เทคนิคทางอณูวิทยาสำหรับวิเคราะห์กลุ่มเชื้อเชิงปริมาณและการติดตามรูปแบบจุลินทรีย์และบทบาทในกระบวนการหมักน้ำปลา

นางสาวนัทธีวรรณ อุดมศิลป์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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MOLECULAR-BASED TECHNIQUES FOR QUANTIFICATION OF STARTER CULTURES AND MONITORING OF MICROBIAL PROFILES AND THEIR ROLES IN FISH SAUCE FERMENTATION

Natteewan Udomsil

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

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MOLECULAR-BASED TECHNIQUES FOR QUANTIFICATION OF STARTER CULTURES AND MONITORING OF MICROBIAL PROFILES AND THEIR ROLES IN FISH SAUCE FERMENTATION

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

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นัทธีวรรณ อุดมศิลป์ : เทคนิคทางอณูวิทยาสำหรับวิเคราะห์กล้าเชื้อเชิงปริมาณและการติดตามรูปแบบจุลินทรีย์และบทบาทในกระบวนการหมักน้ำปลา (MOLECULAR-BASED TECHNIQUES FOR QUANTIFICATION OF STARTER CULTURES AND MONITORING OF MICROBIAL PROFILES AND THEIR ROLES IN FISH SAUCE FERMENTATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 204 หน้า.

วิธีพีซีอาร์เชิงปริมาณ (quantitative polymerase chain reaction, qPCR) ได้พัฒนาขึ้นเพื่อตรวจจับแบบจำเพาะเจาะจงและตรวจวัดปริมาณของ *Virgibacillus* sp. SK37 และ *Tetragenococcus halophilus* MS33 ซึ่งเป็นกล้าเชื้อในกระบวนการหมักน้ำปลา ดีเอ็นเอติดตาม (probe) Vir1086 และ Tet48 ออกแบบมาจากยีน alkaline serine protease-X (*aprX*) และ internal transcribed spacer (ITS) ของ *Virgibacillus* sp. SK37 และ *T. halophilus* MS33 ตามลำดับ แสดงความจำเพาะเจาะจงในระดับสปีชี่ส์ (species-specificity) ต่อ *V. halodenitrificans* และ *T. halophilus* ตามลำดับ โดยไม่ทำปฏิกิริยากับดีเอ็นเอของแบคทีเรียท้องถิ่นชนิดอื่นที่คัดแยกจากกระบวนการหมักน้ำปลา ประสิทธิภาพของดีเอ็นเอติดตาม Vir1086 และ Tet48 สำหรับตรวจจับดีเอ็นเอบริสุทธิ์ของ *Virgibacillus* sp. SK37 และ *T. halophilus* MS33 มีค่าเท่ากับ 100.4% และ 91.7% ตามลำดับ ปริมาณต่ำสุดที่วิเคราะห์ได้ (quantification limits) สำหรับ *Virgibacillus* sp. SK37 และ *T. halophilus* MS33 คือ 10^3 เซลล์/มิลลิลิตร และ 10^2 เซลล์/มิลลิลิตร ตามลำดับ วิธีพีซีอาร์เชิงปริมาณร่วมกับโพรพิเดียมโมโนเอไซด์ (propidium monoazide, PMA) เข้มข้น 100 ไมโครโมลาร์ (PMA-qPCR) สามารถตรวจจับการเปลี่ยนแปลงปริมาณเซลล์ที่มีชีวิตของ *Virgibacillus* sp. SK37 และ *T. halophilus* MS33 ในแบบจำลองกระบวนการหมักน้ำปลาด้วยปลาแมคคาเรล (mackerel) ได้อย่างมีประสิทธิภาพ

ผลการวิเคราะห์เชิงปริมาณของกล้าเชื้อในกระบวนการหมักน้ำปลาที่ระยะเวลา 6 เดือน ด้วยวิธี PMA-qPCR มีค่าสูงกว่าการตรวจนับด้วยอาหารเลี้ยงเชื้อประมาณ 10-10^3 เซลล์/มิลลิลิตร ในช่วง 120-150 วันของการหมัก ($P < 0.05$) ตัวอย่างที่เติมกล้าเชื้อ *Virgibacillus* sp. SK37 เป็นเวลา 1 เดือน ตามด้วย *T. halophilus* MS33 (SK37_1M+MS33) พบการอยู่รอดของ *T. halophilus* MS33 สูงกว่าตัวอย่างที่ใช้กล้าเชื้อเดี่ยว ตัวอย่าง SK37_1M+MS33 และกล้าเชื้อเดี่ยว *Virgibacillus* sp. SK37 (SK37) มีปริมาณฮีสตามีนต่ำกว่าตัวอย่างควบคุม ($P < 0.05$) ปริมาณแอลฟาอะมิโนของตัวอย่างน้ำปลาที่เติมกล้าเชื้อร่วมพร้อมกัน (MS33+SK37) และ SK37_1M+MS33 เท่ากับ 1,160.41 และ 1,185.71 มิลลิโมลาร์ ตามลำดับ เมื่อเปรียบเทียบกับตัวอย่างควบคุม (1,008.29 มิลลิโมลาร์) และมีปริมาณกรดกลูตามิคอิสระ (free glutamic) และทั้งหมด (total glutamic) สูงกว่าตัวอย่างควมคุม ($P < 0.05$) 2-เมธิลโพรพาแนล (2-methylpropanal) 2-เมธิลบิวทาแนล (2-methylbutanal) 3-เมธิลบิวทาแนล (3-methylbutanal) เป็นสารระเหยเด่นที่พบในตัวอย่างน้ำปลาที่เติมกล้าเชื้อ SK37_1M+MS33 ดังนั้นการเติมกล้าเชื้อตามลำดับในระหว่างกระบวนการหมักจึงมีศักยภาพในการปรับปรุงคุณภาพของสารระเหยและองค์ประกอบทางเคมีของน้ำปลา

รูปแบบของแบคทีเรียในน้ำปลาที่เติมกล้าเชื้อเมื่อวิเคราะห์ด้วย 16S rRNA gene sequencing โดย next generation sequencing, Ion Torrent Personal Genome Machine (PGM®) พบว่า แบคทีเรียสกุลเด่น ได้แก่ *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Tetragenococcus*, และ *Virgibacillus* การเติมกล้าเชื้อร่วมพร้อมกัน MS33+SK37 ส่งผลให้พบ *Virgibacillus* เป็นแบคทีเรียสกุลเด่น แต่พบ *T. halophilus* คือ แบคทีเรียเด่นในตัวอย่างน้ำปลา SK37_1M+MS33

สาขาวิชาเทคโนโลยีอาหาร
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NATTEEWAN UDOMSIL : MOLECULAR-BASED TECHNIQUES FOR QUANTIFICATION OF STARTER CULTURES AND MONITORING OF MICROBIAL PROFILES AND THEIR ROLES IN FISH SAUCE FERMENTATION. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 204 PP.

DNA PROBES/ QPCR/*TETRAGENOCOCCUS HALOPHILUS* MS33/ *VIRGIBACILLUS* SP. SK37/COMBINED CULTURES/AMINO ACIDS/VOLATILE COMPOUNDS/FISH SAUCE/BACTERIAL PROFILING

Quantitative polymerase chain reaction (qPCR) methods were developed for the specific detection and quantification of *Virgibacillus* sp. SK37 and *Tetragenococcus halophilus* MS33, which were used as starter cultures for fish sauce fermentation. Vir1086 and Tet48 probes were designed from the alkaline serine protease-X gene (*aprX*) and the internal transcribed spacer (ITS) of *Virgibacillus* sp. SK37 and *T. halophilus* MS33, respectively, showed species-specificity for *V. halodenitrificans* and *T. halophilus*, respectively, without cross reacting with other species of microbiota isolated from fish sauce fermentation. The efficiencies of Vir1086 and Tet48 probes for the detecting purified DNA from *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were 100.4% and 91.7%, respectively. The quantification limits of the method for *Virgibacillus* sp. SK37 and *T. halophilus* MS33 detection were 10^3 Cells/mL and 10^2 Cells/mL, respectively. The qPCR combined with 100 µM of propidium monoazide (PMA-qPCR) method was successfully detected viable cell changes of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in a Spanish mackerel fish sauce fermentation model.

Quantification of starter cultures in fish sauce fermented for 6 months by PMA-qPCR was higher than that of viable plate counts about 10-10^3 Cells/mL at 120-150 days of

fermentation ($P < 0.05$). Fish sauce prepared by adding *Virgibacillus* sp. SK37, followed by *T. halophilus* MS33 after one month (SK37_1M+MS33), showed higher survival rate of *T. halophilus* MS33 than that of the single starter culture treatment. Histamine contents of SK37_1M+MS33 and fish sauce inoculated with *Virgibacillus* sp. SK37 (SK37) were lower than the control ($P < 0.05$). α-Amino contents of fish sauce added simultaneous co-cultures (MS33+SK37) and SK37_1M+MS33 were 1,160.41 and 1,185.71 mM, respectively, compared to the control (1,008.29 mM). Contents of total and free glutamic acid from inoculated fish sauce were also higher than the control ($P < 0.05$). 2-Methylpropanal, 2-methylbutanal, and 3-methylbutanal were major volatile compounds found in SK37_1M+MS33 fermentation. Addition of starter culture, particularly co-cultures in sequential order (SK37_1M+MS33), showed potential to improve volatile compound and chemical compositions of fish sauce to a greater extent than single culture inoculation.

Bacterial profiling of fish sauce inoculated with starter cultures was analyzed by 16S rRNA gene sequencing using next generation sequencing, Ion Torrent Personal Genome Machine (PGM®). Genera *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Tetragenococcus*, and *Virgibacillus* were domonant. *Virgibacillus* was predominant in fish sauce inoculated with MS33+SK37 but *Tetragenococcus* was dominant in fish sauce inoculated with SK37_1M+MS33.

School of Food Technology

Academic Year 2014

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of varience
aprX	=	Alkaline serine protease-X gene
α	=	Alfa
BLAST	=	Basic local alignment search tool
bp	=	Base pair
CFU	=	Colony forming unit
cm	=	Centrimeter
°C	=	Degree Celsius
CRD	=	Completely Randomized Design
DNA	=	Deoxyribonucleic acid
et al.	=	et alia (and others)
(m, µ) g	=	(milli, micro) Gram
h	=	Hour
(m, µ) l	=	(milli, micro) Liter
(m, µ) M	=	(milli, micro) Molar
min	=	Minute
(m, µ) mol	=	(milli, micro) Mole
%	=	Percentage
GC	=	Gas chromatography
HPLC	=	High performance liquid chromatography
PBS	=	Phosphate buffer saline
PMA	=	Propidium monoazide

LIST OF ABBREVIATIONS (Continued)

ITS	=	Internal transcribed spacer
MS	=	Mass spectrometry
NGS	=	Next generation sequencing
PGM	=	Personal Genome Machine
QIIME	=	Quantitative Insights Into Microbial Ecology
qPCR	=	Quantitative polymerase chain reaction
s	=	Second
SAS	=	Statistical analysis system
sp.	=	Species
subsp.	=	Subspecies
SPME	=	Solid phase micro extraction
TN	=	Total nitrogen

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce is an amber liquid with salty taste and distinctive odor. It is widely used as a seasoning in Asian cuisines. Production of fish sauce involves mixing of fish and salt at a ratio 3:1 and putting in a fermentation tank at ambient temperatures for 12-18 months. Protein hydrolysis during fish sauce fermentation is naturally induced by both endogenous from fish viscera and microbial enzymes, leading to formation of small peptides and free amino acids. Due to long fermentation time, production growth is very limited (Akolkar, Durai, and Desai, 2010). Therefore, the addition of suitable starter culture to accelerate fermentation and to improve fish sauce odor has been investigated. In the past, *Staphylococcus xylosus* was added to fish sauce mash (moromi) to improve fish sauce odor (Fukami, Funatsu, Kawasaki, and Watabe, 2004). It reduced dimethyl trisulfide in the finished product. In 2007, Yongsawatdigul, Rodtong, and Ruksakulthai applied proteinase-producing bacterium, namely *Virgibacillus* sp. SK37, in combination with commercial enzymes to reduce fermentation to 4 months. Akolkar et al. (2010) reported that addition of *Halobacterium* sp. SP1(1) as a starter culture in fish sauce showed the highest protease activity, peptides and α-amino acids content at 10 days of fermentation, indicating that fermentation time was accelerated. In 2011, Udomsil, Rodtong, Choi, Hua and Yongsawatdigul reported that *Tetragenococcus halophilus* isolated from *nam pla* produced desirable volatile compounds and increased amino acids, such as glutamic acid which is a main contributor to umami taste. It was also found that *T. halophilus* can reduce dimethyl disulfide, a compound contributing to fecal note.

Moderately halophilic bacterium, *Virgibacillus* sp. SK37, was isolated from 1-month-old Thai fish sauce mashes (Nawong, 2006). *Virgibacillus* sp. SK37 produced extracellular and cell-bound proteinases at high salt concentrations (Sinsuwan, Rodtong and Yongsawatdigul, 2007; 2008). Extracellular proteinases showed an increase in activity at NaCl concentrations up to 20% with the optimum activity at 65 °C, pH 8.0. Cell-bound proteinases exhibited maximum activity at 65 °C, pH 7.0-9.0 and showed high stability at 25% NaCl, 30 °C. Both extracellular and cell-bound proteinases can hydrolyze fish protein at high salt concentrations (Sinsuwan, et al., 2007; 2008). Addition of *Virgibacillus* sp. SK37 in fish sauce fermentation as a starter culture showed ability to hydrolyze fish protein and reduce fermentation time. Furthermore, total amino acids of fish sauce samples added *Virgibacillus* sp. SK37 fermented for 4 months were comparable to commercial fish sauce fermented for 12 months without affecting the sensory characteristics. These results indicated that *Virgibacillus* sp. SK37 showed potential as a starter culture for acceleration of fish sauce fermentation.

Halophilic lactic acid bacteria (halophilic LAB), *T. halophilus* MS33, was also isolated from 1 month-old Thai fish sauce fermentation (Udomsil, Rodtong, and Yongsawatdigul, 2010). It can grow at 0-25% NaCl and hydrolyzes fish proteins containing 25% NaCl. *T. halophilus* was also found in sugar-thick juice (Justé et al., 2008), fermented mustard (Saun-tsai) (Chen, Yanagida, and Hsu, 2006), Japanese-fermented puffer fish ovaries (Kobayashi, Kimura, and Fuji, 2000), Indonesian ‘terasi’ shrimp paste (Kobayashi et al., 2003), and Indonesian soy mash (Roling, and Verseveld, 1996). LAB play an important role in food fermentation, including soybean fermentation (Li, 2003) and fermented mustard (Saun-tsai) (Chen et al., 2006). They produced several kinds of organic acids including lactic acid, acetic acid, propionic acid, which are responsible for distinctive taste in fermented products. In addition, LAB can produce volatile compounds e.g. aldehydes, ketones, and alcohols. These compounds contributed to distinct flavor

characteristics of fermented foods (Liu, Han and Zhou, 2011). Recently, *T. halophilus* was used as a starter culture to improve flavor characteristics in fish sauce fermentation. Inoculated fish sauce samples containing 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal with meaty note and did not contain undesirable sulfurous compounds (Udomsil et al., 2011). It is likely that *T. halophilus* found in fish sauce could play a significant role in flavor formation during fish sauce fermentation.

Action of microorganisms and enzymes during fermentation leads to desirable biochemical and nutritional changes. Generally, several microorganisms are involved in a naturally-fermented food product. For example, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. helvelticus*, and *Lb. fermentum* are starter cultures for cheese production (Cremonesi et al., 2011). These thermophilic LAB are predominant bacteria during cheese making and the earlier stage of ripening. The essential role of thermophilic LAB is acidification. Zhao et al. (2011) reported that combination of *L. pentosus*, *Pediococcus pentosaceus*, and *S. carnosus* as starter cultures in dry fermented sausage production could increase unsaturated free fatty acid and increase shelf-life of the products. Ciani and Comitini, (2015) reported of multi-yeast starter cultures of wine fermentation was synergistic due to proteolytic activity of non-*Saccharomyces* at the initial stage leading to enrichment of nitrogen source. Then, *S. cerevisiae* strains uptake and the consequent consumption of some amino acids. Since *Virgibacillus* sp. SK37 exhibited high proteolytic activity, while *T. halophilus* contributed to desirable volatile compounds in fish sauce, the use of both bacteria for starter cultures would synergistically improve production and quality of fish sauce. The use of co-cultures of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 for fish sauce fermentation should be studied systematically.

Traditional plate count technique has long been used to monitor changes of microbial population of various fermented foods. Precise enumeration is limited by such a technique. The major drawback is that it cannot differentiate starter cultures and microflora

based on colony morphology. Moreover, plate count cannot detect viable but non-culturable (VBNC) cells which occur when cells are under stress conditions such as high osmotic stress, extreme pH, or high salinity (Fakruddin, Mannan, and Andrews, 2013). This leads to underestimate 'true" microbial population. Furthermore, traditional method takes longer detection time with lower sensitivity (Cremonesi, et al., 2007). The accurate determination on the changes of starter cultures would provide critical information of microbiological dynamics during fermentation. Therefore, a better enumeration technique is greatly needed.

Real-time quantitative PCR (qPCR) has been developed for detection of mixed bacteria in food specimens based on specificity and sensitivity of gene detection using specific probes (Johnson, 2000). Specific genes/regions such as protein-encoding gene, ITS region, or virulence gene have been used to design specific probe which can differentiate at the species level. However, qPCR detects both live and dead cells (Delroisse, Boulvin, Parmentier, Dauphin, Vandenbol, and Portetelle, 2008), which could lead to the overestimation of "active" microbial population. Propidium monoazide (PMA) treatment has been used to discriminate live and dead cells. PMA was a derivative of propidium iodide and is a positively charged molecule that can easily penetrate dead or membrane-compromised cells. Inside cells, PMA intercalate into double-stranded nucleic acids, and upon light exposure, cross-linking of the DNA occurs and its PCR amplification is inhibited (Nocker, Cheung, and Camper, 2006). Several studies have been performed by qPCR combined with PMA (PMA-qPCR) for quantification of food-borne bacteria, mainly pathogens such as *Campylobacter* in broiler chicken carcasses (Pacholewicz, et al., 2013) and *Listeria monocytogenes* in fresh-cut vegetable (Elizaquível et al., 2012). However, PMA-qPCR has not been studied for detection and quantification of starter cultures in fish sauce fermentation. There are many factors can affect DNA binding by PMA such as type and concentration of target bacteria (Zhu et al., 2012), fat content of food samples (Yang et

al., 2011), or Mg^{2+} concentration of qPCR reaction (Nocker et al., 2006), Therefore, optimal condition of PMA-qPCR should be investigated.

Only high-throughput and high-resolution detection methods, such as next-generation sequencing (NGS) techniques, thousands-to-many-millions of sequencing reactions are produced in parallel. NGS is a new generation of sequencing technologies has provided unprecedented opportunities for high-throughput functional genomic research. The technologies have been applied in a variety of contexts, including whole-genome sequencing, targeted resequencing, discovery of transcription factor binding sites, and noncoding RNA expression profiling. NGS can adequately assist in the task of extensively and intensively investigating patterns of distribution of microbial communities in environment (Lindström and Langenheder, 2012). Ion Torrent Personal Genome Machine (PGM®) was performed to analyze bacterial profiling, which is the fastest throughput and shortest run time (Nikolaki and Tsiamis, 2013). Ion Torrent PGM® uses semiconductor sequencing technology. When a nucleotide is incorporated into the DNA molecules by the polymerase, a proton is released. Each well holds a different DNA template. The ion changes the pH of the solution, which is detected by an ion sensor. By detecting the change in pH, Ion Torrent PGM® recognized whether the nucleotide is added or not. Each time the chip is flooded with one nucleotide after another, if it is not the correct nucleotide, no voltage will be found. Chip contains hydrogen ion detector that translates released hydrogen ions from each well into a quantitative readout of nucleotide bases that were incorporated in each reaction step. If there are two identical bases on the DNA strand, the output voltage is doubled. Ion Torrent PGM® is the first commercial sequencing machine that does not require fluorescence and camera scanning, resulting in higher speed, lower cost, and smaller instrument size (Flusberg, Webster, and Lee, 2010). The new Ion Torrent PGM® generates read lengths of around 350 bp, which are used to fill gaps in the assembly produced by other technologies (Gupta and Gupta, 2014). The quality of fish sauce greatly depends on

dynamic changes of microflora during fermentation. Because most fermentation processes rely on microorganisms acting in concert to produce the desired product characteristics. Addition of starter cultures might change pattern of microflora in the fermentation. Therefore, the changes of microflora as affected by starter culture inoculation should be elucidated. The Ion Torrent PGM® appears to be the current technology that can be applied to monitor dynamic changes of microbial population during fish sauce fermentation.

The aims of this study are to use *Virgibacillus* sp. SK37 and *T. halophilus* MS33 as co-cultures for improving chemical and microbiological characteristics of fish sauce. The qPCR technique was developed to detect live starter cultures during fish sauce fermentation. Ion Torrent PGM® was used to investigate microbial profiling in fish sauce.

1.2 Research objectives

The objectives of this research were:

(1) To develop qPCR method for specific detection of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in the fish sauce sample.

(2) To apply the combined cultures of *Virgibacillus* sp. SK37and *T. halophilus* MS33 for fish sauce fermentation and to investigate chemical and physico-chemical properties of fish sauce fermented using combined cultures as compared to single culture inoculation.

(3) To apply the developed qPCR method to monitor population changes of the combined starter cultures, *Virgibacillus* sp. SK37 and *T. halophilus* MS33, during fish sauce fermentation.

(4) To detect microbial profiling in fish sauce inoculated with starter cultures using Ion Torrent PGM®

1.3 Research hypotheses

The use of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 as combined starter cultures for fish sauce fermentation would result in different chemical and microbiological characteristics of fish sauce as compared to single culture inoculation. In addition, the use of the combined cultures could improve survival rate of individual cultures. Specific probes designed from specific genes/regions such as protein-encoding genes and ITS region would be specific for detection of respective cultures by real-time qPCR. The developed qPCR technique with propidium monoazide (PMA-qPCR) treatment can detect and quantify live cells of *T. halophilus* MS33 and *Virgibacillus* sp. SK37 during fish sauce fermentation. Ion Torrent PGM® approach could be used to study changes of microbial profiling during fermentation.

1.4 Scope of the study

Virgibacillus sp. SK37 and *T. halophilus* MS33 were used as combined cultures for fish sauce fermentation. Volatile compounds, amino acid profiles and peptides of fish sauce samples were analyzed. Moreover, physico-chemical parameters, such as biogenic amine, total nitrogen, and α-amino acid content were determined. Fish sauce samples added individual cultures were prepared in comparison with combined cultures inoculation. The qPCR technique was developed using ITS region and *aprX* gene of *T. halophilus* MS33 and *Virgibacillus* sp. SK37, respectively. DNA probe was designed from ITS region and *aprX* gene using Primer Express 3.0 software. Specificity and limit of quantification of the method were tested using type strains, microflora isolated from commercial fish sauce tank, pure cultures and spiked sample. Efficiency of DNA extraction was performed. Fish sauce inoculated with starter cultures was quantified for viable cells by PMA-qPCR. Changes of microbial profiling of inoculated fish sauce during 6 months of fermentation were elucidated using Ion Torrent PGM®.

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CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce fermentation

Fish sauce is a condiment widely used in Southeast Asia, produced by mixing fish and salt and fermenting for 12-18 months. Liquid will be 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). 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 obtained from natural hydrolysis by endogenous and microbial proteinases (Lopetcharat et al., 2001). Bacteria found in fish sauce fermentation belong

to *Tetragenococcus*, Archeae bacteria, *Micrococcus*, *Streptococcus*, *Bacillus* and *Staphylococcus* (Akolkar et al., 2010; Fukami et al., 2004; Lopetcharat et al., 2001; Satomi, Furushita, Oikawa, and Yano, 1997). These bacteria play a major role in volatile compounds production and color development. In addition, flavor compounds are derived from protein hydrolysis and chemical reaction, such as lipid oxidation (Lopetcharat et al., 2001). Ammonia, cheesy, and meaty note are major notes of fish sauce. Ketone and aldehyde compounds are the major volatile compounds which contributed to cheesy and meaty note, respectively. Ammonia, amines, and other nitrogenous compounds are responsible for the ammonical note (Fukami et al., 2002). In general, amino acid content increases and most of polypeptides decrease during the fermentation period. Taurine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine are amino acids found in fish sauce (Saisithi, 1994).

Biogenic amines are organic basic compounds which is important quality indices of fish sauce, 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). Many groups of bacteria, such as *Morganella morganii*, *Pseudomonas* sp., *Staphylococcus* sp., and some lactic acid bacteria have been reported as biogenic amines producer. Histamine content in fish sauce is very important for safety.Histidine in fish muscle is the primary source for histamine formation by bacterial histidine decarboxylase. 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). 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 keyfactor in controlling histamine in fish sauce products. 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.

Because of long fermentation time and distinctive odor of fish sauce, many attempts have been made to accelerate the process of fermentation and improve odor. Reduction of salt concentration (Gildberg, Hermes, and Orejana, 1984), increasing temperature (Lopetcharat and Park, 2002), and addition of acid (Gildberg et al., 1984) are reported to accelerate fish sauce fermentation time. Lopetcharat and Park (2002) reported that increasing temperature of fermentation at 50 °C for 15 days could increase total nitrogen equivalent to commercial fish sauce. However, low salt concentration may generate spoilage microorganism, whereas high temperature increases expenditure and give negative sensory characteristics in the finished product.

There are many reports describing the use of starter cultures to reduce and improve fish sauce fermentation. In the past, Aquerreta, Astiasarán, and Bello (2001) tried to develop the fermentation process by using salt-tolerant proteinases produced by *Bacillus subtilis*. They found that fish sauce added *B. subtilis* increased α-amino content to 712 mg/100 ml at 2 days of fermentation. Gildberg and Thongthai (2001)

reported that addition of halophilic lactic acid bacteria (*T. halophilus*) in fish sauce fermentation improved organoleptic quality and increased total nitrogen when compared to the control. In 2005, Uchida et al. reported that use of koji and *T. halophilus* as starter cultures for Chinese silver carp fish sauce fermentation could increase organic acids and amino acids, contributing to the taste of fish sauce. Yongsawatdigul et al. (2007) reported that *Virgibacillus* sp. SK37 isolated from fish sauce showed potential to hydrolyze fish protein. They found that α-amino content of 4-month-old fish sauce inoculated with *Virgibacillus* sp. SK37 was higher than the control (without *Virgibacillus* sp. SK37). Similarly, Akolkar et al. (2010) reported that fish sauce inoculated *Halobacterium* sp. SP1(1) and fermented for 10 days showed an increase in α-amino content and total nitrogen. Moreover, lysine content in fish sauce inoculated with *Halobacterium* sp. SP1(1) was higher than fish sauce without starter culture.

Because of distinctively strong odor of fish sauce, attempts have been made to eliminate undesirable odor. Fukami et al. (2004) reported that dimethyl trisulfide and 2-ethylpyridine content of Japanese fish sauce (Moromi) inoculated with *Staphylococcus xylosus* is lower than that of non-treated fish sauce. These compounds contribute to fecal note. Addition of *S. xylosus* could eliminate unpleasant odor in fish sauce. Recently, Udomsil et al. (2010) isolated 7 strains of *T. halophilus* from fish sauce fermentation and found that these bacteria showed ability to hydrolyze fish protein extract (fish broth) and can produce volatile compounds in fish broth containing 25% NaCl. *T. halophilus* strains are used as starter cultures for fish sauce fermentation (Udomsil et al., 2011). Fish sauce added *T. halophilus* fermented for 6 months contained α-amino content higher than the control.

Moreover, desirable volatile compounds, such as 2-methylpropanal, 2-methybutanal, 3-methybutanal, and benzaldehyde are major compounds in fish sauce added *T. halophilus*. Furthermore, glutamic, proline, and lysine are major amino acids found in fish sauce added *T. halophilus.* These amino acids contribute to flavor characteristic of fish sauce. These studies indicated that the use of starter culture seems to be a promising approach for technological development of fish sauce fermentation. Furthermore, application of the combined starter cultures to accelerate fermentation time and eliminate undesirable odor of fish sauce have not been thoroughly investigated.

2.2 Microbiota and starter cultures in fish sauce fermentation

Microorganisms found during fish sauce fermentation are classified as halophilic bacteria because fish sauce contains high concentration of salt (25–30 %, w/v NaCl) (Lopetcharat et al., 2001). Microorganisms commonly found in fish sauce and can be categorized as halotolerant, moderately halophilic (0.5-2.5, ~3-15% salt), and extremely halophilic (2.5-5.2 M, 15-30% salt). Halotolerant organisms can grow both in high salinity and in the absence of salt. *Bacillus*, *Staphyloccoccus*, *Coryneform* bacteria, and *Micrococcus* are halotolelant bacteria found in initial stage of fermentation (Chaiyanan, 2000). Moderately halophilic bacteria were found such as *Lentibacillus salicampi*, *L. juripiscarius* (Namwong et al., 2005), *Tetragenococcus halophilus* and *T. muriaticus* (Thongsanit, Tanasupawat, Keeratipibul, and Jatikavanich, 2002), and *Virgibacillus* sp. (Sinsuwan et al., 2008).

For extremely halophilic archaea, *Halobacterium salinarum*, *Halococcus*

saccharolyticus, and *Halococcus thailandensis* have been isolated from fish sauce fermentation. (Thongthai et al., 1992; Chaiyanan, et al., 1999).

Halophilic bacteria isolated from fish sauce could produce proteinase e.g. *H. salinarium* and *H. cutirubrum* produced extracellular proteinase at 25% NaCl (Ihara, Wanatabe, and Tamura, 1997; Thongthai et al., 1992; Thongthai and Suntinanalert, 1991). *Halobacterium* sp. showed ability in gelatin and casein hydrolysis, while *Halococcus* sp. hydrolyzed gelatin. Moreover, *T. halophilus* has been reported to produce aminopeptidase toward alanine with the highest activity, followed by leucine, methionine, lysine, ariginine, and glutamic (Udomsil et al., 2010). *Virgibacillus* sp. produced extracellular proteinase at 25 %NaCl (Sinsuwan et al., 2007). The important roles of proteinase-producing bacteria are ability to hydrolyze fish protein to peptides and amino acids by their extracellular and/or cell-bound proteinases.

Naturally food fermentation is spontaneous process by action of several microorganisms and enzymes during fermentation, leading to desirable biochemical and nutritional changes. A desirable flavor, color, aroma, or texture characteristic of fermented product is resulted from metabolic activity of microorganisms and indigenous enzymes (Hammes, 1990). Inconsistency of finish product might occur because it was natural fermentation. Therefore, starter cultures have been applied to fermentation process. Starter cultures were a large numbers of viable microoraganisms, which may be added to accelerate a fermentation process. Quality of fermented product could be improved and controlled by starter cultures (Holzapfel, 1997).

Natural fermentation processes are complex, including interaction among

fermentable substrates and microorganisms. Microbial interactions have an impact on finished product of food fermentation (Smid and Lacroix, 2013). The outcomes or effect of interaction between individuals of different species can be classified positive (+), negative (-), or neutral (0). Types of microbial interactions and mechanisms can be catagorized to 5 types: (i), competition (-/- interaction); (ii), mutualism (+/+ interaction); (iii), commensalism (+/0 interaction); (iv), amensalism (-/0 interaction) and (v) parasitism (+/- interaction) (Hugenholtz, 1986). In a competition, two or more species, strains or subpopulations of microbes compete for one or more growth factors. This type of interaction affects both interacting partners in a negative way but usually leads to a temporary increase in relative abundance of one interacting partner over the other. Mutualism is defined as positive benefit of the interactions between individuals of different species. Commensalism, two organisms living together, one microorganism benefits from another but the other is not affected. For amensalism, it has been described in term of two-species-interaction, one bacterial species was neutral and another one was negative. For parasitism, it is the two-species interaction in which the impact of one species on the other is negative (Hirsch, 2004).

Proteinase-producing bacteria have been used as a starter culture in fermented food production such as cheese, fermented sausage, fermented soybean products and soy sauce (Fermandez-Garcia et al., 1999). *B. subtilis*, *B. pumilus* and *B. licheniformis* isolated from soy-*daddawa*, a traditional soy product of Nigeria (Omafuvbe, Abiose and Shonukan, 2002) have been used as mixed cultures and showed an increased final pH, and resulted in the highest level of proteolytic activity and free amino acid content. For fish sauce fermentation, Fukami et al. (2004)

studied role of *Staphylococcus xylosus* isolated from fish sauce mush (moromi) on odor improvement of fish sauce. They found that 2-ethylpyridine and dimethyl trisulfide were reduced, these compounds contributed to undesirable odor. Uchida et al. (2005) studied the effects of soy sauce koji and lactic acid bacteria on the fermentation of fish sauce from freshwater silver carp (*Hypophthalmichthys molitrix*). The addition of soy sauce koji (*Aspergillus oryzae*) resulted in a remarkable increase of soluble components such as organic acids and amino acids that contributed to taste.

The use of *Virgibacillus* sp. SK37 and commercial enzymes could reduce fermentation time to 4 months (Yongsawatdigul et al., 2007). Addition of *T. halophilus* MS33 in fish sauce fermentation process increased glutamic acid contributing to umami taste and reduces fecal note resulted from dimethyl disulfide (Udomsil et al., 2011). It produced 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde to a greater extent than the control (Udomsil et al., 2010). These volatile compounds have significant effect on overall aroma of fish sauce due to their high odor activity values (OAVs) (Lapsongphon, Cadwallader, Rodtong, and Yongsawatdigul, 2013). 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. Therefore, volatile compounds of fish sauce can be enhanced by applying suitable starter cultures in fermentation process.

2.3 *Virgibacillus* sp. SK37

The genus *Virgibacillus* was firstly reclassified from *Bacillus pantothenticus* Heyndrickx et al. (1998). A phylogenetic group of *B. pantothenticus* species was sufficiently different from other *Bacillus* species to warrant the status of a separate genus for which they had proposed the name *Virgibacillus*. This genus could be distinguished from members of *Bacillus* rRNA group aerobic endospore-forming bacteria, such as *Halobacillus*, *Paenibacillus*, *Brevibacillus* and *Aneurinbacillus*. Thus far, there are 17 species of *Virgibacillus* genus; *V. arcitcus*, *V. carmonnensis*, *V. chiguensis*, *V. halodenitrificans*, *V. halophilus*, *V. kekensis*, *V. koreensis*, *V. litoralis*, *V. marismortui*, *V. necropolis*, *V. olivae*, *V. pantothenticus*, *V. proomii*, *V. salarius*, *V. salexigens*, *V. siammensis*, and *V. zhanjiangensis*. *Virgibacillus* are motile, Gram-positive rods (0.5-0.7 x 2.0-5 µm), and single, pair or long chain of cells arrangement. Their colonies are small, low convex, circular, and slightly transparent-to-opaque. They are aerobic, catalase-positive, motile, and spore forming. *Virgibacillus* grow at pH 6.0-10.0 (optimum at 7.5-8.0), and 10-55°C (optimum at 30-40°C). They are mostly found in saline environment. Members of this genus are moderately halophilic bacteria able to grow at 0-25% (w/v) NaCl with optimum of 5-10% NaCl. The G+C content is 36-43 mol%, compared to 32-69 mol% in *Bacillus* species. Cell wall peptidoglycan of *Virgibacillus* contains *meso*-diaminopimelic acid. Major fatty acids in cell membrane are mostly anteiso-$C_{15:0}$ followed by iso-$C_{15:0}$ (Heyrman et al., 2003).

Virgibacillus sp. SK37 is a moderately halophilic bacterium isolated from Thai fish sauce fermented for 1 month. *Virgibacillus* sp. SK37 is Gram-positive endospore-forming rod, non-motile, and grows at 0-30% NaCl, 20-45 °C, pH 4-12.

Morphology of *Virgibacillus* sp. SK37 is circular, raised, and white to cream color with 2-3 mm diameter. It can grow under aerobic or anaerobic condition. Moreover, it can hydrolyze skim milk on skim milk agar containing 25% NaCl (Nawong, 2006), indicating proteinase activity at high salt concentration. *Virgibacillus* sp. SK37 is used as a starter culture in fish sauce fermentation made from Indian anchovy (*Stolephorus indicus*) at 25% solar salt. *Virgibacillus* sp. SK37 showed potential to accelerate fermentation time (Yongsawatdigul et al., 2007). The reason may be because the presence of extracellular and cell-bound proteinases.

Extracellular proteinases of *Virgibacillus* sp. SK37 could hydrolyze skim milk at 25% NaCl. Optimum activity of crude extracellular proteinase is at 65 °C and pH 8.0 (Sinsuwan et al., 2007). *Salinivibrio* sp. AF-2004 and *Pseudoalteromonas* sp. CP76 isolated from saline lake and saline soil, respectively, showed optimum temperature and pH of extracellular proteinases activity at 55 to 65 °C and pH 8.0 to 8.5 (Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007; Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003). Molecular mass (Mm) of extracellular proteinases of *Virgibacillus* sp. SK37 is estimated to be 81, 67, 63, 50, 38, and 18 kDa, based on native-PAGE activity staining. All proteinases exhibited caseinolytic activity at 25% NaCl (Sinsuwan et al., 2007). Studdert, De Castro, Herrera, and Sánchez (1997) found that crude proteinases from *Natronococcus occultus* showed 7 bands with Mm ranging from 50-120 kDa. In addiiton, crude proteinase from *Halobacillus thailandensis* contained serine proteinase with MW of 100 and 17 kDa, and 42 kDa of metalloproteinase (Chaiyanan, Maugel, Huq, Robb, and Colwell, 1999). Cell-bound proteinase of *Virgibacillus* sp. SK37 was Ca^{2+}-activated serine proteinase with subtilisin-like characteristics. The enzyme exhibited

maximum activity at 65 °C, pH 7.0 and 9.5, based on azocasien as a substrate. Cell-bound proteinases showed high stability at 25% NaCl, 30 °C and effectively hydrolyzed anchovy actomyosin at high NaCl concentration up to 20% (Sinsuwan et al., 2008). Cell-bound proteinases from *Virgibacillus* sp. SK37 showed hydrolysis of casein and required 10 mM Ca^{2+} for complete casein hydrolysis. The result suggested that the enzyme is activated by Ca^{2+} (Sinsuwan et al., 2008). Fernandez-Espla, Garault, Monnet, and Rul, (2000) reported that activity of *Streptococcus thermophilus* CNRZ385 increased 2 to 10 times when Ca^{2+} is increased from 2 to 10 mM. However, Ca^{2+} does not affect activity of cell-bound proteinase of *Lactobacillus helveticus* L89 (Martin-Hernández, Alting, and Exterkate, 1994). Catalytic domain of cell-bound proteinase is N-terminal, while C-terminal region is an anchor to cell envelope (Laan and Konings, 1989). Germond, Delly, Gilbert, and Atlan, (2003) reported that *prtB* is gene encoding cell-bound proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus*. They found that C-terminal contained lysine rich region and forming electrostatic interactions with teichoic acids of cell wall. Cell-bound proteinase has been studied in LAB starter cultures in several fermented dairy products. The enzyme contributed to casein degradation at the initial stage, producing peptides and amino acids for translocation into cell. Amino acids turn into substrates for LAB to produce volatile compounds, such as aldehyde, alcohol, and ketone contributing to flavor characteristics in cheese (Bockelmann, 1995; Smit and Engels, 2004). However, extracellular and cell-bound proteinases produced from *Virgibacillus* sp. SK37 showed ability to hydrolyze fish protein at high salt concentration to peptides and amino acids which might serve as substrates for other bacteria in fish sauce fermentation.

2.4 *Tetragenococcus halophilus* MS33

Collins, Williams, and Wallbanks (1990) reported that *Pediococcus halophilus* is more closely related to enterococci and carnobacteria than to pediococci and lactobacilli on the basis of 16S rRNA sequence data. *Pediococcus halophilus* was proposed to be reclassified in a new genus as *T. halophilus* (Anonymous, 1994). The member of *Tetragenococcus* genus, *T. muriaticus* was found to be a new species of moderately halophilic bacteria isolated from squid liver sauce (Satomi et al., 1997). Lee et al. (2005) reported *T. koreensis* sp. nov. isolated from kimchi. In 2005, Ennahar and Cai 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 *T. solitarius* comb. nov. Justé et al. (2012) reported *T. osmophilus* in concentrated sugar thick juice. At present, the genus *Tetragenococcus* comprises of four species: *T. halophilus, T. muriaticus, T. koreensis, T. solitaries, and T. osmophilus.* Characteristics of *Tetragenococcus* species were shown in Table 2.2.

Tetragenococcus halophilus is halophilic LAB requiring sodium chloride (NaCl) for growth and is tolerant to NaCl concentration more than 18% (Hozapfel, Franz, Ludwig, Back, and Dicks, 2006). Cells are pairs and/or tetrads arrangement 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. They are homofermentative and microaerophile. It cannot grow at 45 °C. Optimum NaCl concentration for growth is between 5-10 % and can grow between pH 5.0-9.0 (Thongsanit, et al., 2002; Udomsil et al., 2010). *T. halophilus* has been found in a

Table 2.2 Physiological and biochemical characteristics of *Tetragenococcus* species.

Characteristics	*T. halophilus*[a] ATCC 33315	*T. muriaticus*[b] JCM 10006	*T. koreensis*[c] DSM 16501	*T. solitarius*[d] JCM 8736	*T. osmophilus*[e] DSM23765
Optimum temperature (°C)	30	25-30	15-30	NA	30
Growth at 40 °C	-	+	-	+	-
45 °C	-	-	-	+	-
Growth range of pH	5.0-9.0	5.0-9.6	NA	NA	NA
Optimum pH	NA	7.5-8.0	9.0	6.5	8.0
Range of NaCl (%)	0-25	1-25	0-8	NA	0-25
Optimum NaCl (%)	5-10	7-10	2-5	NA	5
Gas from D-glucose	-	-	-	NA	NA
Acid from:					
Amygdalin	+	-	+	+	-
L-Arabinose	-	-	-	-	-
D-Cellobiose	+	-	-	+	+
D-Galactose	+	-	-	+	-
Glucose	+	+	+	NA	NA
Glycerol	+	-	NA	-	-
D-Lactose	-	-	-	-	-
D-Maltose	+	-	+	+	+
D-Raffinose	-	-	NA	-	-
L-Rhamnose	-	-	NA	-	NA
D-Mannitol	-	+	+	+	+
D-Mannose	+	+	+	+	NA
D-Melibiose	-	-	NA	-	-
Sucrose	+	-	+	+	W
D-Melezitose	+	-	+	+	-
α-Methyl-D-glucoside	+	-	-	+	+
D-Ribose	+	+	+	-	-
D-Sorbitol	-	-	NA	-	-
Sorbose	+	-	-	-	NA
D-Trehalose	+	-	+	+	+
D-Xylose	+	-	-	-	-
Xylitol	NA	-	+	-	NA
Turanose	NA	-	+	+	+
D-Tagatose	NA	-	-	+	-
D-Arabitol	NA	-	+	+	NA
Gluconate	NA	-	+	+	NA

NA = Not available, w = weak reaction, negative (-), and positive (+) reaction. [a]Thongsanit et al. (2002), [b]Satomi et al. (1997), [c]Lee et al. (2005), [d]Ennahar and Cai (2005), [e]Justé et al. (2012).

variety of fermented foods, including soy sauce (Stiles and Holzapfel, 1997), Indonesian soy mash (Kecap) (Roling et al., 1996), fermented black bean (Chen et al., 2006), and salted anchovies (Villar et al., 1985). Saisithi (1994) reported that halophilic bacteria are predominantly found at the initial stage of fish sauce fermentation (between 1-5 months) whereas halophilic LAB are dominant at the final stage of color, aroma and flavor development. Therefore, *T. halophilus* isolated from fish sauce may play a significant role in desirable flavor and/or aroma formation in fish sauce.

Tetragenococcus halophilus MS33 was isolated from one-month-old fish sauce and the culture has been deposited at the Research Center on Microbial Cultures for Food and Bioplastics Production. It could grow at 0-25% NaCl, pH 4.5-9.0, and optimum NaCl of 5-10%. *T.halophilus* MS33 strain showed ability to grow in fish broth extracted from anchovy containing 25% NaCl and also produced benzaldezyde, 1-propanol, and 2-methylpropanal. Moreover, *T. halophilus* exhibited intracellular aminopeptidase activity towards amino-*p*-nitrianilide, such as alanine, glutamic, arginine, leucine, lysine, and methionine (Udomsil et al., 2010). Other LAB isolated from fermented food including, *Lb. sake* exhibited high activity toward leucine and alanine substrates (Sanz and Toldra, 1997). Similarly, aminopeptidase from *Lb. curvatus* is characterized and showed high activity toward leucine (Magboul and McSweeney, 1999). Macedo, Tavares, and Malcata (2003) characterized intracellular aminopeptidase of *Lb. plantarum* isolated from traditional

Serra da Estrela cheese. They reported that the enzyme is a metallo-enzyme with optimum activity at 37°C, pH 6.5. Mm of the enzyme is 70 kDa, and it is apparently composed of two subunits, Mm of which is 34 kDa. The enzyme is able to cleave lysine-*p*-nitrianilide but does not hydrolyze glutamic-*p*-nitrianilide, glycine-*p*-nitrianilide or proline-*p*-nitrianilide (Macedo et al., 2003). Aminopeptidases are parts of the proteolytic system of LAB. Amino acids resulting from aminopeptidase activity are served as a substrate for amino acid catabolism pathway which could produce volatile compounds. Therefore, *T. halophilus* isolated from fish sauce may play a significant role in desirable flavor and/or aroma formation in fish sauce. Because of this reason, *T. halophilus* MS33 is used as a starter culture for improving volatile compounds in fish sauce (Udomsil et al., 2011). Fish sauce inoculated *T. halophilus* showed increasing level of aldehydes and ketones which contributed to meaty and cheesy note. In contrast, dimethyl disulfide and dimethyl sulfide do not increase in *T. halophilus* MS33 inoculated-fish sauce, indicating that *T. halophilus* MS33 could play a significant role in aroma and flavor development.

2.5 Using of combined starter cultures in food fermentation

Commensalistics interaction, the interaction is the stimulation of growth of proteinase-negative strain of *Lactococcus lactis* in a cheese starter culture by proteinase-positive strain of *L. lactis*. Similarly, Elizabeth, Yeung, and Tong, (2011) studied the survival rate of *Lb. acidophilus* in yogurt. They found that *Lb. acidophilus* showed 100% of relative survival rate throughout storage time (28 days) when they used in combination of *Lb. acidophilus* and *S. thermophilus*. In contrast, *Lb. acidophilus* combined to *Lb. delbrueckii* ssp. *bulgaricus* showed a slight

decrease up to 28 days because *Lb. delbrueckii* ssp. *bulgaricus* produced inhibitory metabolites such as H_2O_2 that can eventually lead to microbial cell death (Talwalkar and Kailasapathy, 2003). Furthermore, yeast-yeast interaction in multi-starter wine fermentation has been reported. Amino acids and vitamins were consumed by non-*Saccharomyces*, resulting in the limited growth of *S. cerevisiae*. However, non-*Saccharomyces* showed proteolytic activity at the initial stage of fermentation, leading to enrichment of nitrogen source. The presence of more yeast species might improve the uptake and the consequent consumption of some amino acids by *S. cerevisiae* strains, resulting in a synergistic mechanism of nitrogen use (Ciani and Comitini, 2015). Osborne and Edwards (2007) reported that peptides produced from *S. cerevisiae* can inhibit growth of *Oenococcus oeni*. On the other hand, certain substances that are released by yeasts, such as amino acids (Fourcassier et al.,1992) and mannoproteins (Guilloux-Benatier et al., 1995), may have a positive impact on bacterial growth and malolactic activity. These studies showed that interaction of co-cultures in food fermentation could improve quality and characteristic of product.

A problem of using of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 as a single starter culture for fish sauce fermentation was their limited survival rate. Viable cells count of *Virgibacillus* sp. SK37 decreased from 5 Log CFU/ml to 3 Log CFU/ml at 60 days and remained constant until 120 days (Yongsawatdigul et al., 2007). In addition, *T. halophilus* MS33 rapidly decreased from 6 Log CFU/ml to 2 Log CFU/ml at 60 days and are not detected thereafter (Udomsil et al., 2011). From previous studies in other products, the combined starter cultures showed ability to improve chemical compositions and survival rate of starter cultures (Uchida et al., 2005; Zhoa et al., 2011; Elizabeth et al., 2011). However, the use of *Virgibacillus* sp.

SK37 and *T. halophilus* MS33 as co-culture starter and in relation to their survival in fish sauce fermentation has not been studied. Moreover, the effect of co-culture on chemical compositions, volatile compounds, and amino acids/peptides profile of fish sauce has not been investigated.

2.6 Monitoring of bacterial starter culture in food fermentation

2.6.1 Culture dependent method

Culture-dependent methods for bacterial detection are based on growth of bacteria on synthetic media that mimic the conditions of the system from which the microorganisms are isolated (Rantsiou and Cocolin, 2006). Step of culture-dependent methods including isolating, culturing, and identifying bacteria according to either morphological, biochemical or genetic characteristics (Ercolini, 2004). These methods introduce biases because some species could not grow under the selected condition (Jany and Barbier, 2008). Culture-dependent methods are used to monitor and identify microorganisms in various fermented foods, such as fermented sausages (Rantsiou and Cocolin, 2006), fermented fish paste (Kasankala, Xiong, and Chen, 2010), and fish sauce (Lopetcharat and Park, 2002; Ijong and Ohta, 1996; Uchida et al., 2005; Udomsil et al., 2011; Yongsawatdigul et al., 2007). Rantsiou and Cocolin (2006) reported that de Man Rogosa, Sharpe (MRS) agar and Mannitol Salt Agar (MRA) are used to isolate LAB and coagulase-negative cocci (CNC) from fermented sausage. After being cultured in the selective media, colonies are randomly selected and several steps for bacterial identification including, morphological characteristics and biochemical identification. These are time-consuming process. Moreover, culture-dependent methods are not able to detect

non-culturable cells, stressed or weakness cells may need specific culture conditions to recover before any quantification is possible. In food matrices, 20-50% of active microbial organisms may not be culturable (Justé et al., 2008). Uchida et al. (2005) monitored changes of *T. halophilus* starter culture added into fish sauce fermentation using MRS agar containing 10% NaCl. Confirmation of *T. halophilus* starter culture during fermentation was not carried out. Methods to overcome the limitations of conventional methods are molecular-based techniques.

2.6.2 Molecular-based method

Molecular methods are based on the direct analysis of DNA without any culturing step (Jany and Barbier, 2008). These methods have overcome problems associated with selective cultivation and isolation of bacteria from samples. Moreover, molecular methods are rapid, reliable, and reproducible.

2.6.2.1 Finger printing techniques

PCR-based fingerprinting techniques, such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE), single strand conformation polymorphism-PCR (SSCP-PCR) and terminal restriction fragment length polymorphism (T-RFLP) have been used to identify and monitor bacterial community changes in fermented foods (Fontana, Vignolo, and Cocconcelli, 2005). PCR-DGGE technique is based on the separation of the same size but different sequence of PCR products in gel electrophoresis containing chemical denaturants. Therefore, these fragments can be separated in denaturing gradient gel based on their differential denaturation profile (Muyzer and Smalla, 1998). SSCP-PCR is a technique using either acrylamide gel based or capillary based automated sequencer, based on the separation of denatured (single-stranded) PCR products (Jany and

Barbier, 2008). T-RFLP is based on digestion of fluorescent end-labelled PCR products with restriction endonucleases. The strength of genetic fingerprinting techniques is that large numbers of samples can be analyzed and compared (Smalla et al., 2007). PCR-DGGE has been used to study the succession and temporal changes of complex microbial ecosystem (Amor, Vaughan, and de Vos, 2007; Walter, Hertel, Tannock, Lis, Munro, and Hammes, 2001). Ercolini (2004) used PCR-DGGE to characterize the dominant spoilage bacteria in beer or chilled pork during refrigerated storage. Fontana et al. (2005) used PCR-DGGE to identify *Staphylococcus* and *Lactobacillus* species in Argentinean fermented sausage. DGGE has been used for identification of bacterial microflora in dairy product such as cheese. Ogier, Son, Gruss, Tailliez, and Buchet (2002) reported that PCR amplification of V3 region of 16S rDNA sequence can be used to identified bacterial community in cheese. Hu et al. (2008) reported that the inhibition of spoilage bacteria in cooked ham by *Lb. sakei* was monitored by PCR-DGGE. In addition, Delbès and Montel (2005) evaluated the structure and dynamics of *Staphylococcus* populations during cheese making by SSCP-PCR. Moreover, the application of T-RFLP to food microbiology has been reported by Rademaker, Peinhopfa, Rijnena, Bockelmannb, and Noordman (2005). They used T-RFLP to detect the surface microflora dynamics of bacterial smear-ripened Tilsit cheese. Although these genotypic fingerprinting methods have been applied for identification and characterization of mixed microbial communities, they do not provide quantitative data of bacterial counts. Limitations of genetic fingerprinting procedures are low sensitivity in detecting rare members of community, reliability and reproducibility in lysis of all bacterial cells as well as the extraction of intact nucleic acids, and some

substances, such as bacterial exopolysaccharides, which may inhibit DNA digestion with restriction enzymes and PCR amplification (Amor et al., 2007).

T-RFLP called fragment analysis is based on digestion of fluorescent end-labelled PCR products with restriction endonucleases. Either one or both 5’ and 3’ ends of the amplicon can be labelled by incorporating a dye on either one or both PCR primers. The digested products are separated by electrophoresis using acrylamide gel- or capillary-based automated sequencer, with laser detection of the labeled fragments (Jany and Barbier, 2008). This system only detects the end-labelled terminal restriction fragments (TRFs) of the digested PCR products and their size can be calculated based on the use of DNA size standards that are run simultaneously with the samples. The data consist of the sizes of the PCR amplicons that contain the labeled primer and are observed as electrophoregram peaks or gel bands (Jany and Barbier, 2008). Apart from the presence or absence of members of the population, shifts in the relative presence of the members at different points in time can be followed. Therefore, the shift of a community member relative to other members of a population can be observed by following the shifts in height or surface area of the peaks present at different points in time (Rademaker, Peinhpf, Rijnen, Bockelmann, and Noordman, 2005). T-RFLP analysis is a rapid and sensitive molecular approach that can assess subtle genetic differences between strains and also provides insight into the composition of microbial communities (Rademaker et al., 2005).

SSCP-PCR is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility using

either acrylamide gel- or capillary-based automated sequencer (Jany and Barbier, 2008). The rapidity and automation of SSCP method by capillary electrophoresis, compared to other molecular techniques, such as TGGE or DGGE, allows the microbial dynamics of different samples to be analyzed without the requirement of several gel conditions to separate sequences (Randazzo et al, 2009). SSCP-PCR is potentially easier to carry out than PCR-DG/TTGE since there is no need for gradient gels or use of GC-clamp primers and it can be performed using an automated sequencer. However, when using an automated sequencer, one of the disadvantages of this technique is difficulty of appending new data to an existing database. Unknown profiles cannot be directly sequenced because they are labeled for detection by automated sequencer.

2.6.2.2 Real-time quantitative PCR (qPCR)

2.6.2.2.1 Principle of qPCR

In conventional PCR, the amplified product has been detected on an agarose gel after the reaction has finished. In contrast, amplified product of real-time PCR is detected and measured as the reaction progresses, that is, in “real time” (Arya et al., 2005) The amplification of a target DNA is detected rather than amount of DNA product accumulated at the end. Real-time detection of PCR products is made by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The cycle in which the fluorescence reaches the detection level of the instrument is known as the threshold cycle (Ct). The fluorescent chemistries used for this purpose include DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection

modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence indicates the amount of amplified product in each cycle (Higuchi et al., 1992; 1993), therefore qPCR can be used for quantitation. The most frequent quantitative application of real-time PCR is in gene expression studies and quantitation of microorganisms using specific gene/region of DNA sequence (Bubner, Gase, and Baldwin, 2004).

The detection methods used in qPCR can be classified into two main groups: (I) Non-specific methods that detect all double stranded DNA (dsDNA) produced in the reaction. The simplest of qPCR is based on non-specific quantification that involve DNA-binding fluorophore such as SYBR green I, SYTO9, SYBR Gold, BEBO, and BOXTO. The dye molecules are DNA minor-groove binders that emit a strong fluorescent signal only when associated with dsDNA and exposed to the appropriate wavelength of light (Longan, Edwards and Saunders, 2009). (II) Amplicon sequence-specific methods that distinguish target sequence amplifications from primer-dimers or non-specific amplifications. Fluorescence probe is used to detect specific sequence. There are two types of probe binding with fluorescence dye. The first type is hybridization of probe to target DNA. The probes include molecular beacon, MGB Eclipse, and Scorpions. Another type is fluorescence emission takes place after hybridization degradation by the 5'-3' exonuclease activity of DNA polymerase during the amplification process. Theses probes are TaqMan oligoprobe and TaqMan-MGB (Longan et al., 2009).

The qPCR is highly specific, reproducible, sensitive and characterized by high discriminatory power, rapid processing time, and low cost. However, it is strongly limited by presence of inhibitors in food. Substances present

in plant and food samples that can be co-extracted with DNA can affect the reaction. These include polysaccharides, proteins, phenolic compounds and other plant secondary metabolites. Additionally, components of DNA isolation buffers can affect the PCR reactions (Holden et al., 2003; Peist, Honsel, Twieling, and Löffert, 2001). The inhibitor compounds can interfere with reaction, leading to a decrease or even a complete inhibition of DNA polymerase (Lipp et al., 2001). Therefore, DNA extracted from different sample matrixes must be evaluated for suitability in quantitative analysis. Each sample should be serially diluted and tested in duplicate PCR runs to determine whether any inhibitors are present. However, the incorporation of an internal control (IC) is the best alternative way (Levin, 2004). This is very useful in the identification of false negative results. In addition, IC can be used to detect the effect of the food matrix on the efficiency of qPCR assays (Levin, 2004).

Internal control (IC) is required for identification of false-negative qPCR (Diez-Valcarce, Kovač, Cook, Rodríguez-Lázaro, Hernández, 2011; Martín, B., Jofré, A. Garriaga, M. Pla, M. Aymerich, 2006; Rodríguez-Lázaro et al., 2006). Bile salt, heme in blood, urea, humic acid and cation have been shown to inhibit PCR, probably interference with the binding and/or polymerization of DNA polymerases (Monteiro, Bonnemaison , Vekris, Petry, Bonnet, and Vidal , 1997; Al-Soud and Radstrom, 2001; Khan, Kangro, Coates, Heath, 1991; Frankenhuyzen, Trevors, Flemming, Lee, and Habash, 2013). Carryover of reagents used for isolation of nucleic acids from samples can also inhibit amplification reactions. Other causes of false-negative results include target nucleic acid degradation, sample processing errors, and thermal cycler malfunction. IC of this study was designed

using the same primers set of targets. Thus, IC is co-extracted and co-amplified for indicating inhibition of amplification. IC is subjected into all samples with the same concentration prior to extraction and showed the consistent of C_q of all experiments. In addition, IC can be used to detect the effect of the food matrix on the efficiency of qPCR assays. This can be measured by spiking IC into samples and performing the assay with serial dilutions of this food (Schneider, Enkerli, and Widmer, 2009).

The main advantage of real-time PCR over conventional PCR is that real-time PCR allows to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). In contrast, conventional PCR is at best semi-quantitative. Additionally, real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for post amplification manipulation is eliminated (Costa, 2004; Kaltenboeck and Wang, 2005; Longo, Berninger, and Hartley, 1990).

2.6.2.2.2 DNA probe development

Species-specific PCR method has been used for identification and quantification of microorganisms. Blaiotta, Pennacchia, Parente, and Villani (2003) investigated the development of species-specific PCR assay for identification of *S. xylosus* used as a starter culture in dry fermented sausage. Two sets of PCR primers, targeting on xylulokinase (*xyl*B gene) and 60 kDa heat-shock protein (*hsp*60 gene) were amplified to obtain 899 and 219 bp fragment, respectively. Moreover, restriction endonuclease analysis was used for confirmation

of the specificity of the amplified fragment. Another gene, such as gene encoding manganese-dependent superoxide dismutase (*sod*A gene) and gene encoding the peptide chain formation (*tuf* gene) have been used for identification of Staphylococci. Species-specific PCR assay is a rapid and reliable technique, and can be applied for monitoring *S. xylosus* strains during food fermentation (Poyart, Quesne, Boumaila, and Trieu-Cout, 2001).

The protein-coding gene, *aprX* gene encodes a novel member of the subtilase super family, alkaline serine protease-X (AprX) enzyme of *Virgibacillus* sp. SK37. Phylogenetic analysis suggested that AprX-SK37 belongs to a novel family of the subtilase superfamily. The AprX-SK37 is intracellular bacterial AprX serine protease. The AprX-SK37exhibited optimal catalytic conditions at pH 9.5 and 55 °C, based on the azocasien assay containing 5 mM of $CaCl_2$ (Phrommao et al., 2011). AprX-SK37 was not categorized into the oxidant stable proteases (OSPs) group. The phylogenetic analysis indicated that most OSPs do not belong to any of the subtilisin families previously described in bacteria or fungi, but appear to belong to a different cluster of phylogenetic tree. Therefore, OSPs should not be classified as the subfamily of subtilisins as previously suggested (Saeki et al., 2002). Phylogenetic analysis of various OSPs producers suggested that the sources of OSPs encompass bacilli from diverse taxon, which could be the result of different mechanisms of oxidant stabilization. Thus, alkaline serine protease X (AprX) is proposed a new family of subtilases superfamily. *aprX* was used to design probe development for *Virgibacillus* sp. SK37 detection. The *aprX* of *Virgibacillus* sp. SK37 showed potential for specific detection at the species level of *Virgibacillus*.

Internal transcribed spacer (ITS) region (or 16S-23S intergenic region) is sequence between 16S rRNA and 23S rRNA region which is effective for detection and species identification (Osorio, Collins, Romalde, Alicia, and Toranzo, 2005). The DNA sequence of ITS show length of variation and the region has been used for differentiating species of prokaryotes (Barry, Colleran, Glennon, Dunican, and Gannon, 1991; Navarro, Simonet, Normand, and Bardin, 1992). DNA of the intergenic region between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon is amplified by PCR using oligonucleotide primers targeted to conserved regions in the 16S and 23S genes. The 16S-23S intergenic region, which may encode tRNAs depending on the bacterial species, displays a high degree of variability between species in both length and nucleotide sequence. Both variations have been extensively used to distinguish bacterial strains and closely related species (Scheinert, Krausse, Ullman, Soller, and Krupp, 1996; Maes et al., 1997). The ITS of *Nocardia crassostreae* was successfully applied for detection at the species level in oysters (Carrasco et al., 2013). ITS sequences can be used to discriminate two subspecies including *Photobacterium damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* (Osorio et. Al., 2005). Yavuz, Gunes, Bulut, Harsa and Yenidunya (2004) reported amplification of ITS region of *rrn* operon and then the amplification products were analyzed by RFLP using a set of restriction enzymes, *Alu*I, *Hae*III, and *Taq*I. The results of restriction pattern analyses clearly differentiated the nine reference strains of *Lactobacillus*.

2.6.2.2.3 PCR chemistry for detection

There are 2 different approaches used for detection of the amplified products in the qPCR. These include:

2.6.2.2.3.1 DNA binding dye

This method uses double strand DNA (dsDNA) intercalating agents such as SYBR®Green, SYBR®Gold, SYTO, and EvaGreen, which allow the detection of specific products, nonspecific products, and primer-dimers product during the PCR reaction (Monis et al., 2005; Gudnason et al., 2007; Wang et al., 2006). The fluorescence is increased and can be measured in the extension phase of each cycle of qPCR when such a dye binds to the minor groove of dsDNA (Wittwer et al., 1997). Nonspecific products and primer dimers can be formed during the PCR process (Chou et al., 1992), a melting curve analysis is needed to check the specificity of the amplified fragments. This analysis consists of applying heat to the sample (from 50 °C to 95 °C) and monitoring the fluorescence emission during real-time PCR. The production of amplified products is monitored by fluorescent reporter molecules during each cycle of the PCR reaction. The most commonly used dye, SYBR® Green I, presents some limitations, including limited dye stability and inhibition of PCR reaction. A third generation dsDNA binding dye is EvaGreen that offers several advantages such as being less inhibitory to PCR than SYBR® Green I. It can be used under saturating conditions to generate greater fluorescent signals. EvaGreen is also well suited for high resolution melt analysis (Mao et al., 2007). These two dyes can be used to detect either single or multiplex assays. SYBR® Green I and EvaGreen are used for pathogen detection, gene expression, SNP detection, mutation detection, GMO detection (Eischied, 2011: Navarro, Serrano-Heras, Castañoa, and Solera, 2015).

2.6.2.2.3.2 Labeled fluorophore oligonucleotides

Fluorophores are attached to oligonucleotides and only specific PCR products are detected. It has been further divided into three subgroups according to the type of fluorescent molecules added to the reaction: (i) probes acting as primers, called primer-probes (e.g. Scorpions, Amplifluor®, LUX™, Cyclicons, Angler®); (ii) hydrolysis probes emitting fluorescent light upon degradation during the extension phase, and hybridization probes that give a fluorescent signal when binding to the DNA target during the amplification reaction (e.g. TaqMan, MGB-TaqMan, Snake assay); and (iii) analogues of nucleic acids (e.g. PNA, LNA®, ZNA™, non-natural bases: Plexor™ primer, Tiny-Molecular Beacon) (Navarro et al., 2015). Donor (or reporter) and accepter (or quencher) are two types of fluorophores. Donor fluorophore is raised to an excited state when it absorbs energy from light. Emission of energy as fluorescence is the process of returning to the ground state. This emitted light from the donor can be transferred to an acceptor fluorophore. Emitted light has a lower energy, lower frequency and a longer wavelength than the absorbed light. At present, there are a wide variety of donors and acceptors with different excitation and emission spectra that can be used in qPCR (Marras, 2006).

(i) **Primer-probes** are oligonucleotides that combine a primer and probe in a single molecule. They can be classified into three groups:

(A) Hairpin primer-probes

Hairpin primer-probes are single strand oligonucleotide which contain loop of the structure binds to target DNA, a short tail

sequence of 6 nucleotides (GC) at the 5'-end, one or two of fluorophores attached at the end, and the probe contains primer linked to the hairpin structure (in some case). There are 3 types of hairpins primer-probes, Scorpions, Amplifluor® and LUX™. In scorpion primer-probes, after binding of the primer-probe to the target DNA, the sequence of nucleotides from the 3′-end of the primer is copied by the polymerase. In the next denaturation step, the complementary region binds to the specific sequence of the probe within the same strand of newly amplified DNA (Figure 2.1A). This hybridization opens the hairpin structure and, as a result, the reporter is separated from the quencher leading to a fluorescent signal proportional to the amount of amplified PCR product (Whitcombe, Theaker, Guy, and Brown, 1999). This system is inexpensive because the primer-probe combines the binding and detection in the same molecule. The formation of primer-dimers and non-specific PCR amplification products is prevented by oligonucleotides with hairpin structures because the intramolecular binding of such structures is kinetically favorable and highly effective. Furthermore, in this system enzymatic breakdown of the primer-probe is not necessary and the fluorescent signals are stronger than those produced when other probes are used (Whitcombe et al., 1999).

For Amplifluor® primer-probes, the hairpin structure is intact and the reporter transfers energy to the quencher via Fluorescence Resonance Energy Transfer (FRET) quenching when the primer-probe is not bound (Figure 2.1B). DNA amplification occurs after binding of the primer-probe to the target sequence. Reporter and quencher are then separated in the next step of denaturation,andthe emitted fluorescence of the donor is measured by the fluorimeter (Nazarenko, Bhatnagar, and Hohman, 1997; Wong and Medrano, 2005). These

probes display the same advantages as those described previously for Scorpion primer-probes.

In LUX™ primer-probes, the hairpin structure shows the ability to increase the fluorescence signal exponentially when the primer probe binds to its target sequence (Figure 2.1C) after the incorporation of LUX™ primer-probes into dsDNA, the maximum fluorescence emission is generated (Nazarenko, Lowe, Darfler, Ikonomi, and Schuster, 2002). Fluorescence is measured during the extension phase. The advantages of this system are similar to those methods that rely on Scorpions and Amplifluor primer-probes. LUX™ primer-probes offer high sensitivity and specificity despite that they contain only a single fluorescent molecule (Nordgren, Bucardo, Dienus, Svensson, and Lindgren, 2008).

(B) Cyclicon primer-probes

Cyclicons have a reporter at the free 3′-end of the modified oligo and a quencher placed on a thymine base at the 5′-position in the primer probe sequence (Kandimalla and Agrawal, 2000). In this technique, reporter and quencher molecules are closely and energy transfer occurs via FRET-quenching in the absence of the target sequence (Figure 2.1D). Cyclic structure is opened by the binding of Cyclicon probes to DNA and leads to extension of the 3′-end primer-probe by DNA polymerase without any interference from the quencher. The 3′-end of the modified oligo is not extendible since it does not bind to the target DNA and its 3′-end is blocked by a reporter. Emission of fluorescence is measured during the extension phase, it occurs by the separation between reporter and quencher molecules (Kandimalla and Agrawal, 2000). The advantages of Cyclicons are that it allows the use of shorter oligonucleotides, reducing the costs of the assay,

simplifies the reaction set up and avoids unnecessary carry-over contaminations. It has been reported that Cyclicons with a 5′-5′-attached structure give less fluorescence background in reactions with polymerases devoid of nuclease activity (Kandimalla and Agrawal, 2000).

(C) Angler® primer-probes

The probe component is a DNA sequence identical to that of the target, which is bound to a reverse primer through a hex-ethylene glycol (HEG) linker (Newton, Holland, and Heptinstall, 1993). The donor fluorescent moiety used for this assay is SYBR® Gold (Lee, Siddle and Page, 2002). In Angler® primer-probes, the primer-probe does not emit fluorescence since there is no donor fluorescent close enough for FRET (E).

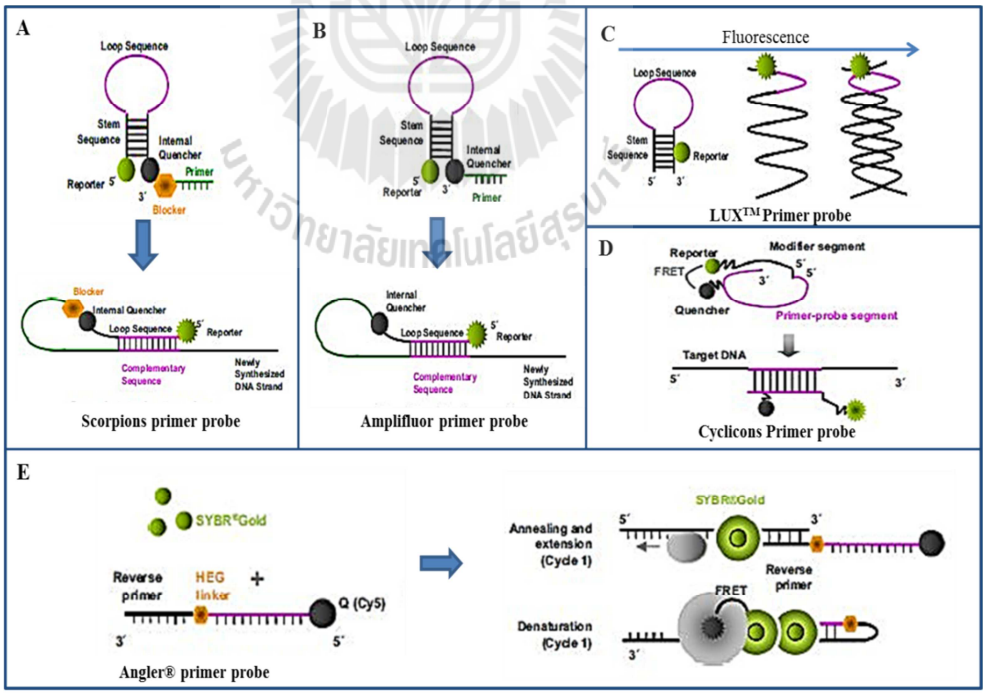

Figure 2.1 Structure and mechanism of Scorpion (A); Ampliflour® (B); LUXT™ (C); Cyclicons (D) and Angler® (E) primer-probes. Adapted from: Navarro et al. (2015).

DNA polymerase starts the extension of the 3′-end reverse primer when the Angler® primer-probe binds to its target DNA during the annealing step. Subsequently, the specific sequence of the probe binds to the complementary region of newly amplified DNA during the denaturation phase, producing a dsDNA fragment in which SYBR®Gold dye can be intercalated to generate fluorescence (Lee et al., 1999; Taylor et al., 2001). Hence, the emitted fluorescence is measured during the denaturation step in each cycle.

(ii) **Probes** are oligonucleotides with connected-donor and/or -acceptor fluorophore. There are two types of probe, (a) hydrolysis and (b) hybridization probes.

(A) Hydrolysis probes

The most widely used probe-based detection chemistry for real-time PCR remains to be the original hydrolysis probes, also known as 5′ nuclease or TaqMan® probe. The probe is generally labelled at 5′ end with a fluorescent reporter dye and at the 3′ end with a quencher. Hydrolysis probes are designed to have melting temperature approximately 10 °C higher than their corresponding primers, causing the probe to anneal to the DNA template prior to the primers. The fluorescent signal of the donor fluorophore is suppressed by the acceptor fluorophore (Heid, Stevens, Livak, and Williams, 1996). During the extension, Taq polymerase hydrolyzes the bound hydrolysis probe by 5′-3′ exonuclease activity, separating the reporter dye from quencher and generating fluorescence from the donor (Figure 2.2A). The exponential synthesis of the PCR products without interfering is repeated in each cycle. The amount of amplified specific product is proportional with measured fluorescence at the end of the

extension phase (Wittwer et al., 1997). The design and synthesis of TaqMan probes are not difficult but primer-dimers might be formed during qPCR assay, if they are not well designed. Other hydrolysis probe, Snake assay, the 5′-nuclease activity of DNA polymerase generate fluorescence signals over the probe (Kutyavin, 2011). In this system, target DNA amplification and detection of fluorescence are two

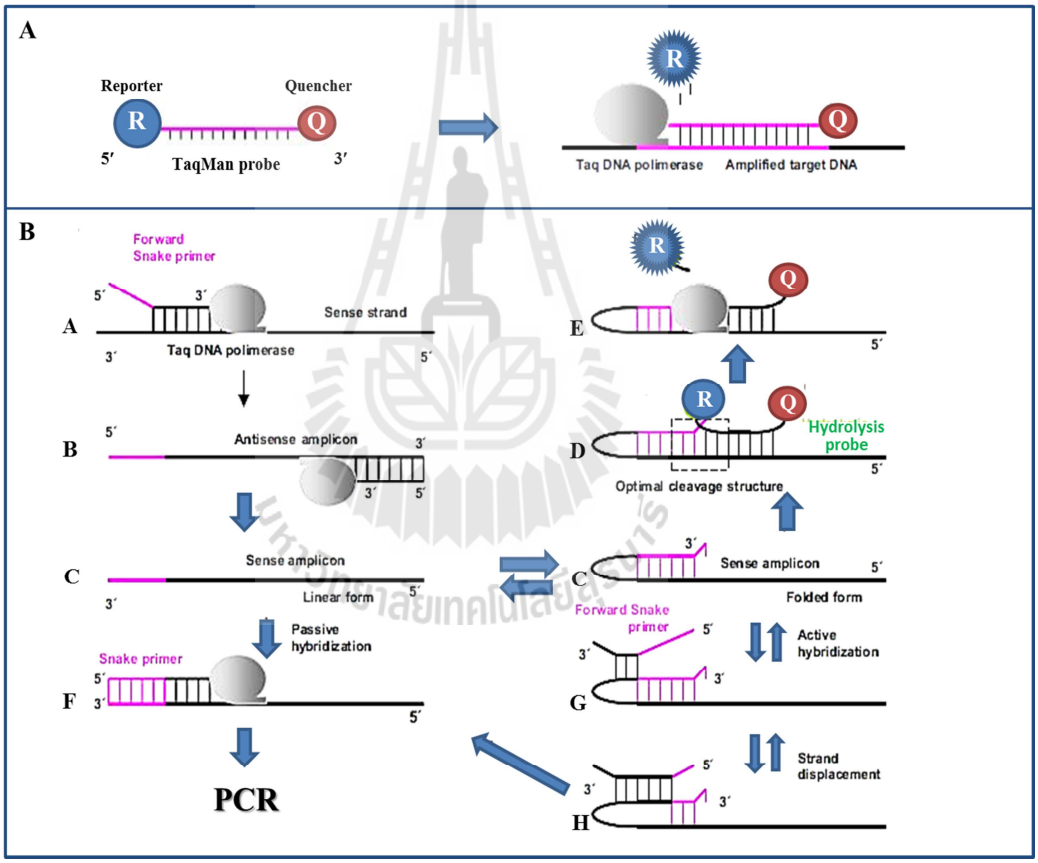

Figure 2.2 Structure and mechanism of TaqMan® probe (A) and scheme of Snake system (B).

Adapted from: Navarro et al. (2015).

separated in time and space as shown in Figure 2.2B. The target DNA sequence binds to a forward primer containing a 5′-flap sequence that located downstream

from the primer binding site. An antisense strand is synthesized by extension of this primer, which provides a double stranded amplicon (stage A). In stage B, a reverse primer hybridizes to the separated antisense strand and DNA polymerase extends the complex, resulting in another double stranded amplicon. Since the 5′-flap of the forward primer functions as a template for DNA synthesis, a complementary sequence appears at the 3′-end of the sense amplicon strand (stage C, linear form). After another round of strand separation, the synthesized sense amplicon in stage B will be folded into a secondary structure as shown at stage C (folded form). Stage D, the sense strand binds to hydrolysis probe creating an optimal cleavage structure for 5′-nuclease. Then, this structure is subsequently cleaved in stage E, releasing a detectable fluorescent signal, stage E (Kutyavin, 2011). Besides, alternative pathway could be taken by the sense amplicon during the PCR assay. Briefly, there is a small fraction of linear form (C) amplicon that might be accessible to a forward5′-flap primer (stage F). The strand DNA replication would be accomplished through a passive hybridization (pathway C to F). In addition, the active hybridization of the forward Snake primer in stage G might be followed by a strand displacement in stage H, which substantially accelerates the replication process (Kutyavin, 2010).

(B) Hybridization probes

During the annealing or the extension phase, the fluorescence emitted by binding hybridization probes can be measured. The main advantage over hydrolysis probes is that the use of these probes allows amplified fragments to be analyzed by performing melting curves. The amount of fluorescent signal detected is directly proportional to the amount of the target amplified during the qPCR reaction. There are many type hybridization probes e.g.

FRET probes, Molecular Beacon Probes, HyBeacon™ probes, MGB-conjugated DNA probes, ResonSense® probes, and Yin-Yang probes or displacing probes.

(iii) **Nucleic acid analogues** are analogous compounds to naturally occurring RNA and DNA. An analogue may have alterations in its phosphate backbone, pentose sugar (either ribose or deoxyribose) or nucleobases (Petersson, Nielsen, and Rasmussen, 2005). Normally, the analogues incorporate all of the advantages of native DNA but are more stable and have increased affinity for complementary nucleic acid targets (Bustin and Nolan, 2004). A variety of nucleic acid analogues have been used e.g. Phosphorodiamidate Morpholino Oligomer (PMO) (Summerton and Weller, 1997), 1,5-anhydrohexitol nucleotides (HNAs) (Verheggen, Van Aerschot, and VanMeervekt, 1995), Locked Nucleic Acids (LNAs) (Singh, Nielsen, Koshkin, and Wengel 1998), Peptide Nucleic Acids (PNAs) (Nielsen, Egholm, Berg, and BuchardtO, 1991), 2′-Fluoro N3-P5′-phosphoramidites (Schulz and Gryaznov, 1996), Zip nucleic acids (ZNAs) (Voirin, Berh, and Kotera, 2007) and Non-natural bases: isoguanine (iG) and 5′-methylisocytosine (iC) (Sherrill, Marshall, and Moser, 2004).

2.6.2.3 Application of qPCR in food

qPCR has been evaluated for the detection and quantification of a wide variety of microorganisms, including bacteria, fungi and viruses, with emphasis on the main food-borne pathogens. Several studies have reported qPCR detection thresholds similar to those obtained with standard plate counts (Aparecida de Oliveira et al., 2010; Chen et al., 2010; Takahashi et al., 2009). In artificially contaminated beef samples, *S. aureus* could be detected by qPCR with the lowest of 5×10^2 CFU/2 g (Alarcon et al., 2006). In baby food, about 60 CFU/mL of *B. cereus*

was detection limit (Martinez-Blanch et al., 2009). In wine, as low as 31 CFU/mL of *Brettanomyces* could be measured (Tessonnière et al., 2009). Low levels of target pathogens can also be detected in matrices contaminated by other dominant microorganisms. For instance, the spoiling agent, *Zygosaccharomyces bailii,* could be detected with a threshold of 6 cells/mL in wine and 2-22 cells/mL in fruit juices even in the presence of 10^7 CFU/mL *Sacharomyces cerevisiae* (Rawsthorne and Phister, 2006). However, in a number of cases, the detection and quantification limit obtained without enrichment of the food samples prior to performing qPCR were in the range of 10^2-10^3 CFU/g (or mL) of food matrix (Hierro et al., 2006, 2007; Takahashi et al., 2009). The major advantage of this molecular method over standard methods is time saving. Detection of *L. monocytogenes* by qPCR methods including an enrichment step takes 2 working days, instead of 7 days with the standard method (Aparecida de Oliveira et al., 2010; O'Grady et al., 2009). Beer-spoilage contaminants of the class Clostridia were identified with an enrichment time reduced from 2-4 to 1-3 days due to higher sensitivity of the PCR reaction over the standard method (Juvonen, Koivula, and Haikara, 2008). Detection of *B. cereus* without enrichment could be achieved within 2 h as compared to 2 days from the standard method with a similar cost (Reekmans, Stevens, Vervust, and De Vos, 2009). In order to meet the microbiological criteria required by national and international legislations for foodstuffs, it is sometimes necessary to associate qPCR with an enrichment step of a few hours. Using this technique, a detection limit <5 CFU/25 g of food was easily reached for Salmonella (Chen et al., 2001).

Linear quantifications were reported in a range of at least 5 logs when qPCR was used as a quantitative tool (Martinez-Blanch et al., 2009; Takahashi

et al., 2009) and very good correlations with plate counts are typically obtained. In some cases, however, difference between microbiological counts and qPCR estimates have been reported, with higher bacterial counts with the molecular method (Hein et al., 2005; Hierro et al., 2007; Makino et al., 2010). Several reasons are likely to explain the differences: (i) the presence of intact DNA from dead cells, (ii) the presence of viable but non culturable forms, which can be quantified by qPCR but not by plate counts, (iii) the fact that one CFU on plate might be generated from more than one cell, and (iv) the use of PCR primers targeting varying numbers of multicopy (e.g. 16S rRNA).

Detection limits (DL) and quantification limits (QL) were difference. DL and QL were calculated by comparing to standard curve. DL or limit of detection (LOD) is the lowest population of microorganisms that can be detected by the method. The limit of quantification (LOQ) is the minimal population that can be accurately quantified. Most of the time, the DL is lower than QL, this is the case when qPCR gives a positive signal but the amount of template is too low and provides a C_q that falls out of the linear range of quantification curves. (Tessonnière et al., 2009). When quantifying pathogens in food, the lower QL in the food matrix should be considered, however, should not be lower than QL that obtained from pure cultures. This takes into account the efficiency of nucleic acid extraction and possible interactions of food components with PCR amplification.

Detection limits of various LAB in fermented milk using 16S rRNA gene were approximately 10^2 and 10^3 CFU/mL by qPCR-SYBR® green, even in the presence of other bacteria and without enrichment (Furet, Quénée, and Tailliez et al., 2004; Grattepanche et al., 2005). Liang et al. (2011) reported limit of detection

of viable *Salmonella* was as low as 2 LogCFU/mL in pure culture and 3 LogCFU/g in lettuce using *safC* gene by qPCR-SYBR® green. The detection limit of *L. sakei* of sausage model was 3 LogCFU/g (Martín et al., 2006) using ITS by qPCR-TaqMan. Ueda et al. (2013) reported that the detection limit of *B. cereus* in food samples (e.g. lettuce, vegetable salad, liquid whole egg, and cow milk) using cereulide synthetase genes (*ces* genes) by qPCR-TaqMan and qPCR-SYBR® green was 4-5 Log CFU/mL of both methods.

16S rRNA gene with qPCR-SYBR® green developed by Monnet et al. (2006) enabled direct and specific quantification of *Corynebacterium casei* in cheeses with a quantification limit of 10^5 CFU/g and a linear range between 10^5 - 10^{10} CFU/g. Although these thresholds are higher than those observed in broth medium or with other types of foods and are likely due to the cheese matrix itself, they remain sensitive enough to study the influence of bacterial populations on the finished product. The detection limit of *Enterococcus gilvus* in cheese was 10^4 CFU/g (Zago et al., 2009). Furet et al. (2004) used conventional method (plate count methods) and real-time PCR to quantify *Lb. delbrueckii* in fermented milk. Detection threshold of real-time PCR method to detect *Lb. delbrueckii* in fermented milk product is 10^3 cells/g of sample. The results are similar to those obtained by the molecular quantification of *S. aureus* in cheese or *Listeria monocytogenes* in milk (Nogva, Rudi, Naterstad, Holck, and Lillehaug 2000; Hein, Lehner, Rieck, Klein, Brandl, and Wagner, 2001). However, the numbers of *Lb. delbrueckii* cells determined by plate count methods were 10 to 100 times lower than that by real-time PCR. This indicated that some *Lb. delbrueckii* lost their viable cells during storage. Therefore, qPCR method may allow the detection of not only the viable but also the

nonviable and dead cells (Delroisse, Boulvin, Parmentier, Dauphin, Vandenbol, and Portetelle, 2008).

Ethidium monoazide (EMA) and propidium monoazide (PMA) derivatives from ethidium bromide and propidium iodide, respectively, have been used for discrimination of live and dead cells. They are a positively charged molecule and it is excluded by intact, negative charge, bacterial cell walls but can enter bacteria with damaged cell wall/membrane (Nocker, Cheung, and Camper, 2006). EMA was firstly reported to be useful to quantify viable bacteria by qPCR (Nocker and Camper, 2006; Rudi, Naterstad, Dromtorp, and Holo, 2005) but can penetrate and be toxic for viable bacteria. Later, PMA was applied to use as a non-toxic alternative (Pan and Breidt, 2007). PMA was able to bind to DNA with covalent bond under light exposure and DNA cannot amplify by PCR (Coffiman, Gaubatz, Yielding, and Yielding, 1982). The use of PMA has been recently applied. Elizaquível, Sánchez, and Aznar (2012) used PMA-qPCR to selectively detect live *Escherichia coli* O157:H7 in vegetables after inactivation by essential oils. In fact, PMA does not completely remove DNA from dead cells (Fittipaldi, Codony, Adrados, Camper, and Morato, 2010). Many factors affect PMA treatment such as, short amplicon size of qPCR assay (Li and Chen, 2013; Schnetzinger, Pan, and Nocker, 2013), high concentration of target bacteria (Zhu, Li, Jia, and Sond, 2012), Mg^{2+} concentration of qPCR reaction (Nocker et al., 2006), or high fat content of food sample (Yang, Badoni, and Gillet, 2011). These substances can bind to PMA, resulting in a decrease of PMA binding.

2.7 Next generation sequencing

The first generation of sequencing was chain-terminating dideoxy nucleotide (ddNTPs) analogs developed for the Sanger sequencing, by Sanger et al. (1977). Maxam and Gilbert (1977) also proposed DNA sequencing by chemical degradation, in which terminally labeled DNA fragments were subjected to base-specific chemical cleavage. The reaction products were separated by gel electrophoresis. The DNA sequencing by chemical degradation is technically complex and requires hazardous chemicals. Sanger sequencing was more efficient and used fewer toxic chemicals (Erwin et al., 2014). The growing DNA strand of chain termination is terminated by A, T, G, and C in different reaction. The amplicons were then run on agarose to detect the sequence. Then, the ddNTPs are labeled with fluorescent dyes to make the fragment readable through a laser light in capillary gels, rather than on the slab gel. Sanger sequencing can produce 800-1,000 bp of sequence read because only one read can be sequenced in one capillary of the sequencer (Gupta and Gupta, 2014). Based on the procedure of the Sanger method, sequencing of 3 billion base pairs of human genome would take a very long time. Therefore, fast and high-throughput technologies are needed to achieve the goal.

Figure 2.3 showed development of DNA sequencing technologies at each timeline. Next-generation sequencing (NGS) technologies were the second generation work on the principle of sequencing millions of DNA fragments simultaneously in massively parallel manner to produce huge amount of sequence data in megabases or gigabases. The cost of NGS technologies is low the cost in which producing of large sequence data in massively parallel model. Overview schematic experiment of next generation sequencing is shown in Figure 2.4.

In 2005, the pyrosequencing method by 454 Life Sciences (now Roche) was the first released NGS technology and generated about 20 Mb of 110 base-pairs (bp) (Margulies et al., 2005). After a year, Solexa/Illumina sequencing plat-form was launched (Illumina acquired Solexa in 2007). In 2007, Sequencing by Oligo Ligation Detection (SOLiD) by Applied Biosys-tems (now Life Technologies) was released (Valouev et al., 2008). The Illumina and SOLiD sequencers generated 30 and 100 million reads, respectively, that much larger numbers but the reads produced were only 35 bp long. In 2010, Ion Torrent released the Personal Genome Machine (PGM). Semiconductor technology was used in the PGM. This resulted in higher speed, lower cost, and smaller instrument size. The first PGM generated up to 270 Mb of sequence with up to 100 bp and slightly shorter than those produced by 454 pyrosequencing.

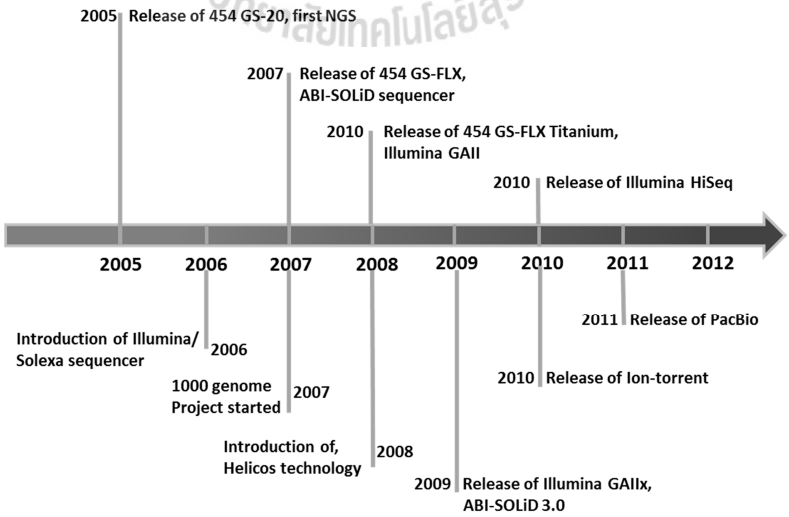

Figure 2.3 DNA sequencing technologies timeline and plate form.

Adapted from: Gupta and Gupta (2014).

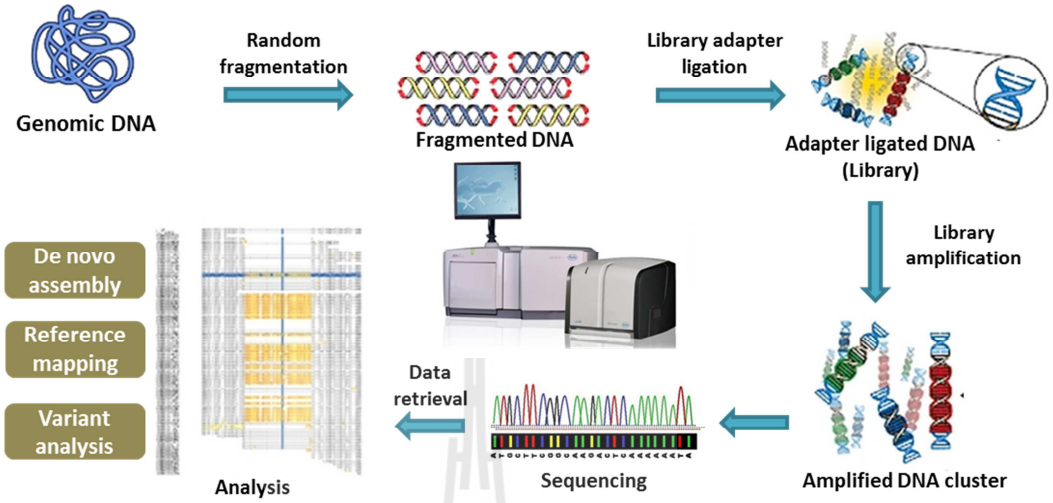

Figure 2.4 Overview schematic experiment of next generation sequencing.

Pacific Biosciences (PacBio) is currently popular in third-generation sequencing technologies which DNA is not amplified before sequencing, which places these methods at the interface between NGS. The PacBio, appeared in 2010 and generated several thousands of up-to-several-kilo-base-long reads. PacBio is based on the detection of natural DNA synthesis by a single DNA polymerase. Incorporation of phosphate-labeled nucleotides leads to base-specific fluorescence, which is detected in real time. Sequencing runs therefore last minutes or hours rather than days (http: //www.pacificbiosciences.com/pro- ducts/). The five platforms are over the past decade: 454 pyrosequencing, Illumina, SOLiD, Ion Torrent, and PacBio (Eid et al., 2009). The comparison of NGS platforms were shown in Table 2.3.

2.7.1 Pyrosequencing technology

Pyrosequencing is a method based on the "sequencing by synthesis" principle. Pyrosequencing relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides.

Table 2.3 Comparison summary of next generation sequencing platforms.

Plateforms	**Roche 454 GS FLX Plus**	**Illumina Solexa HiSeq2500**	**ABI SOLiD 5500xl**	**Ion Torrent**	**Pacific Bio**
Sequencing method	Pyrosequencing	Reversible Dye Terminators	Sequencing by ligation	H+ Detection	ZMW - Single molecule
Read Lengths	700–1000 bp	2x100 bp	75 bp	200 bp	Av 1200, upto 3 kb
Sequencing run time	23 h	11–14 days	6–7 days	2 h	Less than a day
Data generated	700 MB / run	600 GB / run	10–15 GB/day	10–1000 MB (depends upon chip used)	70–140 MB / cell
Advantages	Longer read length,	Huge data	High quality	Low cost,	Fast, longer read
Concerns	Less data, Homopolymer	Short reads, Dephasing, Long run time	Short reads, Long run time	Less data, Small read	Random indel errors

Adapted from: Gupta and Gupta (2014).

A pyrophosphate molecule is released during synthesis of nucleotide growing chain by DNA polymerase. This pyrophosphate is converted into ATP by enzymatic reactions. The ATP is used for conversion of luciferin to oxy-luciferin by enzymatically, which emits fluorescence that is recorded by the camera. By detecting this fluorescence, the incorporation of a nucleotide is confirmed. The identity of the incorporated nucleotide is known, as four dNTPs (dATP, dTTP, dCTP and dGTP) are introduced in the reaction separately in cycles (Ronaghi et al., 1996).

The 454 Sequencing uses a massively parallel pyrosequencing system capable of sequencing up to 1,000 bp of DNA in a 23 h. The method starts with

ligating adaptors to DNA fragments, making a library, and attaching the library to small DNA-capture beads. The bead-bound library is emulsified along with the amplification reagents in a water-in-oil mixture. After amplification, the emulsion is disrupted, and the beads containing clonally amplified template DNA are enriched. Each amplified DNA-bound bead is placed into a tiny well on a PicoTiterPlate consisting of around 3.4 million wells. A mix of enzymes such as DNA polymerase, ATP sulfurylase, and luciferase, are also packed into the well. The PicoTiterPlate is then placed into the sequencer machine for sequencing. The GS FLX platform can produce data of approximately 450 MB with a 400-600 bp read length. The DNA sequence is determined by light emitted. Four dNTP nucleotides are added and only one letter can be incorporated on the single stranded template. The intensity of the light determines if there is more than one of these "letters" in a row. One out of four dNTP is degraded before the next nucleotide letter is added for synthesis and allow for the next nucleotides, resulting intensity of the light, if the next added nucleotides was complementary letters in the sequence. This process is repeated with each of the four letters until the DNA sequence of the single stranded template is determined (Dressman, 2003).

The 454 pyrosequencing has been used to study microbial profiling. Nam, Yi, and Lim (2012a,b,c) used V1-V2 16S rDNA sequencing to analyze bacterial community in Korean fermented food, cheonggukjang, doenjang, and kochjang. They found that all samples seemed to be dominant by *Bacillus* spp. followed by *Lactobacillus* spp., and species of other. Park et al., 2012 reported that *Leuconostoc* spp. and *Weissella* spp. dominate the early stages of kimchi fermentation, while *Lactobacillus* spp. are dominant at the end using V1-V3 16S rDNA sequencing.

Cocolin et al. (2013) also used V1-V3 16S rDNA sequencing to analyze bacterial profiling in fermented olives. The initial stages of fermented olives were dominated by large numbers of *Chromohalobacter* and *Halomonas* approximately 50-60%, while at the end of the fermentation, *Lactobacillus* species surpassed all other microorganisms.

The advantage of 454 technologyis ability to sequence reads in the read length of 700-1000 bp. The method is suitable for downstream bioinformatics, resulting in sequence assembly with longer contigs, higher N50 length, and less gaps, especially in *de novo* sequencing projects. Longer paired-end reads produced by the 454 platform also facilitate construction of better scaffolds.

A major limitation of the 454 technology relates to resolution of homopolymer-containing DNA segments, such as AAA and GGG (Rothberg and Leamon, 2008), producing light that cannot be discriminated after a certain length (> 6 bp) with high accuracy (Mardis, 2008). Because there is no terminating moiety preventing multiple consecutive incorporations at a given cycle, pyrosequencing relies on the magnitude of light emitted to determine the number of repetitive bases. As a consequence, the dominant error type for the 454 platform is insertion-deletion, rather than substitution. Another disadvantage of 454 sequencing platform is that the per-base cost of sequencing is much higher than that of other nextgeneration platforms, e.g., SOLiD and Solexa (Rothberg and Leamon, 2008). Therefore, the method is unsuitable for sequencing targeted fragments from small numbers of DNA samples.

2.7.2 Illumina Solexa

Synthesis reagents consist of primers, DNA polymerase, and four differently labelled, reversible terminator nucleotides. Small oligonucleotide anchors are immobilized on the surfaces of these lanes. The template DNA to be sequenced is fragmented, phosphorylated at the 5′ end, and adenylated to add a single A at the 3′ end. Oligonucleotide adaptors are ligated to the DNA fragment, and the ligation is facilitated by the presence of a single T overhang on the adaptors. The adaptor-ligated oligonucleotides are complementary to the flow-cell anchors, and hence attach to the anchors. After incor-poration of a nucleotide, which is identified by its color, the 30 terminator on the base and the fluorophore are removed, and the cycle is repeated (Voelkerding, Dames, and Durtschi 2009). On denaturation, both strands separate, and again bend and hybridize with their distal ends to adjacent anchors complementary to their distal ends. After multiple amplification cycles, a single DNA template makes a clonally amplified cluster with thousands of clonal molecules. Millions of clusters of different template molecules can be generated per flow cell (Fedurco, Romieu, Williams, Lawrence, and Turcatti 2006).

The Illumina has been used to study microbial in wine using V4 and V5 16S rDNA sequence with 150 bp of amplicon length. The results showed that *Acetobacteriaceae*, *Proteobacteria*, and *Saccharomyces* were dominant organisims (Bokulich, Joseph, Allen, Benson, and Mills, 2102). Bokulich, Bamforth, and Mills (2012) also used V4 16S rDNA sequence to characterized microorganisms in beer. They showed that initially stage dominated by *Enterobacteriaceae* and *Pediococcus* spp. became dominant after few weeks.

The advantage of Illumina is that large data and low cost per base renders the technology a good choice for many sequencing applications where large read length and *de novo* construction of a genome is not required. Because 95 GB of data coming out from nearly 150 bp long reads from both sides (2 x 150 bp) in the most widely cited platform Genome Analyzer IIx, the throughput has been significantly increased up to 600 GB data with 2 × 100 bp reads in newer versions of the platform (e.g. HiSeq2500 or HiSeq 2000), resulting in low cost per base (Nakamura, Oshima, and Morimoto, 2011).

The major of limitations of Illumina technology is that of de-phasing, which means different fragments in a cluster are sequenced with different phases, result in fragments of varying lengths. It reduces precision in base calling at the 3′ ends of the fragments. Increased read length increases de-phasing. It is more common at sequences of invert repeats or GGC (Nakamura et al., 2011). Illumina technology produces reads of short length "micro-reads," hence assembly and downstream bioinformatics could be a challenge, especially for certain *de novo* sequencing. Longer run-time is also a limitation.

2.7.3 ABI SOLiD

Small Oligonucleotide Ligation and Detection (SOLiD) System is based on a sequencing-by-ligation technology. This platform has its origins in the system described by Shendure et al. (2005) and McKernan et al. (2006). The principle of this sequencing, specific incorporate bases depend on the ability of DNA ligation. The emulsion PCR is clonally amplified that DNA fragments attached on beads for ligation sequencing. After PCR, specific primers and adaptor sequence of the amplified templates on the beads were hybridized together and provides a free 5′

phosphate group for ligation to the fluorescently labeled probes instead of providing a 3′ hydroxyl group as in normal polymerase-mediated extension. It is called interrogation probes. Ligation, detection, and cleavage are performed, in multiple cycles of with the number of cycles determining the eventual read length. The extension product is removed and the template is reset with following a series of ligation cycles. A primer complementary will be started for a second round of ligation cycles. Thus the sequencing is divided into library preparation, emulsion PCR, bead deposition, sequencing, and primer reset. A 6-7-day long instrument run in a SOLiD 5500 system generates sequence data at approximately 10-15 GB/day (Voelkerding, Dames, and Durtschi, 2009). SOLiD has been used in transcriptome, targeted sequencing, and whole genome sequencing.

The advantage of this technology is generation higher accuracy of sequencing data of comparatively than other sequencing methods. One of the reasons behind the high accuracy is each nucleotide of the template is sequenced twice due to sequencing with successive offset primer less by one bp (Voelkerding et al., 2009).

The limitations of this technology require close genome sequencing for mapping. The data are output less than with Illumina, and shorter read length. Even the time taken for a whole run is about 6-7 day to complete, especially for bigger genomes (Voelkerding et al., 2009).

2.7.4 Pacific Biosciences

Pacific Biosciences (PacBio) technology works on the Single Molecule Real-Time (SMRT) sequencing technology, and enables the observation of DNA synthesis as it occurs in real time. Zero Mode Waveguide (ZMW) was used as specially designed micro-holes. Approximately 75,000 single molecule sequencing

reactions in parallel enable to detect. Laser light of wavelength of approximately 600 nm cannot pass completely through the ZMW because the small size of the ZMW is a nano-hole made in a 100 nm metal film on a glass surface. Therefore, only the bottom 30 nm of the ZMW applying a laser through the glass into the ZMW become illuminated. A single DNA polymerase molecule is anchored to the bottom glass surface of the ZMW. Nucleotides are labeled with a different colored fluorophore and were then flooded above the ZMWs. As laser light does not excite fluorescently labeled nucleotides present on the upper side of the holes because it cannot penetrate up through the holes. Thus the labeled nucleotides above the ZMWs are dark. Labeled nucleotides fluoresce when they diffuse through the bottom 30 nm of the ZMW. Thus, single nucleotide incorporation can be detected inside the ZMW (Mardis, 2008). PacBio has been used for sequencing difficult DNA regions and for genone scaffolding in metagenomic studies.

The advantages of PacBio sequencing technology do not require the PCR amplification steps. This avoids the usual amplification bias in the sequenced DNA fragments. PacBio generates read lengths of around 1,000-3,000 bp with an average of 1,200 bpThe time taken from sample preparation to sequencing results is also shorter, and can take less than one day. Kinetic information about polymerase activity can be observed by the technology (Mardis, 2011).

Limitation of PacBio technology that is around 70 to 140 MB of data were generated per SMRT cell, depending upon a GC content of the template DNA (www.pacificbiosciences.com) that is significantly less compared to the other technologies. (Mardis, 2011).

2.7.5 Ion Semiconductor Sequencing

For Ion Torrent technology with its Ion Personal Genome Machine (PGM®) sequencer technology, hydrogen ion is released as by-product during incorporation of new nucleotides into the growing DNA template by DNA polymerase. Ion Torrent, with its Ion Personal Genome Machine (PGM®) sequencer, uses a high-density array of micro-machined wells to perform nucleotide incorporation in a massively parallel manner. Each well bottom is an ion-sensitive layer followed by a proprietary ion-sensor and holds a different DNA template. An ion sensor detects changes of the pH of the solution as a result of released ion. The output voltage is doubled if there are two identical bases on the DNA strand and the chip records two identical bases called without scanning, camera, and light. A direct connection between the chemical and digital events is used in Ion Torrent technology, instead of detecting light as in 454 pyrosequencing. Hydrogen ions are detected on ion-semiconductor sequencing chips. The transistors and circuits are then pattern-transferred and subsequently etched onto the wafers using photolithography. A multi-layer system of circuits is created and repeated 20 times. A total data output of around 10-1,000 MB is generated, depending upon the type of ion semiconductor sequencing chip used which are 314, 316 and 318 chips. The chips are different in the number of wells resulting in higher production within the same sequencing time. The Ion 318 chip enables the production of >1Gb data in 2 h. Read length is expected to increase to >400 bp in 2012 until now. The Ion Torrent sequencer can generate up to 4 Mb in 7 h at a moderate cost per Mb. The length of the reads has risen in recent years up to 400 bp.

Ion Torrent technology is useful in small studies, such as the sequencing of

microbial genomes and targeted metagenomics (Rothberg et al., 2011). Galindo-González et al. (2014) conducted experiments to develop an Ion Torrent-based method for discovery of mutation in *Linum usitatissimum* genome. Fujimoto et al. (2012) used 16S rRNA gene amplicon sequence to analyze microbial community in alkali ballast water. They found that the relative abundance of genera *Rheinheimera* and *Pseudomonas* increased in NaOH-treated samples. However, *Alishewanella* became dominant in the NaOH-treated samples. Bacteria from the genera *Escherichia*, *Enterococcus*, and *Vibrio* were not detected in all samples.

The advantages of Ion Torrent are used to fill gaps in the assembly produced by other technologies because the technique generates read lengths of around 200 bp. Ion Torrent can be a reasonable choice in some cases due to lower costs. The short run time of this technique also facilitates multiple runs for generation of more data in a given time (Liu et al., 2012; Metzker, 2010). Limitation of Ion Torrent technology is Ion lags behind in total data output, whereas short read technologies are facilitated by huge data generation. Ion Torrent has to prove itself as a standalone sequencing technique for *de novo* sequencing projects of big genomes (Liu et al., 2012; Metzker, 2010).

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CHAPTER III

DETECTION OF VIABLE BACTERIAL STARTER CULTURES OF *VIRGIBACILLUS* SP. SK37 AND *TETRAGENOCOCCUS HALOPHILUS* MS33 IN FISH SAUCE FERMENTATION BY REAL-TIME QUANTITATIVE PCR

3.1 Abstract

Novel real-time quantitative polymerase chain reaction (qPCR) methods were developed for the specific detection and quantification of *Virgibacillus* sp. SK37 and *Tetragenococcus halophilus* MS33, which are potential starter cultures in fish sauce fermentation. The PCR assays were designed based on the *aprX* gene and the internal transcribed spacer (ITS) of *Virgibacillus* sp. SK37 and *T. halophilus* MS33, respectively. Specificity was evaluated using 6 reference strains and 28 strains isolated from fish sauce fermentation. The developed methods showed species-specificity for both *V. halodenitrificans* and *T. halophilus* without cross reacting. The efficiencies of Vir1086 and Tet48 probes for the detecting purified DNA from *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were 101.1% and 90.2%, respectively. The matrix effect was insignificant when tested using samples fermented for 1-6 months. The quantification limits of the assays were 10^3 Cells/mL and 10^2 Cells/mL in fish sauce samples with good linear correlations (R^2 values of 0.990 and 0.976) over 4 Logs for *V.*

halodenitrificans and *T. halophilus*, respectively. The repeatability and reproducibility of the methods were within 37% and 33% of relative standard deviation. The propidium monoazide (PMA) was used to treat samples to eliminate the effect of dead cell DNA on PCR. The developed PMA-qPCR methods were successfully applied to monitor changes in *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in a mackerel fish sauce fermentation model.

Keywords: qPCR, real-time PCR, PMA treatment, *Virgibacillus* sp. SK37, *T. halophilus* MS33, starter culture, fish sauce fermentation

3.2 Introduction

Fish sauce is considered as a natural flavor enhancing ingredient in many types of cuisines due to its high content of glutamic acid. Fish sauce fermentation normally takes 12-18 months to complete. The addition of starter cultures has therefore been used to accelerate fermentation and to improve fish sauce quality (Udomsil et al, 2011). *T. halophilus* MS33, a starter culture isolated from *nam pla* Thai fish sauce, was found to produce desirable volatile compounds and increase amino acids, such as glutamic acid, thereby contributing to umami taste. *T. halophilus* MS33 was also demonstrated to reduce dimethyl disulfide, a compound contributing to fecal notes (Udomsil et al., 2011). *Virgibacillus* sp. SK37, a moderately halophilic starter culture bacterium isolated from 1-month-old Thai fish sauce mashes was found to produce extracellular and cell-bound proteinases at high salt concentrations (Sinsuwan et al., 2007; 2008). This culture increased protein hydrolysis and increased desirable aldehyde compounds (e.g., 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal) in reduced salted fish sauce, which had an

effect on the overall aroma of the fish sauce. These compounds likely contributed to the strong malty or dark chocolate notes (Lapsongphon et al., 2013). Growth monitoring of these cultures would be necessary for the quality control of the fermentation process.

The spread plate technique is a classical method that is used to monitor microbiological changes during fish sauce fermentation. In the presence of added starter cultures, it is difficult to differentiate between added starter cultures and naturally existing microflora based on selective media and colony morphology. More importantly, the plate count technique cannot be used to detect viable but nonculturable (VBNC) microorganisms. The VBNC state of microorganisms normally occurs under harsh environments, such as extreme temperature, nutrient starvation, oxygen availability, and sharp changes in osmotic stress, pH, or salinity (Fakruddin et al., 2013). This would lead to an underestimation of the "true" population of the starter cultures. Therefore, there is a need to develop methods that can specifically detect starter cultures during fish sauce fermentation.

The qPCR technique based on gene specificity has been widely used for the sensitive and specific detection of microbial species or subtypes (Fonseca et al., 2013). Specific genes/regions, such as protein-encoding genes, ITS regions, or virulence genes, have been used to design specific PCR primers and probes to detect and discriminate between species of bacteria (Tilsala-Timisjärvi and Alatossava, 1997), such as *Staphylococcus aureus* (Kadiroglu et al., 2014), *Enterococcus* spp., and *Escherichia coli* (Noble et al., 2010). The most reported PCR methods for bacteria are based on the detection of DNA from both live and dead cells (Delroisse et al., 2008). This could lead to false interpretations, particularly to monitor starter

cultures where only live cultures are of concern. Dead cells have a damaged membrane that is unable to retain DNA (Cook and Bolster, 2007). Propiodium monoazide (PMA) has been used to covalently bind DNA under light exposure and assist in the selective detection of live cells because PMA-conjugated DNA cannot be amplified by PCR (Coffiman et al., 1982). PMA treatment conditions vary with specific PCR methods and sample matrices. Factors including the type and concentration of target bacteria (Zhu et al., 2012), the fat content of food samples (Yang et al., 2011), or the Mg^{2+} concentration of the qPCR reaction (Nocker et al., 2006) can affect DNA binding by PMA and should be investigated for a specific application.

The PCR methods for the specific detection of the aforementioned starter cultures have not been reported. The objectives of this study were to develop and evaluate qPCR methods to detect viable starter cultures of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 and to apply these PCR methods to monitor changes in starter cultures during fish sauce fermentation.

3.3 Materials and methods

3.3.1 Bacterial strains

Virgibacillus sp. SK37 (accession no. DQ910840) and *T. halophilus* MS33 (accession no. FJ715465) were isolated from fermented fish sauce at 1 month and are potential fish sauce starter cultures. *Virgibacillus* sp. SK37 was cultured in tryptic soy broth containing 5% NaCl (TSB5) and incubated at 35 ºC under aerobic condition for 3 days. *T. halophilus* MS33 was cultured on De Man, Rogosa and Sharp broth containing 5% NaCl and 0.5% $CaCO_3$ (MRS5) and was incubated at 35

ºC under anaerobic conditions for 3 days. These two strains were used as the target strains in this research.

Indigenous bacteria were isolated from commercial fish sauce tanks at various fermentation times between 1 and 8 months. A total of 28 isolates were obtained and identified by 16S rDNA sequence analysis (Table 3.2). Isolates identified to be *Tetragenococcus* and *Virgibacillus* were cultured using the media and conditions described above. The other isolates were grown on tryptic soy agar (TSA) and incubated at 35 ºC under aerobic condition for 24 h. Reference strains, including *T. halophilus* ATCC33315, *T. muriaticus* JCM10006, *Staphylococcus condimenti* JCM6074, *S. carnosus* TISTR833, *S. pisciferm*entans JCM6057, and *V. halodenitrificans* ATCC12304, were cultured using MRS5 and TSA medium. These strains were used for the specificity test.

3.3.2 Fish sauce samples

Fresh Spanish mackerel (*Scomberomorus maculatus*) was purchased from a local supermarket (Mississauga, ON, Canada) and ground into paste using a blender (Cuisinart®, Woodbridge, ON, Canada). The ground samples (250 g) were added with NaCl to a final concentration of 25%. Cells of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 (10^6-10^7 CFU/mL) were added at 5% for co-culture fermentation. For single culture fermentation, each of the inoculum cultures (10^6-10^7 CFU/mL) was added to a final concentration of 10%. Fermentation was performed at 35 ºC for 90 days. Samples (10 g) were taken at 0, 7, 14, 30, 60, 75, and 90 days for bacterial enumeration by plate count and qPCR analysis.

Commercial fish sauce samples fermented for 1, 3, 6, 8 and 12 months were collected from the Rayong Fish Sauce Industry (Rayong, Thailand). The

samples were taken from 3 fermentation tanks at each of the sampling points and were used as matrices to evaluate the qPCR methods for the quantification of *Virgibacillus* sp. SK37 and *T. halophilus* MS33.

3.3.3 Plate count enumeration

Mackerel fish sauce samples were enumerated by the standard plate count technique. Mackerel fish sauce (10 g) was taken and mixed with 90 mL of 0.85% NaCl. Samples were diluted and enumerated using plate count agar containing 10% NaCl (PCA10) and modified JCM168 agar containing 10% NaCl (JCM10) for total halophilic bacteria and *Virgibacillus* sp. SK37, respectively. MRS5 medium was used to enumerate *T. halophilus* MS33. PCA10 and JCM10 were incubated under aerobic conditions, while MRS5 agar was incubated under anaerobic conditions; all media were incubated at 35 ºC for 3-5 days. For commercial samples, 100 µL of undiluted fish sauce was spread on MRS5 and JCM10 agar and incubated under the same conditions described above.

3.3.4 DNA extraction

Pure cultures of *T. halophilus* MS33 and *Virgibacillus* sp. SK37 (100 µL) equivalent to 10-10^7 CFU/mL were centrifuged in 1.5-mL microcentrifuge tubes at 10,000 ×g for 10 min to collect cell pellets that were then washed twice with 1 mL of 1× Phosphate Buffered Saline (1× PBS). The pellets were resuspended in 180 µL of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme) and incubated at 37 °C for 30 min. DNA was extracted from the cell lysates using the DNeasy® tissue kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's instructions. DNA was eluted in 100 µL of AE buffer (10 mM Tris-HCL, 0.5 mM EDTA, pH 9.0). DNA concentrations and quality

were assessed based on the OD_{260} and the ratio of OD_{260}/OD_{280}, respectively, using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

Fish sauce samples (100 µL) were washed twice with 1 mL of 1×PBS and centrifuged at 10,000 ×g for 10 min. The pellets were resuspended in 500 µL of 1×PBS, and propiodium monoazide (PMA) was then added to a final concentration of 100 µM. The mixture was incubated in the dark at a shaking speed of 600 rpm for 20 min, followed by light exposure on ice for 5 min at a distance of 20 cm. The samples were subsequently washed and centrifuged at 10,000 ×g for 10 min to collect the pellets. An internal control culture was added to the pellets at 1.66 x 10^4 CFU/sample prior to DNA extraction. The DNA was extracted using the DNeasy® tissue kit and evaluated as described above.

3.3.5 Polymerase chain reaction (PCR)

The alkaline serine protease-X gene (*aprX*) and the internal transcribed spacer (ITS) were used to target *Virgibacillus* sp. SK37 and *T. halophilus* MS33, respectively. The *aprX* gene sequence of *Virgibacillus* sp. SK37 (accession no. HM587897) was retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The ITS region sequence of *T. halophilus* MS33 (accession no. KP638354) was obtained by PCR and sequencing of the PCR product using the forward primer 5'-CTACAACCCCAGAATGCAAG-3' and the reverse primer 5'-CAATGGGAAGTACAACGAGC-3, both of which were designed from the 16S and 23S rDNA sequence of *T. halophilus* NBRC12172 (accession no. NC_016052). Primers and probes (Table 3.1) were then designed using Primer Express® 3.0 software (Life Technologies, Foster City, CA, USA) and synthesized at Laboratory Services, University of Guelph (Guelph, ON, Canada) using an ABI

3900 HT DNA synthesizer (Life Technologies). The primers and probes were purified using oligo purification cartridges (Life Technologies). The probes (Vir1086 and Tet48) were labeled at the 5'-end with the reporter dye FAM and at the 3'-end with the quencher Black Hole Quencher®-1 (Biosearch Technologies, Inc., Novato, CA, USA). Internal controls (IC) were constructed by cloning artificial fragments (Table 3.1) into a plasmid using the One Shot® Top10 Cloning® kit (Invitrogen, Carlsbad, CA, USA) and then transforming the recombinant plasmid into *E. coli* DH5α following the instructions provided by the kit manufacturer. The artificial IC fragments were flanked with the same sequences of the primers of their respective targets to allow for the amplification of a target and the IC with a single primer pair. The IC probes were labeled with the reporter dye CAL Fluor Orange560 (Biosearch Technologies) at the 5'-end and with the quencher Black Hole Quencher®-1 (Biosearch Technologies) at the 3'-end. The recombinant IC cells were grown in 5 mL of TSB containing 100 µg/mL of ampicillin, stored in 20% glycerol in a -80 °C freezer, and added to all samples prior to cell lysis for DNA extraction.

The amplification was carried out in 20-µL reactions in a PCR MicroAmp™ Fast 96-well Reaction Plate sealed with MicroAmp™ Optical Adhesive Film (Life Technologies). Each reaction contained 3 µL of genomic DNA, 10 µL of 1× iTaq Universal Probe Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada), 500 nM of each primer, and 300 nM of each probe. The amplification was conducted using a 7500 Fast Real-Time PCR system (Life Technologies). The cycling conditions were 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 30 s and then 60 ºC for 30 s. Fluorescence was read at 60 ºC using 7500 software V2.0.1 (Life

Table 3.1 Primers and probes designed for detection of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 using *aprX* and ITS, and internal control used in this study.

Target gene/ region	Primers/ Probes name	Sequences (5'-3')	*T*m (°C) of primers/ probes	Position of primers/ probes	Amplicon size (bp)	Accession number
aprX	Vir_F1029	GGATCGGCGCTAGAAAAACA	58.9	1029-1048	131	HM587897
	Vir_R1140	GCTGAGGATTTGCCTCAAGC	58.6	1140-1159		
	Vir1086	FAM-ACTATCTGGCACTTCGAT-BHQ1	69.0	1086-1103		
	Internal control for SK37	**GGATCGGCGCTAGAAAAACA*CTTGTCCCTCCTGTTGGTACTAGAGAGGGGGAAAGGGCGAATTCTACAAGATG AAAGGGCCCTACAGATTCGCAGAATTCGCGTGATG*GCTTGAGGCAAATCCTCAGC*				
ITS	Tet_F20	GGTCAAGGGTTTCTCGAAGGT	58.0	20-40	110	KP638354
	Tet_R107	AATCAACACCAACCGAGAATCC	59.0	107-128		
	Tet48	FAM-TTGAGCGATCAAAGCCCTTCGAACA-BHQ1	68.0	48-72		
	Internal control for MS33	*GGTCAAGGGTTTCTCGAAGGT*CTTGTCCCTCCTGTTGGTACTAGAGAGGGGGAAAGGGCGAATGCTGCAAGAT GAAAGGGCCCTACAGATTCGCAGAATTCGCGTGATG*GGATTCTCGGTTGGTGTTGATT*				

*Forward and reverse primers were shown in italic alphabets. Underline was IC probe sequence.

Technologies). The quantification cycle (C_q) values were assigned automatically using the system software. The delta C_q (ΔC_q) between the C_q of the samples and the C_q of IC was used for normalization.

For each experiment, samples were prepared in duplicate, and PCR was carried out in duplicate for each sample. Cultures or DNA of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were used as positive controls. The PCR master mix without a DNA template was used as a negative control. IC was included in each PCR reaction to monitor PCR inhibition or PCR failure to safe guard against false negative results.

3.3.6 Evaluation of the qPCR assays

The specificity of the primers and probes were tested with the genomic DNA (15ng/PCR) of 28 isolates of bacterial microflora isolated from fish sauce plants and the genomic DNA from 6 reference strains (Table 3.2). The quantification limit/range and the matrix effect of the optimized qPCR methods were assayed using fish sauce mashes taken at various fermentation times of 1, 3, and 6 months. Fish sauce samples (100 µL) were added to 1.5-mL microcentrifuge tubes, and pellets were collected by centrifuging at 10,000 ×g for 10 min. The pellets were washed twice with 1 mL of 1× PBS and were spiked with *Virgibacillus* sp. SK37 or *T. halophilus* MS33 at various cell concentrations between 10-10^7 CFU/mL. DNA was then extracted from the spiked fish sauce using the same protocol as described in section 3.3.4. The qPCR reactions were prepared as described in section 3.3.5.

Repeatability and reproducibility of the PCR methods were also evaluated using 100 µL of fish sauce mash fermented for 1, 3, and 6 months. Fish sauce pellets were prepared and spiked with the pure cultures of *Virgibacillus* sp.

SK37 or *T. halophilus* MS33 at cell concentrations between 10^2-10^6 CFU/mL. The DNA extraction and PCR reactions were carried out as described above. Repeatability and reproducibility were determined by calculating the standard deviation (SD) and the relative standard deviation (RSD) of C_q and the CFU of each qPCR reaction within and between experiments, respectively.

3.3.7 Optimization of propidium monoazide (PMA) treatment conditions for spiked fish sauce

The parameters of PMA treatment, including the PMA concentration, incubation time, and light exposure time, were investigated. *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were cultured in broth as described in section 3.3.1. To prepare dead cells, live cells (100 µL) from *Virgibacillus* sp. SK37 or *T. halophilus* MS33 suspensions at 10^6 CFU/mL were inoculated into 0.9 mL of fish sauce and incubated at 35 °C for 7 days. To confirm that the spiked samples contained dead cells from *Virgibacillus* sp. SK37 and *T. halophilus* MS33, the spiked samples were spread on agar plates using the same medium and conditions as described in section 3.3.3. Samples (90 µL) containing dead cells were transferred into 1.5-mL microcentrifuge tubes and were then spiked with respective live cells (10 µL) from *Virgibacillus* sp. SK37 or *T. halophilus* MS33 at concentrations ranging from 10 to 10^6 CFU/mL. Prior to DNA extraction, PMA was added to fish sauce containing live and dead cells at final concentrations between 100 and 300 µM. Subsequently, the samples were placed in a Thermomixer (Thermo Scientific, Wilmington, DE, USA) in the dark at room temperature for times ranging from 10 to 40 min with a shaking speed of 600 rpm. The samples were then placed horizontally on ice under light for times from 5 to 20 min at a distance of 20 cm using a 500 W halogen lamp

(Canadian Tire, Guelph, ON, Canada). When the light exposure time was complete, the samples were centrifuged at 10,000 ×g for 10 min. Pellets were collected and IC was added. The samples were then tested by the qPCR procedures as described in previous sections to determine the optimal PMA treatment condition.

3.4. Results and discussion

3.4.1 qPCR assay development and specificity

TaqMan-based qPCR methods for specific detection and quantification of *Virgibacillus* sp. and *T. halophilus* have been developed in this research. The *aprX* gene encodes alkaline serine proteinase-X (AprX), a novel member of the family of the subtilase superfamily, which was used to design the primers (Vir_F1029/Vir_R1140) and the probe (Vir1086) to detect *Virgibacillus* sp. SK37. The sequence of the *aprX* gene was deposited in GenBank under accession number HM587897. The primers and the probe showed 100% homology to only the *aprX* gene of *Virgibacillus* sp. SK37 and the complete genome of *Virgibacillus* sp. SK37 (accession no. CP007161) in the Genbank®. The ITS sequence of *T. halophilus* MS33 (accession no. KP638354) was used to design the primers (Tet_F20/Tet_R107) and the probe (Tet48) for the detection of *T. halophilus*. The primers and the probe showed 100% homology to only the ITS sequence of *T. halophilus* NBRC12172. None of the other species of *Tetragenococcus* and *Virgibacillus,* or any other genus of bacteria, showed homology with the primers and probes when tested using the BLAST tool against the public database.

The rationale behind the selection of the ITS region and the protein-coding gene for the design of specific primers and probes for detection of the

bacterial starter cultures in this study is that these target sequences exhibit higher genetic variation than the more conserved 16S rRNA gene and therefore can be utilized for differentiating species of closely related taxa (Fox et al., 1992). The ITS region is a highly variable region among species in both base length and sequence (Boyer et al., 2001; Osorio et al., 2005). Both variations have been extensively used to distinguish closely related species and even bacterial strains (Scheinert et al. 1996; Maes et al., 1997), e.g., *Bacillus* species (Saikaly et al., 2007; Xu and Côté, 2003) and *Lactobacillus sakei* in meat and fermented sausages (Martín et al., 2006). The ITS of *Nocardia crassostreae* was successfully applied for detection at the species level in oysters (Carrasco et al., 2013).

The protein-coding gene (*aprX*) of *Virgibacillus* sp. SK37 showed potential for specific detection at the species level of *Virgibacillus*. Protein-coding genes or specific genes have been used for the detection of various pathogens at a species level in foods. Gyrase subunit B gene (*gyrB*) was used to design the specific primers for *Photobacterium phosphoreum* detection in salmon steaks (Macé et al., 2013). *Vibrio cholerae* has been detected by outer membrane protein (*ompW*), hemolysin (*hlyA*) and the regulatory protein (*toxP*) genes. Garrido-Maestu et al. (2015) reported that the outer membrane lipoprotein (*lolB*) gene has been used as a new detection target for *V. cholerae* in foods.

The internal control (IC) sequences for the detection of each target (*Virgibacillus* sp. SK37 and *T. halophilus* MS33) were artificially constructed in this study (Table 3.1). The optimal primer, target probe, and IC probe concentrations were 500, 300 and 300 nM, respectively. The annealing temperature was 60 °C. The template concentrations of IC for *Virgibacillus* sp. SK37 and *T. halophilus* MS33

were optimized to be 10^4 CFU/PCR, which were equivalent to C_q values of 25-26 and 24-25, respectively. In this study, the internal controls were designed using the same primer set of the target, which provides same or similar amplification efficiency between the target and the IC in addition to simplifying the assay reagent components.

The specificity of the qPCR assays was tested using the genomic DNA of 28 isolates from fish sauce plants and 6 reference strains; the results are shown in Table 3.2. The qPCR results showed that the qPCR assay targeting *Virgibacillus* sp. SK37 also detected other strains of *V. halodenitrificans* isolated from the commercial fish sauce during fermentation (Table 3.2). Similarly, the qPCR assay targeting *T. halophilus* MS33 also detected other strains of *T. halophilus*. There was no cross reaction of either the Vir PCR or the Tet PCR to other tested species. The results indicate that the PCR assays were specific at a species level, but not at a strain level. The *Virgibacillus* assay was specific for *Virgibacillus* sp., as demonstrated by the detection of strains of SK37 and SK33 as well as strains of *V. halodenitrificans* SK1-3-7 and ATCC12304. The PCR method for *T. halophilus* detection also showed specificity towards all of the tested strains of *T. halophilus* (e.g. MCD10-5-10 and MS33). *V. halodenitrificans* and *T. halophilus* may grow as microflora in fish sauce, but were likely to be lower than the quantification limit of both PCR assays. The results suggested that these methods can be used to monitor *Virgibacillus* sp. SK37 and *T. halophilus* MS33 added into commercial fish sauce without being interfered with by naturally existing microflora. The PCR efficiencies were estimated using DNA from pure cultures of the target organisms at concentrations of 10-10^6 Cells/mL, which were equivalent to 0.5-4.5 LogCells/PCR.

Table 3.2 Specificity of the qPCR assays toward bacteria isolated from fish sauce fermentation and type strains.

Source*	Bacterial name	qPCR result	
		Vir PCR	Tet PCR
CFT-1	*Salinicoccus hispanicus* M1P10	-	-
CFT-1	*Salinicoccus roseus* M1J10	-	-
CFT-1	*Tetragenococcus halophilus* M1M5	-	+
CFT-1	*Virgibacillus* sp. SK33	+	-
CFT-1	*Staphylococcus* sp. M5-1-3	-	-
CFT-1	*Staphylococcus* sp.SK24-1	-	-
CFT-1	*Tetragenococcus halophilus* MS33	-	+
CFT-1	*Tetragenococcus halophilus* MRC10-1-3	-	+
CFT-1	*Virgibacillus sp.* SK37	+	-
CFT-2	*Bacillus* sp. M2J10	-	-
CFT-3	*Tetragenococcus halophilus* M3M5	-	+
CFT-3	*Virgibacillus halodenitrificans* SK1-3-7	+	-
CFT-3	*Virgibacillus halodenitrificans* M3P10	+	-
CFT-4	*Salinicoccus roseus* M4P10	-	-
CFT-4	*Virgibacillus halodenitrificans* M4J10	+	-
CFT-4	*Bacillus* sp. M4J10	-	-
CFT-4	*Bacillus vietnamensis* M4J10	-	-
CFT-5	*Salinicoccus hispanicus* M5G-P10	-	-
CFT-5	*Staphylococcus epidermidis* M5B-P10	-	-
CFT-5	*Salinicoccus hispanicus* M5G-J10	-	-
CFT-5	*Staphylococcus* sp. M5J10	-	-
CFT-5	*Staphylococcus* sp. M5B-M5	-	-
CFT-6	*Staphylococcus* sp. M6B-P10	-	-
CFT-6	*Virgibacillus halodenitrificans* M6J10	+	-
CFT-7	*Salinicoccus hispanicus* M7GJ10	-	-
CFT-7	*Staphylococcus* sp. M7B-M5	-	-
CFT-8	*Salinicoccus roseus* M8P10	-	-
CFT-8	*Bacillus* sp. M8J10	-	-
ATCC	*Tetragenococcus halophilus* ATCC33315	-	+
ATCC	*Virgibacillus halodenitrificans* ATCC12304	-	-
JCM	*Staphylococcus condimenti* JCM6074	-	-
JCM	*Tetragenococcus muriaticus* JCM10006	-	-
JCM	*Staphylococcus piscifermentans* JCM6057	-	-
TISTR	*Staphylococcus carnosus* TISTR833	-	-

*CFT, commercial fish sauce tank, numbers indicate fermentation time in month, ATCC, American Type Strain Culture Collection; JCM, Japan Collection of Microorganisms; TISTR, Thailand Institute of Scientific and Technological Research. Negative (-), No C_q; positive (+), C_q was 25-26.

3.4.2 qPCR efficiency

The linear regression between C_q and the bacterial concentration (Log Cells/PCR) (Figure 3.1) showed qPCR efficiencies of 100.4% and 91.7% for *Virgibacillus* sp. SK37 and *T. halophilus* MS33, respectively. The efficiencies were also tested in the presence of background DNA that was prepared from uninoculated fish sauce samples, which were 101.1% and 90.2% for Vir PCR and Tet PCR, respectively. The determination coefficients (R^2) were 0.99 in all cases, indicating a high linearity between target bacterial count and the C_q values. The PCR efficiency of the developed qPCR methods was in the range of 90 to 110%. This result is comparable to studies of pathogenic bacteria. Garrido-Maestu et al. (2015) reported that the efficiency of qPCR to detect the *lolB* gene of *V. cholera* was 95.2%.

The acceptable PCR efficiency range was reported to be 85 to 110% (Postollec et al., 2011). Background DNA was DNA from other substances in the fish sauce, e.g., DNA from fish and non-target microflora. Background DNA can reduce the PCR efficiency by capturing nucleic acids and/or by inactivating DNA polymerase (Fortine et al., 2004). However, the efficiency of the developed qPCR assays, even with the presence of the background DNA, was still in the same acceptable range (Figure 3.1). The results indicated that the efficiency of the developed qPCR methods was not affected by background DNA.

3.4.3 Quantification limit/range and matrix effect

The quantification limit/range and matrix effect of the optimized qPCR methods were determined using fish sauce samples spiked with known cell concentrations (10-10^6 Cells/mL) of the target organisms. Linear correlations (R^2 values = 0.9899 and 0.9764) were obtained between the qPCR results and bacterial

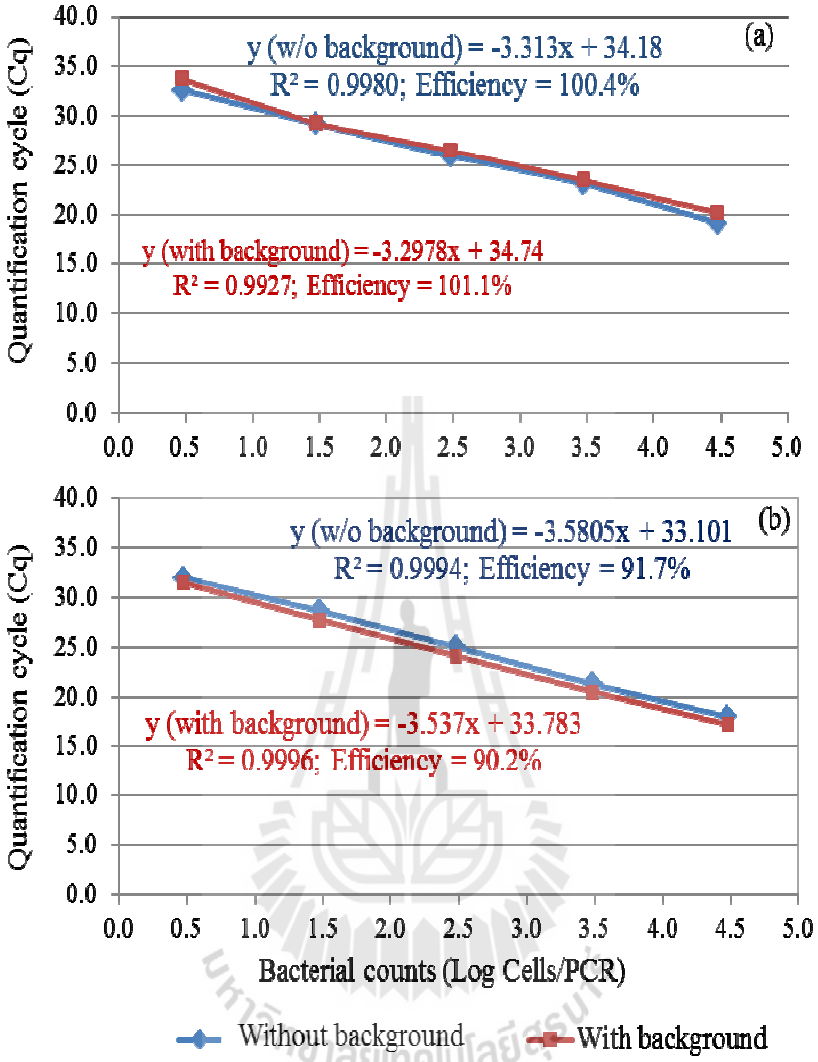

Figure 3.1 Linearity of qPCR efficiency of Vir1086 for *Virgibacillus* sp. SK37 (a) and Tet48 probe for *T. halophilus* MS33 (b) detection. The linear correlation between $\log_{10}$ of cell suspension and C_q with slope and R^2 (square regression coefficient after the linear regression). Efficiency of qPCR was calculated using the equation:

$$\text{Efficiency} = (-1 + 10^{(-1/\text{slope})}) \times 100$$

counts of *Virgibacillus* sp. SK37 and *T. halophilus* MS33, which were added to the samples (Figure 3.2). The low quantification limits were 3 LogCells/mL for

Virgibacillus sp. SK37 with C_q values of 29-30 and 2 LogCells/mL for *T. halophilus* MS33 with C_q values of 31-34 (Figure 3.2). At 7 LogCells/mL, the qPCR assays also showed positive results with both targets (data not shown). These results indicate that the qPCR assay for *T. halophilus* detection was more sensitive than the PCR for *V. halodenitrificans*.

The quantification limit of the Vir PCR and Tet PCR for the detection of targets in fish sauce mash samples was 10^3 Cells/mL and 10^2 Cells/mL, respectively, which are similar to those in published PCR assays. The limit of detection of viable *Salmonella* was as low as 2 LogCFU/mL in pure cultures and 3 LogCFU/g in lettuce (Liang et al., 2011). The detection limit of *L. sakei* in a sausage model was 3 LogCFU/g (Martín et al., 2006). Ueda et al. (2013) reported that the detection limit of *B. cereus* in food samples (e.g., lettuce, vegetable salad, liquid whole egg, and cow milk) was 4-5 Log CFU/mL. The linear correlations (R^2 values) of the Vir PCR and the Tet PCR were also similar to others that have been previously reported. For example, the R^2 for the quantification of *Brochothrix thermosphacta* in seafood was 0.911 (Mamlouk et al., 2012). A higher sensitivity was observed in the detection of *T. halophilus* MS33 compared to the *aprX* qPCR assay. The reason for this is likely because the *aprX* gene of *Virgibacillus* sp. SK37 contained only 1 copy per genome (Phrommao, 2010), while the ITS regions contained at least 4 copies per genome. There are 4 and 5 copies per genome of the 16S rRNA and 23S rRNA genes, respectively, in *T. halophilus* NBRC12172 (NCBI database, http://www.ncbi.nlm.nih.gov). This implies that the ITS region of *T. halophilus* was supposed to have at least 4 copies per genome.

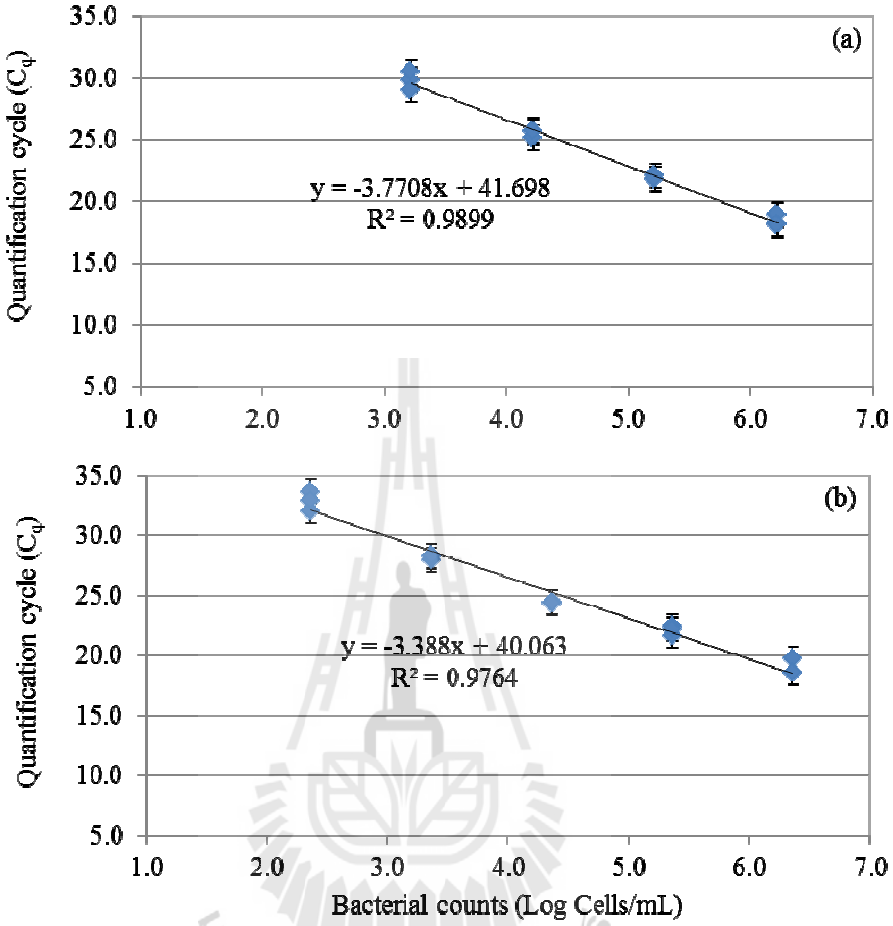

Figure 3.2 Quantification limit of qPCR assays for *Virgibacillus* sp. SK37 (a) and *T. halophilus* MS33 (b).

To determine the matrix interference on the qPCR assays, fish sauce samples fermented at 1, 3, and 6 months were spiked with the target organisms at concentrations between 10-10^6 Cells/mL and tested as fish sauce samples from the different stages of fermentation presenting with different physical, chemical, and microbiological characteristics. DNA extracts from the spiked fish sauce fermented for 1 and 3 months had very high DNA concentrations and were diluted 10 times with AE buffer prior to the qPCR assays, while those extracted from 6-month-old

samples were used directly in PCR. No amplification signal was observed in the uninoculated samples (negative controls). Good linearity was observed in all the spiked fish sauce samples from different stages of fermentation (Figure 3.3). C_q values were comparable at a given target concentration among the fish sauce matrices from 1, 3 and 6 months of fermentation. The results indicated that matrix interference effects on DNA amplification were negligible when using the DNA extraction and qPCR procedures developed in this research.

Fish sauce is a complex system, and the matrix interferences of the qPCR assays are likely to vary with fermentation time. Samples from the initial period of fermentation (the first 3 months) contain undigested fish tissue. Most fish protein, with the exception of connective tissue and other stroma proteins, was not completely hydrolyzed to small peptides and amino acids compared to the later stages of fermentation (Lopetcharat et al., 2001). Therefore, samples of different periods of fermentation would present with varied organic components and different sizes of fish tissue. A high DNA yield (>400 ng/μL) was obtained from the initial period due to a high amount of fish tissue particles. To overcome this problem, fish sauce mash was washed twice with 1× PBS prior to DNA extraction and DNA was then diluted 10-fold before qPCR determination to decrease the effect of background DNA. After dilution, the linear quantification of qPCR was still maintained, confirming that there was no negative effect on the PCR performance due to dilution. Luna et al. (2012) reported that a 100-fold dilution of DNA was free from inhibitors, indicating that the washing steps before DNA extraction in combination with dilution was a simple strategy to remove interference from marine sediment samples. Inhibitory substances, such as fats, proteins and a high concentration of Ca^{2+}, can

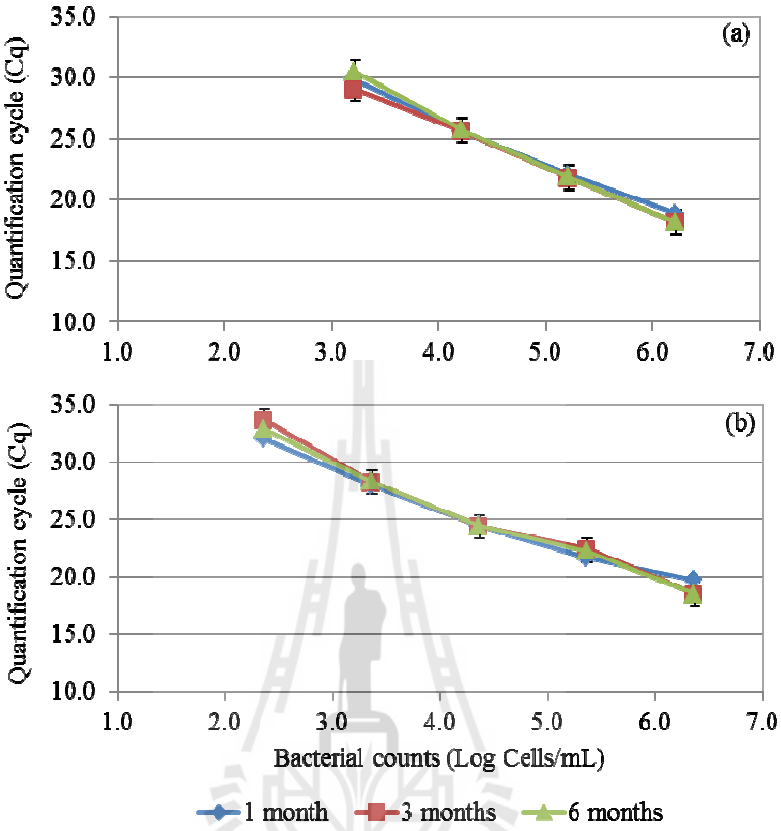

Figure 3.3 Fish sauce fermented for 1, 3, and 6 months as representative matrices for qPCR assay of *Virgibacillus* sp. SK37 (a) and *T. halophilus* MS33 (b).

reduce the efficiency of extraction and PCR (Cresmonesi et al., 2006). The procedure for DNA extraction from fish sauce samples was an important step for starter culture detection using qPCR.

3.4.4 Repeatability and reproducibility

The repeatability and reproducibility of the developed qPCR assays were determined using intra-assay and inter-assay results, respectively. The repeatability was calculated at each of the spiking levels among the C_q values of duplicate fish sauce samples and duplicate PCRs for each of the fish sauce samples. The relative

standard deviations (% RSD) ranged from 15 to 37% for the *Virgibacillus* PCR assay and from 17 to 32% for the *Tetragenococcus* PCR assay (Table 3.3). The reproducibility was calculated at each of the spiking levels among the data from 3 separate experiments conducted on different days. The relative standard deviations were within 33% for both of the qPCR assays (Table 3.3).

To ensure the reliability and sensitivity of the qPCR technique, inhibitory substances from foods or carry-over of reagents used for the isolation of nucleic acids must be removed; internal controls (IC) are required for inhibition monitoring during qPCR (Diez-Valcarce et al., 2011). Since the internal controls were in the form of recombinant cells, they were co-extracted and co-amplified, and were used effectively not only for monitoring inhibition or failure of amplification, and also for monitoring DNA extraction and normalizing the qPCR results.

Table 3.3 Repeatability and reproducibility of qPCR assay in spiked fish sauce.

Bacteria	**Spiking level (CFU/mL)**	**Repeatability (within experiment)**		**Reproducibility (between experiment)**	
		C_q ± SD	**%RSD**	**CFU ± SD**	**%RSD**
Virgibacillus sp. SK37	1,650,000	18.8 ± 0.08	15.32	1,504,167 ± 103119.75	8.90
	165,000	22.2 ± 0.44	36.76	156,987± 5665.89	15.40
	16,500	25.7 ± 0.14	21.69	15,990 ± 360.91	33.33
	1,650	29.8 ± 0.03	23.69	1,140 ±360.38	28.20
	165	ND	ND	ND	ND
T. halophilus MS33	2,310,000	18.19 ± 0.12	29.30	1,598,960 ± 502781.15	33.33
	231,000	22.48 ± 0.28	17.17	147,363 ± 59140.63	27.45
	23,100	25.42 ± 0.21	12.25	19,488 ± 2553.77	10.01
	2,310	28.85 ± 0.74	32.42	2,116 ± 137.38	27.36
	231	32.87 ± 0.21	31.06	155 ± 54.12	33.33
	NTC	ND	ND	ND	ND

ND, not detected. NTC, negative template control.
C_q, quantification threshold; SD, standard deviation; RSD, relative standard deviation.

3.4.5 Effect of PMA treatment

PMA was used in the sample preparation to prevent amplification of DNA from dead target cells and to ensure that the PCR assays measured live cell concentrations. The effectiveness of the PMA treatment was tested using fish sauce samples spiked with live and dead target cells. The dead cells were prepared by incubating the target cells (10^6 CFU/mL) in anchovy fish sauce at 35 °C for 7 days and confirmed by plate counting. The optimal conditions of PMA treatment for both *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were found to be 100 µM PMA, 20-min incubation in the dark with shaking at 600 rpm and 5-min light exposure time prior to DNA extraction. Figure 3.4 shows that without PMA treatment, the qPCR assays resulted in readings of 6 Log Cells/mL of the target cells, mainly from dead cells, regardless of the numbers of live cells present, indicating that the qPCR assays detected both live and dead cells. When the samples were treated with PMA, only live cells were detected by the PCR assays at concentrations proportional to live cells, which were added to the fish sauce samples (Figures 3.4a and 3.4b). These results demonstrated that the qPCR assays coupled with PMA treatment could be used to selectively quantify viable cells of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in fish sauce samples. The main disadvantage of DNA quantification methods is the inability to distinguish between viable and non-viable cells, as dead cells may also retain a significant amount of DNA. To overcome this limitation, PMA as a cell membrane-impermeable DNA intercalating agent was applied to avoid amplification of DNA from dead cells, as reported previously (Mamlouk et al., 2012). Quantitative analysis of starter cultures in Spanish mackerel fish sauce by PMA-qPCR showed different results from those by the plate count technique. The bacterial count of the

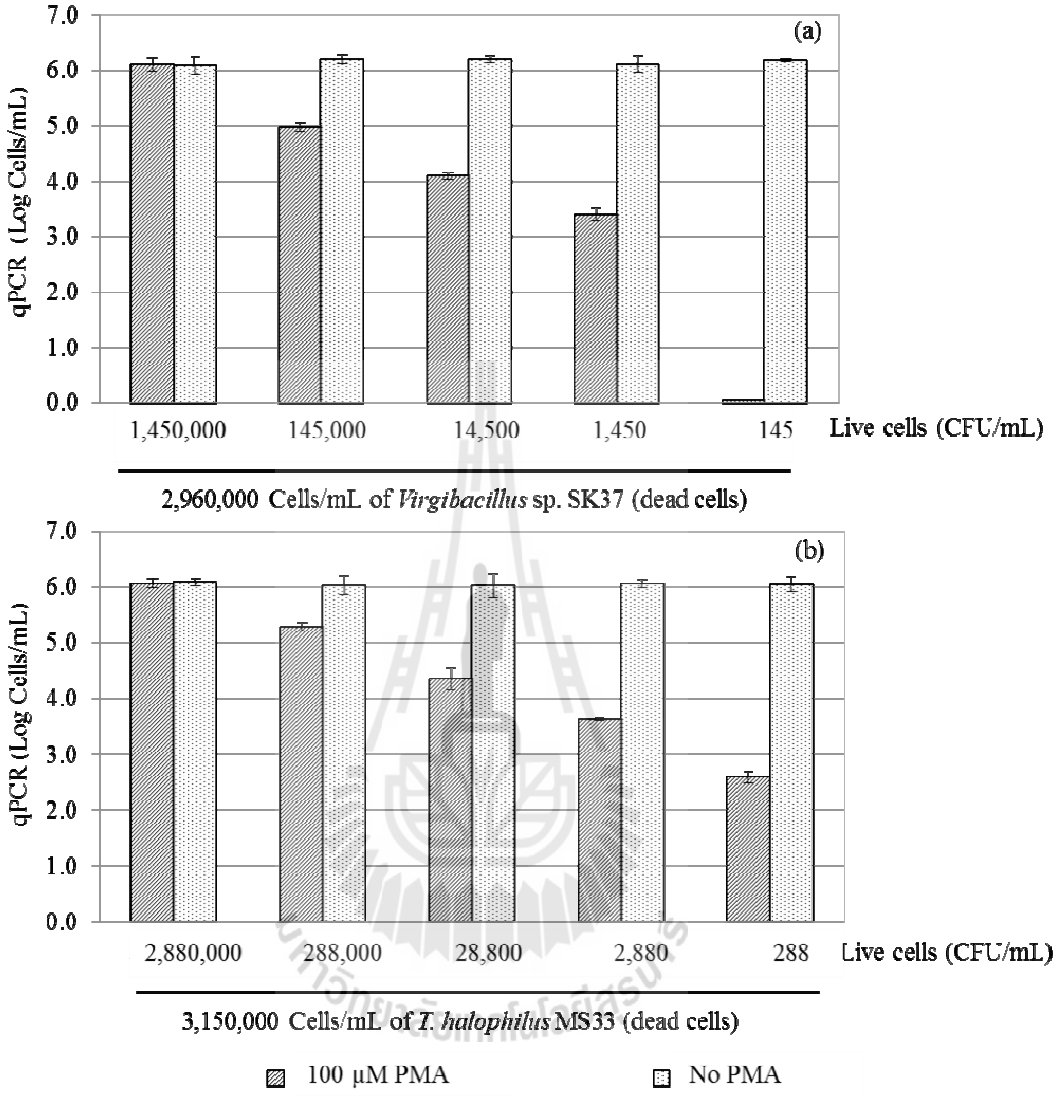

Figure 3.4 qPCR results of fish sauce containing 10^6 Cells/mL of dead cells and various live cells (10^2-10^6 CFU/mL) of *Virgibacillus* sp. SK37 (a) and *T. halophilus* MS33 (b) at the optimal PMA treatment.

control sample was not detected on agar plates during the first 7-30 days and detected after 30 days, indicating that the bacterial microflora in the initial 30 days might be VBNC.

3.4.6 Application of the qPCR assays for quantification of starter cultures during fish sauce fermentation

The qPCR assays were used to quantify the changes in the starter cultures during laboratory scale fermentation of Spanish mackerel fish sauce. The starter cultures of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 were added into the fish paste at 10^7 CFU/mL as a single culture separately and also as co-cultures. The PCR results were compared to conventional plate counts. As illustrated in Figure 3.5a, no amplification signal was observed in the uninoculated samples during 90 days of fermentation, while bacterial plate counts were 2-3 LogCFU/mL during the course of fermentation. Fish sauce inoculated with *T. halophilus* MS33 showed counts of 5-7 Log Cells/mL based on the Tet PCR during 90 days of fermentation, corresponding to enumeration obtained from MRS5 and JCM10 (Figure 3.5b). Although JCM agar showed bacterial counts of 4-7 Log CFU/mL throughout the fermentation, no amplification of Vir PCR was detected in this sample. These results indicated that *Virgibacillus* sp. microflora were not present in the sample and/or that the presence of *Virgibacillus* sp. in the sample was lower than the limit of detection (LOD) of the method.

No signal was found from the Tet PCR in fish sauce inoculated with *Virgibacillus* sp. SK37 (Figure 3.5c). The bacterial count on JCM10 agar was 7 LogCFU/mL at day 0 and decreased to 3 LogCFU/mL by 7 days; no colonies were found during the 14-60 days of fermentation. A small number of counts were recorded at 75 and 90 days on JCM10 (Figure 3.5c). When the Vir PCR was applied, the sample showed 7 LogCells/mL at day 0, gradually decreased to 5 LogCells/mL by day 7 and remained at 3-4 LogCells/mL during 90 days of fermentation. The

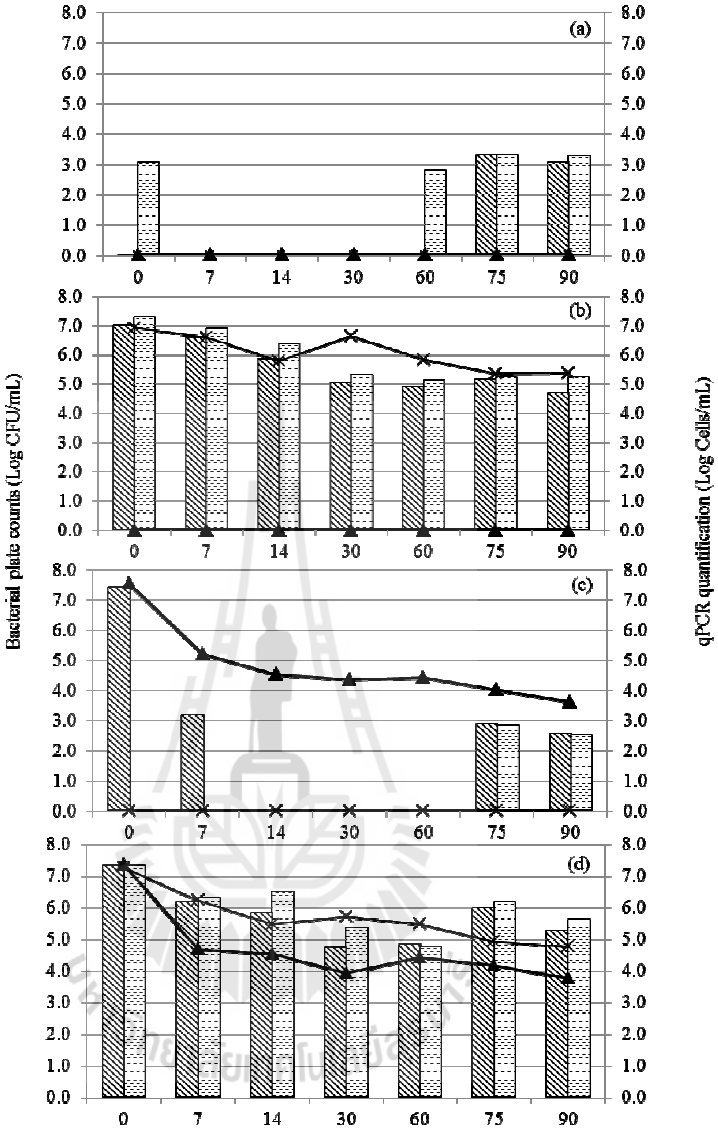

Figure 3.5 Detection of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in fish sauce inoculated with single or co-culture starters and incubated at 35 ºC for 3 months by qPCR with Vir1086 and Tet48 probe (line) and enumerated by spread plate technique (bar). (a), the control (C); (b), fish sauce inoculated with *T. halophilus* MS33 (MS33); (c), fish sauce inoculated with *Virgibacillus* sp. SK37 (SK37); (d), fish sauce inoculated with *Virgibacillus* sp. SK37 and *T. halophilus* MS33 (SK37+MS33).

bacterial counts detected by qPCR were higher than those obtained from the plate count technique (Figure 3.5c), indicating that some cells of *Virgibacillus* sp. SK37 may undergo a viable but nonculturable state (VBNC). Moreover, the Tet PCR showed negative amplification results throughout the fermentation period. However, the bacterial counts on MRS5 agar were 2 LogCFU/mL. The results imply that the *T. halophilus* microflora of this sample were not present or were lower than LOD, 2 LogCFU/mL; or the colonies presented on the MRS5 agar were not *T. halophilus*.

For co-cultured sample, bacterial detection by both qPCR assays was 7 LogCells/mL at day 0 (Figure 3.5d). The bacterial counts based on the Vir PCR were slightly lower than that obtained from the plate counts. The Vir PCR detection revealed counts of 7 LogCells/mL at day 0 that decreased by 2-3 LogCells/mL thereafter. The Tet PCR also indicated 7 LogCells/mL at day 0 and slightly decreased by 1-2 LogCells/mL at 60-90 days (Figure 3.5d). In this sample, the qPCR and the plate count showed comparable results. In addition, no cross reaction was detected with these two assays. Therefore, the developed qPCR methods were more specific and enable detection of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in the co-culture inoculation system.

Based on the qPCR assays, both *Virgibacilus* sp. SK37 and *T. halophilus* MS33 gradually decreased during 3 months of fermentation. *T. halophilus* MS33 appeared to have higher retention than *Virgibacilus* sp. SK37 in co-culture fermentation. This could be due to small peptides and oligopeptides being digested by the proteinases of *Virgibacilus* sp. SK37 and then serving as nutrients for *T. halophilus* MS33. However, the survival rate of starters in single and co-culture fermentation was comparable when detected by qPCR. Traditional culture-dependent

techniques failed to detect *Virgibacilus* sp. SK37 in a VBNC state and were also unable to differentiate these two starter cultures from other microflora even with the use of selective media. The molecular methods offer more specific detection of viable bacterial species of interest, particularly in a complex system, such as that found in fish sauce fermentation, which contains not only starter cultures but also various types of background microflora. Traditional methods (plate count) cannot provide accurate information regarding changes in viable starter cultures during fish sauce fermentation, even though this information is critically vital for process control and development. This is the first report to develop qPCR methods for the monitoring of the starter cultures in fish sauce.

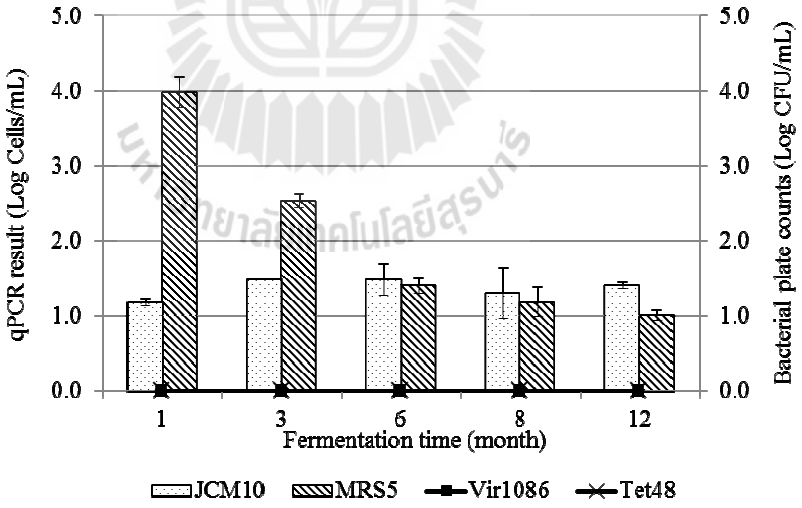

Figure 3.6 *Virgibacillus* sp. SK37 and *T. halophilus* MS33 counts based on qPCR (line) and plate count technique (bar) using JCM10 and MRS5 agar of commercial fish sauce samples fermented for 1-12 months.

Because the qPCR assays showed species specificity, but not strain specificity, it was necessary to know whether they detect the naturally existing

microflora of fish sauce fermentation like *V. halodenitrificans.* DNA extracted from all of the commercial fish sauce samples showed negative results with either the Vir PCR or Tet PCR (Figure 3.6). However, the bacterial plate count on MRS5 ranged from 1 to 4 LogCFU/mL, while the halophilic bacterial count on JCM10 was approximately 1.5 LogCFU/mL throughout the course of fermentation. The colonies on MRS5 and JCM10 reflected cultured halophilic bacteria, including *T. muriaticus, Bacillus,* and *Salinicoccus*. *T. halophilus* and *Virgibacillus* sp., if naturally present in commercial fish sauce, would likely be lower than LOD of both the PCR methods. Thus, the developed qPCR assays can be applied to monitor changes of the starter cultures *Virgibacillus* sp. SK37 and *T. halophilus* MS33 during fish sauce fermentation at an industrial scale without being interfered with by *V. halodenitrificans* and *T. halophilus* naturally present in the fermentation system.

The quantification of *Virgibacillus* sp. and *T. halophilus* microflora in commercial fish sauce fermented for 12 months did not exhibit any signal during fermentation, implying that there was not *T. halophilus* or *Virgibacillus* sp. in the control and/or their amount was lower than the LOD of the methods. In samples inoculated with *Virgibacillus* sp. SK37, a bacterial count by the spread plate technique was not found, but the PMA-qPCR assay revealed the presence of *Virgibacillus* sp. SK37. This indicated the VBNC state of *Virgibacillus* sp. SK37 which likely underwent a dormant state in response to a high salt environment. VBNC cells have an intact membrane, a low metabolic rate, and still respire. In general, VBNC cells have higher chemical and physical resistance than culturable cells. This might be due to their metabolic rate being reduced and their cell wall being strengthened by increased peptidoglycan cross-linking (Signoretto et al.,

2000). The VBNC cells that are still alive can be resuscitated when the environmental conditions improve (Pinto et al., 2011). Detection using the spread plate technique would underestimate the population of cultures in the VBNC state. *T. halophilus* MS33 did not show a VBNC state as the PMA-qPCR assay provided comparable results to the plate count technique. The VBNC state of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 might be different. The conditions that trigger the induction of a VBNC in these bacteria need to be further investigated.

3.5 Conclusions

The developed qPCR methods are specific, sensitive, and fast techniques that can be used to quantitatively determine viable *Virgibacillus* sp. SK37 and *T. halophilus* MS33 inoculated during fish sauce fermentation. Limit of quantification of Vir1086 and Tet48 probe was 3 and 2 LogCFU/mL for *Virgibacillus* sp. SK37 and *T. halophilus* MS33, respectively.

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CHAPTER IV

ENUMERATION OF STARTER CULTURES *TETRAGENOCOCCUS HALOPHILUS* MS33 AND *VIRGIBACILLUS* SP. SK37 DURING FISH SAUCE FERMENTATION BY QPCR AND THEIR ROLES ON VOLATILE COMPOUNDS AND AMINO ACID CHARACTERISTICS

4.1 Abstract

The potential of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 as combined cultures and single culture on chemical characteristics of fish sauce was investigated. Fish sauce added starter cultures in sequential order by inoculating *Virgibacillus* sp. SK37 followed by addition of *T. halophilus* MS33 after 1 month (SK37_1M+MS33) showed higher survival rate of bacterial growth than single starter culture treatments, approximately 0.5-1 Log CFU/mL by qPCR. α-Amino contents of fish sauce added co-cultures were the highest of 1,160-1,185 mM. Histamine of SK37_1M+MS33 showed lower amount than that of the control ($P < 0.05$). Fish sauce inoculated with either sequential cultures or single culture showed higher content of total glutamic acid and their sum of total amino acids was higher than the control ($P < 0.05$). The amount of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal of

SK37_1M+MS33 was higher than that of the control ($P < 0.05$). These compounds were an odor active compound in fish sauce. Moreover, fish sauce inoculated with starter cultures did not increase surfur-containing compounds that contributed to undesirable note. Addition of starter culture, particularly in sequential order, showed potential to improve volatile compound and chemical compositions of fish sauce.

Key words: Fish sauce fermentation, *Virgibacillus* sp. SK37, *T. halophilus* MS33, qPCR, α-amino acids, biogenic amines, glutamic acids, volatile compounds

4.2 Introduction

Fish sauce is one of important Asian fermented food which requires long fermentation time of 12-18 months to attain optimal aroma and taste characteristics. Addition of starter cultures has been proposed as a means to reduce fermentation time and improve product quality. Hariono, Yeap, Kok, and Ang (2005) reported that addition of koji and commercial protease derived from *Aspergillus oryzae* to fish sauce fermentation increased glutamic acid and umami of the product. *Staphylococcus xylosus* added to mackerel fish sauce reduced fecal note resulted from dimethyl disulfide and dimethyl trisulfide (Fukami, Funatsu, Kawasaki, and Watabe, 2004). *Vigibacillus* sp. SK37, a moderately halophilic bacterium, has been reported to reduce fermentation time from 12 months to 4 months (Yongsawatdigul et al., 2007). Halophilic lactic acid bacteria (LAB) have also been reported to be dominant microorganisms during flavor and color development at the final stage of fish sauce fermentation (Saisithi, 1994). *T. halophilus* MS33 isolated from Thai fish sauce produced various desirable aldehydes, including 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde, (Udomsil, Rodtong,

Tanasupawat, and Yongsawatdigul, 2010). The use of *T. halophilus* as a starter culture could improve flavor quality of fish sauce.

The use of combined starter cultures in fish sauce fermentation would provide more benefits than a single culture. It could improve various quality aspects simultaneously. Uchida et al. (2005) reported that combination of koji and *T. halophilus* increased organic acids and amino acids of freshwater silver carp fish sauce as compared to the use of *T. halophilus* alone. Zhao et al. (2011) reported that combination of *Lactobacillus pentosus*, *Pediococcus pentosaceus*, and *S. carnosus* as starter cultures in dry fermented sausage production increased unsaturated free fatty acids and extended shelf-life of the product. As *Virgibacillus* sp. SK37 exhibits high proteolytic activity, and *T. halophilus* MS33 produces desirable volatile compounds, the combination of these 2 cultures in the fermentation would shorten fermentation time and improve aroma quality of fish sauce. The practice of combined cultures could be carried out in 2 different means, namely co- or sequential inoculation. In co-inoculation, cultures are inoculated simultaneously to the fermentation system. Letort, Nardi, Garault, Monnet, and Juillard (2002) reported that co-cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* stimulated growth and acid production of the latter to a greater extent than a single-strain culture. For sequential inoculation, starter culture was inoculated for a month and followed by one another added. Wen et al. (2014) reported the sequential inoculation of *Clostridium thermocellum* for 5 days and followed by *C. beijerinckii*. The former provided sugar accumulation as a carbon source for the latter to for acetone-butanol-ethanol (ABE) production. The use of combined cultures either co- or sequential inoculation has never been explored in fish sauce fermentation.

Detection of starter cultures in fish sauce fermentation is a challenge. The selective media cannot provide accurate results due to the inability to discriminate starter cultures and microflora based on colony morphology. Moreover, bacteria under stress like high salt content would undergo viable but nonculturable (VBNC) state, which cannot be detected by plate count technique. Therefore, molecular method, quantitative real-time PCR (qPCR) has been proposed as a better approach to for detection and enumeration of starter cultures based on specific probes. The use of qPCR combined with propidium monoazide (PMA-qPCR) has been developed for detection and quantification of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 based on alkaline serine protease X gene (*aprX*) and Internal Transcribed Spacer (ITS), respectively (Udomsil et al. 2015). The application of the developed qPCR technique to monitor changes of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 during fermentation would accuracy and The objectives of this study were to investigate the use of combined cultures of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in fish sauce fermentation with regarding to changes of starter cultures based on the PMA-qPCR technique and key quality indicators of fish sauce.

4.3 Materials and methods

4.3.1 Preparation of starter cultures

Virgibacillus sp. SK37 and *T. halophilus* MS33 were isolated from fish sauce fermentation as described by Udomsil et al. (2010) and Yongsawatdigul et al. (2007) with accession number DQ910840 and FJ715465 (www.ncbi.nlm.nih.gov/genbank), respectively, and have been stored at the Microbial Cultures Research Center for Food and Bioplastics Production in

Suranaree University of Technology. *Virgibacillus* sp. SK37 was cultured in 25 mL of Tryptic Soy Broth (TSB) containing 5% NaCl and incubated at 35 ºC under aerobic condition for 3 days. *T. halophilus* MS33 was cultured in 25 mL of De Man, Rogosa and Sharp (MRS) broth containing 5% NaCl, added 0.5% $CaCO_3$, and incubated at 35 ºC under anaerobic condition for 3 days. After incubation time was attained, cell suspensions of individual cultures (10%) were transferred into 100 mL of fish broth containing 10% NaCl prepared according to Yongsawatdigul et al. (2007) and incubated at each respective culture condition to obtain the initial cells approximately 10^6-10^7 CFU/mL.

4.3.2 Fish sauce fermentation

One kg of Indian anchovy (*Stolephorus indicus*) was mixed with 25% solar salt and 10% of either *Virgibacillus* sp. SK37 or *T. halophilus* MS33, which was referred to as "SK37" or "MS33", respectively. These two samples were considered as single culture fermentation. For the combined cultures fermentation, 5% of each starter culture, *Virgibacillus* sp. SK37 and *T. halophilus* MS33, was added to anchovy containing 25% salt. This was co-culture inoculation and referred to as "MS33+SK37". For the sequential inoculation, 5% of *Virgibacillus* sp. SK37 was inoculated to salted anchovy, and fermentation was allowed to proceed for 1 month before addition of 5% of *T. halophilus* MS33 inoculum, and was denoted as "SK37_1M+MS33". All samples contained comparable counts of inoculum of 10^7-10^8 CFU/mL and incubated at 35 ºC for 180 days. The control was added with 10% of fish broth without starter cultures. Chemical analyses and bacterial enumeration by qPCR and plate count techniques were performed at 0, 30, 60, 90, 120, 150 and 180 days.

4.3.3 Monitoring of starter cultures during fermentation

4.3.3.1 qPCR

At each time interval, fish sauce (100 µL) was transferred to a 1.5-mL microcentrifuge tube. Samples were washed twice with 1×PBS and pellets were collected. PMA was added to a final concentration of 100 µM. The samples were incubated in dark for 20 min with shaking at 600 rpm and were then put on ice under light (500 W) for 5 min with 20 cm distance. Subsequently, the samples were centrifuged at 10,000×g for 10 min and pellets were collected. Internal control (IC) of each probe was constructed by cloning artificial fragments as described by Udomsil et al. (2015) and added to all samples at 1.66 x 10^4 CFU/sample prior to cell lysis. The DNA was extracted using the DNeasy tissue kit (QIAGEN, Toronto, Ontario, Canada) according to the manufacturer's procedure. Extraction of DNA from each subsampling was carried out in duplicate.

The forward and reverse primers of *Virgibacillus* sp. SK37 were 5'-GGATCGGCGCTAGAAAAACA-3' and GCTGAGGATTTGCCTCAAGC, while the forward and reverse primers of *T. halophilus* MS33 were 5'-GGTCAAGGGTTTCTCGAAGGT-3' and 5'-AATCAACACCAACCGAGAATCC-3', respectively. The developed probe of *Virgibacillus* sp. SK37, Vir1086, was FAM-ACTATCTGGCACTTCGAT-BHQ1, while that of for *T. halophilus* MS33, Tet48, was FAM-TTGAGCGATCAAAGCCCTTCGAACA-BHQ1. The probe were designed using Primer Express® 3.0 software (Life Technologies, Foster City, CA, USA) as described in details by Udomsil et al. (2015). The qPCR reaction of each sample and each probe was performed in duplicate. Negative and positive control of qPCR were carried out using master mix without DNA template and with DNA

template extracted from pure culture, respectively. Standard curves were constructed in duplicate of spiked samples using uninoculated fish sauce added with *Virgibacillus* sp. SK37 or *T. halophilus* MS33 and was run duplicate for qPCR assay. The reaction contains 10 µL of 1× iTaq Universal Probe Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada), 500 nM of each primer, and 300 nM of each probe. Each reaction was added 3 µL of genomic DNA. The amplification were run under the reaction condition: 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 30 s and then 60 ºC for 30 s using a 7500 Fast Real-Time PCR system (Life Technologies, Foster city, CA, USA). Quantification cycle (C_q) values were assigned automatically using 7500 software V2.0.1 (Life Technologies, Foster city, CA, USA). Delta C_q (ΔC_q) between C_q of the samples and C_q of internal control (IC) was used to normalize data for bacterial quantification. The IC cells were added into all samples as described in 4.3.3.1.

4.3.3.2 Plate count

Fish sauce mash (25 g) was added with 225 mL of 0.85% NaCl. *Virgibacillus* sp. SK37 was counted on the modified JCM 168 containing 10% NaCl (JCM10). MRS gar containing 5 %NaCl (MRS5) and 0.5% $CaCO_3$ was used for *T. halophilus* MS33 enumeration. Total halophilic bacteria were enumerated using PCA containing 10% NaCl (PCA10). The JCM10 and PCA10 were incubated at 35 °C under aerobic condition for 3-5 days, while MRS5 was incubated at 35 °C under anaerobic condition for 3-5 days.

4.3.4 α-Amino acid content

Liquid samples (100 µL) obtained from various time intervals were determined for α-amino content based on trinitrobenzenesulfonic acid (TNBS) using

L-leucine as a standard (Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul, 2011). Absorbance was measured at 420 nm using a spectrophotometer (SmartSpecTM Plus, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

4.3.5 Biogenic amine content

Biogenic amine content of fish sauce samples fermented for 6 months was analyzed according to Yongsawatdigul, Udomporn, and Choi (2004). Briefly, 100 µl of fish sauce sample were derivatized using dansyl chloride. Biogenic amines were separated using HPLC (Agilent 1260 Infinity series, Agilent Technologies Inc., Santa Clara, CA, U.S.A.) equipped with a Hypersil BDS column C_{18} (3 µm, 100 × 4 mm, Agilent Technologies Inc., Palo Alto, CA, U.S.A.) with solvent A (acetonitrile: 0.02 M acetic acid, 1: 9) and solvent B (0.02 M acetic acid: acetonitrile: and methanol, 1:4.5:4.5) at a flow rate of 1 ml/min. The solvent B (50%) 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. Multi-wavelength detector was set at 254 nm and 550 nm as a reference wavelength.

4.3.6 Amino acid profiles

Total and free amino acid profiles of fish sauce samples were determined as described by Udomsil et al. (2011). For total amino acid profile, 2 mL of diluted 6 month-old fish sauce was added 2 mL of 12 M HCl containing 1% phenol and hydrolyzed at 110 °C for 24 h using an autoclave. Subsequently, acid was evaporated and the precipitates were dissolved in deionized water and filtered through a 0.22-µm membrane. Total amino acids were measured by amino acid analyzer (Biochrom 30, Pharmacia-Biotech, Buckinghamshire, UK).

Performic acid oxidation with acid hydrolysis-sodium metabisulfite method was used for methionine and cysteine determination. The protocol was slightly modified method from AOAC (2000). Briefly, 2 mL of diluted fish sauce (1:10) were placed on ice bath for 15 min. Subsequently, 5 mL of cold performic solution (2 mg of phenol, 0.5 mL of 30% H_2O_2, and 4.5 mL of 88 %formic acid) was added into sample tubes. The solution was stirred for 15 min at room temperature and was then left in 4 °C for 16 h. After that the samples were added 0.84 g of sodium matabisulfite and stirred for 15 min. Then, 5 mL of 12 N HCl mixed with 1 %phenol were added in all sample tubes. All tubes were autoclaved at 110 °C for 24 h. Subsequently, the samples were dried on heating bath and were then added with 20 mL of deionized water. The samples were filtered through a 0.22-μm membrane before injection.

Tryptophan was determined after alkaline hydrolysis using modified protocol from AOAC (2006). Two mL of diluted fish sauce (1:10) was added with 10 mL of 4.2 M NaOH and 3 drops of 1-octanol. The samples were frozen immediately in liquid nitrogen. Subsequently, the samples were flooded by N_2 and melted in water at room temperature. The samples were hydrolyzed at 110 ºC for 20 h. After hydrolysis, the samples were added with 3 mL of sodium citrate buffer solution (pH 4.25). Neutralize the samples with 3.5 mL of HCl and adjusted pH to 4.25 ± 0.05. Then, the samples were transferred into 25-mL volumetric flask and adjusted volume by deionized water. Subsequently, 40 mL of the samples were centrifuged at 1,150 ×g for 20 min and collected supernatant. The samples were then filtered through a filter paper (WhatMan® No. 1), and centrifuged at 23,000 ×g for 10 min. Tryptophan was measured by amino acid analyzer.

For free amino acid profiles, 100 mg of 5'-sulfosalicylic acid (SSA) was added into 2 mL of fish sauce samples diluted 10 times with deionized water, left at room temperature for 1 h and centrifuged at 1,500×g for 10 min (Tungkawachara et al., 2003). The supernatant was collected for free amino acid analysis. Amino acids content was measured using an amino acid analyzer. Lithium and sodium buffer was used for determination of free amino acid and total amino acid, respectively. Four lithium buffers with various pHs were used in this experiment: Li-1 buffer (pH 2.8), Li-2 buffer (pH 3.0), Li-3 buffer (pH 3.15), and Li-4 buffer (pH 3.50). For sodium buffers with various pHs were Na-1 buffer (pH 2.65), Na-2 buffer (pH 3.2), Na-3 buffer (pH 3.35), Na-4 buffer (pH 4.25), Na-5 buffer (pH 6.45), and Na-6 buffer (pH 8.6). Norleucine was used as internal standard at 250 µmol of final concentration. Spectrophotometric detection was set at 440 nm and 570 nm. The amounts of amino acids were calculated as mg/ 100 ml of fish sauce.

4.3.7 Volatile compounds

Fish sauce samples fermented for 6 months were analyzed for volatile compounds by SPME-GC-MS. Fish sauce (2 mL) was added with cyclohexanol as an internal standard to contain final concentration of 1 mg/L. The samples were filled in a 20-mL vial and sealed with septum in an aluminum cap. The samples were heated at 50 ºC in a heating block for 30 min to obtain equilibrium headspace. Volatile compounds in the headspace were absorbed by a 3-phase SPME fiber (1 cm-50/ 30 µm StableFlex Divinyl-benzene/Carboxen/PDMS, Supelco, Bellefonte, PA, U.S.A.). The fiber was introduced into the injection port of gas chromatography-mass spectrometry (GC-MS, Varian Inc., Walnut Creek, CA, U.S.A.) and were then desorbed at 250 ºC for 3 min. The desorbed volatile compounds were separated using

GC-MS equipped with a quadrupole mass detector and a capillary column (DB-WAX, 60 m x 0.25 mm x 0.25 μm Agilent Technologies, Redwood, CA, U.S.A.) as described by Udomsil et al. (2010). Mass spectra were analyzed using the database of National Institute Standard and Technology (NIST). The relative amount of volatile compounds was calculated from peak area relative to the internal standard.

4.3.8 Physico-chemical properties

The 6-month-old fish sauce samples were analyzed for total nitrogen and salt content (AOAC, 2000). 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 (SmartSpecTM Plus, Bio-Rad Laboratories, Hercules, CA, U.S.A.). The pH was measured using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland).

4.3.9 Preference test

The 6-month-old fish sauce samples were evaluated by trained panelists. The panel consisted of 10 expert individuals from fish sauce industry. 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 were asked to give acceptance scores for 4 attributes: color, odor, taste and overall acceptance, using the 7-point hedonic scale. The panelists compared odor by opening the lid of the cup and sniffing. Taste preferences were assessed 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.

4.3.10 Statistical analysis

The fermentation was prepared independently in triplicate. All chemical experiments were analyzed in duplicates. The samples from each of 1-6 months of fermentation were extracted in duplicate and were run in duplicate for qPCR reaction. Statistical analysis was evaluated in Completely Randomized Design (CRD) with Statistical Analysis System (SAS) (SAS Inst. Inc., Cary, NC, U.S.A.). Means comparison by Duncan's Multiple Range Test (DMRT) were used to determine differences between mean at $P < 0.05$.

4.4 Results and discussion

4.4.1 Changes of starter culture during fermentation

Based on the developed qPCR method, *Virgibacillus* sp. SK37 detected by Vir1086 probe of SK37_1M+MS33 and MS33+SK37 was 4.4-4.8 LogCells/mL after 1 month fermentation (Figure 4.1a). Population of *Virgibacillus* sp. SK37 in the SK37 sample was 3.87 LogCells/mL. Growth of *Virgibacillus* sp. SK37 in fish sauce added with combined culture (SK37_1M+MS33 and MS33+SK37) was higher than that of fish sauce added only *Virgibacillus* sp. SK37 (SK37) ($P < 0.05$). The result indicated that adding of combined cultures improve survival rate of *Virgibacillus* sp. SK37. No qPCR readings were obtained from the MS33 sample and the control (Figure 4.1a), indicating that these samples contained no *V. halodenitrificans* microflora or they were present in lower than limit of quantification (LOQ) of 10^3 Cells/mL (Udomsil et al., 2015). After 1 month of fermentation, *Virgibacillus* sp. SK37 gradually decreased to 1.5 LogCells/mL at day 180 in all samples (Figure 4.1a). Population of *Virgibacillus* sp. SK37 in SK37_1M+MS33 at 120 and 150 days

was higher than MS33+SK37 and MS33 treatment ($P < 0.05$). The results implied that fish sauce added with SK37_1M+MS33, *T. halophilus* MS33 might stimulate the growth of *Virgibacillus* sp. SK37.

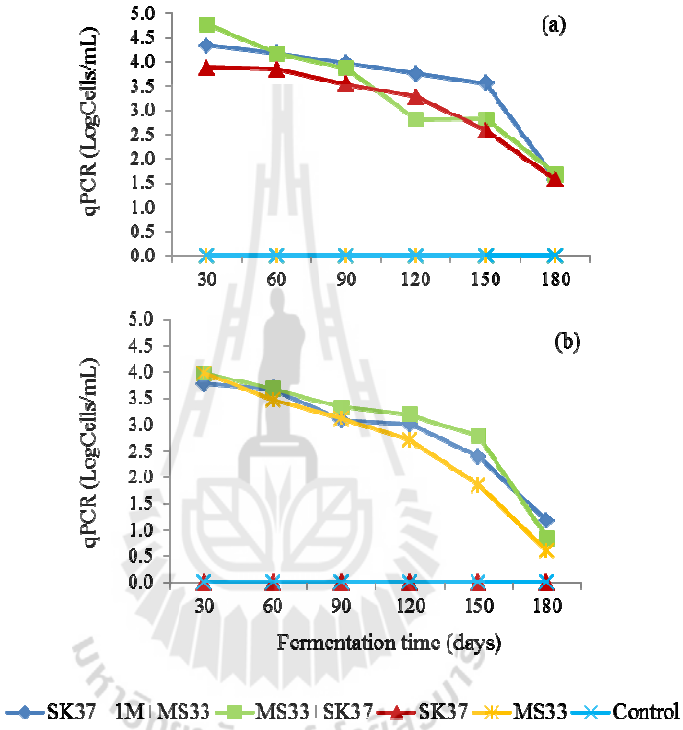

Figure 4.1 Changes of *Virgibacillus* sp. SK37 (a) and *T. halophilus* MS33 (b) inoculated to anchovy fermentation for 180 days at 35 °C by qPCR using Vir1086 and Tet48 probe, respectively.

Population of *T. halophilus* MS33 detected by Tet48 probe in *T. halophilus* MS33 inoculated samples was 3.5-4.0 LogCells/mL at 30 days and slightly decreased to 0.5-1.0 LogCells/mL at 180 days (Figure 4.1b). Fish sauce added with *Virgibacillus* sp. SK37 (the SK37) and the control did not show any signal of qPCR by Tet48 probe, indicating the control and the SK37 sample did not have *T. halophilus* microflora or T. *halophilus* in these samples might be lower than LOQ of

2 LogCells/mL. These results confirmed that also indicated that confirmed that no cross-reaction between these 2 probes. Survival rate of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 detected by qPCR with Vir1086 and Tet48 probe was not different (Figure 4.1a, b). Based on qPCR results, population of *T. halophilus* MS33 of MS33+SK37 and SK37_1M+MS33 at 120 and 150 days of fermentation was higher than that of fish sauce inoculated with the single culture, MS33 ($P < 0.05$). It should be noted that survival rate of *T. halophilus* MS33 in fish sauce inoculated with SK37_1M+MS33 and MS33+SK37 was not different ($P > 0.05$, Figure 4.1b).

Halophilic bacterial plate count (Figure 4.2a) on JCM10 agar of SK37_1M+MS33, MS33+SK37, and SK37 was 6 LogCFU/mL at the initial day (0 day), implying the initial count of *Virgibacillus* sp. SK37 (Figure 4.2a). At 30 and 60 days of fermentation, counts of SK37_1M+MS33 and MS33+SK37 decreased to 4 LogCFU/mL and of SK37 decreased to 3 LogCFU/mL. These results were similar to the qPCR results ($P > 0.05$, Figure 4.1 a). At 90 days, the count on JCM10 agar of MS33+SK37 and SK37 sample was 1-2 LogCFU/mL. These results were quite lower than those obtained from the qPCR, which were reported to be 3.5-4 LogCells/mL (Figure 4.1a). Similar to the result of 120-180 days of fermentation, the conventional plate count of these 3 samples was 1-2 LogCFU/mL lower than results obtained the qPCR (Figure 4.1a). The results implied that it would be possible that inoculated *Virgibacillus* sp. SK37 may undergo viable but non culturable (VBNC) state at the extended fermentation time. VBNC is one of the morphological adaptations of bacteria when they are under extreme environments, such as high or low temperature, nutrient starvation, oxygen availability, and sharp changes in osmotic stress, pH, or salinity (Frankenhuyzen et al., 2013).

In addition, the control showed the count approximately 1.0-4.0 LogCFU/mL on JCM10 agar (Figure 4.2a) throughout the fermentation. These were unlikely to be *Virgibacillus* sp. SK37 due to negative result of qPCR detection with Vir1086 probe (Figure 4.1a). These results confirmed that the culture-dependent technique based on selective medium is not always specific for the culture of interest, leading to inaccurate quantification. Based on the fish sauce model, the developed qPCR offered more accurate detection and quantification of *Virgibacillus* sp. SK37.It should be noted that MS33 showed count on JCM10 agar approximately 5.8 LogCFU/mL at the initial and decreased to 1.0 LogCFU/mL at 180 days of fermentation (Figure 4.2a), suggesting that *T. halophilus* MS33 could also grow on JCM10 agar. From the result, colonies appearance on JCM10 agar was very small, indicating *T. halophilus* MS33 did not grow well on JCM10 agar. Since JCM10 agar plates were incubated under aerobic condition. *T. halophilus* was microaerophilic bacteria, it needs oxygen to survive, but requires environments containing lower level of oxygen than are present in the atmosphere (Christensen, Dudley, Pederson, and Steele, 1999). Therefore, it could grow on agar under aerobic condition. Leisner et al. (2001) reported that bacteria isolated from acid-fermented condiment (tempoyak) using plat count agar (PCA) were LAB. Colonies of LAB on PCA were small, catalase negative, grey or white, and able to grow under aerobic condition. Furthermore, Udomsil (2008) found that bacterial colonies from fish sauce added with *T. halophilus* MS33 grown on JCM 168 medium were likely to be the *T. halophilus* due to its growth under anaerobic condition after re-streaked on MRS agar. Moreover, it was catalase negative, Gram-positive cocci with pairs/tetrads cell arrangement.

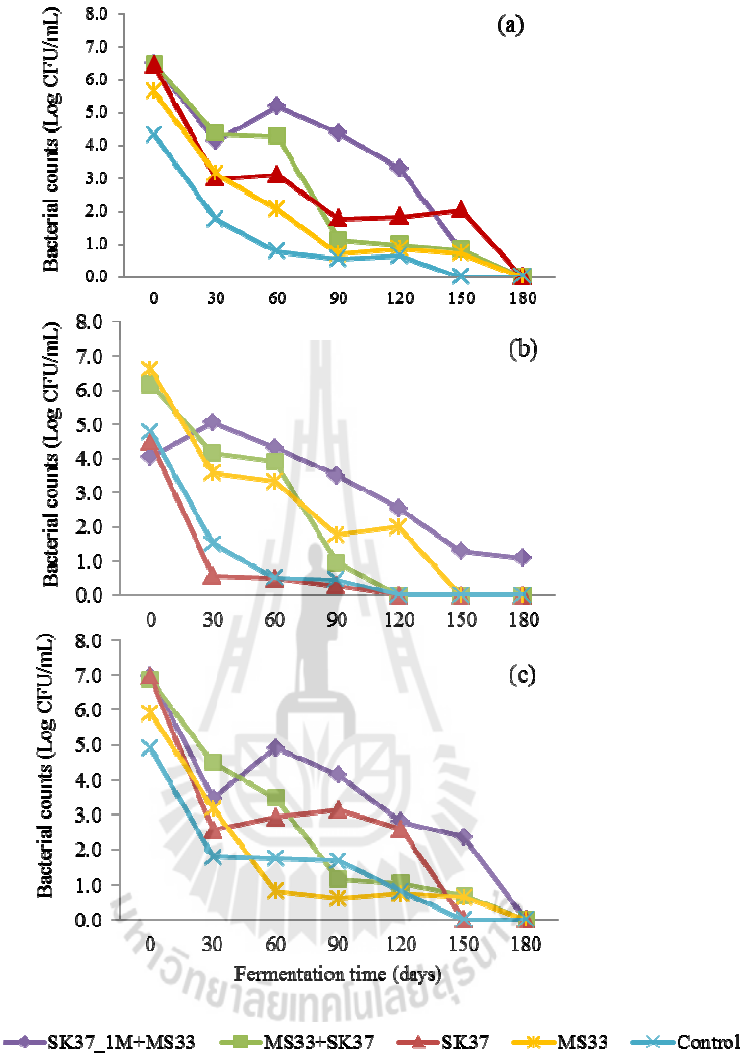

Figure 4.2 Changes of halophilic bacteria on modified JCM 168 containing 10% NaCl (a), lactic acid bacteria on MRS containing 5% NaCl and added 0.5% $CaCO_3$ (b), total halophilic bacteria on PCA containing 10% NaCl (c), and of fish sauce inoculated with starter cultures and fermented at 35 °C for 6 months.

For enumeration of *T. halophilus* MS33 based on MRS5 agar, MS33 and SK37+MS33 contained the initial count approximately 6 LogCFU/mL (Figure 4.2b). SK37_1M+MS33, SK37 and the control showed the initial count approximately 4 LogCFU/mL, despite of no inoculation of *T. halophilus* MS33 in these samples at

day 0 and these samples were only inoculated with *Virgibacillus* sp. SK37. Colonies grown on MRS5 agar were likely to be *Virgibacillus* sp. SK37 because *Virgibacillus* sp. SK37 was facultative anaerobe, it can grow under aerobic and anaerobic condition (Nawong, 2006). These results were confirmed by qPCR based on Vir1086 probe shown in positive signal in SK37_1M+MS33 and SK37 sample. For the control, qPCR results of both Vir1086 and Tet48 probe were negative throughout the fermentation process. Indicating that colonies appeared on all media was not *V. halodenitrificans* and *T. halophilus*. Population of *T. halophilus* MS33 detected by plate was lower than qPCR ($P < 0.05$). Therefore, bacterial quantification of fish sauce fermentation was underestimated based on culture-dependent technique.

Based on the qPCR results, sequential inoculation (SK37_1M+MS33) showed higher counts of both *Virgibacillus* sp. SK37 and *T. halophilus* MS33 as compared to single culture inoculation at later stage of fermentation (120-180 days, Figure 4.1a,b). The qPCR readings of both probes of sequential inoculation (SK37_1M+MS33) were approximately 1 LogCells/mL higher than those of plate count at 120-150 days. This could be attributed to VBNC state of both *Virgibacillus* sp. SK37 and *T. halophilus* MS33. A bacterium in the VBNC state is considered as metabolically active, but incapable of cellular differentiation on a medium (Oliver, 1993). The physiological significance of the VBNC state is still unclear. It could be an adaptive response of long-term survival under adverse conditions (Oliver, 2010).

Total halophilic bacteria of fermentation were enumerated on PCA containing 10 %NaCl (Figure 4.2c). The SK37_1M+MS33showed total halophilic count higher than that of fish sauce samples inoculated with single culture (SK37 and MS33) at 30-60 days (Figure 4.2c). Similarly, halophilic population on JCM10 agar

of fish sauce samples inoculated with SK37_1M+MS33 was higher than that of single culture counterpart at 30-120 days (Figure 4.2a). In addition, sequential inoculation, SK37_1M+MS33, showed the highest total halophilic bacterial counts at 150 days and thereafter as compared to others (Figure 4.2c). Moreover, halophilic LAB count of fish sauce inoculated SK37_1M+MS33 using MRS5 agar showed the highest population at 90 days and thereafter ($P < 0.05$, Figure 4.2b). Probably, *Virgibacillus* sp. SK37 hydrolyzed fish protein to oligopeptides/small peptides serving as substrates for *T. halophilus* MS33 growth. The interactions of mixed cultures in food fermentation have been reported. During mutualism, both participating microorganisms derive a benefit from the interaction. Mutualism is defined as positive benefit of the interactions between individuals of different species (Hugenholtz, 1986). Many food fermentations rely on mutualistic interactions. Mutualism interaction of *Streptococcus salivarius* subsp. *thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in yogurt fermentation is well-known relationship (Sieuwerts et al., 2008). Several long-chain fatty acids are stimulatory to *L. delbrueckii* subsp. *bulgaricus* but it lacks part of the biosynthetic machinery for long-chain fatty acids. Therefore, *S. thermophilus* is also able to supply *L. delbrueckii* subsp. *bulgaricus* with long-chain fatty acids (Partanen et al., 2001).

In commensalism interaction of proteolytic positive strain of *L. lactis* stimulated growth of negative proteolytic strain in cheese by supplying of peptides and amino acids to support growth of the latter (Smid and Lacroix, 2013). *L. lactis* strains produced extracellular proteases (PrtP), leading to peptides released from milk protein, then *L. lactis* strains without protease activity benefit from the peptides while the protease positive strains did not seem to be directly affected (Juillard et al.,

1997). Interaction of LAB and propionic acid bacteria (PAB) in Swiss-type cheese is another possible form of commensalism. Additional metabolites of LAB and PAB might support synergistic growth effects of each other (Van Hijum, Vaughan, and Vogel, 2013). Synergistic interactions on growth of combined cultures inoculation, *Virgibacillus* sp. SK37 and *T. halophilus* MS33 fermentation might occur during fish sauce fermentation. Further studies of such interactions are needed.

4.4.2 Biogenic amine content

Histamine was predominant biogenic amine in all samples (Table 4.1). Histamine content of fish sauce inoculated with *Virgibacillus* sp. SK37 (SK37) and sequential culture (SK37_1M+MS33) was lower than the control ($P < 0.05$). The

Table 4.1 Biogenic amines (mg/100mL) of fish sauce samples prepared by single or combined cultures inoculation.

Biogenic amine	SK37	MS33	MS33+ SK37	SK37_1M+ MS33	Control
Putrescine	ND	ND	0.95 ± 0.02a	ND	ND
Cadaverine	0.48 ± 0.08b	0.17 ± 0.03c	1.48 ± 0.11c	0.36 ± 0.09bc	1.59 ± 0.22a
Histamine	3.11 ± 0.24b	3.55 ± 0.62ab	3.57 ± 0.05ab	2.85 ± 0.15b	4.22 ± 1.26a
Tyramine	ND	ND	ND	ND	2.43 ± 0.43a

Different letters within a row indicate significant differences ($P < 0.05$).
ND = Not Detected.
Fish sauce inoculated with single culture, *Virgibacillus* sp. SK37 (SK37) or *T. halophilus* MS33 (MS33); co-cultures (MS33+SK37) or sequential co-cultures (SK37_1M+MS33) and the control without starter cultures.

control contained the highest histamine, cadaverine and tyramine ($P < 0.05$). Tyramine was found in anchovy fish sauce in a range of 33.7 to 73.9 mg/100 mL (Kirschbaum, Rebscher, and Brückner, 2000). Tyramine content of this study was very low and was only detected in the control. Saaid et al. (2009) reported that

tyramine was occasionally found in some samples and was very low amount. Low level of cadaverine and tyramine indicating freshness quality of raw material used (Yongsawatdigul et al., 2007). Putrescine was only found in fish sauce inoculated with co-cultures (MS33+SK37). LAB especially lactobacilli and staphylococci were able to produce putrescine and cadaverine (Kim, Mah and Hwang, 2009). Putrescine was converted from ornithine by ornithine decarboxylase activity. Low level of putrescine in MS33+SK37 indicated that bacteria producing ornithine decarboxylase was probably low population. Yongsawatdigul et al. (2007) and Udomsil et al. (2011) reported that putrescine of fish sauce added with *Virgibacillus* sp. SK37 or *T. halophilus* MS33 was not detected, indicating that these bacteria did not produce putrescine. However, putrescine presented in MS33+SK37 might be produced from other microflorabacteria.

The SK37_1M+MS33 showed the low amount of histamine and cadaverine content (Table 4.1, $P < 0.05$). Our study indicated that addition of MS33, SK37, and MS33+SK37 in fish sauce did not increase cadaverine and histamine in fish sauce fermentation. Histamine was considered low content and did not exceed international standard.

T. halophilus MS33 inoculated in fish sauce did not increase histamine content (Table 4.1). Similar to previous report, Udomsil et al. (2011) showed that histamine accumulation of *T. halophilus* MS33 inoculated fish sauce was comparable to the control sample. *T. halophilus* has been reported as histamine producer, such as *T. halophilus* strain H isolated from fish sauce was predominant histamine producing bacteria during fish sauce fermentation (Satomi et al., 2008). This strain contains 30 kbp plasmid (pHDC) encoding a single copy of the pyruvoyl dependent histidine

decarboxylase gene (*hdc*). However, gene characterization of *T. halophilus* MS33 has not been investigated.

The US Food and Drug Administration established the maximum limit of histamine in fishery products to be 50 mg/kg (FDA, 2011), while the European regulation set the limit of 100-200 mg/kg (European commission, 2005); According the Canadian Food Inspection Agency (CFIA), the maximum allowable histamine in fish sauce is 20 mg/100 g (CFIA 2003). All samples contained histamine lower than international standards and the sequential inoculation resulted in the lowest biogenic amines in the finished product (Table 4.1). Moon et al. (2013) reported that *B. licheniformis* isolated from fermented fish product, anchovy sauce, and sand lance sauce, showed the presence of *hdc* gene in chromosome and produced 15-22 mg/L of histamine in decarboxylating broth containing 0.5 %histidine. Lysine decarboxylase gene (*cadA*) was detected in *Virgibacillus* sp. SK37 genome, however histidine decarboxylase (*hdc*), tyrosine decarboxlyase, and aromatic-L-amino acid decarboxylase (*ddc*) were not detected (Phrommao, 2010). Implying, *Virgibacillus* sp. SK37 was not histamine producing bacteria.

4.4.3 α-Amino content

α-Amino contents of all inoculated samples at 30 days were approximately 644-656 mM and comparable to that of the control (Table 4.2, $P > 0.05$). However, α-amino content of inoculated samples gradually increased after 30 days. The MS33 showed the highest α-amino content at 60 days ($P < 0.05$). When the fermentation progressed to 180 days, α-amino content of all inoculated samples was higher than that of the control ($P < 0.05$). α-Amino content of fish sauce inoculated with combined cultures (MS33+SK37 and SK37_1M+MS33) appeared to

Table 4.2 Changes of α-amino content of fish sauce samples produced by single or combined cultures inoculation.

Fermentation time	α-Amino content (mM)*				
(day)	SK37	MS33	MS33+SK37	SK37_1M+MS33	Control
30	644.02 ± 37.3	648.08 ± 39.47	656.20 ± 27.99	645.37 ± 45.56	580.55 ± 5.17
60	733.11 ± 10.81ab	779.08 ± 36.25a	724.32 ± 28.30ab	743.42 ± 47.99ab	663.50 ± 21.75b
90	920.83 ± 5.89c	1004.58 ± 10.02ab	1058.76 ± 15.89a	957.08 ± 52.44bc	911.25 ± 26.51c
120	1096.18 ± 26.41ab	1160.85 ± 18.84a	1144.01 ± 53.98a	1143.77 ± 50.11a	998.52 ± 20.29b
150	1015.18 ± 15.86	1007.57 ± 1.64	1153.17 ± 52.04	1047.31 ± 36.35	1003.45 ± 47.87
180	1085.85 ± 7.99b	1141.36 ± 38.89ab	1160.41 ± 29.39a	1185.71 ± 11.80a	1008.29 ± 33.07c

* Different letters indicate significant differences within a row ($P < 0.05$).
Abbreviations are the same as Table 4.1.

be the highest ($P < 0.05$). The combined inoculation resulted in the highest proteolytic degradation of fish proteins. *Virgibacillus* sp. SK37 might improve the uptake and the consequent consumption of some amino acids by *T. halophilus* MS33, resulting in a synergistic mechanism of amino acids use. Since increasing of α-amino content appeared to correlate with microbial population. Ciani and Comitini (2015) reported that mixed yeast species in wine fermentation improved amino acid consumption of *Saccharomyces cerevisiae* and supported its growth. Although bacterial count of all inoculated samples gradually declined and reached the lowest at 180 days (Figures 4.2), α-amino content continually increased until the end of fermentation, implying that released proteinase activity from the cells was still remained.

4.4.4 Total and free amino acid profiles

The contents of total aspartic acid, glutamic acid, isoleucine, tyrosine, and arginine of SK37_1M+MS33 were higher than those of the control ($P < 0.05$,

Table 4.3). Glutamic acid was important, contributing to meaty flavor and umami taste. Phenylalanine and tyrosine have also been reported as umami-related components (Lioe, Wada, Aoki, and Yasuda, 2007). Phenylalanine of all samples was comparable ($P > 0.05$). However, MS33, MS33+SK37, and SK37_1M+MS33 showed higher tyrosine content than the control ($P < 0.05$). It should be noted that leucine and arginine of MS33 and SK37_1M+MS33 was higher than that of the control ($P < 0.05$) and the sum of total amino acids of MS33 and SK37_1M+MS33 were the highest ($P < 0.05$), except fish sauce added with SK37 did not increase ($P > 0.05$).

Table 4.3 Total amino acid content of fish sauce samples inoculated with single or combined cultures of *Virgibacillus* sp. SK37 and *T. halophilus* MS33.

Total amino acid (mg/100ml)	**SK37**	**MS33**	**MS33+SK37**	**SK37_1M +MS33**	**Control**
Aspartic acid	1376.67b	1461.38a	830.53c	1461.13a	739.79d
Threonine	639.02d	698.90c	1121.65a	695.74c	782.43b
Serine	543.53c	617.59b	758.49a	612.08b	395.07d
Glutamic acid	2051.41a	2178.82a	1656.45b	2153.68a	1779.05b
Proline	540.03b	640.44a	531.99b	585.15ab	572.85b
Glycine	728.53ab	775.95a	640.64b	721.18ab	748.03a
Alanine	978.78	1041.47	1107.76	970.04	1037.39
Valine	786.16	840.39	778.31	779.64	879.21
Methionine	529.66	528.36	520.75	525.21	544.53
Isoleucine	465.58a	500.93a	464.62a	459.83a	374.81b
Leucine	541.29ab	574.06a	520.70ab	530.26ab	467.78b
Tyrosine	84.36b	102.86a	94.38ab	101.56a	85.08b
Phenylalanine	428.40	467.41	406.17	442.13	452.08
Histidine	656.35	694.28	667.22	676.75	714.95
Lysine	1375.37c	1542.50bc	1441.26bc	1554.24bc	1664.23a
Arginine	346.54c	345.60c	374.90bc	474.67a	427.23ab
Tryptophan	72.81	82.32	90.79	85.87	90.58
Total	12480.41b	13420.05a	12433.73b	13263.72a	12065.27b

Different letters within a row indicate significant differences ($P < 0.05$).
Abbreviations are the same as described in Table 4.1.

These results suggested that inoculation of *T. halophilus* MS33 and the sequential inoculation (SK37_1M+MS33) increased total amino acid content to the greatest extent. These results corresponded to α-amino acids content. Addition of *Virgibacillus* sp. SK37 might produce small peptides by its proteinases. These small peptides might serve as nitrogen source for *T. halophilus* MS33 for their growth and produced amino acids and oligopeptides. The result of total amino acid content corresponded to qPCR quantification, survival rate of *T. halophilus* MS33 in MS33+SK37 and SK37_1M+MS33 detected by Tet48 probe was higher that fish sauce added with single culture (Figure 4.1b).

For free amino acid profile, all samples contained similar contents of free amino acids except free glutamic acid of all starters culture-inoculated fish sauce was higher than the control ($P < 0.05$, Table 4.4). Glutamic acid provides the umami taste through a synergistic effect with 5'-nucleotides. Udomsil et al. (2011) reported that fish sauce inoculated with *T. halophilus* MS33 increased total of free glutamic acid and also increased of total leucine and phenylalanine in fish sauce. *T. halophilus* MS33 has been reported to produce intracellular aminopeptidases activity toward glutamic acid, alanine, methionine, leucine, lysine and arginine, resulting in increasing of free amino acids and small peptide residues in fish sauce (Udomsil et al., 2010). The sum of total amino acids was lower than the sum of free amino acids, suggesting that amino acids in inoculated fish sauce existed in the form of free amino acids rather than peptides, which was important for the taste of fish sauce (Table 4.3 and 4.4). Furthermore, amino acids might be destroyed during sample analysis with high concentration acid and temperature.

Table 4.4 Free amino acid content of inoculated fish sauce with single and combined culture starter fermented at 35 °C for 6 months.

Free amino acid (mg/100ml)	**SK37**	**MS33**	**MS33+SK37**	**SK37_1M +MS33**	**Control**
Aspartic acid	892.19	893.73	910.82	896.75	933.24
Threonine	996.45	1004.17	1016.43	998.22	1028.77
Serine	808.22	818.70	819.81	816.93	843.93
Glutamic acid	1937.12a	1943.07a	1960.89a	1942.12a	1805.22b
Proline	650.36	651.39	680.25	642.60	702.90
Glycine	499.00	486.79	498.18	491.41	504.92
Alanine	920.64	915.64	921.52	912.23	920.23
Valine	1272.80	1257.95	1271.84	1249.42	1291.85
Methionine	310.41	294.19	263.21	275.10	245.25
Isoleucine	778.09	795.06	802.28	789.93	815.78
Leucine	745.17	758.60	756.40	751.96	760.41
Tyrosine	202.08	214.18	214.22	219.90	213.73
Phenylalanine	795.13	819.49	824.20	807.41	846.09
Histidine	793.25	808.41	814.61	808.70	834.25
Lysine	1231.90	1240.54	1248.70	1249.15	1256.02
Arginine	204.04	205.93	216.64	227.27	204.04
Total	13036.85	13107.85	13219.98	13079.09	13206.62
Amino derivative					
Cysteine	71.35b	66.99b	70.59b	67.34b	82.73a
Ammonia	648.16	649.09	655.60	642.79	657.66
Taurine	242.43	257.65	255.84	248.94	263.97
Hydroxyproline	50.76a	31.15b	33.46b	32.18b	45.85ab
Ornithine	581.82a	596.32a	592.74a	517.06b	595.75a
Citrulline	749.79b	745.93b	760.18b	849.73a	821.70ab
Total	2344.31	2347.14	2368.42	2358.05	2467.65

Different letters within a row indicate significant differences ($P < 0.05$).
Abbreviations are the same as Table 4.1.

Free amino contents of aspartic acid, alanine, threonine, valine, isoleucine, leucine, and lysine were comparable to the control ($P > 0.05$, Table 4.4). Branch-chain amino acids (isoleucine, leucine, and valine) are precusors for volatile

compounds in fish sauce e.g. aldehydes (3-methylbutanal, 2-methylbutanal, and 2-methylpropanal) via amino acid catabolism pathway of bacteria (Smit, Engels, and Smit, 2009). Amino acids are important taste contributor in food e.g. lysine and proline are sweet taste with bitter after taste. Isoleucine, leucine, arginine, cysteine, methionine, phenylalanine, tryptophane and histidine contributed to bitter taste (Shallenberger, 1993). However, free amino acids and the sum of free amino acids of fish sauce inoculated with starter cultures did not show significant difference from the control sample, except, glutamic acid was increased in all inoculated fish sauce (Table 4.4). These results indicated that inoculation of single and combined cultures treatment could increase glutamic acids. Amino derivatives found in samples included cysteine, hydroxyproline, ornithine, and citrulline. Correspondingly, Udomsil et al. (2011) reported that ammonia, ornithine and citrulline were major amino derivatives in fish sauce added *T. halophilus* MS33. These amino derivatives content were not higher than the control. Ammonia, ornithine and citrulline were product of arginine degradation by arginine deiminase pathway (ADI) which was commonly found in bacteria (Arena, Saguir, and Manca de Nadra, 2001; Quintero, Muro-Pastor, Herrero, Flores, 2000).

4.4.5 Volatile compounds

Five major groups of volatile compounds, including aldehyde, ketone, alcohol, sulfur-containing, and nitrogen-containing compounds were found in fish sauce samples (Table 4.5). Propanal and 2-methylbutanal content of fish sauce prepared from sequential inoculation was highest ($P < 0.05$). Inoculation with single *T. halophilus* (MS33) and the sequential culture inoculation (SK37_1+MS33) resulted in higher 2-methylpropanal, 3-methylbutanal, and 3-(methylthio)propanal

Table 4.5 Volatile compounds of fish sauce inoculated with single and combined cultures and fermented for 6 months.

		Relative peak area*				
RI**	**Volatile compounds**	**SK37**	**MS33**	**MS33+SK37**	**SK37_1M +MS33**	**Control**
	Aldehyde					
841	Propanal	0.068b	0.041b	0.048b	0.110a	0.021c
851	2-Methylpropanal	0.465a	0.509a	0.495a	0.669a	0.328b
904	2-Methylbutanal	0.004c	0.018b	0.012b	0.059a	0.011b
908	3-Methylbutanal	0.004c	0.021a	0.013b	0.024a	0.011b
1463	Benzaldehyde	0.012c	0.284a	0.018c	0.056b	0.036b
1597	3-(Methylthio)propanal	0.006c	0.100a	0.039b	0.128a	0.040b
	Ketone					
853	Acetone	0.066b	0.081b	0.081b	0.100a	0.136a
897	2-Butanone	0.011b	0.014b	0.006c	0.075a	0.050a
954	2-Pentanone	0.005b	0.006b	0.026a	0.014a	0.003b
890	Ethyl Acetate	0.005b	0.007b	0.020a	0.007b	0.020a
	Alcohol					
938	Ethanol	0.005b	0.008b	0.028a	0.008b	0.004b
1119	1-Butanol	0.003b	0.008b	0.069a	0.043a	0.008b
1131	1-Penten-3-ol	0.009c	0.048b	0.029b	0.171a	0.026b
1168	3-Methyl-1-butanol	0.005b	0.023a	0.011a	0.011a	0.004b
1200	1-Pentanol	0.011d	0.066b	0.167a	0.079b	0.042bd
1265	(E)-2-Penten-1-ol	0.004b	0.056a	0.018b	0.050a	0.058a
1270	(Z)-2-Penten-1-ol	0.030b	0.020b	0.003c	0.083a	0.067a
	Sulfur-containing compound					
1036	Dimethyl disulfide	0.025b	0.014b	0.006c	0.011b	0.078a
1324	Dimethyl trisulfide	0.005	0.004	0.007	0.005	0.010
	Nitrogen-containing compound					
1215	Methylpyrazine	0.020b	0.056a	0.022b	0.051a	0.013b
1281	2,6-Dimethylpyrazine	0.004c	0.067a	0.031b	0.080a	0.020b

*Different letters within a row indicate significant differences (P < 0.05).
**RI, retention indices.
Abbreviations are same as Table 4.1.

than others (P < 0.05). All inoculated fish sauce samples contained 2-methylpropanal higher than did the control (P < 0.05). The highest content of benzaldehyde was also found in fish sauce inoculated with MS33 (P < 0.05),

corresponding with the study of Udomsil et al. (2011), reporting that *T. halophilus* MS33 produced benzaldehyde during fish sauce fermentation. Valine and isoleucine are precursors of 2-methylpropanal and 2-methylbutanal, respectively (Pripis-Nicolau et al., 2000). High amount of these aldehydes corresponded with high contents of free valine and isoleucine (Table 4.4). 3-(Methylthio)propanal is a Strecker aldehyde of methionine, which is a process mediated by amino transferase and α-ketoacid decarboxylase (Amarita, Fernandez-Espla, Requena, and Pelaez, 2001). This aldehyde contributes to cooked potato note. 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 catalyzed by Mn^{2+} results in 2-methylpropanal (Smit and Engels, 2004). The activity can be modulated by the Mn^{2+} and oxygen concentration. It should be noted that 2- methylbutanal and 3-methylbutanal of co-cultures sample were comparable to the control ($P > 0.05$). 2-Methylpropanal, 2-methylbutanal, and 3-methylbutanal contributed to malty, nutty, almond and dark chocolate note, which is one of important notes of fish sauce (Giri et al., 2010; Lapsongphon et al., 2013). Lapsongphon et al. (2013) reported that 2-methylpropanal, 2-methylbutanal, 3-methylbutanal and 3-(methylthio)propanal showed the highest odor-activity values (OAVs), suggesting that they have strong influence on the overall aroma of fish sauce. Amino acid catabolism has been found in whole genome of *Virgibacillus* sp. SK37 (Phrommoa et al., 2013). Therefore, those volatile compounds might be produced from metabolism of *Virgibacillus* sp. SK37. *Virgibacillus* sp. SK37 has been reported to increase 2-

methylpropanal, 2-methylbutanal, and 3-methylbutanal in fish sauce fermented under reduced salt content condition.

The content of acetone and 2-butanone of sequential inoculated fish sauce was comparable to the control ($P > 0.05$). 2-Butanone is responsible for a cheesy note. However, these compounds were unlikely to be the important odor because of their high threshold value of 1.55-7.76 mg/L (Michihata, Yano, and Enomoto, 2002). The content of 1-butanol, 1-penten-3-ol, and 3-methyl-1-butanol of fish sauce inoculated with combined cultures (MS33+SK37 and SK37_1M+MS33) was higher than others ($P < 0.05$), whereas fish sauce prepared from co-cultures (MS33+SK37) showed the highest amount of 1-pentanol. 3-Methyl-1-butanol contributed to rancid and pungent odor note, while 1-penten-3-ol contributed to a meaty, grassy, burnt, and green odor (Giri et al., 2010). These compounds were found in fish miso, soy miso, soy sauce, and fish sauce. Leucine was precursor for 3-methyl-1-butanol formation in cheese via transamination pathway of *Lactococcus lactis* (Smit et al., 2005). However, the pathway of amino acid conversion of *T. halophilus* has not been investigated thus far. The role of alcohols on overall acceptance in fish sauce has not been reported because their odor threshold was relatively high (Michihata et al., 2002).

Dimethyl disulfide of fish sauce inoculated with starter cultures was lower than the control ($P < 0.05$). The content of dimethyl trisulfide was relatively low in both single and combined cultures. Dimethyl trisulfide contributed to cooked cabbage, fishy, sulfury and fecal note with its low threshold value of 0.015 µg/L, thus, it was considered as odor active compound (Devos, Patte, Roualt, Laffort, and Gemert, et al., 1995; Gire et al., 2010). Dimethyl disulfide and dimethyl trisulfide

were products from methionine catabolism via tranamination (Yvon and Rijnen, 2001). *Virgibacillus* sp. SK37 and *T. halophilus* MS33 have been reported to reduce these sulfur-containing compounds in fish sauce (Lapsongphon et al., 2013; Udomsil et al., 2011; Yongsawatdigul et al., 2007). Methylpyrazine and 2,6-dimethylpyrazine of fish sauce prepared from sequential inoculation (SK37_1+MS33) were the highest ($P < 0.05$). These compounds played less important role in overall of fish sauce due to their high threshold (Giri el al., 2010). Our result indicated that sequential inoculation efficiently improved volatile characteristic of fish sauce. However, the interactions of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 on production of volatile compounds deserved further investigation.

4.4.6 Physico-chemical properties

Physico-chemical properties of fish sauce samples were shown in Table 4.6. Salt content of fish sauce inoculated with starter cultures and the control were comparable and in the range of 27-28% (Table 4.6, $P > 0.05$). The pH values of fish sauce samples were 5.54-5.80, and the sample SK37_1+MS33 exhibited the lowest pH of 5.54. Acidity of fish sauce is due to organic acids, such as lactic acid and acetic acid during fermentation. Although starter cultures were added, they did not drastically decrease pH ($P > 0.05$). This was because fish sauce fermentation did not contain sugar (glucose in particular), an important substrate for lactic acid production of *T. halophilus* MS33.

Total nitrogen (TN) contents of all fish sauce samples were comparable (Table 4.6, $P > 0.05$). Ammonia, free amino acids, nucleotide, peptides, urea, and trimethylamine (TMA) were TN in fish sauce (Shahidi, Sikorski, and Pan,

Table 4.6 Physico-chemical properties of fish sauce samples inoculated with single and combined cultures fermented for 6 months.

Samples	Salt (%)	Total nitrogen (%)	pH	Browning index
Control	27.37 ± 0.20	2.29 ± 0.1	5.80 ± 0.12	0.44 ± 0.24
SK37	27.95 ± 1.21	2.29 ± 0.04	5.67 ± 0.24	0.44± 0.24
MS33	27.74 ± 0.40	2.36 ± 0.02	5.67 ± 0.38	0.43 ± 0.24
MS33+SK37	28.45 ± 0.81	2.42 ± 0.06	5.62 ± 0.34	0.45± 0.24
SK37_1M+MS33	28.52 ± 0.40	2.44 ± 0.02	5.54 ± 0.23	0.44 ± 0.24

Abbreviations are same as Table 4.1.

1994). TN has been used to classify 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 at 6 months fermentation. The browning index was comparable among samples (Table 4.6, $P > 0.05$). Maillard reaction is responsible for brown color of fish sauce during ripening step (Lopetcharat et al., 2001).

4.4.7 Preference test of fish sauce

Preference test based on color, odor, taste and overall acceptance of all fish sauce was shown in Table 4.7. Color and odor of fish sauce samples inoculated with single- and co-cultures were comparable to the control ($P > 0.05$). The sample SK37_1M+MS33 was more preferable in terms of taste and overall acceptance ($P < 0.05$). Since SK37_1M+MS33 contained high content the sum of total amino acids ($P < 0.05$) and more desirable volatile compounds, particularly aldehydes, these compounds could likely contribute to higher preference in taste characteristic and

overall acceptance. In addition, some panelists who were familiar with fish sauce tasting revealed that SK37_1M+MS33 received greater taste preference than others.

Table 4.7 Hedonic score of color, odor, taste, and overall acceptance of fish sauce samples inoculated with single and combined cultures fermented for 6 months.

Samples	Attribute			
	Color	**Odor**	**Taste**	**Overall acceptance**
Control	4.44 ± 0.88	3.89 ± 1.05	3.56 ± 0.88^{b}	3.56 ± 0.53^{b}
MS33	4.78 ± 0.67	4.11 ± 0.78	3.56 ± 1.24^{b}	4.00 ± 0.71^{b}
SK37	4.44 ± 0.73	3.89 ± 0.93	3.89 ± 0.78^{b}	4.00 ± 0.50^{b}
SK37+MS33	4.44 ± 0.88	4.33 ± 0.71	4.22 ± 0.97^{ab}	4.00 ± 0.71^{b}
SK37_1M+MS33	4.78 ± 0.83	4.89 ± 1.27	5.00 ± 1.00^{a}	4.78 ± 1.09^{a}

Acceptance score: 7 = extremely like; 4 = neither like nor dislike; 1 = extremely dislike.
Different superscripts within a column indicate significant difference ($P < 0.05$).
Abbreviations are same as Table 4.1.

4.5 Conclusions

Sequential inoculation of *Virgibacillus* sp. SK 37 for 1 month, followed by *T. halophilus* MS33 (SK37_1M+MS33) contained high content of α-amino content at 6 months. Moreover, SK37_1M+MS33 contained higher 2-methylbutanal, 3-methylbutanal, and 3-methylpropanal contributing to malty, nutty, almond and dark chocolate notes, than others. Single and combined cultures also increased both free and total glutamic acid. The use of the MS33 alone also increased total amino acids of fish sauce. Therefore, the use of sequential inoculation could improve volatile

compounds and amino acid profile, rendering improvement of flavor characteristic and overall acceptance.

4.6 References

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CHAPTER V

DETECTION OF MICROBIAL PROFILING OF FISH SAUCE INOCULATED WITH STARTER CULTURES BY 16S rRNA GENE SEQUENCING

5.1 Abstract

16S rRNA gene sequencing approaches were used to monitor changes in starter cultures and microflora populations of fish sauce inoculated with combined cultures, *Virgibacillus* sp. SK37 and *T. halophilus* MS33, during 6 months of fermentation. DNA was extracted and sequenced using Ion Torrent Personal Genome Machine (PGM®), yielding a total of 528,814 reads, with an average read length of 400 bp. Quantitative Insights Into Microbial Ecology (QIIME) was used to analyze output data. Based on 16S rRNA genes from the metagenome, 12 genera were found in inoculated fish sauce. The results indicated that microbial profiling in inoculated fish sauce was dominated by *Bacillus, Brevibacillus, Staphylococcus, Tetragenococcus,* and *Virgibacillus.* In contrast, *Halomonas, Prochlorococcus, Rhodococcus,* and *Sediminibacterium* were prominent bacteria in uninoculated fish sauce. Inoculation of *Virgibacillus* and *Tetragenococcus* as a single culture notably suppressed abundance of microflora. Simultaneous co-cultures inoculation (MS33+SK37) predominantly showed *Virgibacillus,* but *Tetragenococcus* was found in low population. In contrast, *Tetragenococcus* was predominant in sequential inoculation. Addition of sequential starter culture might promote growth of *T. halophilus* MS33.

Keywords: Inoculated fish sauce, co-cultures, Ion Torrent PGM®, 16S rRNA gene sequencing, *Virgibacillus*, *Tetragenococcus*

5.2 Introduction

Recently, *Virgibacillus* sp. SK37 and *T. halophilus* MS33 have been proved as potential starter cultures for fish sauce fermentation since they shortened fermentation time and improved aroma quality (Yongsawatdigul et al., 2007; Udomsil et al., 2011). Many microflora bacteria founded in fish sauce included *Bacillus* (Uchida et al., 2004), *Staphylococcus* (Fukami *et al*., 2004), *Micrococcus*, *Streptococcus* (Ijong and Ohta, 1996), *Halobacterium* (Thongthai and Suntinanalert, 1991), *Halobacillus* (Chaiyanan et al., 1999), *Virgibacillus* (Nawong, 2006), and *Tetragenoccus* (Udomsil et al., 2010). These microorganisms diversity were isolated by culture-dependent method. However, culturing method is not complete for microbial characterization. Microorganisms may require unknown growth factors and/or growth conditions present in natural habitats that are not reproduced by media. (Amann, Ludwingg, and Schleifer, 1995). Such limitations lead these techniques to underestimate microbial diversity, and sometimes even the failure to detect the majority microbial groups (Ercolini, Moschetti, Blaiotta, and Coppola, 2004). Therefore, next-generation sequencing (NGS) has been used to describe microbial constituent of ecosystems, that help overcome the problems most of which have been used extensively in food systems. These techniques allow the identification and, in some cases, quantification of food-associated microbial groups, and provides sensitive and rapid methods for determining the composition and diversity of complex microbial communities. NGS technique is based on nucleic acid sequencing without culturing step.

DNA of microorganisms was extracted directly from samples and DNA sequence fragments for the purpose of microbial diversity were amplified using universal primers targeting known marker gene. The most commonly used genes include 16S rRNA gene for prokaryotes, 18S and 28S rRNA gene for eukaryotes, and Internal Transcribed Spacer (ITS) regions for fungi (Anderson, Campbell, and Prosser, 2003). This method is high-throughput and has been used to analyze food microbial ecology. Pyrosequencing was the first NGS technique has been used to study in food microbiology such as meju (dried fermented soybeans) (Kim et al., 2011), natto, doengjang and tempeh (Nam, Lee, and Lim, 2012). *Bacillus* was dominant throughout fermentation, followed by *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Weissella*, and *Tetragenococcus*. Jung et al. (2011) reported that bacterial community of kimchi was dominated by *Leuconostoc*, *Lactobacillus*, and *Weisella*. Pyrosequencing showed that *Leuconostoc* and *Weissella* dominate the early stages of fermentation, while *Lactobacillus* was dominant at the end (Part et al., 2012). Dynamics of starter cultures and diversity of fish sauce microflora were still ambiguous. Understanding changes of bacterial community during fermentation with starter cultures would lead to obtain knowledge about role of starter cultures on microflora in fish sauce.

Ion Torrent PGM® has received considerable attention. Characterization of bacterial 16S rRNA gene pools through massively parallel amplicon sequencing is a method of choice which can replace previously used clone library sequencing techniques (DeSantis et al., 2007). Ion Torrent PGM® has been used to characterize the microbial diversity in commercial fish sauce using 16S rRNA gene sequencing. *Halanaerobium*, *Staphylococcus*, and *Tetragenococcus* were dominated bacteria found

in commercial Thai fish sauce fermented for 1-12 months based on Ion Torrent PGM® (Chuea-nongthon, 2013). Ion Torrent PGM® sequencing is determined by measuring pH changes due to hydrogen ion liberation as nucleotides are incorporated during strand synthesis in picoliter wells (Rothberg et al., 2011). The increasing numbers, quality and length of reads per run, together with the possibility of ‘‘barcode-tagging’’ amplicons with sample-specific adaptors are leading to be multiplesamples (Parameswaran et al., 2007). In this study, we investigated microbial profiling of fish sauce inoculated with combined cultures fermented for 1-6 months using Ion Torrent PGM® with sample-specific barcoded primers targeting the hyper-variable regions V2 of the 16S rRNA gene.

5.3 Materials and Methods

5.3.1 Starter culture preparation and sample collection

One kilogram of fresh anchovy was mixed with 25 % of solar salt and inoculated with 10% of *Virgibacillus* sp. SK37 (SK37) or *T. halophilus* MS33 (MS33) with initial cell counts of 10^6-10^7 CFU/mL. For co-cultures, 5% of *T. halophilus* MS33 and *Virgibacillus* sp.SK37 were added simultaneously (MS33+SK37). In addition, sequential inoculation was carried out by initial inoculation of 5% *Virgibacillus* sp. SK37 inoculum for 1 month and following 5% of *T. halophilus* inoculum (SK37_1M+MS33). The control was added with 10% of fish broth without starter cultures. All treatments were incubated at 35 ºC for 6 months. Ten gram of the samples were aseptically taken every month (1-6 months) and stored at -20 ºC until further use.

5.3.2 DNA extraction

Bacterial DNA was extracted from 1 mL of fish sauce mash by DNeasy® tissue kit (QIAGEN, Mississauga, ON, Canada). Briefly, 1 mL of fish sauce mash was added with 500 µL of 1xPBS and transferred into filter bag (Labplas Inc., Sye-Julie, QC, Canada). Filtrate was collected to a 1.5-mL tube and fish particles were centrifuged at 500×g for 5 min. Supernatants were collected, and further centrifuged at 10,000×g for 10 min. Subsequently, cell pellets were washed twice with 1 mL of 1xPBS. Genomic DNA was isolated using DNeasy® tissue kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's procedure. Quantification and purity of DNA were carried out using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, U.S.A) and extracted DNA was stored at -20 ºC.

5.3.3 16S rRNA gene amplicon and emulsion PCR

To analyze the bacterial community, the fragment size ~ 450 bp of bacterial 16S rRNA genes hypervariable regions (V2) were amplified using primers reported in Table 5.1. These primers were connected to additional PGM adaptors, unique error correcting Golay Barcode, and 'CAT' spacer (Hamady et al., 2008). DNA was amplified in 40 µL containing 20 µL of Qiagen HotStar master mix (2x) (QIAGEN, Mississauga, ON, Canada), 0.8 µL of 20 µM of each primer, 0.16 µL of 10 mg/mL of BSA and 1 µL of DNA template. The PCR conditions were 95 ºC for 15 min, followed by 32 cycles of 94 ºC for15 s, 52 ºC for 30 s, 72 ºC for 40 s and a final extension at 72 ºC for 8 min. Amplicon product was verified in 2% agarose gel by electrophoresis and purified by MinElute PCR purification kit (QIAGEN, Mississauga, ON, Canada). Concentration and quality of DNA was assessed on

BioAnalyzer 2100 (Agilent, Santa Clara, CA, U.S.A). For the sequence run, all DNA libraries from different samples were adjusted to 26 pM and pooled in an equal volume before clonal amplification by emulsion PCR which was carried out by Ion One touch™ Instrument (Life Technologies, Foster city, CA, USA) and attached to Ion Sphere Particles (ISPs) using Ion PGM® Template OT2 400 kit (Life Technologies, Foster city, CA, USA) according to manufacturer's protocol. Unenriched template was measured after emulsion PCR amplification by Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

5.3.4 Sequencing and microbial profiling analysis

The enrichment templated-ISPs (>95%) were sequenced on 316 chip V2 using Ion Torrent PGM® and employing the Ion Sequencing 400 kit (Life Technologies, Foster city, CA, USA) according to the supplier's manual. After sequencing, the individual sequence reads were filtered by PGM® software to remove low quality and polyclonal sequences. Sequences matching the PGM® 3'-adaptor were automatically trimmed. FastQ, and *.fasta, files were exported and processed using QIIME pipeline as described by Caporaso et al. (2010). The sequences with ≥ 97% homology were clustered and defined as percent of 16S rRNA Operational Taxonomic Units (OTUs). QIIME was used for all sequence reads (~4.5 Mb) which were classified to the lowest possible taxanomic rank.

5.4 Results and discussion

5.4.1 Microbial profiling in fish sauce inoculated with starter cultures

A filtered yield of 528,814 reads was obtained from 30 samples with an average read length of 400 bp. For complete lists of 24 genera in Table 5.2-5.6.

Table 5.1 Primers used to amplify V2 hypervariable region of bacteria in inoculated and non-inoculated fish sauce fermented for 1-6 months.

Primer	Adaptor	Barcode	Spacer	16S forward primer
Forward	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAAGGTAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAAGGAGAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGAGGATTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TACCAAGATC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAAGGAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGCAAGTTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCGTGATTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCCGATAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGAGCGGAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGACCGAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCCTCGAATC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAGGTGGTTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAACGGAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGAGTGTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAGAGGTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTGGATGAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTATTCGTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGGCAATTGC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTAGTCGGAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATCCATC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGCAATTAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCGAGACGC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGCCACGAAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACCTCATTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CCTGAGATAC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTACAACCTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACCATCCGC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCCGGAATC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGACCACTC	GAT	agagtttgatcctggctcag
	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAGGTTATC	GAT	agagtttgatcctggctcag
Reverse	CCTCTCTATGGGCAGTCGGTGATtgctgcctcccgtaggagt			

Habitats of these 24 genera were saline environment.

Therefore, they might suppose to be found in fish sauce fermentation. There were 12 genera of bacteria detected as dominant microorganisms in fish sauce inoculated with starter cultures and the control, which were *Salimicrobium, Salinicoccus, Sediminibacterium, Rhodococcus, Serinicoccus, Halomonas,*

Prochlorococcus, *Staphylococcus*, *Brevibacterium*, *Bacillus*, *Tetragenococcus*, and *Virgibacillus* (Figure 5.1a, b). The predominant genera in the control of the 1st batch at 1 month of fermentation were *Prochlorococcus*, *Rhodococcus*, and *Sediminibacterium* (Figure 5.1a) while dominant bacteria of the 2nd batch at 1 month was *Halomonas*, *Bacillus*, and *Prochlorococcus* (Figure 5.1b). Minor population (some as low as <10% of total relative abundance) detected in the control at 2-6 months of fermentation include *Salimicrobium*, *Salinicoccus*, *Staphylococcus*, *Brevibacterium*, *Bacillus*, and *Serinicoccus*. *Virgibacillus* and *Tetragenococcus* were not detected in both control samples (Figure 5.1a, b). High variation of bacterial population in the controls was observed. *Prochlorococcus* (accession no. DQ366718), *Rhodococcus* (accession no. KJ028076), and *Sediminibacterium* (accession no. FM179320) were isolated from saline environment. *Salimicrobium* was isolated from Korean fermented seafood (myeolchi-jeot) (Choi, Jin, Kim, and Jeon, 2014). *Salinicoccus* is a moderate halophile isolated from saline soil sample (Chen et al., 2007).

Virgibacillus was only predominant genus throughout the course of fermentation inoculated with *Virgibacillus* sp. SK37 (SK37, Figure 5.1a, b). *Virgibacillus* was dominant genus and its cumulative abundance of 50-80%. *Virgibacillus* was the moderately halophilic bacteria and widely distributed in environments containing high NaCl concentrations, such as saline lakes and fish sauce (Amziane et al., 2013;Tanasupawat et al., 2000). *Bacillus*, *Brevibacillus*, and *Tetragenococcus* were found with low relative abundance in SK37 (Figure 5.1b). *Tetragenococcus* was the most dominant bacteria detected in fish sauce added *T. halophilus* MS33 at 1-6 months, with *Virgibacillus* being the second abundance (Figure 5.1a, b).

Table 5.2 Relative abundance (%) of bacterial composition of fish sauce without starter culture (control) at 1-6 months.

		Control											
		1		2		3		4		5		6	
No.	Genus	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1	*Virgibacillus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	*Tetragenococcus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	*Bacillus*	3.28	6.89	3.28	34.53	9.28	28.57	31.92	7.35	33.55	7.06	5.16	26.31
4	*Brevibacterium*	2.22	3.37	2.22	5.59	4.86	2.45	8.78	5.44	0.30	10.59	4.97	4.43
5	*Staphylococcus*	5.01	3.65	5.01	6.59	0.72	1.58	4.72	3.04	0.22	3.02	36.46	1.84
6	*Prochlorococcus*	23.70	3.21	23.70	22.75	0.44	10.00	4.39	3.21	0.26	26.22	1.61	27.11
7	*Halomonas*	4.06	74.75	4.06	11.18	2.59	18.57	37.33	2.56	0.73	19.66	5.29	35.05
8	*Serinicoccus*	0.78	0.28	0.78	0.13	0.04	30.82	0.04	0.09	0.02	2.18	0.13	0.05
9	*Rhodococcus*	21.19	0.11	21.19	0.06	2.95	0.08	0.06	0.02	0.04	0.07	6.28	0.00
10	*Sediminibacterium*	24.72	0.00	24.72	0.00	0.64	0.00	0.02	0.05	0.05	0.00	0.45	0.29
11	*Salinicoccus*	6.88	0.22	6.88	0.45	1.06	0.04	3.56	0.16	0.07	3.36	2.13	0.38
12	*Salimicrobium*	0.18	1.23	0.18	0.84	0.50	7.14	0.04	0.18	1.03	3.25	0.42	0.81
13	*Alkalibacterium*	1.34	0.39	6.80	4.07	0.21	0.08	0.88	76.45	2.39	1.22	0.63	0.14
14	*Psychrobacter*	0.18	0.84	0.00	0.00	75.67	0.08	0.00	0.27	55.84	1.20	0.48	0.38
15	*Paracoccus*	0.85	1.12	0.00	4.39	0.17	0.04	1.67	0.14	0.38	0.35	0.17	0.40
16	*Janibacter*	2.72	0.33	1.17	0.06	0.03	0.00	4.89	0.30	0.01	3.01	2.71	0.02
17	*Rhodobacter*	1.02	0.11	0.00	0.06	0.24	0.00	0.02	0.23	1.22	3.50	6.93	0.10
18	*Oceanimonas*	0.00	1.23	0.00	0.06	0.00	0.04	0.00	0.02	1.21	0.04	13.46	1.48
19	*Salinivibrio*	0.00	2.10	0.00	8.38	0.00	0.00	0.00	0.05	0.03	0.04	10.59	0.00
20	*Streptococcus*	0.49	0.06	0.00	0.06	0.18	0.48	0.02	0.02	2.20	0.60	0.25	0.55
21	*Enterobacter*	0.71	0.11	0.00	0.78	0.14	0.04	0.58	0.14	0.11	0.32	0.63	0.07
22	*Lentibacillus*	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00	14.29	0.00	0.00
23	*Salinibacterium*	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.43
24	*Micrococcus*	0.04	0.00	0.04	0.00	0.30	0.00	1.08	0.02	0.33	0.04	1.25	0.17
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

R1, R2 = fish sauce samples from first (R1) and second (R2) batch.

Table 5.3 Relative abundance (%) of bacterial composition of fish sauce inoculated with *Virgibacillus* sp. SK37 (SK37) at 1-6 months.

		SK37											
		1		2		3		4		5		6	
No.	**Genus**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
1	*Virgibacillus*	85.33	44.55	54.42	54.60	63.17	86.45	77.13	49.29	44.19	57.08	71.24	44.35
2	*Tetragenococcus*	0.02	0.66	1.12	0.87	2.58	0.16	0.29	1.77	2.65	0.58	0.27	2.39
3	*Bacillus*	2.99	6.46	5.85	19.25	7.91	8.12	3.14	14.49	2.96	13.20	2.63	28.24
4	*Brevibacterium*	3.67	9.52	0.60	3.36	6.17	0.51	2.21	11.81	5.02	14.29	15.86	5.51
5	*Staphylococcus*	0.88	0.58	0.24	5.75	0.90	0.40	7.78	4.09	3.54	0.70	0.25	2.34
6	*Prochlorococcus*	0.20	0.29	0.04	1.63	0.58	0.70	0.27	0.40	1.16	1.52	0.33	1.79
7	*Halomonas*	0.16	26.98	2.89	2.20	6.87	0.88	2.32	12.76	6.52	3.68	1.44	6.06
8	*Serinicoccus*	0.00	0.89	2.63	2.12	0.25	0.03	0.05	2.83	2.61	1.08	0.14	0.85
9	*Rhodococcus*	0.02	0.07	0.01	1.72	0.01	0.04	0.05	0.08	0.60	0.12	0.07	0.06
10	*Sediminibacterium*	0.00	0.00	31.95	1.59	9.12	0.01	3.06	0.00	0.01	0.47	0.02	0.06
11	*Salinicoccus*	0.00	3.39	0.06	2.97	0.18	1.25	0.11	0.16	0.05	0.12	0.61	3.72
12	*Salimicrobium*	0.00	0.29	0.00	0.19	0.16	0.02	1.91	0.16	0.02	3.68	0.22	0.13
13	*Alkalibacterium*	0.18	2.66	0.06	0.56	0.07	0.22	0.67	0.24	5.77	0.23	0.16	0.26
14	*Psychrobacter*	0.00	0.06	0.00	2.09	0.40	1.00	0.00	1.51	24.32	3.03	3.20	0.39
15	*Paracoccus*	6.44	0.22	0.00	0.37	0.24	0.01	0.00	0.08	0.10	0.00	0.04	0.00
16	*Janibacter*	0.00	0.07	0.00	0.25	0.69	0.05	0.05	0.08	0.00	0.12	0.07	2.62
17	*Rhodobacter*	0.00	2.65	0.00	0.06	0.08	0.07	0.00	0.08	0.01	0.00	0.02	0.00
18	*Oceanimonas*	0.11	0.07	0.12	0.06	0.43	0.01	0.93	0.16	0.27	0.00	0.01	0.58
19	*Salinivibrio*	0.00	0.07	0.00	0.12	0.08	0.03	0.00	0.00	0.00	0.00	0.01	0.32
20	*Streptococcus*	0.00	0.07	0.00	0.19	0.00	0.01	0.00	0.00	0.15	0.00	0.01	0.06
21	*Enterobacter*	0.00	0.44	0.00	0.06	0.06	0.03	0.00	0.00	0.03	0.12	0.02	0.26
22	*Lentibacillus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	*Salinibacterium*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	*Micrococcus*	0.00	0.00	0.00	0.00	0.07	0.00	0.01	0.00	0.00	0.00	3.35	0.00
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

R1, R2 = fish sauce samples from first (R1) and second (R2) batch.

Table 5.4 Relative abundance (%) of bacterial composition of fish sauce inoculated with *T. halophilus* MS33 (MS33) at 1-6 months.

		MS33											
		1		2		3		4		5		6	
No.	Genus	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1	*Virgibacillus*	20.30	32.80	0.14	0.00	0.00	0.00	76.48	65.91	76.67	0.00	0.54	0.00
2	*Tetragenococcus*	74.14	30.73	49.91	47.61	5.12	4.22	0.64	0.45	4.78	50.86	72.40	15.94
3	*Bacillus*	0.19	10.81	47.45	4.50	2.68	4.14	2.31	11.15	0.75	10.98	7.93	35.62
4	*Brevibacterium*	0.08	4.92	1.20	1.60	84.27	8.27	1.49	1.99	8.10	1.84	0.62	8.97
5	*Staphylococcus*	0.15	1.63	0.11	0.46	0.27	2.81	4.83	2.09	0.59	1.40	1.87	5.35
6	*Prochlorococcus*	0.78	1.61	0.33	2.47	0.26	3.04	2.70	0.33	1.94	1.78	2.56	3.24
7	*Halomonas*	0.18	3.86	0.21	41.19	2.84	68.07	5.04	3.54	1.99	6.60	0.62	15.44
8	*Serinicoccus*	0.04	1.76	0.21	0.08	0.65	2.44	0.07	1.09	0.16	0.62	0.02	0.09
9	*Rhodococcus*	0.05	0.04	0.03	0.08	0.03	0.07	1.03	0.07	0.01	1.46	0.07	0.05
10	*Sediminibacterium*	2.23	3.13	0.02	0.08	0.27	0.00	1.28	8.34	0.25	0.00	0.13	0.00
11	*Salinicoccus*	0.24	4.60	0.04	0.49	0.08	1.48	0.62	2.54	0.04	1.40	0.17	2.50
12	*Salimicrobium*	0.03	1.38	0.07	0.26	1.79	1.25	0.30	0.22	0.27	6.07	7.31	9.09
13	*Alkalibacterium*	0.04	1.02	0.21	0.23	0.16	1.41	1.53	0.07	0.04	0.21	0.12	0.19
14	*Psychrobacter*	0.09	0.00	0.00	0.16	0.37	1.25	0.00	0.33	0.64	0.55	2.32	2.04
15	*Paracoccus*	0.33	0.86	0.01	0.39	0.34	0.59	0.96	1.09	0.60	0.14	0.05	0.28
16	*Janibacter*	0.08	0.23	0.00	0.13	0.01	0.11	0.02	0.04	0.01	6.54	0.02	0.05
17	*Rhodobacter*	0.01	0.04	0.00	0.03	0.13	0.07	0.09	0.04	0.05	0.03	0.06	0.05
18	*Oceanimonas*	0.00	0.08	0.00	0.08	0.01	0.18	0.46	0.41	0.03	0.03	0.03	0.14
19	*Salinivibrio*	0.01	0.27	0.00	0.08	0.03	0.33	0.02	0.04	0.01	8.89	2.94	0.05
20	*Streptococcus*	0.00	0.08	0.04	0.03	0.06	0.04	0.02	0.15	0.02	0.03	0.01	0.05
21	*Enterobacter*	0.03	0.12	0.00	0.05	0.61	0.07	0.07	0.11	0.02	0.28	0.20	0.84
22	*Lentibacillus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	*Salinibacterium*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	*Micrococcus*	1.00	0.04	0.00	0.03	0.03	0.15	0.02	0.00	3.01	0.28	0.01	0.05
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

R1, R2 = fish sauce samples from first (R1) and second (R2) batch.

Table 5.5 Relative abundance (%) of bacterial composition of fish sauce inoculated with co-cultures (MS33+SK37) at 1-6 months.

		MS33+SK37											
		1		2		3		4		5		6	
No.	Genus	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1	*Virgibacillus*	44.07	20.66	53.86	38.24	51.64	26.20	87.70	88.70	24.46	95.62	87.44	35.44
2	*Tetragenococcus*	11.91	44.27	1.09	1.89	2.75	0.21	1.62	0.28	2.42	0.63	2.78	21.81
3	*Bacillus*	40.18	8.26	36.65	16.67	1.28	5.58	1.55	0.53	0.12	0.56	1.99	14.56
4	*Brevibacterium*	0.30	2.24	0.33	9.78	1.92	4.86	1.34	2.98	0.30	0.54	0.45	2.42
5	*Staphylococcus*	0.58	5.67	0.18	6.23	0.08	2.43	0.06	0.31	0.58	0.20	1.13	6.81
6	*Prochlorococcus*	0.10	0.27	2.44	7.69	0.17	0.40	0.14	0.15	0.10	0.10	0.01	1.87
7	*Halomonas*	0.33	4.25	2.92	9.32	39.77	6.03	6.09	1.00	0.33	1.24	0.22	9.78
8	*Serinicoccus*	0.12	0.71	1.65	0.24	0.31	2.36	0.02	0.03	68.05	0.06	0.07	2.16
9	*Rhodococcus*	0.01	0.21	0.01	0.03	0.01	42.12	0.00	0.01	0.01	0.04	0.17	0.12
10	*Sediminibacterium*	0.00	0.00	0.02	1.01	0.12	0.09	0.01	0.00	0.00	0.00	0.03	0.20
11	*Salinicoccus*	0.50	3.78	0.01	0.70	0.05	0.18	1.15	0.20	0.55	0.04	1.46	0.40
12	*Salimicrobium*	0.01	0.21	0.24	0.24	0.10	0.85	0.10	0.03	0.01	0.22	0.37	0.60
13	*Alkalibacterium*	0.02	2.34	0.29	3.22	0.02	0.18	0.00	0.17	0.29	0.27	0.15	0.28
14	*Psychrobacter*	1.61	5.90	0.00	1.76	0.08	1.55	0.00	0.55	2.42	0.25	2.67	0.60
15	*Paracoccus*	0.18	0.14	0.20	0.20	1.45	0.37	0.01	0.00	0.28	0.04	0.12	0.40
16	*Janibacter*	0.01	0.07	0.01	0.84	0.01	0.12	0.11	0.01	0.01	0.04	0.02	1.87
17	*Rhodobacter*	0.01	0.41	0.00	0.07	0.01	0.30	0.00	0.00	0.01	0.01	0.05	0.04
18	*Oceanimonas*	0.01	0.21	0.00	0.00	0.01	0.06	0.06	0.50	0.01	0.01	0.02	0.04
19	*Salinivibrio*	0.00	0.14	0.02	0.10	0.12	0.22	0.00	0.01	0.00	0.04	0.02	0.04
20	*Streptococcus*	0.03	0.07	0.00	0.03	0.07	0.06	0.00	0.03	0.03	0.03	0.03	0.08
21	*Enterobacter*	0.02	0.07	0.07	1.67	0.03	5.75	0.01	4.51	0.02	0.05	0.77	0.08
22	*Lentibacillus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	*Salinibacterium*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	*Micrococcus*	0.00	0.14	0.00	0.07	0.01	0.06	0.01	0.00	0.00	0.02	0.02	0.40
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

R1, R2 = fish sauce samples from first (R1) and second (R2) batch.

Table 5.6 Relative abundance (%) of bacterial composition of fish sauce inoculated with sequential cultures (SK37_1+MS33) after 1 month at 1-6 months.

		SK37_1M+MS33											
		1		2		3		4		5		6	
No.	Genus	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1	*Virgibacillus*	84.32	1.67	35.35	23.83	5.82	58.98	0.89	4.63	15.07	6.89	75.51	9.63
2	*Tetragenococcus*	0.32	63.31	28.11	36.46	84.11	36.70	86.77	76.19	71.63	75.27	20.32	73.09
3	*Bacillus*	1.01	1.75	26.85	12.64	4.78	1.93	3.56	5.17	4.25	4.10	1.68	2.84
4	*Brevibacterium*	0.78	22.32	0.96	9.39	0.57	0.61	0.78	5.28	1.17	1.09	0.97	3.51
5	*Staphylococcus*	0.64	2.63	0.32	0.72	0.28	0.27	0.64	2.48	0.56	3.27	0.10	1.08
6	*Prochlorococcus*	0.32	0.50	0.30	5.78	0.07	0.23	1.42	0.40	0.52	1.45	0.15	0.54
7	*Halomonas*	10.8	1.87	7.50	4.33	0.28	0.44	0.78	1.51	4.95	1.38	0.20	3.61
8	*Serinicoccus*	0.57	0.63	0.02	2.96	0.07	0.08	0.57	0.28	0.66	0.41	0.34	1.64
9	*Rhodococcus*	0.04	0.06	0.02	0.34	0.00	0.04	0.04	0.37	0.06	0.61	0.07	0.12
10	*Sediminibacterium*	0.00	0.03	0.25	0.24	0.04	0.04	0.00	0.18	0.02	0.10	0.08	0.04
11	*Salinicoccus*	0.04	0.25	0.01	0.72	2.48	0.25	0.04	0.18	0.56	0.10	0.07	1.01
12	*Salimicrobium*	0.27	0.53	0.03	0.96	0.04	0.16	3.58	0.28	0.04	1.23	0.07	0.23
12	*Alkalibacterium*	0.11	2.47	0.03	0.24	0.34	0.08	0.11	0.46	0.00	0.61	0.17	0.58
11	*Psychrobacter*	0.36	0.22	0.00	0.24	0.19	0.08	0.36	0.28	0.00	0.41	0.15	0.19
16	*Paracoccus*	0.04	1.35	0.03	0.44	0.01	0.04	0.04	0.09	0.08	0.31	0.02	1.27
17	*Janibacter*	0.04	0.03	0.03	0.00	0.16	0.00	0.08	0.18	0.04	0.51	0.00	0.12
19	*Rhodobacter*	0.00	0.03	0.01	0.00	0.01	0.00	0.00	0.00	0.02	0.00	0.00	0.15
14	*Oceanimonas*	0.18	0.03	0.16	0.24	0.09	0.08	0.18	0.09	0.00	0.20	0.06	0.04
20	*Salinivibrio*	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.04
21	*Streptococcus*	0.00	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.15
10	*Enterobacter*	0.18	0.00	0.00	0.48	0.62	0.00	0.18	1.94	0.31	2.04	0.03	0.12
24	*Lentibacillus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	*Salinibacterium*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	*Micrococcus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

R1, R2 = fish sauce samples from first (R1) and second (R2) batch.

When fermentation time increased to 4 and 5 months, *Virgibacillus* became the predominant genus with 76% relative abundance in MS33 (Figure 5.1b), demonstrating that *T. halophilus* MS33 might promote growth of *Virgibacillus.*

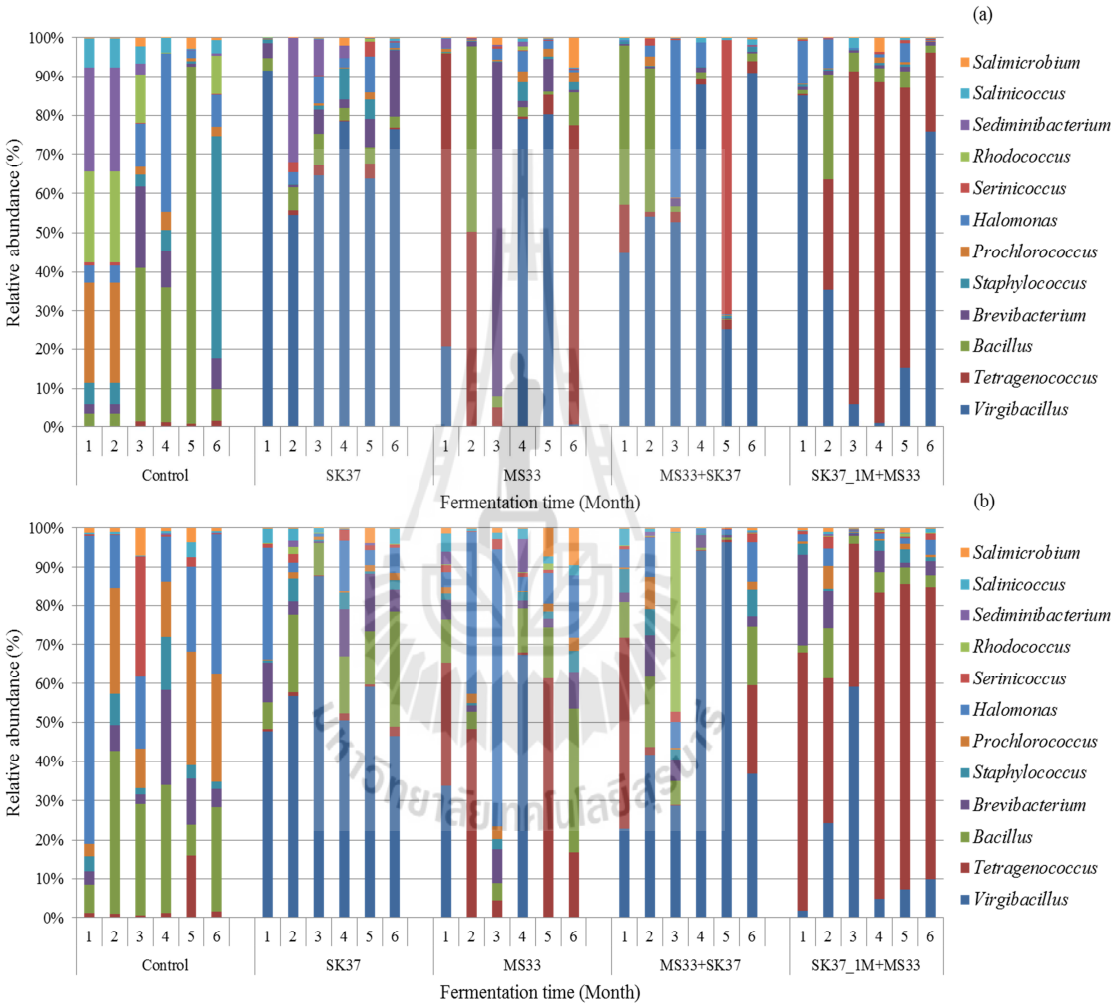

Figure 5.1 Relative abundance of bacterial 16S rRNA gene from fish sauce inoculated with single and co-culture at genus level identified with 16S rRNA database from QIIME. Fish sauce samples were prepared in the first (a) and second batch (b).

For simultaneous co-cultures (MS33+SK37), variation of %relative abundance of *Virgibacillus* and *Tetragenococcus* was found during fermentation and %relative

abundance of *Virgibacillus* was higher than that of *Tetragenococcus*. The MS33+SK37 sample was covered by *Virgibacillus*, *Bacillus*, and *Halomonas* when fermentation progressed. It should be noted that *T. halophilus* MS33 was relatively low abundance in MS33+SK37, except the 2nd batch of fermentation at 1 and 6 months (Figure 2b). For sequential inoculation (SK37_1M+MS33), *Tetragenococcus* showed relatively higher abundance than *Virgibacillus* (Figure 5.1a, b). Since *Virgibacillus* sp. SK37 produced extracellular proteinase to hydrolyze fish protein to small peptides/ oligopeptides which acted as a substrate for *T. halophilus* MS33 and promoted growth of *T. halophilus* MS33. *Bacillus, Brevibacillus, Staphylococcus, Prochlorococcus, and Halomonas* were found as minor bacterial population (Figure 5.1a, b). This study demonstrated the application of Ion Torrent PGM® sequencing for the detection of microbial profiling and diversity in fish sauce inoculated with starter culture. Ion Torrent PGM® sequencing has also been used to assess microbial community diversity in alkaline water treatment and piggery waste (Fujimoto et al., 2014; Whiteley et al., 2012). They found that bacterial community obtained from Ion Torrent PGM® sequencing was comparable to that obtained from 454 pyrosequencing.

Commercial Thai fish sauce fermented for 1-12 months is dominated by *Halanaerobium, Staphylococcus, Tetragenococcus, Salinivibrio, Lactobacillus, Bacillus, Salinicoccus, Pseudomonas, Flavobacterium, Virgibacillus,* and *Lentibacillus* (Chuea-nongthon, 2013). However, *Halanaerobium, Lactobacillus, Pseudomonas* and *Flavobacterium* were not found in this study. *Lentibacillus* and *Salinivibrio* were also found to be insignificant (Table 5.2-5.6). Differences between these 2 studies could be due to variation in fish sauce samples. In this study, samples were prepared in laboratory, while those of commercial fish sauce were collected from

fish sauce plant. Accumulation of microflora of industrial fermentation could be regarded as back-slopping and serve as natural bacteria in the new batch. In contrast, inoculated samples were prepared in clean containers. Therefore, diversity of microorganisms would be different. *Bacillus* and *Salinicoccus* were found in marine fish and fish sauce fermentation and were considered as halotolerant and halophilic, respectively (Noguchi et al., 2004; Chamroensaksri et al., 2009; Tanasupawat et al., 2000). In addition, abundance of *Halomanas*, *Prochlorococcus*, *Sediminibacterium*, and *Rhodococcus* have not been previously reported in fish sauce. It might be due to different source of raw material, particularly anchovy fish and solar salt), leading to different microflora diversity in fish sauce. Nam, Park, and Lim (2012) reported that predominant microbial population of *kochujang* (Korean traditional food made from fermented soybeans, wheat, and red pepper powder) analyzed by pyrosequencing included *Bacillus subtilis* and *B. licheniformis* but *B. amyloliquefaciens*, *B. pumilus*, and *B. sonorensis* were broadly distributed to *kochujang* samples prepared from meju (dried fermented soybeans). Variations in microbial profiling were due to different sources of raw material and manufacturing environment.

Table 5.7 showed majority bacteria found in all samples. *Virgibacillus*, *Tetragenococcus*, *Bacillus*, *Brevibacillus*, *Staphylococcus*, and *Prochlorococcus* were 6 genera detected in all inoculated samples. *Virgibacillus* and *Tetragenococcus* were not found in the controls (Table 5.7), indicating that they were minority microflora. *Virgibacillus* in fish sauce inoculated with *Virgibacillus* sp. SK37 as single culture were predominant bacteria throughout the fermentation process. *Virgibacillus* and *Tetragenococcus* were identified as *V. halodentitrificans* and *T. halophilus*, respectively, based on 16S rRNA sequencing by QIIME database. Based on %relative

Table 5.7 Relative abundance of majority bacteria found in all fish sauce samples inoculated with single and co-cultures and fermented for 1-6 months.

Sample	Fermentation time (month)	Relative abundance (%)					
		Virgibacillus	*Tetragenococcus*	*Bacillus*	*Brevibacterium*	*Staphylococcus*	*Prochlorococcus*
Control	1	0.00	0.00	19.81	10.89	16.87	52.42
	2	0.00	0.00	31.63	12.15	40.52	15.70
	3	0.00	0.00	65.37	12.62	3.97	18.04
	4	0.00	0.00	57.03	20.65	11.27	11.04
	5	0.00	0.00	50.00	13.41	3.99	32.60
	6	0.00	0.00	29.17	8.71	35.50	26.62
SK37	1	83.71	0.43	6.09	8.50	0.94	0.32
	2	73.80	1.34	16.99	2.68	4.05	1.13
	3	84.22	1.54	9.02	3.76	0.73	0.72
	4	73.21	1.19	10.21	8.12	6.87	0.39
	5	68.94	2.20	11.00	13.15	2.88	1.83
	6	65.98	1.52	17.62	12.20	1.48	1.21
MS33	1	29.81	58.87	6.18	2.81	1.00	1.34
	2	0.09	62.60	33.35	1.80	0.37	1.80
	3	0.05	8.12	5.93	80.42	2.67	2.87
	4	83.57	0.64	7.90	2.04	4.06	1.78
	5	48.02	34.84	7.34	6.22	1.25	2.33
	6	0.35	56.98	28.09	6.19	4.66	3.74
MS33+ SK37	1	36.26	31.47	27.13	1.43	3.50	0.21
	2	52.61	1.70	30.46	5.77	3.66	5.79
	3	79.82	3.04	7.03	6.95	2.58	0.58
	4	95.16	1.02	1.12	2.33	0.20	0.16
	5	95.58	2.43	0.54	0.67	0.62	0.16
	6	69.54	13.92	9.37	1.62	4.49	1.07
SK37_1M+ MS33	1	47.88	35.43	1.54	12.87	1.82	0.46
	2	32.75	35.73	21.85	5.73	0.58	3.37
	3	33.34	62.16	3.45	0.60	0.29	0.16
	4	2.93	86.58	4.64	3.22	1.66	0.97
	5	11.85	79.29	4.51	1.22	2.07	1.06
	6	44.95	49.31	2.39	2.37	0.62	0.36

abundance of majority bacteria found in all samples, *Virgibacillus* was predominant in fish sauce added with *Virgibacillus* sp. SK37. *Tetragenococcus* was predominant in MS33 at 1, 2, 5, and 6 months (Table 5.7). Interestingly, *Virgibacillus* was predominant in fish sauce inoculated with simultaneous co-cultures (MS33+SK37), but *Tetragenococcus* was found in low population. In contrast, fish sauce with

sequential inoculation showed predomiant population of *Tetragenococcus*. The results indicated that addition of *T. halophilus* MS33 after *Virgibacillus* sp. SK37 might promote growth of the former as fish proteins were digested by *Virgibacillus* sp. SK37 leading to small peptides serving as substrates for *T. halophilus* MS33 as describe above. Both bacteria were classified as halophilic bacteria. Thongsanit et al. (2002) reported that *T. halophilus* and *T. muriaticus* were dominated at 1-7 months of fish sauce fermentation. However, *T. muriaticus* was not found in this study. This might be due to inoculation limited bacterial diversity. Addition of *Virgibacillus* sp. SK37 in fish sauce suppressed growth of microflora such as *Bacillus*, *Brevibacterium*, *Staphylococcus*, and *Prochlorococcus* at 2-6 months of fermentation (Table 5.7).

5.5 Conclusions

This is the first study to investigate microbial profiling of fish sauce inoculated with starter cultures using Ion Torrent PGM® sequencing. Uninoculated fish sauce was dominated by various genera at the first month, without *Virgibacillus* and *Tetragenococcus*. Inoculation of starter cultures suppressed growth of microflora and became the majority throughout fermentation. Addition of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in sequential order seemed to promote growth of *T. halophilus* MS33. Understanding microbial community dynamics of starter culture and their impact on microflora would help in the development of starter culture technology.

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CHAPTER VI

CONCLUSIONS

The developed qPCR methods showed specific detection and quantification of *Virgibacillus* sp. SK37 and *Tetragenococcus halophilus* MS33 used as starter cultures in fish sauce fermentation. Vir1086 and Tet48 probe were designed from alkaline serine protease-X gene (*aprX*) of *Virgibacillus* sp. SK37 and internal transcribed spacer (ITS) of *T. halophilus* MS33, respectively, using specific TaqMan hydrolysis probes. Vir1086 and Tet48 probe were species-specific for *V. halodenitrificans* and *T. halophilus* without cross reacting, respectively. They also did not cross react with microflora isolated from fish sauce plant. The qPCR methods showed good correlation efficiency of 101.1% for *V. halodenitrificans* and 90.2% for *T. halophilus.* The repeatability and reproducibility of the methods were within 37% and 33% of relative standard deviation, respectively. PMA was used to eliminate DNA of dead cell on qPCR. The quantification limits of the assays were 10^3 Cells/mL and 10^2 Cells/mL for *V. halodenitrificans* and *T. halophilus,* respectively, when tested in fish sauce samples. The developed PMA-qPCR methods were successfully applied to monitor changes in *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in a Spanish mackerel fish sauce fermentation model.

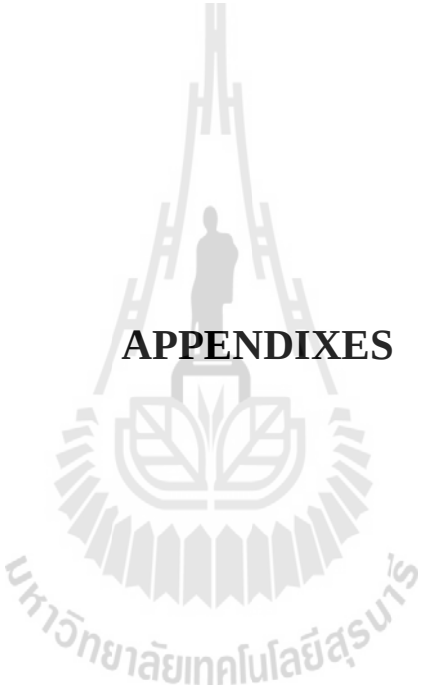
After the qPCR method was developed and validated, it was applied to monitor changes of *Virgibacillus* sp. SK37 and *T. halophilus* MS33 in anchovy sauce fermented for 180 days. Based on the qPCR results, fish sauce prepared by sequential inoculation of *Virgibacillus* sp. SK37 followed by *T. halophilus* MS33 after one month (SK37_1M+MS33) showed higher survival rate of bacteria growth than single culture inoculation approximately 0.5-1.0 LogCells/mL at 150-180 days of fermentation. Bacterial

count by spread plate technique was slightly lower than the qPCR due to the limitation of detecting viable but nonculturable (VBNC) cells. Fish sauce inoculated with either single culture of *Virgibacillus* sp. SK37 or *T. halophilus* MS33 or the combined cultures showed higher content of free glutamic acid than the control ($P < 0.05$). α-Amino contents of fish sauce added with combined cultures were the highest ($P < 0.05$). The sample SK37_1M+MS33 showed lower amount of histamine than the control ($P < 0.05$) and contained higher levels of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal content when compared to the control ($P < 0.05$). The use of combined cultures did not increase sulfur-containing compounds that contributed to undesirable note. Addition of starter culture, particularly co-cultures in sequential order (SK37_1M+MS33) showed potential to improve volatile compound and chemical compositions of fish sauce. Accurate enumeration of starter cultures during fermentation can be achieved using specific probes and qPCR.

Ion Torrent Personal Genome Machine (PGM®) technique was successfully applied to monitor changes in starter cultures and microflora population of fish sauce inoculated with starter cultures. Inoculation of *Virgibacillus* sp. SK37 and *Tetragenococcus halophilus* MS33 as a single or combined cultures suppressed abundance of microflora. Based on 16S rRNA genes from the metagenome, 12 genera were found in all inoculated fish sauce samples. Dominant bacteria found throughout fermentation of all samples were *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Tetragenococcus*, *Virgibacillus*, and *Prochlorococcus*. In contrast, *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Halomonas*, *Prochlorococcus*, *Rhodococcus*, and *Sediminibacterium* were predominantly found in the control (without starter inoculation). *Virgibacillus* sp. was predominant in the *Virgibacillus* sp. SK37-inoculated samples throughout the course of fermentation. *Tetragenococcus* was predominant in fish sauce inoculated with *T. halophilus* MS33 at 1, 2, 5, and 6 months. It should be noted that *Virgibacillus* was predominant in the fish sauce inoculated with co-

culture should be noted that *Virgibacillus* was predominant in the fish sauce inoculated with co-cultures (MS33+SK37), but *Tetragenococcus* was found in low population. In contrast, fish sauce with sequential inoculation (SK37_1M+MS33) showed predominant population of *Tetragenococcus*. The use of sequential inoculation can increase survival rate of *T. halophilus* MS33.

APPENDIXES



APPENDIX A

Preparations of culture media

1. Culture media

1.1 De Man, Rogosar and Sharpe broth (MRS broth) containing 5 %NaCl

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
Sodium chloride	50.0	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.

1.2 De Man, Rogosar and Sharpe agar (MRS agar) containing 5 %NaCl

The components were similar with MRS broth containing 0.5% $CaCO_3$ and 5 %NaCl with added 15.0 g/L agar. The medium was autoclaved at 115 °C for 10 min.

1.3 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.

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	100.0	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.

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

Tryptone	5.0	g
Yeast extract	2.5	g
Dextrose	1.0	g
Sodium chloride	100.0	g
Agar	15.0	g
Added distilled water and brought volume up to	1,000.0	ml

pH 7.0 $\pm$ 0.2

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

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

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

pH 7.0 $\pm$ 0.2

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

1.6 JCM 168 broth

Casamino acids	5.0
Yeast extract	5.0
Sodium glutamate	1.0
tri-Sodium citrate	3.0
Potassium chloride	2.0
$MgSO_4.7H_2O$	20.0
Sodium chloride	50.0
$FeCl_2.4H_2O$	36.0
$MnCl_2.4H_2O$	0.36
Add distilled water to bring volume up to	1,000.0

pH 7.0 $\pm$ 0.2

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

APPENDIX B

Deposition of internal transcribe spacer (ITS) in GenBank

(U.S.A.)

Table 1B Internal transcribe spacer (ITS) of *Tetragenococcus halophilus* MS33 in GenBank (U.S.A.).

Bacterial strain	**Internal transcribe spacer (ITS)**	**Length of sequence (bp)**	**NCBI acession no.**
T.halophilus MS33	ITS1	468	KP638351
	ITS2	599	KP638352
	ITS4	538	KP638353
	ITS6	660	KP638354

APPENDIX C

Volatile compound chromatograms of fish sauce

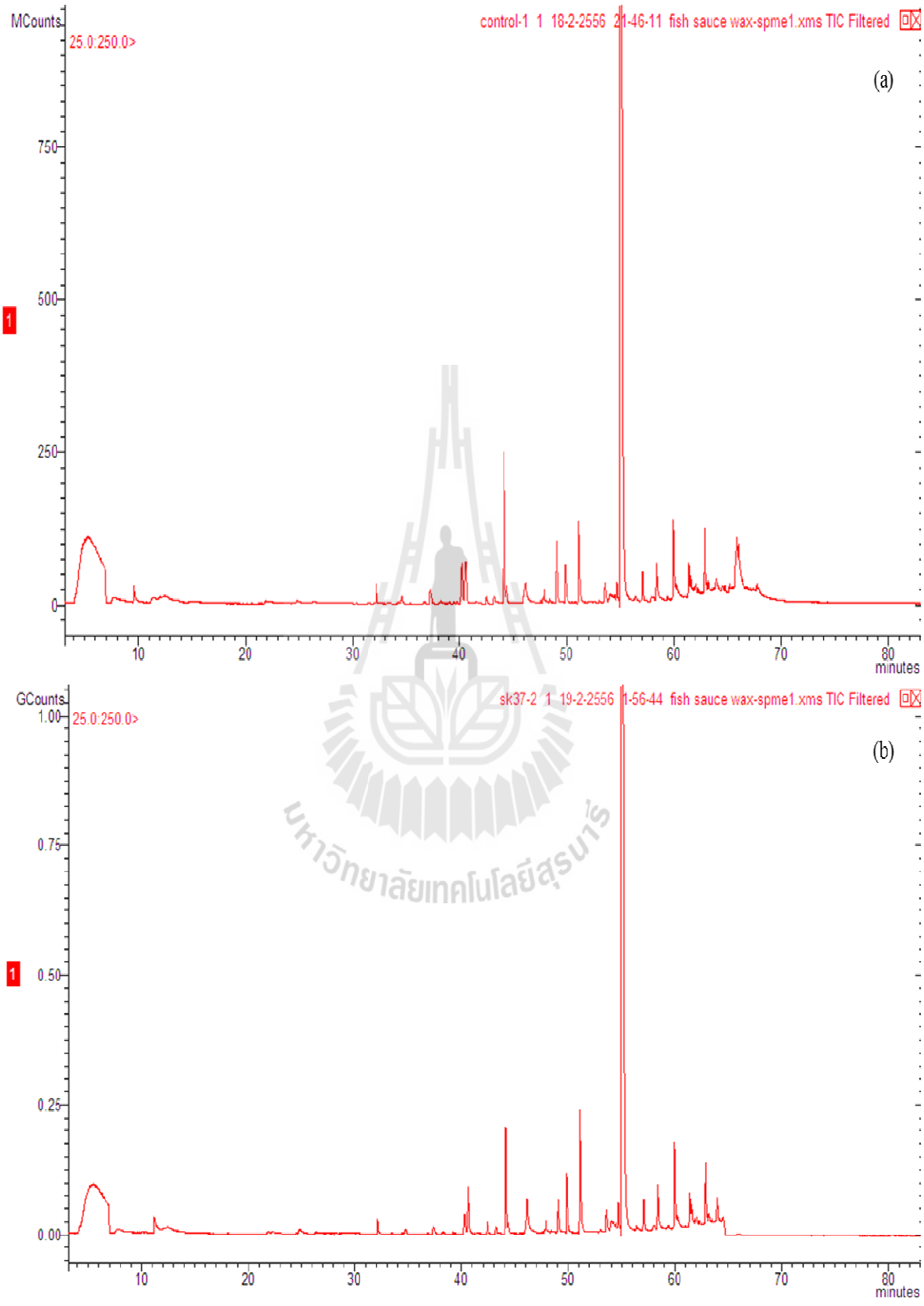


Figure 1C Chromatograms of volatile compounds of control (a) and fish sauce samples inoculated with *Virgibacillus* sp. SK37 (b) fermented for 6 months.

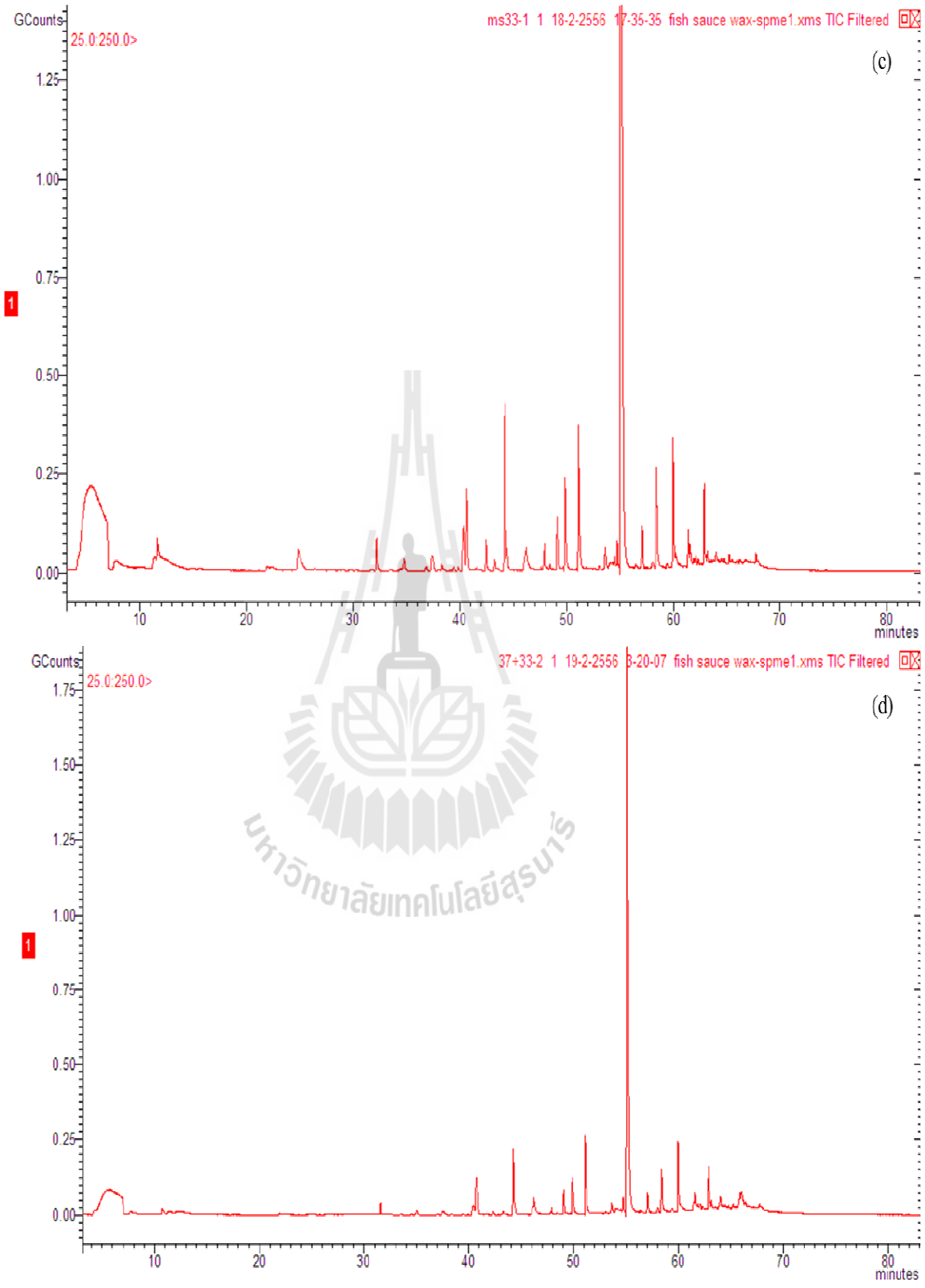


Figure 2C Chromatograms of volatile compounds of fish sauce samples inoculated with *T. halophilus* MS33 (c) and co-cultures (*T. halophilus* MS33 and *Virgibacillus* sp. SK37, MS33+Sk37) (d) fermented for 6 months.

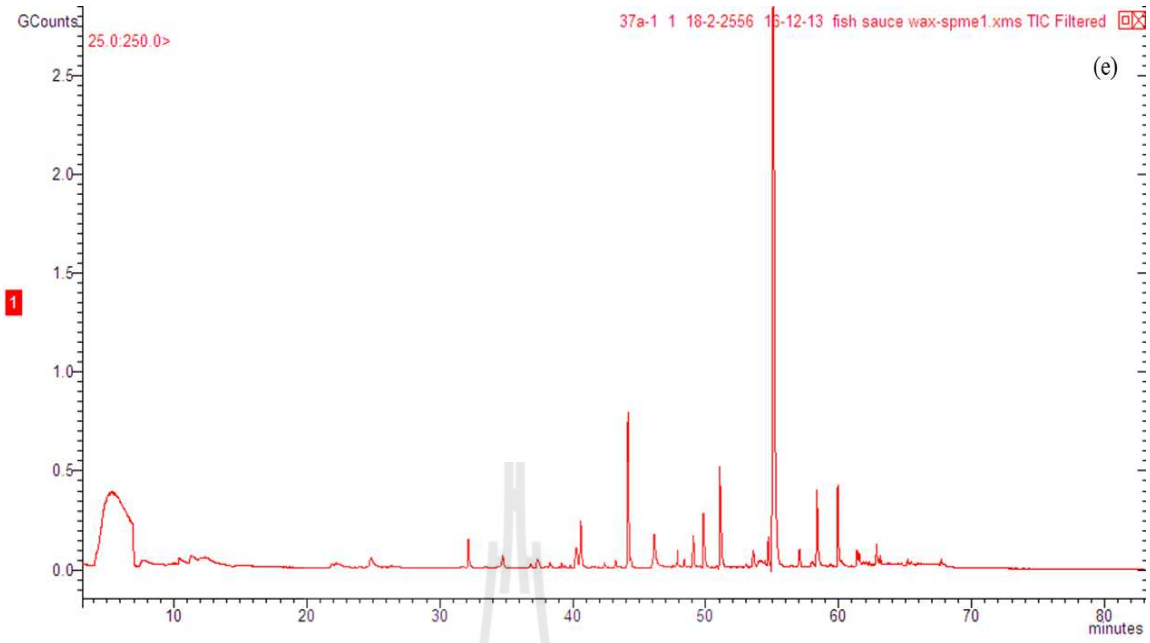


Figure 3C Chromatograms of volatile compounds of fish sauce samples inoculated with sequential cultures (added *Virgibacillus* sp. SK37 for a month, followed by *T. halophilus* MS33, SK37_1M+MS33) (e) fermented for 6 months.

BIOGRAPHY

Natteewan Udomsil was born in November 30, 1980 in Nan. She received Bachelor Degree in B.Sc. (Food Technology) from Institute of Agricultural Technology, Suranaree University of Technology (SUT), Thailand in 2003. 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. She presented her research work in the 7th Agro-industrial conference, June 22-24, 2005, BITEC, Bangkok, Thailand with excellent poster presentation award. In 2005-2008, she was granted a scholarship by National Center for Genetic Engineering and Biotechnology (BIOTEC) for Master program of Food Technology at SUT. Her Master Thesis received 2nd prize of Best Thesis in Food Science held by The Federation of Thai Industries in June 18-19, 2009. She also published 2 articles of her research work in highly regarded international journals. In 2009-2014, she received the Higher Education Commission Scholarship from Ministry of Education for her Ph.D. study. During her graduate study, she had opportunities to present her research works at Institute of Food Technologists (IFT) Annual Meeting and Food Expo (Anaheim, CA, U.S.A., June 6-10th, 2009) and European Federation of Food Science and Technology (EFFoST) Annual Meeting 2012 (Montpellier, France, November 21-23, 2012).