (2002). Characteristics of fish sauce made from Pacific whiting and surimi by-products during fermentation stage. **J. Food Sci.** 67: 511-516.

Macedo, A. C., Vieira, M., Pocas, R., and Malcata, F. X. (2000). Peptide hydrolysate system of lactic acid bacteria isolated from Serra da Estrela cheese. **Int. Dairy J.** 10: 769-774.

Macedo, A. C., Tavares, T. G., and Malcata, F. X. (2003). Purification and characterization of an intracellular aminopeptidase from a wild strain of *Lactobacillus plantarum* isolated from traditional Serra da Estrela cheese. **Enz. Micro. Technol**. 32: 41-48.

Magboul, A. A., and McSweeney, P. (1999). Purification and characterization of an aminopeptidase from *Lactobacillus curvatus* DPC2024. **Int. Dairy. J.** 9: 107-116.

McIver, R. C., Brooks, R. I., and Reineccius, G. A. (1982). Flavor of fermented fish sauce. **J. Agric. Food Chem**. 30: 1017.

Manome, A., Okada, S., Uchimura, T., and Komagata, K. (1998). The ratio of L-form to D-form of lactic acid as a criteria for the identification of lactic acid bacteria. **J. Gen. Appl. Microbiol.** 44: 371-374.

Marilley, L., and Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. **Int. J. Food Microbiol**. 90: 139-159.

Masson, F., Hinrichsen, L., Talon, R., and Montel, M. C. (1999). Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*. **Int. J. Food Microbiol**. 49:173-178.

Mathews, C. K., and van Holde, K. E. (1990). **Biochemistry**. Redwood City, Calif.: Benjamin Cummings.

McSweeney, L. H., and Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening. **Oral communication at the symposium on quality and microbiology of traditional and raw milk cheeses of the cost 95 meeting, Dijon, France.** 293-324.

Michihata, T., Sado, Y., Yano, T., and Enomoto, T. (2000). Preparation of Ishiru (fish sauce) by a quick ripening process and changes in the composition of amino acids, oligopeptides and organic acids during processing. **J. Japan Soc. Food Sci**. **Technol**. 47(5): 369-77.

Michihata, T., Yano, T., and Enomoto, T. (2002). Volatile compounds of headspace gas in the Japanese fish sauce *ishiru*. **Biosci. Biotechnol. Biochem.** 66: 2251-2255.

Monstein, H. J., Ahrne´ , S., Molin, G., Nikpour-Badr, S., and Jonasson, J. (2001). Identification of enterococcal isolates by temperature gradient gel electrophoresis and partial sequence analysis of PCRamplified 16S rDNA variable V6 regions. **APMIS.** 109: 209-216.

Moral, A., Severin, J., Ramos-Cormenzana, A., Truper, H. G., and Galinski, E. A. (1994). Compatible solutes in new moderately halophilic isolates. **FEMS Microbiol. Lett**. 122: 165-172.

Nakagawa, A., and Kitahara, K. (1959). Taxonomic studies on the genus *Pediococcus*. **J. Gen. Appl. Microbiol**. 5: 95-126.

Norberg, P., and Hofsten, B. V. (1968). Proteolytic enzymes from extremely halophilic bacteria. **J. Gen. Microbiol**. 55: 251-256.

Omafuvbe, B. O., Abiose, S. H., and Shonukan, O. O. (2002). Fermentation of soy bean (*Glycine max*) for soy-*daddawa* production by starter cultures of *Bacillus*. **J. Food Microbiol**. 19: 561-566.

Otsuka, M., Okada, S., Uchimura, T., and Komagata, K. (1994). A simple method for the determination of stereoisomers of lactic acid by HPLC using an enantiomeric resolution column, and its application to identification of lactic acid bacteria (in Japanese). **Seibutsu-kogaku Kaishi**. 72: 81-86.

Parvathy, S. N., and Puthuvallil, K. S. (2005). Biochemical characterization of lactic acid bacteria isolated from fish and prawn. **J. Culture Col.** 4: 48-52.

Park J. N., Fukumoto, Y., Fujita, E., Tanaka, T., Washio, T., Otsuka, S., Shimazu, T., Watanabe, K., and Abe, H. (2001). Chemical composition of fish sauces produced in Southeast and East Asian countries. **J. Food. Comp. Anal**. 14: 113-125.

Patel, R., Piper, K. E., Rouse, M. S., Steckelberg, J. M., Uhl, J. R., Kohner, P., Hopkins, M. K., Cockerill, F. R., and Kline, B. C. (1998). Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. **J. Clin. Microbiol**. 36: 3399-3407.

Peralta, R., Shimoda, M., and Osajima, Y. (1996). Further identification of volatile compounds in fish sauce. **J. Agric. Food Chem**. 44: 3606-3610.

Pons-Sánchez-Cascado, S., Bover-Cid, S., Veciana-Nogués, M. T., and Vidal-Carou, M. C. (2005). Amino acid-decarboxylase activity of bacteria isolated from ice-preserved anchovies. **Eur. Food Res. Technol.** 220: 312-315.

Orejana, F. M., and Liston, J. J. (1982). Agents of proteolysis and its inhibition in patis (fish sauce) fermentation**. J. Food Sci.** 47: 198-209.

Raksakulthai, N., and Haard, N. F. (1992). Peptides and amino acids and the flavor of fish sauce. **Asean Food. J.** 7-86.

Robert, M. (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. **Saline Syst**. 1-30.

Robert, H., Marrec, C. L., Blanco, C., and Jebbar, M. (2000). Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophila*. **Appl. Eviron. Microbiol**. 66: 509-517.

Roling, W. F. M and Verseveld, H. W. (1996). Characterization of *Tetragenococcus halophilus* populations in Indonesian soy mash (Kecap) fermentation. **J. Appl. Environ. Microbiol**. 62: 1203-1207.

Roling, W. F. M and Verseveld, H. W. (1997). Growth, maintenance and fermentation pattern of the salt-tolerant lactic acid bacterium *Tetragenococcus halophila* in anaerobic glucose limited retention cultures. **Antonie van Leeuwenhoek**. 72: 239-243.

Ruoff, K. L., de la Maza, L., Murtagh, M. J., Spargo, J. D., and Ferraro, M. J. (1990). Species identities of enterococci from clinical specimens. **J. Clin. Microbiol**. 28: 435-437.

Sakaguchi, K., and H. Mori. (1969). Comparative study on *Pediococcus halophilus, P. soyae, P. homari, P. urinaeequi* and related species. **J. Gen. Appl. Microbiol.** 15: 159-167.

Saisithi, P. (1994). Traditional fermented fish: Fish sauce production. Ch. 5. In A.M. Martin (ed.). **Fisheries Processing: Biotechnological Applications** (pp. 111-129). Chapman and Hall, London.

Saisithi, P., Kasemsarn, B., Liston, J., and Dollar, A. M. (1966). Microbiology and chemistry of fermented fish. **J. Food Sci**. 31: 105-110.

Sambrook, J. and Russell, D. W. (2001). **Molecular Cloning: A Laboratory Manual** (3rd ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanceda, N. G., Suzuki, E., and Kurata, T. (2001). Development of normal and branched chain volatile fatty acids during the fermentation process in the manufacture of fish sauce. **J. Sci. Food Agric.** 81: 1013-1018.

Sanceda, N. G., Kurata, T., and Arakawa, N. (1986). Study on the volatile compounds of fish sauce-shottsuru, nam-pla, and noucmam. **Agric. Biol. Chem**. 50: 1201-1208.

Sanceda, N. G., Kurata, T., and Arakawa, N. (1990). Overall quality and sensory acceptance of a lysine-fortified fish sauce. **J. Food Sci.** 55: 983-988.

Sanceda, N. G., Kurata, T., Suzuki, Y., and Arakawa, N. (1992). Oxygen effect on volatile acids formation during fermentation in manufacture of fish sauce. **J. Food Sci.** 57(5): 1120-1123.

Sanceda, N. G., Suzuki, E., and Kurata, T. (2003). Quality and sensory acceptance of fish sauce partially substituting sodium chloride or natural salt with potassium chloride during the fermentation process. **Int. J. Food Sci. Tech**.38: 435-443.

Sands, A., and Crisan, E. V. (1975). Microflora of four fermented fish sauces. **Appl. Environ. Microbiol.** 29(1): 106-108.

Sanz, Y., and Toldra, F. (1997). Purification and characterization of aminopeptidase from *Lactobacillus sake*. **J. Agric. Food Chem.** 45: 1552-1558.

Sato, H., Yanagida, F., Shinohara, T., and Yokotsuka, K. (2000). Restriction fragment length polymorphism analysis of 16s rRNA genes in lactic acid bacteria isolated from red wine. **J. Biosci. and Bioeng**. 90(3): 335-337.

Satomi, M., Kimura, B., Mizoi, M., Sato, T., and Fuji, T. (1997). *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. **Int. J. Syst. Bact**. 47: 832-836.

Schleifer, K. H., Ehrmann, M., Beimfohr, C., Brockmann E., Ludwig, W, and Amann, R. (1995). Application of molecular methods for the classification and identification of lactic acid bacteria. **Int. Dairy J.** 1081-1094.

Schillinger, U., and Lücke, F. K. (1987). Identification of lactobacilli from meat and meat products. **J. Food Microbiol**. 4: 199-208.

Shahidi, F., Sikorski, Z. E, and Pan, B. S. (1994). Proteins from seafood processing discards (pp. 171-193). **Seafood Protein**. Chapman and Hall, New York.

Sharpe, M. E. and Fryer, T. F. (1965). **Media for Lactic Acid Bacteria** (pp. 697-701). National Institute for Research in Dairying (N.I.R.D).

Sheldon, R. M., and Lindsay, R. C. (1971). Chemical nature of malty flavor and aroma produced by *Streptococcus lactis* var. maltigenes. **Appl. Microbiol.** 22(3): 263-266.

Shimoda, M., Peralta, R., and Osajima, Y. (1996). Headspace gas analysis of fish sauce. **J. Agric. Food Chem**. 44: 3601-3605.

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. **Int. J. Food Microbiol**. 29:213-31.

Simpson, W. J., and Taguchi, H. (1995). The genus *Pediococcus*, with notes on the genera *Tetragenococcus* and *Aerococcus*. In B. J. B. Wood and W. H. Holzapfel (eds.) **The Genera of Lactic Acid Bacteria** (pp. 125-172). Blackie Academic and Professional, London, UK.

Singleton, P. and Sainsbury, D. (1987). **Dictionary of Microbiology and Molecular Biology** (2nd ed.) John Wiley and Sons, New York.

Smit, B. A., and Engels, W. J (2004). Chemical conversion of alpha-keto acids in relation to flavor formation in fermented foods. **J. Agric. Food Chem.** 52(5): 1263-1268.

Smit, B. A., Engels, W. J. M., and Smit, G. (2009). Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods. **Appl. Microbiol. Biotechnol**. 81: 987-999.

Smit, G., Smit, B. A., and Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. **FEMS. Microbiol**. 29: 591-610.

Steinhaus, P., and Schieberle, P. (2007). Characterization of the key aroma compounds in soy sauce using approaches of molecular sensory science. **J. Agric. Food Chem**. 55(15): 6262-6269.

Stiles, M., and Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. **Int. J. Food Microbiol**. 36: 1-29.

Stratton, J. E., Hutkins, R. W., and Taylor, S. L. (1991). Biogenic amines in cheese and other fermented foods: a review. **J. Food Prot**. 54: 460-470.

Tanasupawat, S., and Daengsubha, W. (1983). *Pediococcus* species and related bacteria found in fermented foods and related materials in Thailand. **J. Gen. Appl. Microbiol**. 29: 487-506.

Tanasupawat, S., Thongsanit, J., Okada, S., and Komagata, K. (2003). Lactic acid bacteria isolated from soy sauce mash in Thailand. **J. Gen. Appl. Microbiol**. 48: 201-209.

Taira, W., Funatsu, Y., Satomi, M., Takano, T., and Abe, H. (2007). Changes in extractive components and microbial proliferation during fermentation of fish sauce from underutilized fish species and quality of final products. **Fisheries Sci**. 73: 913-923.

Teuber, M. (1995). The genus *Lactococcus*. In B.J.B. Woods and W.H. Holzapfel (eds.). **The Genera of Lactic Acid Bacteria** (Vol. 2., pp. 173-234). Aspen Publishers, Gaithersburg.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. **Nucleic Acids Res**. 25: 4876-4882.

Thongsanit, J., Tanasupawat, S., Keeratipibul, S., and Jitikavanich, S. (2002). Characterization and identification of *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* strains from fish sauce (Nam-pla). **Japan J. Lactic Acid Bact**. 13(1): 46-52.

Thongthai, C., McGenity, T. J., Suntinanalert, P., and Grant, W. D. (1992). Isolation and characterization of an extremely halophilic archeaebacterium from fermented Thai fish sauce (nam pla). **Lett. Appl. Microbiol**. 14: 111-114.

Thongthai, C., and Suntinanalert, P. (1991). Halophiles in thai fish sauce (Nam pla) **General and applied aspects of halophilic microorganism**. Plenum Press. New York.

Tran, L. H., and Nagano, H. (2002). Isolation and characteristics of *Bacillus subtilis* CN2 and its collagenase production. **J. Food Sci.** 67(3): 1184-1187.

Tsakris, A., Woodford, N., Pournaras, S., Kaufmann, M., and Douboyas, J. (1998). Apparent increased prevalence of high-level aminoglycoside-resistant *Enterococcus durans* resulting from false identification by a semiautomated software. **J. Clin. Microbiol**. 36: 1419-1421.

Tungkawachara, S., Park, J. W., and Choi, Y. J. (2003). Biochemical properties and consumer acceptance of pacific whiting fish sauce. **J. Food Sci.**. 68 (3): 855-860.

Uchida, K. (1982). Multiplicity in soy pediococci carbohydrate fermentation and its application for analysis of their flora. **J. Gen. Appl. Microbiol.** 28: 215-225.

Vassu, T., Smarandache, D., Stoica, I., Sasarman, E., Fologea, D., Musat, F., Csutak, O., Nohit, A., Iftime, O., and Gherasim, R. (2002). Biochemical and genetic characterization of *Lactobacillus Plantarum* cmgb-1 strain used as probiotic. **Biotechnol. Lett**. 7: 585-598.

Ventosa, A., Nieto, J. J., and Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev**. 62: 504-544.

Villar, M., Ruiz-Holgado, A. P., Sanchez, J. J., Trucco, R. E., and Oliver, G. (1985). Isolation and characterization of *Pediococcus halophilus* from salted anchovies (*Engraulis anchoita*). **Appl. Environ. Microbiol**. 49: 664-666.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S Ribosomal DNA amplification for phylogenetic study. **J. Bacteriol.** 173: 697-703.

Williams, A. M., Rodrigues, U. M., and Collins, M. D. (1991). Intragenic relationships of enterococci as determined by reverse transcriptase sequencing of small-subunit rRNA. **Res. Microbiol.** 142: 67-74.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., and Murray, R. G. E. (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. **Int. J. Syst. Bacteriol.** 37: 463-464.

Yongsawatdigul, J. (2006). **Acceleration of fish Sauce fermentation using starter culture and proteinases.** Nakhon Ratchasima. (pp. 66). National Center for Genetic Engineering and Biotechnology (BIOTEC).

Yongsawatdigul, J., Choi, Y. S., and Udomporn, S. (2004). Biogenic amines formation in fish sauce prepared from fresh and temperature-abused Indian anchovy (*Stolephorus indicus*). **J. Food Sci.** 69(4): 312-319.

Yongsawatdigul, J., Rodtong, S., and Raksakulthai, N. (2007). Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. **J. Food Sci.** 72(9): 382-390.

Yvon, M., and Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism.**Int. Dairy. J.** 11(7): 185-201.

APPENDIXES

APPENDIXE A

Reagent preparations and culture media

1. Reagents

1.1 Acetone alcohol

Alcohol (95%)	700.0	ml
Acetone	300.0	ml

1.2 Crystal violet (Gram stain)

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

1.3 Hydrogen peroxide (3% solution)

Hydrogen peroxide	3.0	g
Distilled water	100.0	ml

1.4 Iodine solution (Gram's iodine)

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

1.5 Safranin (Gram stain)

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

1.6 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100.0	g
Add distilled water to bring volume up to	1,000.0	ml

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

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

1.8 Tetramethyl-p-phenylenediamine dihydrochloride (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1.0	g
Add distilled water to bring volume up to	100.0	ml

1.9 Tris-Borate buffer

Tris-Base	10.77	g
EDTA (di-Sodium salt)	0.93	g
Boric acid	5.54	g
Add distilled water to bring volume up to	1,000.0	ml

2. Culture media

2.1 De Man, Rogosar and Sharpe broth (MRS broth)

Peptone	10.0	g
Meat extract	10.0	g
Sodium chloride	50.0 or 100.0	g
Yeast extract	5.0	g
Dextrose or Glucose	20.0	g
di-Potassium hydrogen phosphate	2.0	g
Tween-80	1.0	ml
di-Ammonium hydrogen citrate	0.2	g
Sodium acetate	5.0	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
$CaCO_3$	5.0	g
Agar	15.0	g
Add distilled water to bring volume up to	1,000.0	ml

pH 7.0

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

2.2 De Man, Rogosar and Sharpe agar (MRS agar)

The components were similar with MRS broth containing 0.5% $CaCO_3$ with added 15.0 g/L agar. The medium was autoclaved at 115 °C for 10 min.

2.3 JCM 168 broth

Casamino acids	5.0	g
Yeast extract	5.0	g
Sodium glutamate	1.0	g
tri-Sodium citrate	3.0	g
Potassium chloride	2.0	g
$MgSO_4.7H_2O$	20.0	g
Sodium chloride	180.0	g
$FeCl_2.4H_2O$	36.0	ml
$MnCl_2.4H_2O$	0.36	ml
Add distilled water to bring volume up to pH 7.0 $\pm$ 0.2	1,000.0	ml

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

2.4 JCM 168 agar

The components were similar with JCM168 broth with added 18.0 g/L agar. The medium was autoclaved at 121 °C for 15 min.

2.5 Modified Glucose Yeast Peptone medium (mGYP medium)

(Bover-Cid and Holzapful, 1999)

Glucose	10.0	g
Yeast extract	10.0	g
Peptone	5.0	g

Tween 80	5.0	g
Amino acid of each concentration (L-lysine, L-histidine, L-tyrosine and L-ornithine)	2.5	g
Sodium acetate trihydrate	2.0	g
$MgSO_4.7H_2O$	0.2	g
$MnSO_4.7H_2O$	0.01	g
$FeSO_4.7H_2O$	0.01	g
Sodium chloride	50.0 or 250.0	g
Add distilled water to bring volume up to	1,000.0	ml

pH 7.0 $\pm$ 0.2

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

2.6 Rogosa agar containing 1% Skim milk (modified from Rogosa SL agar; Atlas, 2004)

1) Rogosa agar

K_2HPO_4	6.0	g
Yeast extract	4.0	g
tri-Ammonium citrate	1.0	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Glucose	10.0	g
Sodium chloride	50.0 or 100.0	g

Agar	15.0	g
Add distilled water to bring volume up to	900.0	ml

pH 7.0 ± 0.2

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

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

and autoclaved at 110 °C for 10 min

Mix reconstituted skim milk (10% solids) and Rogosa agar

2.7 Rogosa agar containing 1% soluble starch or 1% Tween 80 (modified from Rogosa SL agar; Atlas, 2004)

Tryptone	5.0 or 10.0	g
K_2HPO_4	6.0	g
Yeast extract	3.0	g
tri-Ammonium citrate	1.0	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Soluble starch or Tween 80	10.0	g
Sodium chloride	50.0 or 100.0	g
Agar	15.0	g
Add distilled water to bring volume up to	1,000	ml

pH 7.0 ± 0.2

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

APPENDIX B

Nucleotide sequence alignment of 16S rRNA gene

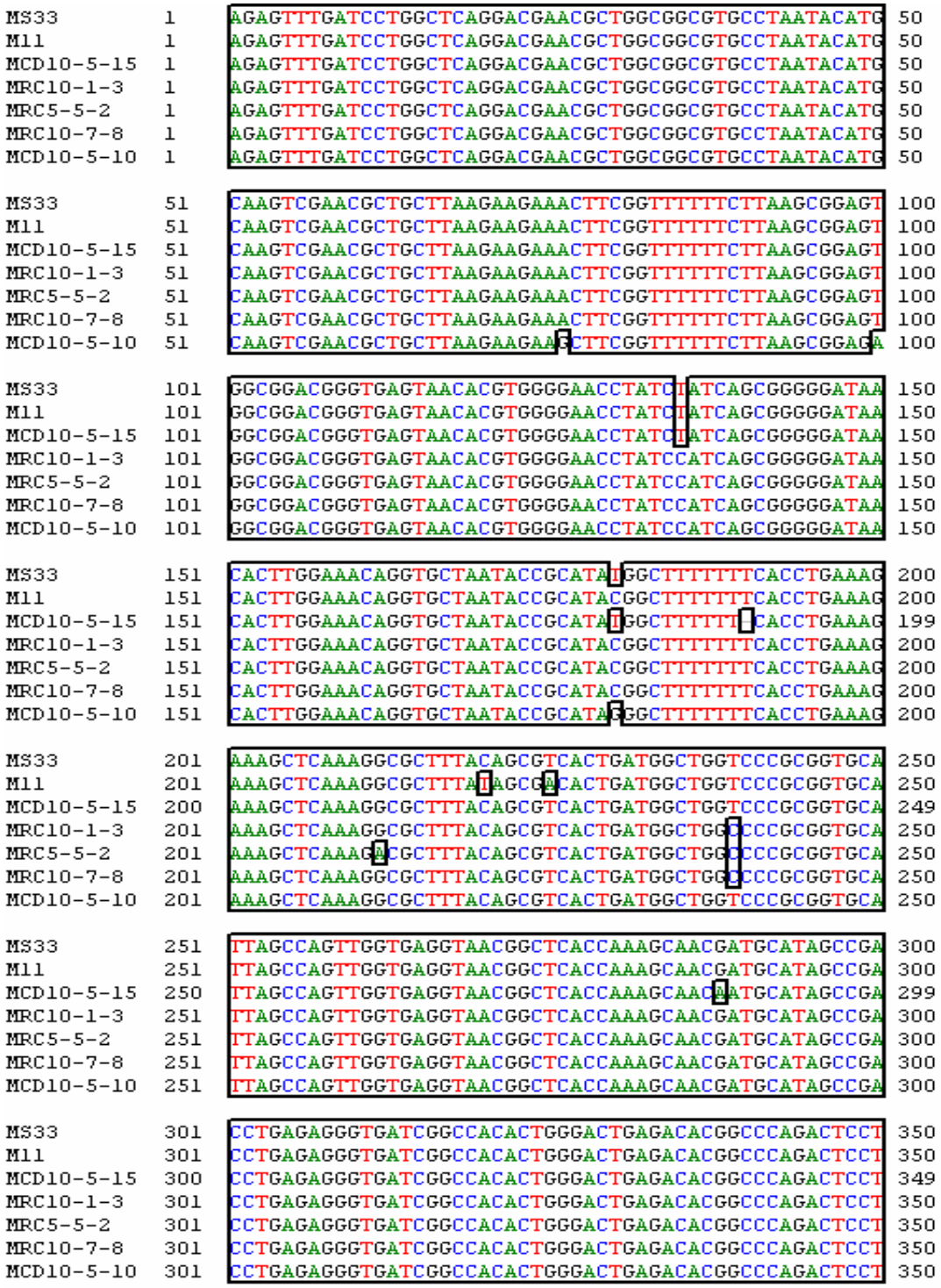

Figure 1B Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of lactic acid bacteria isolated from fish sauce fermentation.

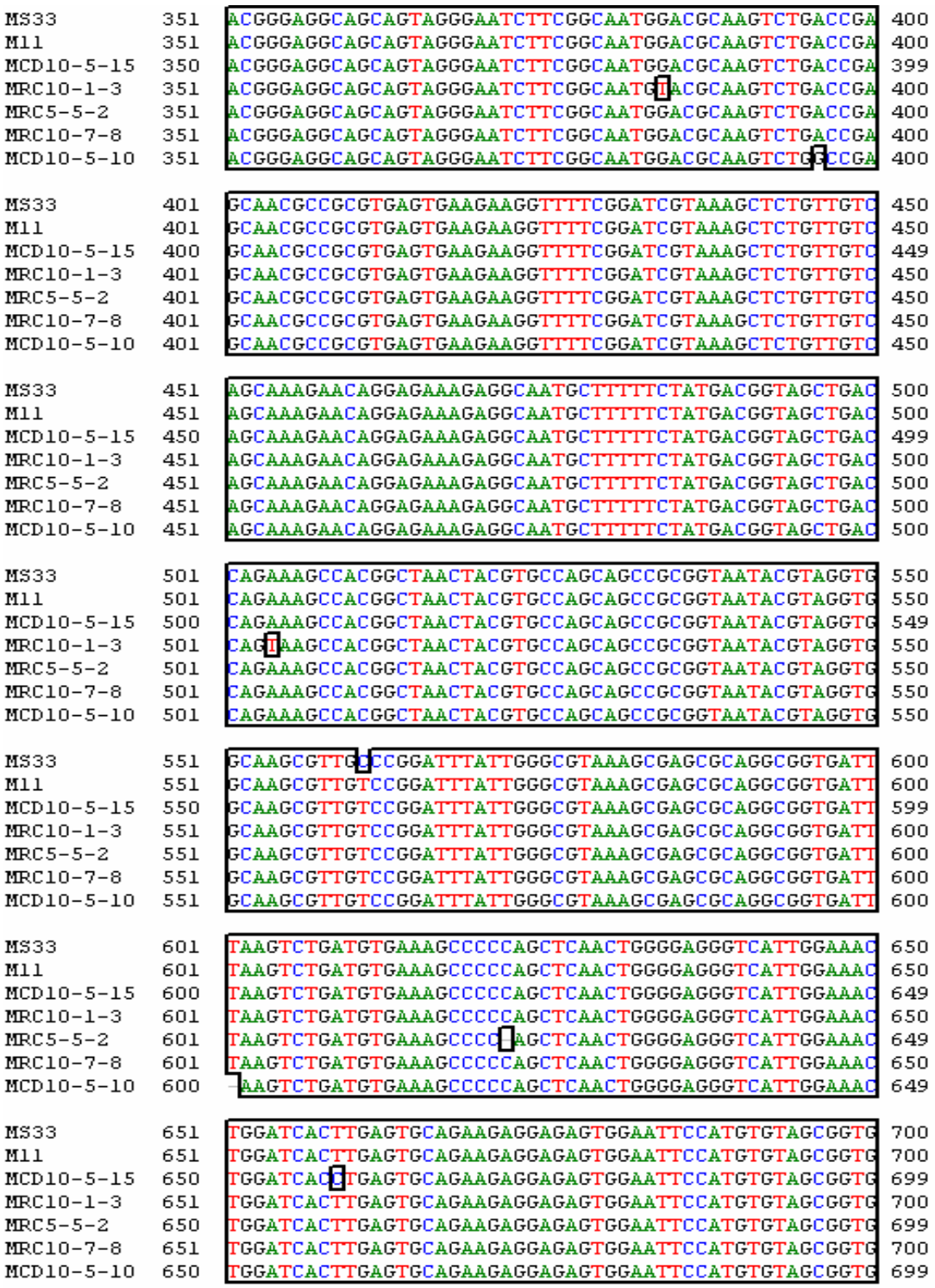

Figure 2B (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of lactic acid bacteria isolated from fish sauce fermentation.

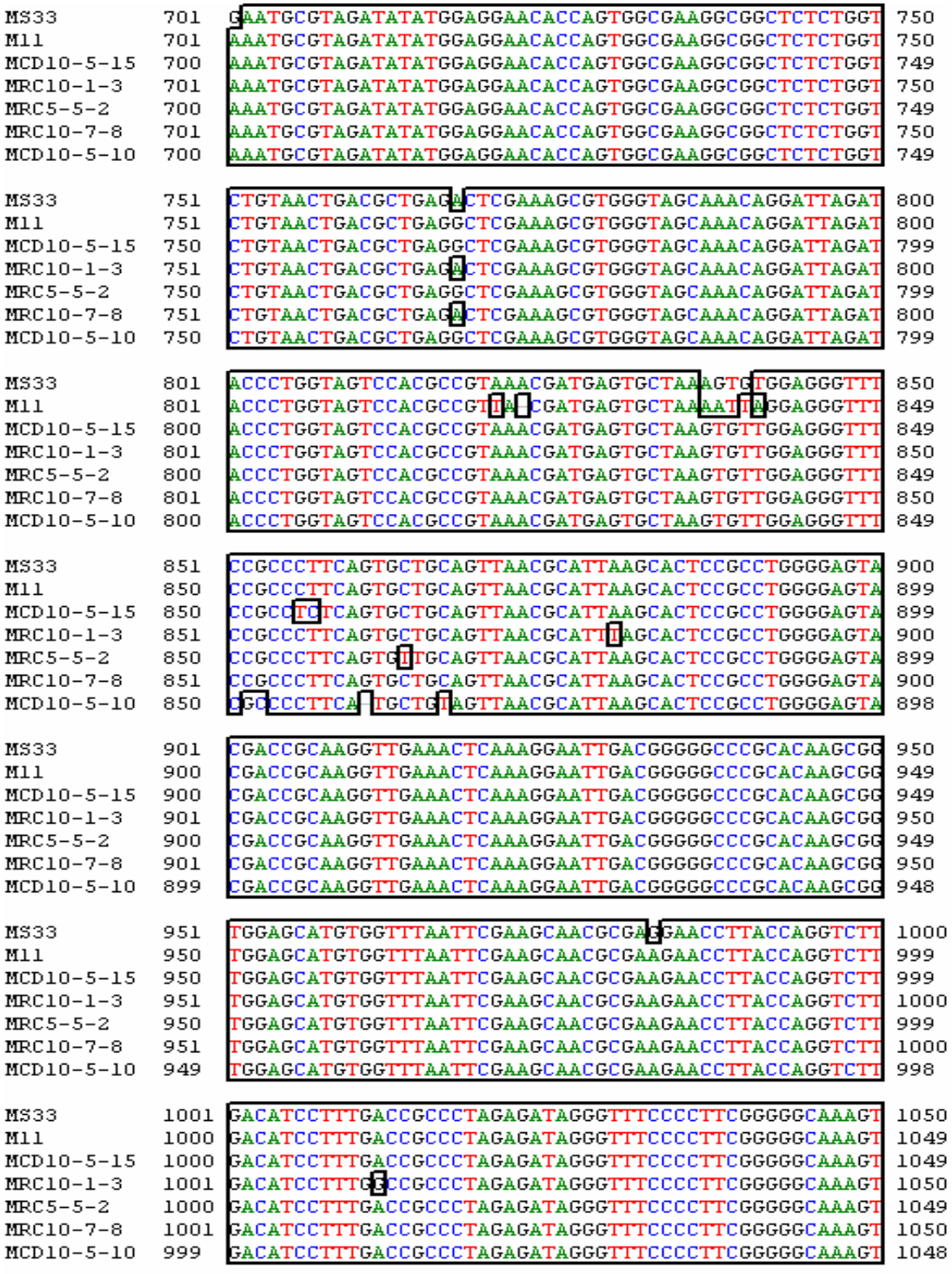

Figure 3B (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of lactic acid bacteria isolated from fish sauce fermentation.

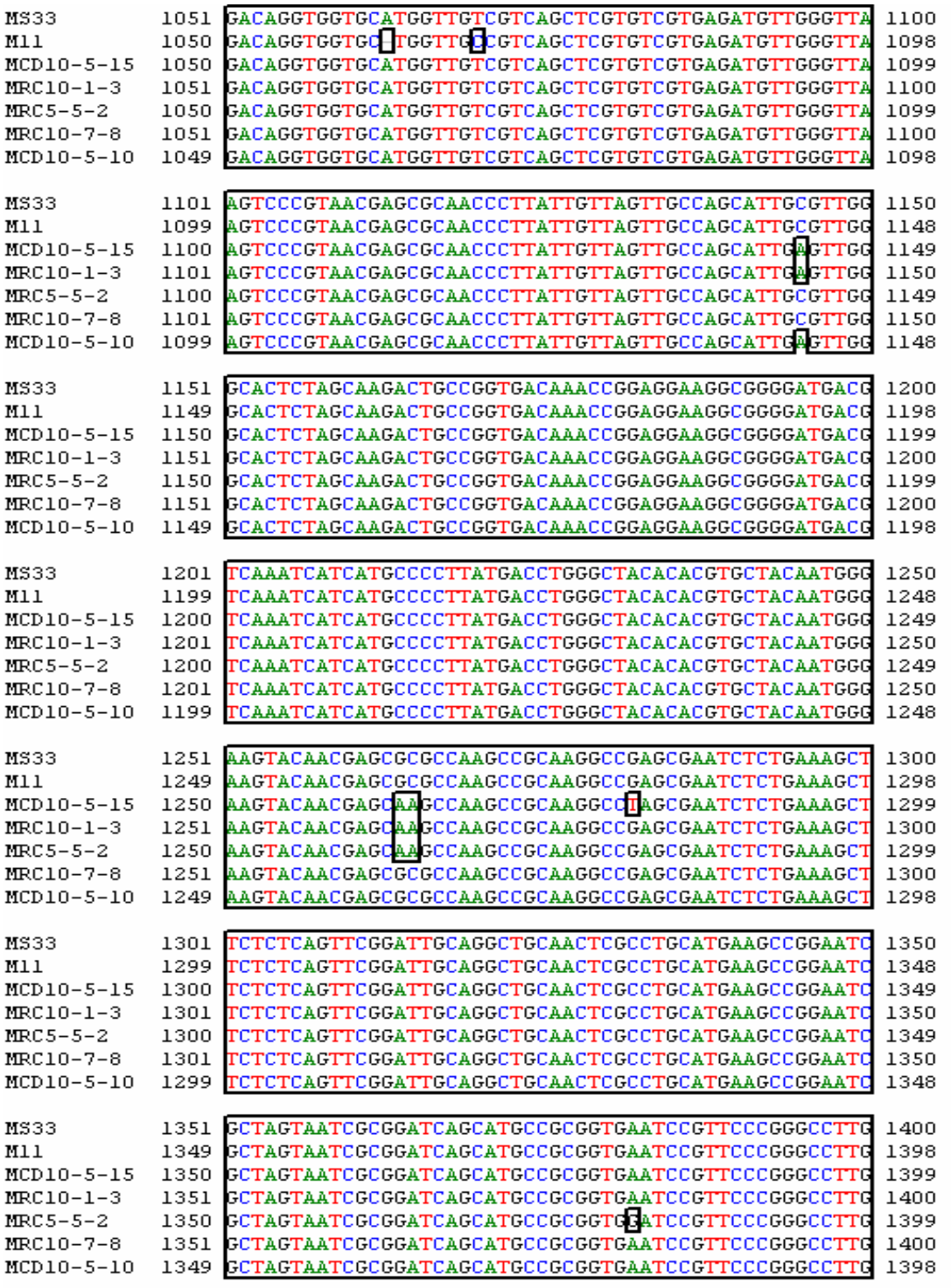

Figure 4B (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of lactic acid bacteria isolated from fish sauce fermentation.

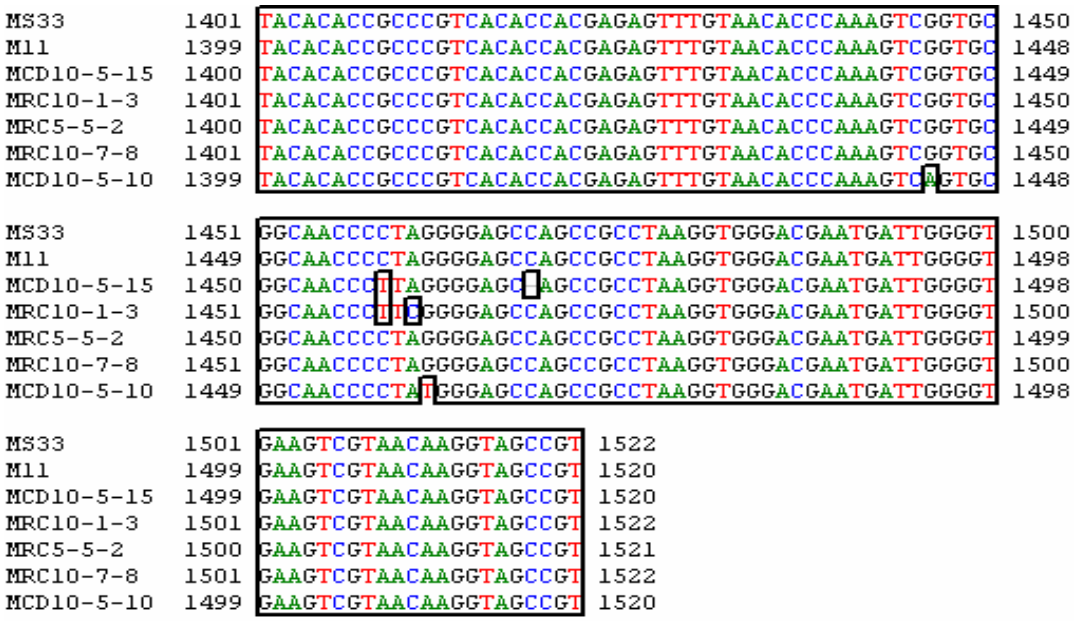

Figure 5B (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of lactic acid bacteria isolated from fish sauce fermentation.

APPENDIX C

Nucleotide sequence accession number in GenBank (U.S.A.)

Table 1C Nucleotide sequence accession number in GenBank (U.S.A.).

Isolation source	Isolate code	Nucleotide sequence submission		
		Identification result	Length of sequence (bp)	NCBI Accession no.
Sample from fish sauce fermentation process at 1st month	M11	*T. halophilus* M11	1520	FJ715464
	MS33	*T. halophilus* MS33	1522	FJ715465
	MRC10-1-3	*T. halophilus* MRC10-1-3	1522	FJ715466
5th	MRC5-5-2	*T. halophilus* MRC5-5-2	1521	FJ715467
	MCD10-5-10	*T. halophilus* MCD10-5-10	1520	FJ715468
	MCD10-5-15	*T. halophilus* MCD10-5-15	1520	FJ715469
7th	MRC10-7-8	*T. halophilus* MRC10-7-8	1522	FJ715470

APPENDIX D

Volatile compound chromatograms

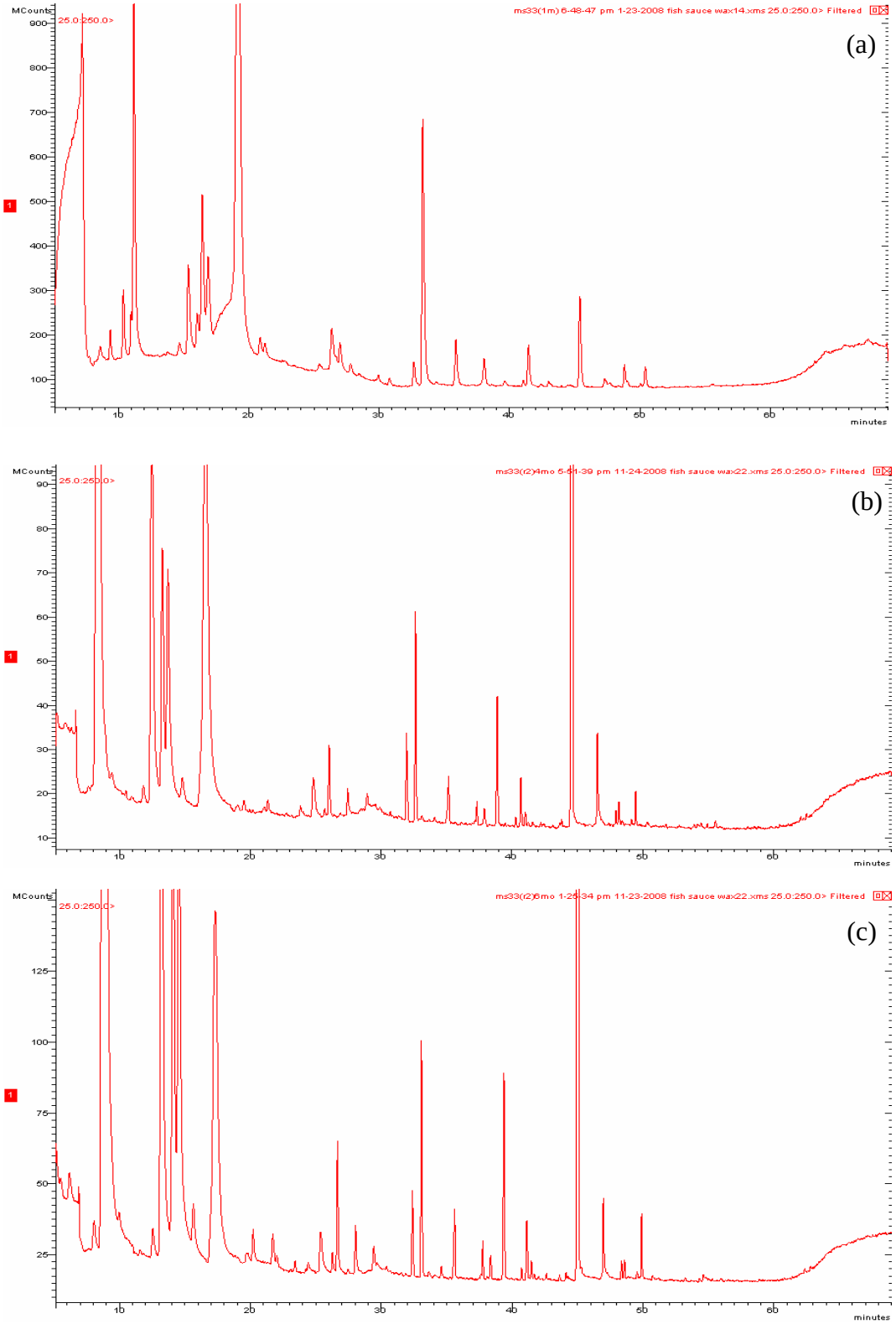

Figure 1D Chromatograms of volatile compounds of fish sauce samples inoculated with *T. halophilus* MS33 fermented for 1 month (a), 4 month (b), and 6 month (c).

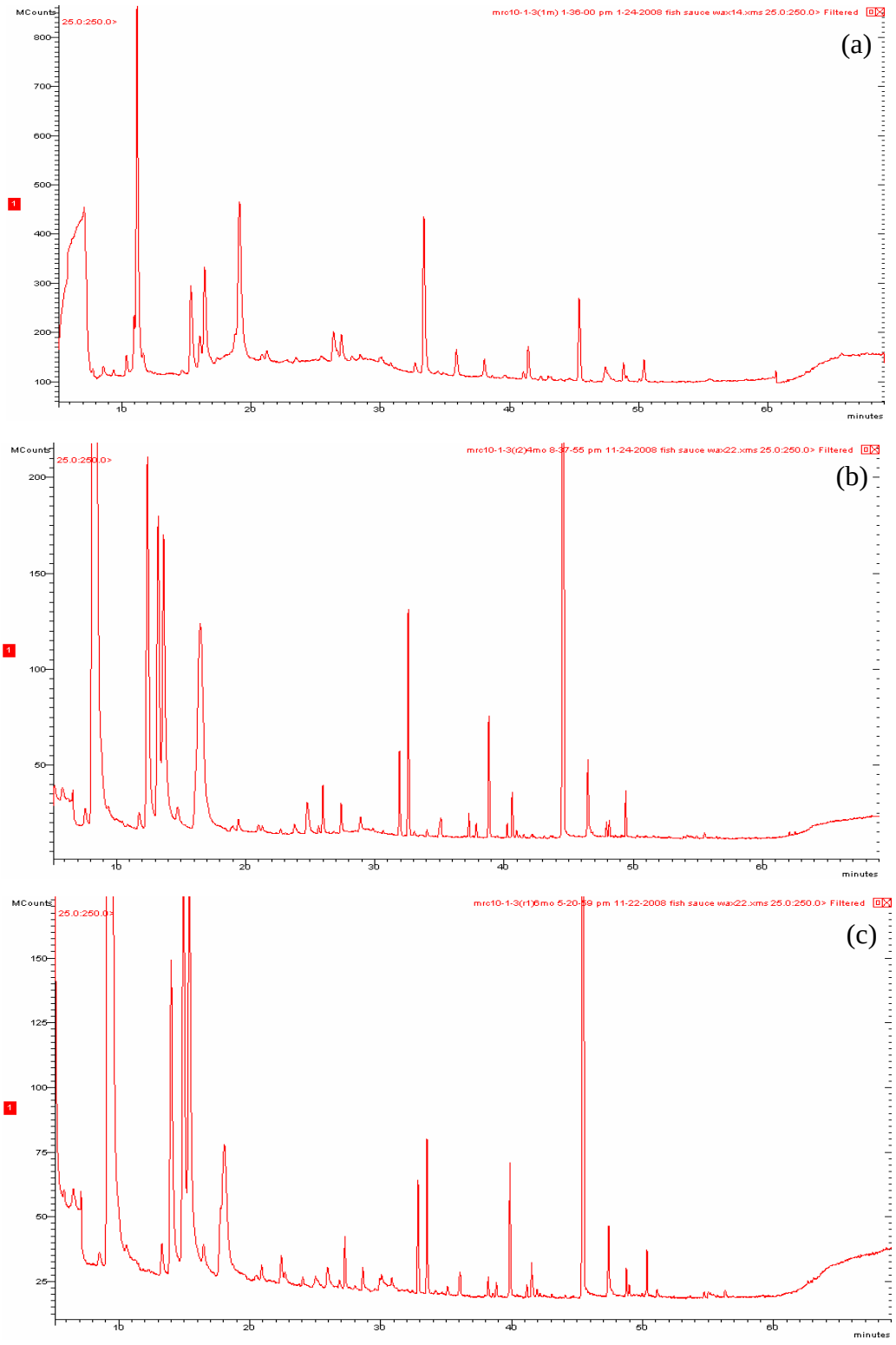

Figure 2D Chromatograms of volatile compounds of fish sauce samples inoculated with *T. halophilus* MRC10-1-3 fermented for 1 month (a), 4 month (b), and 6 month (c).

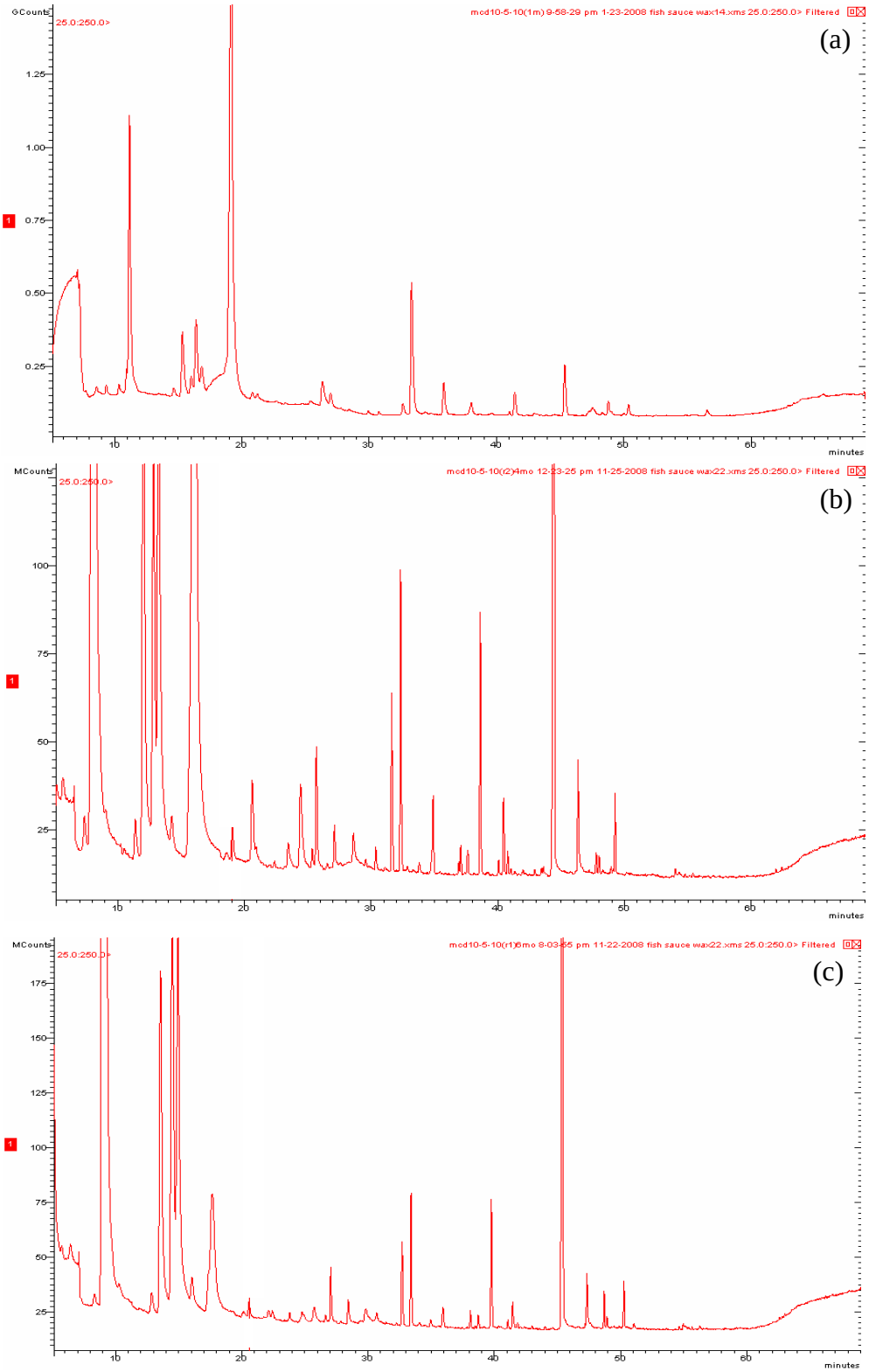

Figure 3D Chromatograms of volatile compounds of fish sauce samples inoculated with *T. halophilus* MCD10-5-10 fermented for 1 month (a), 4 month (b), and 6 month (c).

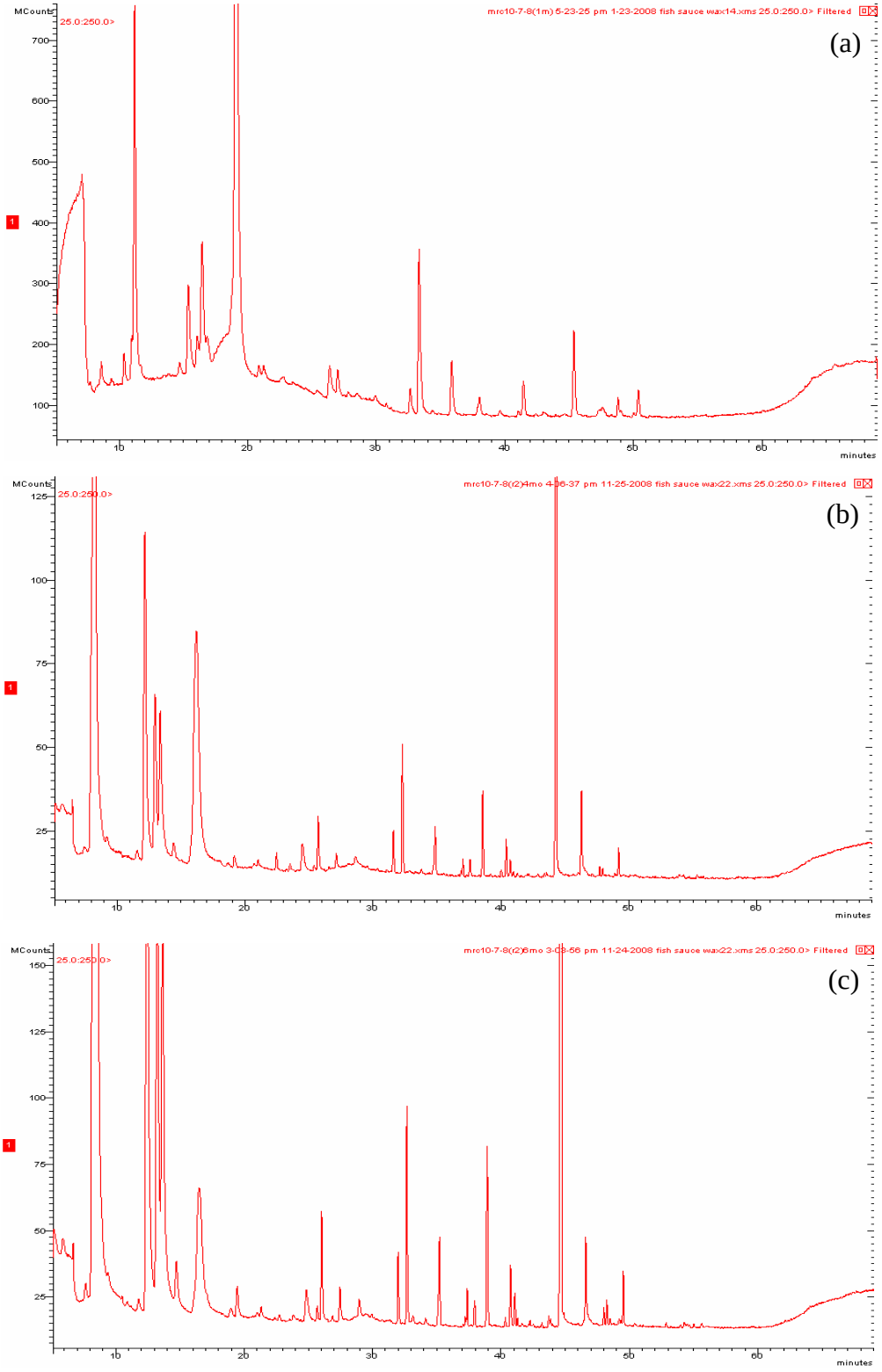

Figure 4D Chromatograms of volatile compounds of fish sauce samples inoculated with *T. halophilus* MRC10-7-8 fermented for 1 month (a), 4 month (b), and 6 month (c).

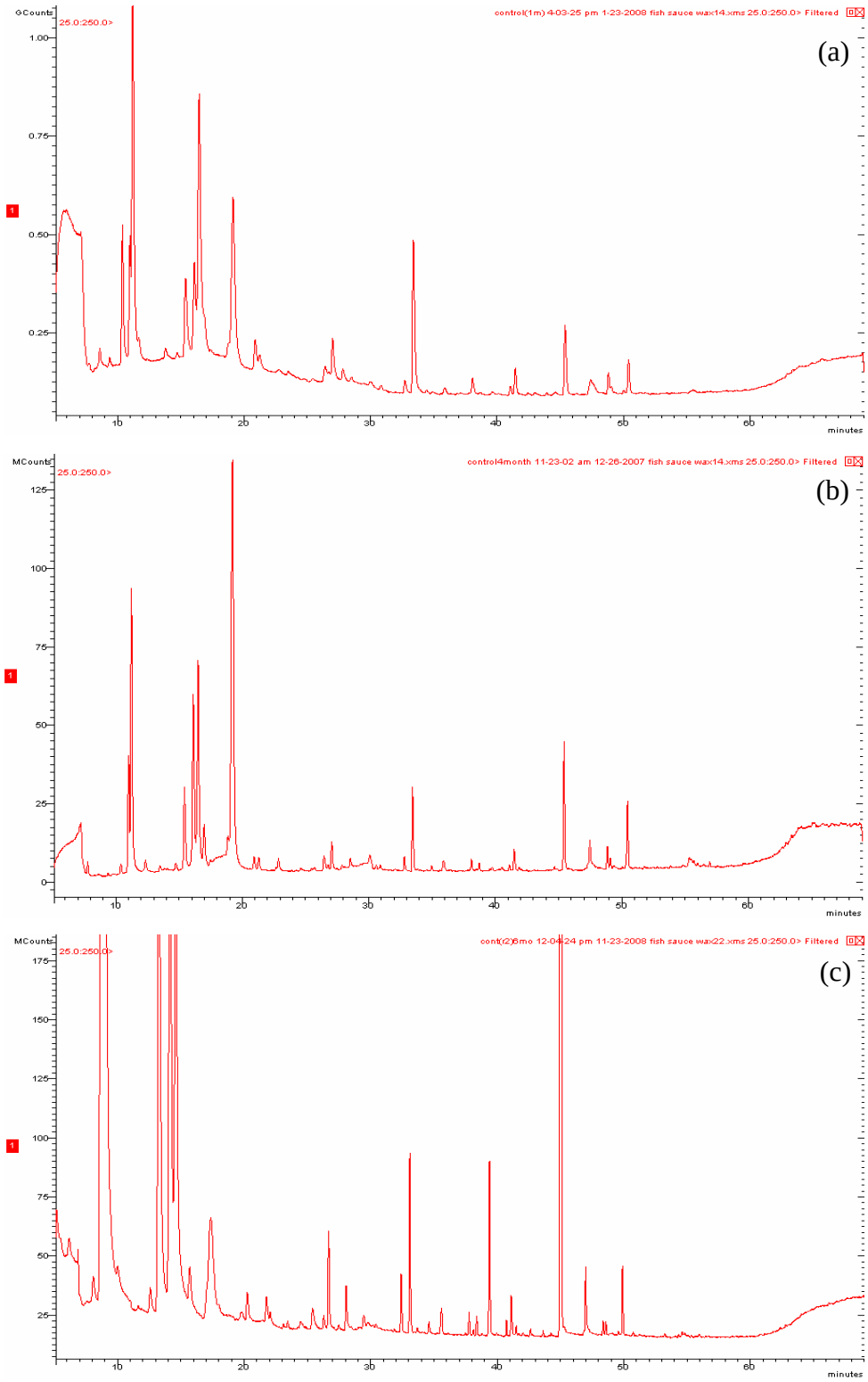

Figure 5D Chromatograms of volatile compounds of fish sauce samples without starter culture (control) fermented for 1 month (a), 4 month (b), and 6 month (c).

BIOGRAPHY

Ms. Natteewan Udomsil was born in November 30, 1980 in Nan. She received Bachelors Degree in B.Sc. (Food Technology) from Institute of Agricultural Technology, Suranaree University of Technology, Thailand in 2003. After graduation, she worked at NutriCreate Food (NC Food) Co., Ltd. as a manager trainee. In 2004, she worked as a research assistant of Assoc. Prof. Dr. Jirawat Yongsawatdigul in the topic of biogenic amines formation in fermented fish products. The results from some part of this research have been presented as a poster presentation at The 7th Agro-industrial conference, June 22-24, 2005, BITEC, Bangkok, Thailand. She got excellent poster presentation award. In 2005, she enrolled in a Food Technology master program, Suranaree University of Technology, Thailand and she was granted a scholarship by National Center for Genetic Engineering and Biotechnology (BIOTEC) under the grant No. BT-B-01-FT-19-5014.