GENOME, PHYSICOCHEMICAL PROPERTIES AND ESTERASE GENE CLONING OF NOVEL *LENTIBACILLUS* AND *HALOBACILLUS* SPECIES

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ปีชีส์ใหม่

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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อัฐพร บุญเจริญ : จีโนม คุณสมบัติทางเคมีกายภาพ และการโคลนยีนเอสเทอเรสของเลนติบาซิลลัสและฮาโลบาซิลลัสสปีชีส์ใหม่. (GENOME, PHYSICOCHEMICAL PROPERTIES AND ESTERASE GENE CLONING OF NOVEL *LENTIBACILLUS* AND *HALOBACILLUS* SPECIES) อ.ที่ปรึกษาหลัก : ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาร่วม : ดร.วรรณพ วิเศษสงวน,ดร.เพลินพิศ ลักษณะนิล

แบคทีเรียชอบเค็มปานกลางจำนวน 831 ไอโซเลทที่คัดแยกได้จากตัวอย่างหลายชนิดในประเทศไทยถูกนำมาคัดกรองกิจกรรมของเอนไซม์ย่อยไขมัน พบว่า 349 ไอโซเลทมีกิจกรรมบนอาหารแข็ง และ 322 ไอโซเลทมีการผลิตเอนไซม์ในอาหารเหลว การศึกษาการจัดกลุ่มและพิสูจน์เอกลักษณ์โดยการใช้ผลลายพิมพ์ดีเอ็นเอโดยใช้คู่ของไพรเมอร์ $(GTG)_5$+ERIC2 และผลการวิเคราะห์ 16S rRNA ของ 322 ไอโซเลท สามารถจัดกลุ่มได้ 180 รูปแบบ และพิสูจน์เอกลักษณ์ได้เป็นแบคทีเรียในจีนัส *Bacillus*, *Halobacillus, Lentibacillus, Marinobacter, Thalassobacillus* และ *Virgibacillus* จากผลการศึกษาลักษณะอนุกรมวิธานแบบโพลีฟาสิกและการวิเคราะห์จีโนมของสายพันธุ์ที่คัดเลือก ได้แก่ สายพันธุ์ SSKP1-9^T มีรูปร่างแท่ง ชอบอากาศ สร้างสปอร์ ย้อมติดสีแกรมบวก การวิเคราะห์ยีนบริเวณ 16S rRNA พบว่าใกล้เคียงกับ *Lentibacillus juripiscarius* TISTR 1535^T (98.7%) และ *L. halophilus* TISTR 1549^T (97.2%) โดยผลการศึกษาชี้ให้เห็นว่าสายพันธุ์ SSKP1-9^T เป็นแบคทีเรียชอบเค็มปานกลางสปีชีส์ใหม่ในจีนัส *Lentibacillus* จึงตั้งชื่อว่า *Lentibacillus lipolyticus* (SSKP1-9^T=JCM 32625^T=TISTR 2597^T) ส่วนสายพันธุ์ SKP4-6^T มีรูปร่างแท่ง สร้างสปอร์ ชอบอากาศ ย้อมติดสีแกรมบวก ผลการวิเคราะห์ยีนบริเวณ 16S rRNA ใกล้เคียงกับ *Halobacilus salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T และ *H. yeomjeoni* KCTC 3957^T เท่ากับ 98.6% ผลการศึกษานี้และการวิเคราะห์จีโนมชี้ให้เห็นว่าสายพันธุ์ SKP4-6^T เป็นแบคทีเรียสปีชีส์ใหม่ในจีนัส *Halobacillus* จึงตั้งชื่อว่า *Halobacillus fulvus* (SKP4-6^T=JCM 32624^T=TISTR 2595^T)

ผลการวิเคราะห์ข้อมูลจีโนมของสายพันธุ์ SSKP1-9^T และ SKP4-6^T พบยีนที่เกี่ยวข้องกับการผลิตเอนไซม์เอสเทอเรสที่น่าสนใจจำนวน 8 และ 5 ยีนตามลำดับ จึงได้นำยีนดังกล่าวมาทำการโคลนเข้าสู่พลาสมิด pSaltExSePR5 และถ่ายโอนเข้าสู่ *Bacillus subtilis* WB800 รีคอมบิแนนท์โคลน APNFEMBD_02578-C112-40 จากสายพันธุ์ SSKP1-9^T และ NKILIEJB_01195-C9-35 จากสายพันธุ์ SKP4-6^T แสดงกิจกรรมของเอนไซม์เอสเทอเรสสูงที่สุด จึงนำไปศึกษาต่อ ยีน APNFEMBD_02578-C112-40 มีความคล้ายคลึงกับโปรตีนในแฟมิลี acyl-CoA thioesterase ที่พบในจีนัส *Lentibacillus* และยีน >NKILIEJB_01195-C9-35-40 มีความคล้ายคลึงกับโปรตีนในแฟมิลี alpha/beta hydrolase fold ที่พบในจีนัส *Halobacillus* ยีนทั้งสองมีน้ำหนักโมเลกุลจากการคำนวณลำดับกรดอะมิโนเท่ากับ 16.2 kDa และ 27.7 kDa ตามลำดับ และแสดงกิจกรรมสูงสุดที่ความเข้มข้นเกลือโซเดียมคลอไรด์ในช่วง 15-30% มีกิจกรรมสูงสุดที่ความเข้มข้น 22% เอนไซม์จากโคลน APNFEMBD_02578-C112-40 แสดงกิจกรรมในช่วง 25-60 องศาเซลเซียส มีกิจกรรมสูงสุดที่ 40-55 องศาเซลเซียส ในขณะที่เอนไซม์จากโคลน NKILIEJB_01195-C9-35 มีกิจกรรมสูงที่สุดที่ 55 องศาเซลเซียส และสูญเสียกิจกรรมอย่างรวดเร็วที่อุณหภูมิสูงกว่า 65 องศาเซลเซียส เอนไซม์ทั้งสองแสดงกิจกรรมที่สูงในช่วงพีเอชตั้งแต่ 6.0 ถึง 9.0 และมีกิจกรรมสูงสุดพีเอช 7.0-8.0 ตามลำดับ เอนไซม์มีความเสถียรหลังจากบ่มที่ 55 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ในสภาวะที่มีความเข้มข้นเกลือโซเดียมคลอไรด์สูงกว่า 17% และยังคงทำงานได้ประมาณ 80% ในช่วงอุณหภูมิ 5-45 องศาเซลเซียส พีเอช 6.0 ถึง 9.0 ทั้งในสภาวะที่มีหรือไม่มีเกลือโซเดียมคลอไรด์ นอกจากนี้ยังพบว่ารีคอมบิแนนท์เอนไซม์ทั้งสองชนิดมีความจำเพาะสูงต่อสารตั้งต้นประเภทเอสเทอร์ชนิดคาร์บอนสายสั้น จากผลการศึกษาเบื้องต้นจึงน่าจะระบุได้ว่ารีคอมบิแนนต์เอนไซม์ที่ผลิตจากทั้งสองโคลนมีความน่าจะเป็นเอนไซม์เอสเทอเรส ชอบเกลือ และทนอุณหภูมิสูง

สาขาวิชา	เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5776453233 : MAJOR PHARMACEUTICAL CHEMISTRY AND NATURAL PRODUCTS
KEYWORD: moderately halophilic bacteria, Lentibacillus lipolyticus, Halobacillus fulvus, esterase, DNA-Fingerprinting

Auttaporn Booncharoen : GENOME, PHYSICOCHEMICAL PROPERTIES AND ESTERASE GENE CLONING OF NOVEL *LENTIBACILLUS* AND *HALOBACILLUS* SPECIES. Advisor: Prof. SOMBOON TANASUPAWAT, Ph.D. Co-advisor: Wonnop Vissessanguan, Ph.D., Plearnpis Luxananil, Ph.D.

A total of 831 moderately halophilic bacteria isolated from various samples in Thailand were screened for their lipolytic activity. Three hundred and forty-nine isolates showed activity on agar plate while 322 isolates exhibited in broth. The grouping and identification of 322 isolates based on DNA-fingerprinting using $(GTG)_5$+ERIC2 primer and 16S rRNA gene sequence, 180 DNA patterns could be generated. The result indicated that the representative isolates belonged to genera *Bacillus, Halobacillus, Lentibacillus, Marinobacter, Thallossobacillus*, and *Virgibacillus*. On the basis of polyphasic taxonomic study and genome analysis, a selected moderately halophilic strain SSKP1-9^T, Gram-staining-positive, aerobic, endospore-forming, rod-shaped was closely related to *Lentibacillus juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T, 98.7 and 97.2%, respectively based on 16S rRNA gene sequence similarity. On the basis of these results, the strain represents a novel species of the genus *Lentibacillus*, for which the name *Lentibacillus lipolyticus* is proposed (SSKP1-9^T=JCM 32625^T=TISTR 2597^T). A Gram-staining-positive, aerobic, endospore-forming, rod-shaped, strain SKP4-6^T was closely related to *Halobacillus salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T and *H. yeomjeoni* KCTC 3957^T, with 98.6% respectively, based on 16S rRNA gene sequence similarity. On the basis of these results taxonomy and whole-genome analysis, strain SKP4-6^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus fulvus* is proposed (SKP4-6^T =JCM 32624^T=TISTR 2595^T).

Based on whole-genome analysis, strain SSKP1-9^T and SKP4-6^T contained protein-encoding esterase for 8 and 5 genes, respectively. The genes were then cloned into the pSaltExSePR5 plasmid and transferred to *Bacillus subtilis* WB800. Two recombinant, >APNFEMBD_02578-C112-40 from strain SSKP1-9^T and >NKILIEJB_01195-C9-35 from strain SKP4-6^T showed the highest esterase activity and were therefore studied. These two genes identified major matches with closely related to a member of family acyl-CoA thioesterase and the alpha/beta hydrolase fold founded in genus *Lentibacillus* and *Halobacillus*, respectively. Of the two recombinant enzymes, the first one has a molecular weight of 16.2 kDa, and the other was 27.7 kDa, identical to the predicted protein size from the amino acid sequence. Two recombinant enzymes reached the maximum activity at 15-30% NaCl, and the highest activity was shown at 22 % NaCl. The recombinant >APNFEMBD_02578-C112-40 displayed the activity around 25-60 °C, and the maximum activity was observed at 40-55 °C while >NKILIEJB_01195-C9-35 had optimal activity at 55 °C and lost its activity rapidly above 65 °C of the remaining activity. They stable in NaCl concentration above 17% (w/v) after incubated at 55 °C for 1 hr. Two enzymes showed good stability and remained activity about 80% in a temperature range of 5-45 °C after 1 hr of incubation at 50 °C with or without NaCl. Theses enzymes reached high activity over a wide range of pH from 6.0 to 9.0, recording maximal activity at pH 7.0-8.0 and displayed excellent stable in with or without NaCl at various pH ranges (pH 6.0 to 9.0). Besides, the two recombinant enzymes were found to have higher esterase activity against *p*-nitrophenyl butyrate (C4:0) comparing to other esters substrate. Based on finding results, two recombinant enzymes were classified as halophilic and thermophilic esterase

Field of Study:	Pharmaceutical Chemistry and Natural Products	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Auttaporn Booncharoen

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	=	American Type Culture Collection
bp	=	Base pairs
BLAST	=	Basic Local Alignment Search Tool
℃	=	Degree celsius
C.I.	=	Confidence intervals
DDBJ	=	DNA Data Bank of Japan
dNTPs	=	Deoxynucleotide triphosphates
dATP	=	2'-deoxyadenosine 5'-triphosphate solution
DPG	=	Diphosphatidylglycerol
DSMZ	=	German Collection of Microorganisms and Cell Cultures GmbH
EDTA	=	ethylenediaminetetraacetic acid
EMBL	=	European Molecular Biology Laboratory
EtBr	=	Ethidium bromide
g	=	Gram
G+C	=	Guanine-plus-cytosine
GenBank	=	NIH genetic sequence database
HCl	=	Hydrochloric acid
H_2O	=	Water
hr	=	Hour
*is*DDH	=	*in silico* DNA-DNA hybridization
JCM	=	Japan Collection of Microorganisms
KCl	=	Potassium chloride
KCTC	=	Korean Collection for Type Cultures
KDa	=	Kilo Dalton
L	=	Liter
Log	=	logarithm
M	=	Molar
mM	=	Millimolar
MEGA	=	Molecular Evolutionary Genetics Analysis

meso-DAP	=	*meso*-Diaminopimelic acid
mg	=	Milligram
min	=	Minute
MK	=	Menaquinone
ML	=	Maximum likelihood
mL	=	Milliliter
MP	=	Maximum parsimony
µg	=	Microgram
µL	=	Microliter
MW	=	Molecular weight
N	=	Normal
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NCBI	=	National Center for Biotechnology Information
NJ	=	Neighbor-joining
nm	=	Nanometer
Nov.	=	Novel
PAO	=	Phenylarsine oxide
PG	=	Phosphatidylglycerol
PMSF	=	Phenylmethylsulfonyl fluoride
DEPC	=	diethyl pyrocarbonate
p-NPA	=	para-Nitrophenyl acetate (C2)
p-NPB	=	para-Nitrophenyl butyrate (C4)
p-NPV	=	para-Nitrophenyl valerate (C5)
p-NPH	=	para-Nitrophenyl hexanoate (C6)
p-NPO	=	para-Nitrophenyl octanoate (C8)
p-NPD	=	para-Nitrophenyl decanoate (C10)
p-NPL	=	para-Nitrophenyl laurate (C12)
p-NPM	=	p-nitrophenyl myristate (C14)
p-NPP	=	para-Nitrophenyl palmitate (C16)
OD	=	Optical density

p-NP	=	p-Nitrophenol
PAGE	=	Polyacrylamide gel electrophoresis
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PL	=	Phospholipids
%	=	Percentage
PCR	=	Polymerase chain reaction
rRNA	=	Ribosomal ribonucleic acid
RPM	=	Round per minute
Sec	=	second
SEM	=	Scanning electron microscope
SDS	=	Sodium dodecyl sulfate
sp.	=	Species
SSC	=	Standard sodium citrate
TISTR	=	Thailand Institute of Scientific and Technological Research
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
v/v	=	volume/volume
w/v	=	weight/volume

CHAPTER I
INTRODUCTION

Lipolytic enzymes are known as one of the main enzymes with the most significant potential in various industries and have attracted the interest of researchers. They have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion-dollar lipid technology bio-industry and still growing up. Additionally, lipolytic enzymes have potential applications in many biotechnological fields. These enzymes are widely distributed in nature, from microorganisms to plant and animal tissues. Among these, lipolytic enzymes gained from microorganisms are receiving more and more attention because of their stability, safety, a wide range of temperature and pH, comfortable of produce by fermentation and purification.

Reported microorganisms producing mesophilic lipolytic enzymes included bacteria, yeast, and fungi, while lipolytic producing halophilic bacteria are now considered to be an excellent source for novel enzymes and diverse biologically active compounds, which are employed directly or modified for biotechnological exploitation. Since industrial processes are typically carried out under severe conditions (i.e., high temperature and pH, high salt concentration and low water activity, and organic solvents), which can lead to low hydrolytic activity of enzymes and low efficiency in the industrial process, in this sense, the lipolytic enzymes isolated from halophilic microorganisms constitute an excellent alternative in the industrial processes.

Generally, the hypersaline environments represent an essential source for isolating halophilic bacteria that produced biomolecule like haloenzymes, which are an attractive option in industrial biotechnology due to their applicability as biocatalysts. Based on its unique physiological characteristic, the function of enzymes from halophilic bacteria show tolerance to high salinity, high temperature, and organic solvents. They retain their optimal activity at the severe conditions of industrial processes where mesophilic enzymes lose their functions. These extraordinary

enzymes are deemed cost-effective due to the elimination of environmental pollution, which is extensively used in a variety of biotechnological fields. These reason making halophilic bacteria a right candidate for selecting novel lipolytic enzymes which have potential applications in harsh industrial processes.

Halophilic bacteria are salt-loving microorganisms widely distributed in environments and characterized by salinity requirement (mainly NaCl concentration) from negligible content until saturation. They are spread in saline environments such as the crystallizer ponds (ponds where NaCl precipitate), salterns, salt mines, salted soil, rocks, water and sediment from hypersaline lakes, water from solar salterns, fermented food, and other sources. They accumulate the organic osmolytes named compatible solutes such as glycine betaine, ectoine, carbohydrates or their derivatives, amino acids, and others in the cytoplasm, which function as osmoprotectants, providing osmotic balance without disturbing vital cellular functions and the correct folding of proteins.

During the past few decades, many researchers have given considerable attention to lipolytic enzymes produced from halophilic microorganisms and their biotechnological capabilities. Many reported were studies about lipolytic production from various species of halophilic bacteria, but there were scarcely reported for lipolytic enzymes produced from the genus *Halobacillus*. Additionally, there is no report on the production of lipolytic enzymes from the genus *Lentibacillus* until now. Although halophiles are still not commonly exploited, the number of lipolytic enzymes derived from halophiles still steadily increasing. Some technical difficulties prevent industrial scale from the use of halo-enzymes produced by halophilic bacteria and archaea inhabiting saline environments. The halophilic enzymes are not feasible due to various reasons, e.g., cost of the chemicals, longer cultivation time, and the requirement of specific equipment with high energy requirements, which increase the overall cost of the enzyme production. In the past, the optimization of lipolytic enzymes production by halophilic bacterial strains was operated by empirical approaches like studying the effects of medium compositions on the production yields. This scarcity in these problems is, to a large extent, due to difficulties in producing a sufficient amount of the enzyme during the cultivation of the halophilic bacteria. A

more economical for halophilic lipolytic enzyme production with an improved product yield should be developed. This difficulty is solved by the cloning of halophilic lipase encoding genes using mesophiles. The purpose of such a treatment is to obtain the overproduction of recombinant lipolytic enzyme and improvement of its properties for specific commercial applications.

Previously, several researchers have focused on the recombinant technology and overexpression of lipolytic genes from halophilic bacteria into the new host. *Escherichia coli*, *Bacillus subtilis,* and yeast were successfully used as mesophilic hosts, where the expression of genes coding lipolytic enzymes was achieved. However, *E. coli* is still the first most commonly choice use as a host for protein expression as it is genetically most accessible even, the expressed enzymes have been accumulated as inclusion bodies inside the cells. Despite the secreted foreign proteins usually remain in biologically active forms, and the downstream purification is greatly simplified, whereas the outer cell membrane of *E. coli* contains lipopolysaccharides (LPS), generally referred to as endotoxins, which are pyrogenic in humans and other mammals. Besides, the over-expressed protein formed an inactive structure in inclusion bodies and the end-product should be completely endotoxin-free. Since the evolution of recombinant DNA technology, various attempts have been made to exploit secretion systems to secreted recombinant proteins into the culture medium. As far as the capability of secreting extracellular enzymes directly into the culture medium is concerned, a new host with potentially efficient expression host will be expected.

The choice of an appropriate host for the secretion of target proteins and suitable production conditions is crucial for the downstream processing. A Generally Recognized as Safe (GRAS) status approved by the U.S. Food and Drug Administration (FDA) and well-known gram-positive bacteria, *Bacillus subtilis*, is a more attractive host because it has a naturally high secretory ability and expressed extracellular proteins directly into the culture medium. Notwithstanding these clear blemish of the *E. coli* system, the use of the highly proficient Bacillus secretion hosts has remained limited for industrial enzyme production. Incredibly, one of the significant preference of using *B. subtilis* for cloning and as an expression host instead of *E. coli* is its ability to secrete

proteins directly into the culture medium and collect them to a high level in a relatively pure state. Accordingly, the use of *B. subtilis* as a host for the production of recombinant proteins is highly preferred above the use of *E. coli*. Hence, *B. subtilis* is considered pleasurable as a "host cell" for the secretion of recombinant proteins.

Since the significance of halophilic enzyme in the development of new biological tactics and the wide-ranging versatility of lipolytic enzyme in biotechnological applications, the requirement for new sources of lipolytic enzyme continues to stimulate the screening and identification of novel lipolytic bacteria with the highest ability for the production of lipolytic enzymes. As a result of these extraordinary properties and skills, the significance of halophiles and their enzymes to both basic and applied research is likely to enlarge in the future.

Thus, this study will focus on the isolation and identification of lipolytic-producing moderately halophilic bacteria (LPMHB) from Thai traditional fermented food and salted area in Thailand. The isolated strains will be chosen for screening their ability to extracellular lipolytic producing to be used for the biotechnology applications. The lipolytic-producing novel strain will be identified by using phenotypic, chemotaxonomic, genotypic, and whole-genome analysis, which will indicate their extraordinary situation. Of particular interest of the strain tested, the novel strain that displayed the lipolytic activity will be cloned into a new host, *Bacillus subtilis* WB800, to improved lipolytic production. Then, the recombinant enzymes that are secreted from the selected clone will be partially purified and also biochemically characterized.

As a result of this study, the LPMHB could be isolated from various sources in Thailand. Some of the isolated and the novel strain would be taxonomically characterized for the diversity and collected for further studies. The recombinant clone containing the selected lipolytic gene can be constructed, and the partial purification and characterization of the recombinant enzyme secreted from the selected clone would be studies. The results of this research may be employed to use in diverse applications in the future.

CHAPTER II

LITERATURE REVIEW

2.1. Halophilic bacteria

2.1.1. History of halophilic bacteria

Halophiles (from the Greek, *hal*, meaning sea or salt, and *philos*, meaning loving) are outstanding by their requirement of salt, mainly in sodium chloride (NaCl) for growth. They are found in all three domains of life, i.e., archaea, bacteria, and eukarya (Figure 2.1).

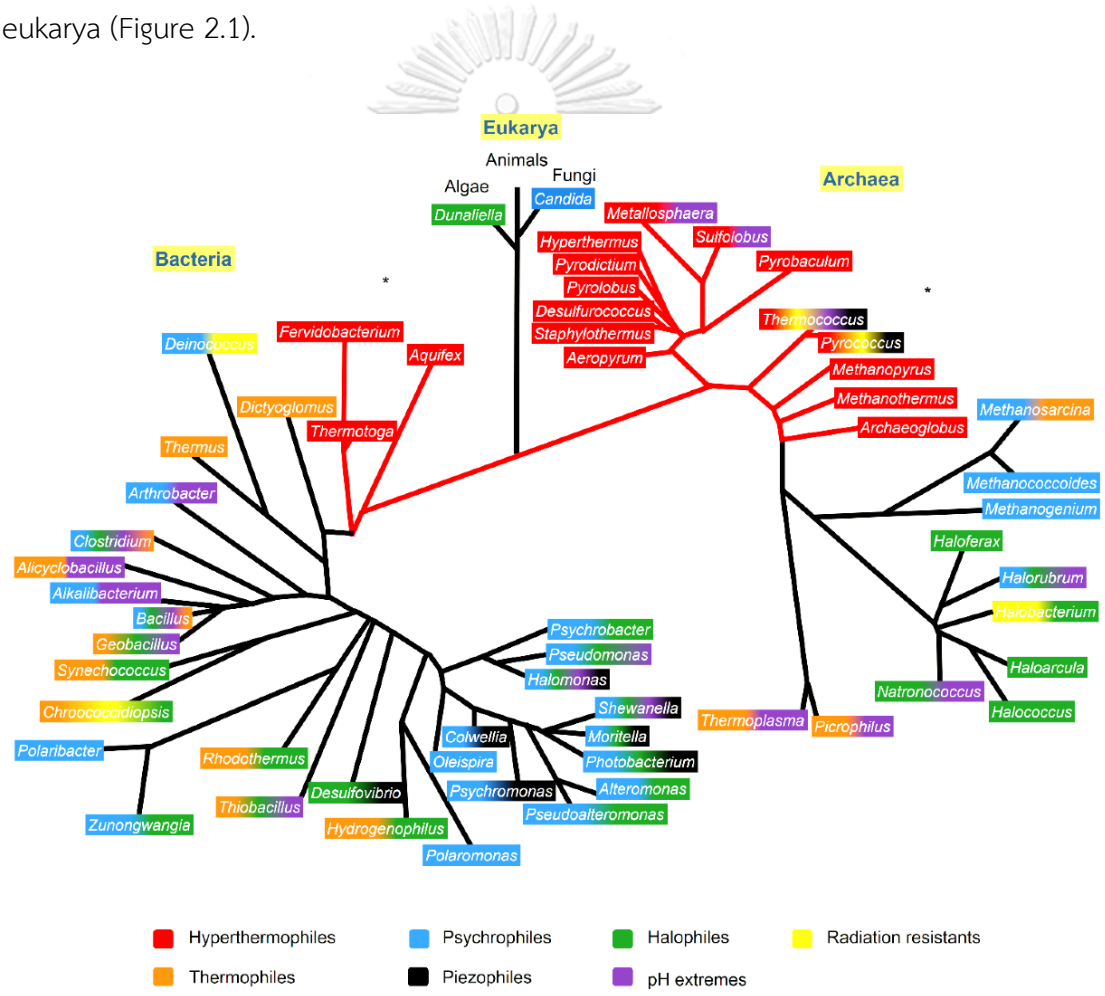


Figure 2.1. Phylogenetic tree showing the extremophiles and the resistant characteristics that appear in at least one species of each genus, identified with the color code.

Reference: Dalmaso *et al.*, 2015.

Back to 2700 BC, the first known of halophilic microorganisms has been found in hypersaline environments, which are isolated from red brine. In 1914, Pierce (1914) had described the isolation of a halophile that viable in ancient Egyptian times. Between the 1920s-1940s, halophilic bacteria were isolated from various sources such as fish, animal hides, and anchovies (Baumgartner, 1937). The studies of Petter and Hof (Petter, 1932; Hof, 1935) impelled new attentiveness in microorganisms that live in salt-saturated environments. Until 1936, the extreme halophiles, *Halobacterium trapanicum* and *Micrococcus morrhuae,* and the moderate halophiles, *Chromohalobacterium marismortui*, *Pseudomonas halestrogus*, and *Flavobacterium halmephium,* were isolated from the Dead Sea (Wilkansky, 1936; Volcani, 1940). However, this first discovery was not followed up until in the 1980s, Antonio Ventosa and co-workers initiated extensive studies of microbial life in the Dead Sea again (Ventosa *et al.*, 1999, 2015). Besides, Oren and colleagues also have studied the microorganisms in hypersaline environments in terms of ecology, physiology, and biochemistry (Oren, 1983 & 2015; Oren & Gurevich, 1995; Oren & Litchfield, 1998).

Nowadays, the renewed interest in halophiles is still increasing because of the advantage in technology, whole-genome sequencing. The first reported of the whole-genome sequence was published in the 2000s for the halophilic archaeon, *Halobacterium* sp. NRC-1 (Ng *et al.*, 2000) and still continuously increasing. Preferably, genome sequences of halophiles can be systematically analyzed *in silico*, which encourages the understanding of their biology (Oren, 2014). In this sense, whole-genome analysis and subsequent genetic engineering of halophiles have led to the improvement of biochemical pathways for the aim of optimizing their growth and for adaptations to use in industrial and environmental biotechnology (Edbeib *et al.*, 2016).

2.1.2. Physiology of halophilic bacteria

Conventional microorganisms cannot survive under saline conditions. However, halophilic microorganisms have adapted to grow optimally in a vast range of salt concentrations (0.5-5.2 M NaCl) (Valentine, 2007; Acikgoz & Ozcan, 2016). They may be divided depending on the degree of their salt requirement: non-halophiles (<0.2 M, approx. 1%), slight or mild halophiles (0.2-0.5 M, approx. 1-3%), moderate halophiles (0.5-2.5 M, approx. 3-15%), borderline extreme halophiles (1.5-4.0 M, approx. 9-23%),

and extreme halophiles (2.5-5.1 M, approx. 15-32%) (Kushner & Kamekura, 1988, Kushner, 1993; Schreck & Grunden, 2014; Yin *et al.*, 2015). For the halotolerant, these organisms can adapt to survive in the conditions of high salinity. They can grow in either absence or the high salt concentration; if they viable at 2.5 M salt, it is classified as extremely halotolerant (Edbeib *et al.*, 2016). Numerous halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations, with the requirement or resistance for salts perhaps dependent on environmental and nutritional support.

Halophiles have the capacity of the adaptation for balancing the osmotic stress between inside the cell and the external medium, which could resist the denaturing effects of salts. They usually adopt either of the two strategies: 'compatible solute' strategy and 'salt-in' strategy (Ventosa *et al.*, 1998; Oren, 2002b; Oren, 2008). They synthesize compatible solutes in cells or uptake that solutes help them to survive in harsh conditions. The compatible solutes such as amino acids (e.g., proline, glutamic acid, and aspartic acid), methylated quaternary ammonium compounds (e.g., glycine betaine, ectoine, hydroxyectoine, alanine betaine), polyols (e.g., mannitol, glycerol, and sorbitol), reducing sugars (e.g., glucose), and non-reducing sugars (e.g., trehalose) have been reported in halophiles to use as stress protectants and stabilizers of enzymes (Ventosa *et al.*, 1998; Shivanand & Mugeraya, 2011).

The salt-in strategy is founded in extremely halophilic bacteria and archaea. These microorganisms are adapted to high salt concentrations and lifeless when the salinity of the environment is lowered. Generally, they do not synthesize compatible solutes to maintain the osmotic equilibrium. This adaptation is related to the selective influx of potassium ions into the cell cytoplasm. All proteins and structural cell components must be modified to high salt concentrations for appropriate cell function (Shivanand & Mugeraya, 2011).

2.1.3. Taxonomy of halophilic bacteria in Thailand

Most studies on halophilic bacteria in Thailand have been focused on taxonomy, environmental and ecological researches. During the past few decades, the adaptation of halophilic microorganisms to the saline environment has been the subject of expanding interest with the methodology for culturing, manipulation, and

genetic engineering steadily advancing. Many halotolerant and halophilic microorganisms have been isolated from diverse saline environments. However, new species of halophilic bacteria isolated and identified from high salt-fermented foods in Asian foods are mostly from Korea, Japan, and Southeast Asia, and Thailand. Most of them are mainly isolated from fish sauce, seasoning for Thai cuisine (almost 80~90% of the total population of halophilic bacteria and archaea) (Lee, 2013). Also, other traditional fermented foods, especially in Thailand, such as fermented fish (*Pla-ra*), fermented shrimp paste (*Ka-pi*), salted crab (*Poo-khem*), and conventionally fermented fish sauce from Southern of Thailand (*Bu-du*) often contain halophilic bacteria and archaea. There is a wide diversity of halophilic bacterial species that have biotechnology important compounds, including hydrolytic enzymes.

Halophilic bacteria and archaea have been isolated from Thai traditional fermented food. Almost of the total population are known to consume in fish sauce. In particular, Thai fish sauce is known as a seasoning for Thai recipes. The new strains of halophilic bacteria and archaea have been isolated from Thai fish sauce such as *Lentibacillus halophilus* (Tanasupawat *et al.*, 2006); *Halococcus thailandensis* (Namwong *et al.*, 2007); *Natrinema gari* (Tapinkae *et al.*, 2008), *Haloarcula salaria* (Namwong *et al.*, 2011) and *Haloacular tradensis* (Namwong *et al.*, 2011). Most of these strains show similar characteristics since a minimum salt concentration of 15% (w/v) is needed for growth, and the optimal salt concentration for evolution is at least 20% (w/v).

There are various fermented foods with high sodium concentration in Thailand, and the likelihood of the existence of moderately halophilic bacteria has been reported. Thai researchers have reported that the novel strain of moderately halophilic bacteria has been isolated from several fermented foods that are produced and sold commercially in Thailand. Some of them have been isolated from *Pla-ra* included *L. juripiscarius* (Namwong *et al.*, 2005); *Piscibacillus salipiscarius* (Tanasupawat et al., 2007); *Salinivibrio siamensis* (Chamroensaksri *et al.*, 2009); *Gracilibacillus thailandensis* (Chamroensaksri *et al.*, 2010); *Virgibacillus siamensis* (Tanasupawat *et al.*, 2010); and *Idiomarina piscisalsi* (Sitdhipol *et al.*, 2013). Some moderately halophilic bacteria have been isolated from shrimp pastes (*Ka-pi*) including, *L. kapialis* (Pakdeeto *et al.*, 2007a);

Salinicoccus siamensis (Pakdeeto *et al.*, 2007b); *Oceanobacillus kapialis* (Namwong *et al.*, 2009); *V. halodenitrificans* (Tanasupawat *et al.*, 2011) and *V. kapii* (Daroonpunt *et al.*, 2016a), and *B. salacetis* (Daroonpunt *et al.*, 2019b). Besides, *L. halophilus* (Tanasupawat *et al.*, 2006), *B. siamensis* (Sumpavapol *et al.*, 2010), and *B. piscicola* (Daroonpunt *et al.*, 2016b) also identified from salted crab (*Poo-khem*) and fish sauce, respectively. These moderately halophilic bacteria showed that Thai fermented foods are a good source for the isolation of the novel moderately halophilic bacteria.

2.1.3.1. Genus *Lentibacillus*

Lentibacillus (Len.ti.ba.cil'lus. L. adj. *lentus* slow; L. dim. n. *bacillus* small rod; N.L. masc. n. *Lentibacillus* slowly growing bacillus/rod) was firstly described by Yoon and co-workers in 2002. The type species of the genus was first proposed for a single strain described as *L. salicampi* SF-20^T (Yoon *et al.*, 2002). Cells are rod-shaped, Gram stain-positive, and form spherical or oval endospores that swell the terminal of sporangia. Moderately to extremely halophilic. Catalase-positive, oxidase variable, and urease-negative. The cell-wall peptidoglycan contains *meso*-DAP, and the predominant menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol.

At the time of writing, the genus contained 17 validly named species (Parte *et al.*, 2018), including the recently described *L. sediminis* 0W14^T from a marine saltern (Guo *et al.*, 2017). *L. amyloliquefaciens* LAM0015^T from saline sediment (Wang *et al.*, 2016). *L. kimchii* K9^T from kimchi (Oh *et al.*, 2016), *L. populi* WD4L-1^T from a poplar tree (Sun *et al.*, 2016), *L. garicola* MJ1^T from a Korean fermented anchovy sauce (Jung *et al.*, 2015), *L. jeotgali* Grbi^T from traditional Korean fermented seafood (Jung *et al.*, 2010), *L. persicus* Amb31^T from a saline lake (Sánchez-Porro *et al.*, 2010), *L. salinarum* AHS-1^T from a marine solar saltern in the Republic of Korea (Lee *et al.*, 2008a), *L. salis* BH113^T (Lee *et al.*, 2008b) and *L. halodurans* 8-1^T from a salt lake in PR China (Yuan *et al.*, 2007), *L. lacisalsi* BH260^T from a saline lake (Lim *et al.*, 2005), and *L. salarius* BH139^T from saline sediment in PR China (Jung *et al.*, 2005). Additionally, *L. halophilus* TISTR 1549^T (Tanasupawat *et al.*, 2006) and *L. juripiscarius* TISTR 1535^T (Namwong *et al.*, 2005) was isolated from fish sauce and *L. kapialis* PN7-6^T from *Ka-pi* (Pakdeeto *et al.*, 2007a) for purposed as new species isolated from Thailand.

Table 2.1 Characteristics of *Lentibacillus* species

Characteristics	1	2	3	4	5	6	7	8
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore shape/position	S/O, T	S/O, T	O, T	S, T	S, T	S/O, T	S, T	O
Motility	+	+	-	+	+	-	-	+
Length/ Width(μm)	2.0-4.0/0.4–0.7	1.5-3.0/0.2-0.3	1.5-6.0/0.4-0.5	1.2-3.0/0.4-0.6	1.0-3.0/0.4-0.6	0.5-1.5/1.5-2.5	0.2-0.4/0.8-2.5	0.7-1.2/2.0-4.0
Colony color	Cream	Cream	White to cream	Cream	White to cream	white	Red	Cream to yellow
Growth pH								
Range	5.5-8.5	6.0-8.0	5.0-9.0	7.0-9.5	6.0-8.0	6.0-9.0	5.0-9.0	6.0-9.5
Optimum	6-8	7.0-7.5	7.0	8.0	7.0-7.5	7.0-7.5	7.0	6.5-7.0
Growth temperature (°C)								
Range	15-40	15-50	10-45	15-40	15-42	22-45	15-45	15-45
Optimum	30	30-35	37	30-32	30-37	30	37	37-40
NaCl conc. for growth (%, w/v)								
Range	2-23	1-20	3–30	5-25	12-30	5-30	5-30	3-24
Optimum	4-8	12-14	10	12-15	20-26	8-12	10	10-12
Nitrate reduction	ND	+	+	+	-	-	+	+
Oxidase	+	-	+	+	+	+	+	+
Hydrolysis of:								
Aesculin	-	+	-	-	-	-	-	+
Casein	+	-	+	-	-	-	-	-
Tween 80	+	-	+	-	-	-	-	-
Acid production from:								
D-glucose	+	+	+	-	-	+	+	+
D-fructose	+	+	+	+	-	+	+	-
D-Cellobiose	+	ND	-	ND	-	ND	-	-
D-Galactose	+	ND	-	ND	-	ND	+	w
Lactose	+	+	-	-	-	ND	-	-
Maltose	+	+	-	-	-	ND	-	-
D-Ribose	+	+	+	+	-	ND	+	+
D-Sucrose	+	ND	w	ND	-	ND	w	-
D-xylose	+	+	+	+	-	ND	-	-
Quinone/Cell wall	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP
Major fatty acids	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$, $C_{15:0\ iso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{15:0\ iso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$, $C_{17:0\ anteiso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ iso}$, $C_{16:0\ iso}$
Major polar lipids	DPG, PG,	PG, DPG	PG, DPG, GL	PG, DPG	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL
G+C content (mol%)	44	43.0	43.4	44.0	42.1-43.1	43.4	41.2-41.6	49.0

Table 2.1 (Cont.) Characteristics of *Lentibacillus* species

Characteristics	1	2	3	4	5	6	7	8
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore shape/position	S/O, T	S/O, T	O, T	S, T	S, T	S/O, T	S, T	O
Motility	+	+	-	+	+	-	-	+
Length/ Width(μm)	2.0-4.0/0.4–0.7	1.5-3.0/0.2-0.3	1.5-6.0/0.4-0.5	1.2-3.0/0.4-0.6	1.0-3.0/0.4-0.6	0.5-1.5/1.5-2.5	0.2-0.4/0.8-2.5	0.7-1.2/2.0-4.0
Colony color	Cream	Cream	White to cream	Cream	White to cream	white	Red	Cream to yellow
Growth pH								
Range	5.5-8.5	6.0-8.0	5.0-9.0	7.0-9.5	6.0-8.0	6.0-9.0	5.0-9.0	6.0-9.5
Optimum	6-8	7.0-7.5	7.0	8.0	7.0-7.5	7.0-7.5	7.0	6.5-7.0
Growth temperature (°C)								
Range	15-40	15-50	10-45	15-40	15-42	22-45	15-45	15-45
Optimum	30	30-35	37	30-32	30-37	30	37	37-40
NaCl conc. for growth (%, w/v)								
Range	2-23	1-20	3–30	5-25	12-30	5-30	5-30	3-24
Optimum	4-8	12-14	10	12-15	20-26	8-12	10	10-12
Nitrate reduction	ND	+	+	+	-	-	+	+
Oxidase	+	-	+	+	+	+	+	+
Hydrolysis of:								
Aesculin	-	+	-	-	-	-	-	+
Casein	+	-	+	-	-	-	-	-
Tween 80	+	-	+	-	-	-	-	-
Acid production from:								
D-glucose	+	+	+	-	-	+	+	+
D-fructose	+	+	+	+	-	+	+	-
D-Cellobiose	+	ND	-	ND	-	ND	-	-
D-Galactose	+	ND	-	ND	-	ND	+	w
Lactose	+	+	-	-	-	ND	-	-
Maltose	+	+	-	-	-	ND	-	-
D-Ribose	+	+	+	+	-	ND	+	+
D-Sucrose	+	ND	w	ND	-	ND	w	-
D-xylose	+	+	+	+	-	ND	-	-
Quinone/Cell wall	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP
Major fatty acids	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$, $C_{15:0\ iso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{15:0\ iso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$, $C_{17:0\ anteiso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ iso}$, $C_{16:0\ iso}$
Major polar lipids	DPG, PG,	PG, DPG	PG, DPG, GL	PG, DPG	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL
G+C content (mol%)	44	43.0	43.4	44.0	42.1-43.1	43.4	41.2-41.6	49.0

Strain 1, L. salicampi SF-20T (Yoon et al., 2002); 2, L. salarius BH139T (Jung et al., 2005); 3, L. juripiscarius IS40-3T (Namwong et al., 2005); 4, L. lacisalsi BH260T (Lim et al., 2005); 5, L. halophilus PS112T (Tanasupawat et al., 2006); 6, L. halodurans 8-1T (Yuan et al., 2007); 7, L. kapialis PN7-6T (Pakdeeto et al., 2007); 8, L. salinarum AHS-1T (Lee et al., 2008a). Note: +, positive; - negative; w, weak positive; ND, no data; A, aerobic; FA, facultative aerobic; E, Ellipsoidal; O, Oval; S, spherical; C, central; T, terminal; ST, subterminal; DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; GL, unknown glycolipid(s); PL, unknown phospholipids.

Table 2.1 (Cont.) Characteristics of *Lentibacillus* species

Characteristics	1	2	3	4	5	6	7	8
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore shape/position	S/O, T	S/O, T	O, T	S, T	S, T	S/O, T	S, T	O
Motility	+	+	-	+	+	-	-	+
Length/ Width(μm)	2.0-4.0/0.4–0.7	1.5-3.0/0.2-0.3	1.5-6.0/0.4-0.5	1.2-3.0/0.4-0.6	1.0-3.0/0.4-0.6	0.5-1.5/1.5-2.5	0.2-0.4/0.8-2.5	0.7-1.2/2.0-4.0
Colony color	Cream	Cream	White to cream	Cream	White to cream	white	Red	Cream to yellow
Growth pH								
Range	5.5-8.5	6.0-8.0	5.0-9.0	7.0-9.5	6.0-8.0	6.0-9.0	5.0-9.0	6.0-9.5
Optimum	6-8	7.0-7.5	7.0	8.0	7.0-7.5	7.0-7.5	7.0	6.5-7.0
Growth temperature (°C)								
Range	15-40	15-50	10-45	15-40	15-42	22-45	15-45	15-45
Optimum	30	30-35	37	30-32	30-37	30	37	37-40
NaCl conc. for growth (%, w/v)								
Range	2-23	1-20	3–30	5-25	12-30	5-30	5-30	3-24
Optimum	4-8	12-14	10	12-15	20-26	8-12	10	10-12
Nitrate reduction	ND	+	+	+	-	-	+	+
Oxidase	+	-	+	+	+	+	+	+
Hydrolysis of:								
Aesculin	-	+	-	-	-	-	-	+
Casein	+	-	+	-	-	-	-	-
Tween 80	+	-	+	-	-	-	-	-
Acid production from:								
D-glucose	+	+	+	-	-	+	+	+
D-fructose	+	+	+	+	-	+	+	-
D-Cellobiose	+	ND	-	ND	-	ND	-	-
D-Galactose	+	ND	-	ND	-	ND	+	w
Lactose	+	+	-	-	-	ND	-	-
Maltose	+	+	-	-	-	ND	-	-
D-Ribose	+	+	+	+	-	ND	+	+
D-Sucrose	+	ND	w	ND	-	ND	w	-
D-xylose	+	+	+	+	-	ND	-	-
Quinone/Cell wall	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP	MK-7/*meso*-DAP
Major fatty acids	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$, $C_{15:0\ iso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$	$C_{15:0\ anteiso}$, $C_{17:0\ anteiso}$, $C_{15:0\ iso}$, $C_{16:0\ iso}$	$C_{15:0\ anteiso}$, $C_{16:0\ iso}$, $C_{17:0\ anteiso}$, $C_{14:0\ iso}$	$C_{15:0\ anteiso}$, $C_{17:0\ iso}$, $C_{16:0\ iso}$
Major polar lipids	DPG, PG,	PG, DPG	PG, DPG, GL	PG, DPG	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL
G+C content (mol%)	44	43.0	43.4	44.0	42.1-43.1	43.4	41.2-41.6	49.0

Strain: 9, L. salis BH113T (Lee et al., 2008b); 10, L. persicus Amb31T (Sánchez-Porro et al., 2010); 11, L. jeotgali GrbiT (Jung et al., 2010); 12, L. garicola MJ1T (Jung et al., 2015); 13, L. kimchii K9T (Oh et al., 2016); 14, L. amyloliquefaciens LAM0015T (Wang et al., 2016); 15, L. populi WD4L-1T (Sun et al., 2016); 16, L. sediminis 0W14T (Guo et al., 2017). Note: +, positive; - negative; w, weak positive; ND, no data; A, aerobic; FA, facultative aerobic; E, Ellipsoidal; O, Oval; S, spherical; C, central; T, terminal; ST, subterminal; DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; GL, unknown glycolipid(s); PL, unknown phospholipids.

2.1.3.2. Genus *Halobacillus*

The genus *Halobacillus* (Ha.lo.ba.cil'lus. Gr. n. *hals* salt; L. n. *bacillus* rod; N.L. masc. n. *Halobacillus* salt-loving rod) belongs to the family *Bacillaceae* within the phylum *Firmicutes*. Members of the genus are moderately halophilic, Gram-positive, motile or non-motile, rod, cocci or spherical to oval cells, strictly aerobic, and chemo-organotrophic. Endospores were produced with ellipsoidal or spherical shaped at the central or subterminal positions and could resist at 75°C for at least 10 min. Colonies are circular, smooth, slightly raised, and opaque. The nondiffusible pigment is variable, ranging from cream to white or pale yellow to bright orange. *Halobacillus* can be separated from other related genera based on the cell-wall peptidoglycan type, L-Orn-D-Asp (A4β type according to the murein key of Schleifer & Kandler (1972) and Spring with co-workers (1996), but not included for *H. campisalis* and *H. seohaensis*, which is based on *meso*-DAP. The cellular fatty acid pattern is characterized by significant amounts of branched fatty acids, especially $C_{15:0\ anteiso}$, and a substantial quantity of $C_{16:1\ \omega 7c}$ alcohol.

In the beginning, the genus *Halobacillus* was proposed, *H. litoralis* DSM 10405^T and *H. trueperi* DSM 10404^T as two novel species. Besides, *Sporosarcina halophila* has been isolated from salt marsh soils of the North Sea coasts of Germany (Claus *et al.*, 1983) and was reclassified as the type species of the genus *Halobacillus,* which the name *H. halophilus* DSM 2266^T (Spring *et al.*, 1996). In recent times, *Halobacillus* comprised 21 species with validly published names (Parte *et al.*, 2018). Due to their ability to grow at high salt concentrations, members of the genus *Halobacillus* have been predominantly isolated from saline environments such as solar saltern, *H. locisalis* KCTC 3788^T (Yoon *et al.*, 2004a), *H. yeomjeoni* KCTC 3957^T (Yoon *et al.*, 2005), *H. campisalis* ASL-17^T (Yoon *et al.*, 2007), *H. seohaensis* ISL-50^T (Yoon *et al.*, 2008), *H. salsuginis* JSM 078133^T (Chen *et al.*, 2009b), *H. sediminis* NGS-2^T (Kim *et al.*, 2015a), *H. salicampi* TGS-15^T (Kim *et al.*, 2016); from saline sediment or saline soil, *H. litoralis* DSM 10405^T, *H. trueperi* DSM 10404^T, and *H. halophilus* DSM 2266^T (Claus *et al.*, 1983), *H. karajensis* MA-2^T (Amoozegar *et al.*, 2003), *H. andaensis* NEAU-ST10-40^T (Wang *et al.*, 2015a); from salt lake, *H. salinus* JCM 11546^T (Yoon *et al.*, 2003), *H. aidingensis* AD-6^T and *H. dabanensis* D-8^T (Liu *et al.*, 2005), *H. alkaliphilus* FP5^T

(Romano *et al.*, 2008); from deep-sea carbonate rock (*H. kuroshimensis* IS-Hb7^{T} and *H. profundi* IS-Hb4^{T} (Hua *et al.*, 2007); from the surface of black mangrove leaves (*H. mangrovi* MS10^{T} by Soto-Ramírez *et al.*, 2008); from mangrove-growing soil, *H. faecis* IGA7-4^{T} (An *et al.*, 2007) and from the sea anemone, *H. naozhouensis* JSM 071068^{T} (Chen *et al.*, 2009a). The differential characteristics of *Halobacillus* species are shown in Table 2.2.

Table 2.2 Characteristics of *Halobacillus* species

Characteristics	1	2	3	4	5	6	7
Cell morphology	Cocci or oval-shaped	Rods	Rods	Rods	Rods	Rods	Long rods filamentous
Flagellation	Single peritrichous	Peritrichous	Peritrichous	Absent	Peritrichous	Single	Single
Spore shape/position	S/C	E,S/C,ST	E,S/C,ST	E,S/C,ST	E	E	E/C, ST
Colony colour	Orange	Orange	Orange	Cream or white	Pale orange- yellow	Light orange- yellow	Light yellow
Max. temp for growth (°C)	40	43	44	49	45	42	48
Growth at:							
4 °C	-	-	-	-	-	-	-
pH 5.0	-	-	-	-	+	+	-
pH 5.5	-	-	-	-	+	+	-
0.5% NaCl	-	+	+	-	+	-	+
25% NaCl	-	+	+	-	-	-	-
Hydrolysis of:							
Aesculin	-	-	-	+	+	+	-
Casein	+	-	-	+	+	-	+
Gelatin	+	+	+	+	+	-	+
Starch	+	-	-	+	-	+	-
Tween 80	+	+	+	+	+	+	-
Acid production from:							
D-Fructose	-	+	+	+	+	+	-
D-Galactose	-	+	+	-	w	-	-

Strain 1, *H. halophilus* (Spring et al., 1996); **2**, *H. litoralis* (Spring et al., 1996); **3**, *H. trueperi* (Spring et al., 1996); **4**, *H. karajensis* (Amoozegar *et al.*, 2003); **5**, *H. salinus* (Yoon *et al.*, 2003); **6**, *H. locisalis* (Yoon *et al.*, 2004a); **7**, *H. yeomjeoni* (Yoon *et al.*, 2005). **Note:** +, positive; - negative; w, weak positive; ND, no data; E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal.

Table 2.2 (Cont.) Characteristics of *Halobacillus* species

Characteristics	1	2	3	4	5	6	7
Cell morphology	Cocci or oval-shaped	Rods	Rods	Rods	Rods	Rods	Long rods filamentous
Flagellation	Single peritrichous	Peritrichous	Peritrichous	Absent	Peritrichous	Single	Single
Spore shape/position	S/C	E,S/C,ST	E,S/C,ST	E,S/C,ST	E	E	E/C, ST
Colony colour	Orange	Orange	Orange	Cream or white	Pale orange- yellow	Light orange- yellow	Light yellow
Max. temp for growth (°C)	40	43	44	49	45	42	48
Growth at:							
4 °C	-	-	-	-	-	-	-
pH 5.0	-	-	-	-	+	+	-
pH 5.5	-	-	-	-	+	+	-
0.5% NaCl	-	+	+	-	+	-	+
25% NaCl	-	+	+	-	-	-	-
Hydrolysis of:							
Aesculin	-	-	-	+	+	+	-
Casein	+	-	-	+	+	-	+
Gelatin	+	+	+	+	+	-	+
Starch	-	-	-	+	+	+	+
Tween 80	-	-	-	-	+	-	+
Acid production from:							
D-Fructose	-	+	+	+	+	+	-

Strain: 1, *H. halophilus* (Spring et al., 1996); **2**, *H. litoralis* (Spring et al., 1996); **3**, *H. trueperi* (Spring et al., 1996); **4**, *H. karajensis* (Amoozegar *et al.*, 2003); **5**, *H. salinus* (Yoon *et al.*,-2003); **6**, *H. locisalis* (Yoon *et al.*, 2004a); **7**, *H. yeomjeoni* (Yoon *et al.*, 2005). **Note:** +, positive; - negative; w, weak positive; ND, no data; E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal.

Table 2.2 (Cont.1) Characteristics of *Halobacillus* species

Characteristics	8	9	10	11	12	13	14
Cell morphology	Rods	Cocci or oval-shaped	Rods	Rods	Rods	Rods	Cocci
Flagellation	Peritrichous	Peritrichous	Peritrichous	Absent	Absent	Absent	ND
Spore shape/position	E/C,ST	S/C	E/C,ST	E,S/C	E,S/C	E,S/C	S
Colony colour	Orange	Light yellow	Cream to Orange	Orange	Yellow-orange	Pale yellow	Pale Orange
Max. temp for growth (°C)	40	41	50	45	48	47	45
Growth at:							
4 °C	-	+	-	-	-	-	-
pH 5.0	-	-	+	-	-	-	-
pH 5.5	-	+	+	+	+	+	-
0.5% NaCl	+	+	+	+	+	+	+
25% NaCl	-	-	+	-	+	+	-
Hydrolysis of:							
Aesculin	-	+	-	ND	+	+	ND
Casein	+	+	+	ND	+	+	ND
Gelatin	+	-	-	+	+	-	ND
Starch	+	-	-	ND	+	+	ND
Tween 80	+	-	-	ND	+	+	ND
Acid production from							

Strain 8, *H. aidingensis* (Liu et al., 2005); 9, *H. campisalis* (Yoon et al., 2007); 10, *H. dabanensis* (Hua et al., 2005); 11, *H. faecis* (An et al., 2007); 12, *H. kuroshimensis* (Hua et al., 2007); 13, *H. profundi* (Hua et al., 2007); 14, *H. alkaliphilus* (Romano et al., 2008). Note: +, positive; - negative; w, weak positive; ND, no data; E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal.

Table 2.2 (Cont.1) Characteristics of *Halobacillus* species

Characteristics	15	16	17	18	19	20	21
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Flagellation	ND	Single	ND	Peritrichous	Peritrichous	Peritrichous	ND
Spore shape/position	ND	E/C,ST	E/T	E/ST	E	E	E
Colony color	Cream	Yellowish white	Orange-yellow	Cream	Cream	light yellow	Pale yellow
Max. temp for growth (°C)	50	38	35	45	35	60	30
Growth at:							
4 °C	-	+	-	-	-	+	-
pH 5.0	-	-	-	-	-	-	+
pH 5.5	-	-	-	-	-	-	+
0.5% NaCl	-	-	-	-	-	-	-
25% NaCl	-	-	-	-	-	-	-
Hydrolysis of:							
Aesculin	-	-	+	+	-	ND	+
Casein	+	+	-	-	-	-	-
Gelatin	+	-	-	+	+	-	-
Starch	+	+	+	+	+	-	-
Tween 80	+	-	ND	-	-	+	ND
Acid production from							
D-Fructose	-	-	+	-	-	+	+
D-Galactose	-	-	-	-	-	-	-

Strain 15, *H. mangrovi* (Soto-Ramirez *et al.*, 2008); **16**, *H. seohaensis* (Yoon *et al.*, 2008); **17**, *H. salicampi* (Kim *et al.*, 2016); **18**, *H. salsuginis* (Chen *et al.*, 2009b); **19**, *H. naozhouensis* (Chen *et al.*, 2009a); **20**, *H.* andaensis (Wang *et al.*, 2015); **21**, *H. sediminis* (Kim *et al.*, 2015a). **Note:** +, positive; - negative; w, weak positive; ND, no data; E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal.

2.1.4. DNA fingerprinting for identification of halophilic bacteria

Identification techniques based on phenotypes such as traditional method and API systems (i.e., API 50 CH, API Zym, and API 20NE, and so on) showed discrepancies between phenotypic and genotypic identifications. However, both methods may be economically unviable for the analysis of large amounts of isolates. Reliable identification of bacterial species using traditional biochemical testing is becoming difficult nowadays due to the increasing number of known bacterial subspecies, often revealing considerable phenotypic similarity. Several molecular methods have been proposed for differentiating and typing bacteria, which have many advantages, including specificity and reliability, as well as the abandonment of the use of non-sensitive and time-consuming phenotypic techniques.

Numerous molecular typing methods have been used previously for typing, including amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), denaturing gradient gel electrophoresis (DGGE), internal transcribed spacer-homoduplex-heteroduplex polymorphism (ITS-HHP) fingerprinting, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA PCR (RAPD-PCR), PCR restriction fragment length polymorphism (RFLP), single-nucleotide polymorphism (SNP) and tRNA-intergenic spacer PCR. Also, the repetitive DNA element sequence fingerprinting (rep-PCR) is promising.

Rep-PCR technique has been devised for the characterization of bacteria and is widely employed to distinguish species, strains, and serotypes. This method is based on PCR amplification of genomic elements between interspersed repeated DNA sequences in bacterial genomes, resulting in a specific DNA band pattern after electrophoresis (Versalovic et al., 1991; Versalovic et al., 1994). The oligonucleotide primers such as BOX (22 bp), ERIC (22 bp), REP (18 bp), and $(GTG)_5$ (15 bp), which have been designed to match the non-coding repetitive DNA sequences distributed throughout the bacterial genomes, are most frequently used in rep-PCR assays applied in bacterial taxonomic studies (Versalovic *et al.*, 1994; Rademaker *et al.*, 2008). These primers amplify genomic regions located between repetitive sequences. During PCR, multiple DNA amplicons with different sizes are generated to produce a unique Rep-PCR profile for each bacterial strain. Differences in these Rep-PCR profiles can

distinguish among strains and have proven extremely useful in the study of microbial diversity. (Versalovic *et al.*, 1991).

The repetitive sequence-based PCR or rep-PCR DNA fingerprint technique has proven to be a valuable tool for studies, identity, track and examine the diversity of microbial ecology, environmentally important microorganisms, molecular diagnostics, medical microbiology, and epidemiological analyses (Versalovic *et al.*, 1998; Rademaker *et al.*, 2008; Ishii & Sadowsky, 2009). Furthermore, rep-PCR is an accurate technique, fast, easy to perform, and decreases time-consuming than the conventional method. The useful approach can be separate inter- and intra-species relatedness (Versalovic *et al.*, 1994). It has been shown that molecular methods can be applied to a wide range of microorganisms. These methods have been successfully applied for typing and identification of several groups of bacterial taxa, e.g., *lactobacilli* (Gevers et al., 2001), *enterococci* (Švec et al., 2005), *streptomycetes* (Lanoot et al., 2004), *geobacilli* (Meintanis et al., 2008), acetic acid bacteria (De Vuyst et al., 2008), Staphylococcus (Švec et al., 2010). It allows the differentiation at a species, subspecies, and sometimes even strain level (Gevers et al., 2001).

Several previous reports have shown the potential of DNA fingerprinting as a technique for study phylogenetic relationships of the species and subspecies of halophilic bacteria, and our results advocate their findings. An example of this was recently shown by Irshad and colleagues (Irshad *et al.*, 2014) for the typing of 200 halophilic bacteria in Korea's foreshore soils using BOX-PCR analysis. The rep-PCR genomic fingerprinting using BOX- and $(GTG)_5$-primer set show is a powerful technique for analysis of genetic microdiversity within the genus *Thioalkalivibrio.* From this point, the correlation between fingerprint clustering, geographic origin, and the characteristics of the lake of birth of 85 haloalkaliphilic bacterial isolates belonging to the genus *Thioalkalivibrio* was found (Foti *et al*., 2006). Xiang and team-mate (Xiang *et al.*, 2008) extended this principle to analyze extremely halophilic bacteria's genetic relationships in southwestern China using ISR-PCR fingerprints. Some studies applied 5S rRNAs fingerprinting analysis to identify the marine bacteria and halophilic archaea populations (Casamayor *et al.*, 2000). Cleland and collaborators (Cleland *et al*., 2008) evaluated the DiversiLab Archaebacteria Kit that utilizes a kit-based repetitive

sequence-based (rep-PCR) method that has been optimized for genotyping and strain identification of Archaea. The two different PCR-based methods, f-ITS and RAPD (using primer D11344) were used to discriminate and cluster the halophilic isolates from Capuchin Catacombs in Palermo, Italy (Pinăr *et al.*, 2014).

A few studies are available to combine multiple primers for rep-PCR fingerprinting (Versalovic *et al.*, 1994). In doing this, Nick and co-workers (Nick et al., 1999) recently represented the usefulness of rep-PCR fingerprinting using $(GTG)_5$ primer in the colleague with REP, ERIC, and BOX primers for biotyping of genus *Rhizobium*. A combined REP+ERIC+BOX+$(GTG)_5$ dendrogram was generated when a maximized specificity of the patterns was preferred (Gevers *et al.*, 2001). However, numerous study of DNA fingerprinting was used as a single primer and different molecular techniques but no report for using the combination of primers. This study aimed to assess DNA fingerprinting using the combination of primer for accurate identification of a group of strains containing multiple representatives of LPMHB. In this study, the rep-PCR applicability was demonstrated as a useful tool in the diversity study among the LPMHB originating from different Thailand sources.

2.2 Potential application of halophilic enzyme

The potential application of halophilic bacteria can be discussed in several fields. As regards, the halophilic bacteria can tolerate high concentrations of salt and have low nutritional requirements, which confers them a significant potential in harsh industrial processes. Over the last decade, halophilic bacteria have been the focus of intensive study; the main discovery is that they can produce enzymes with potential in different biotech industries. The survey of halophilic enzymes is concentrated on their unique structural characteristics and possible applications in various industrial areas (Liszka *et al.*, 2012).

In comparison with other extremophiles such as thermophilic and alkaliphilic extremophiles, halophilic microorganisms are the most successful biotechnological applications. Halophiles may serve as a source of many unique biomolecules, such as stable enzymes, biopolymers, and compatible solutes, and they may also be valuable

for various industrial biotechnological processes and medicine (DasSarma *et al.*, 2010). In biotechnology, several beneficial halophilic enzymes produced from halophilic microorganisms have been described, i.e., alcohol dehydrogenase, aldehyde dehydrogenase, amylases, α-amylases, amylopullulanase, cellulases, chitinase, cyclodextrin glycosyltransferase, DNase, esterases, lipases, pectinases, proteases, pullulanase, endo-1,4-β-xylanase, endo-β-xylanase, β-xylanase, β-xylosidase, 2-hydroxy acid dehydrogenase, etc.

The halo tolerance enzymes derived from halophilic bacteria can be exploited wherever enzymatic transformations are required to function under physical and chemical conditions, such as in the presence of organic solvents and extremes in temperature and salt content. Their enzymes acquire a relatively large number of negatively charged amino acid residues on their surface to prevent precipitation (Danson & Hough, 1997; Demirjian *et al.*, 2001; Burg, 2003). The amino acid composition of enzymes revealed that in addition to the higher acidic amino acid residues, they have higher hydrophobic and lower aliphatic residues compared to their non-halophilic counterparts (Madern & Zaccai 1997). Most halophilic enzymes are inactivated and denatured at a concentration of NaCl below 1 M. Enzymes produced by halophilic bacteria, in contrast, are salt-tolerant, and salt was required due to their need to maintain high intracellular ion concentrations for balancing the osmotic pressure in hypersaline environments (Aljohny, 2015). Consequently, in surroundings with lower salt concentrations, the solubility of halophilic enzymes is often very poor, which could limit their applicability (Madern *et al.*, 2000). In the case of halophilic eubacteria, the intracellular salt concentration is low, and they maintain an osmotic balance between their cytoplasm and external medium by accumulating high concentrations of various organic osmotic solutes, namely ecotine and hydroxyecotine (Margesin & Schinner, 2001). By this mechanism, their intracellular enzymes have no special features for salt tolerance (Madern *et al.*, 2000; Margesin & Schinner, 2001; Kumar *et al.*, 2011; Gupta *et al.*, 2014).

2.2.1. Lipolytic enzymes

Lipolytic enzymes naturally included esterases (EC 3.1.1.1, carboxyl ester hydrolases) and “true” lipases (EC 3.1.1.3, triacylglycerol hydrolases), they are

members of the α/β-fold group of hydrolases (Bhatnagar *et al.*, 2005). Lipases catalyze the hydrolysis of water-soluble long acyl chain esters (≥C10) in fats and oils into fatty acids and glycerol and are inactive against water-insoluble short-chain triacylglycerols (≤C10), which, in turn, are mainly hydrolyzed by esterases. (Gupta *et al.*, 2004; Treichel *et al.*, 2010; Feng *et al.*, 2013). This hydrolysis takes place at the interface between the insoluble substrate phase and the aqueous phase, the region where the enzyme dissolves (Fig. 2.2). Indeed, they share a common structural motif that contains a serine residue within the consensus sequence Gly-X-Ser-X-Gly involved in the hydrolytic activity (Gupta *et al.*, 2004; Pérez *et al.*, 2011). The ability of lipases to catalyze reactions in a broad range of substrates without the addition of expensive cofactors and their stability in organic solvents resulted in the enzymes being listed as the third-largest group of commercialized enzymes after proteases and carbohydrases (Hasan *et al.*, 2006; Casas-Godoy *et al.*, 2012).

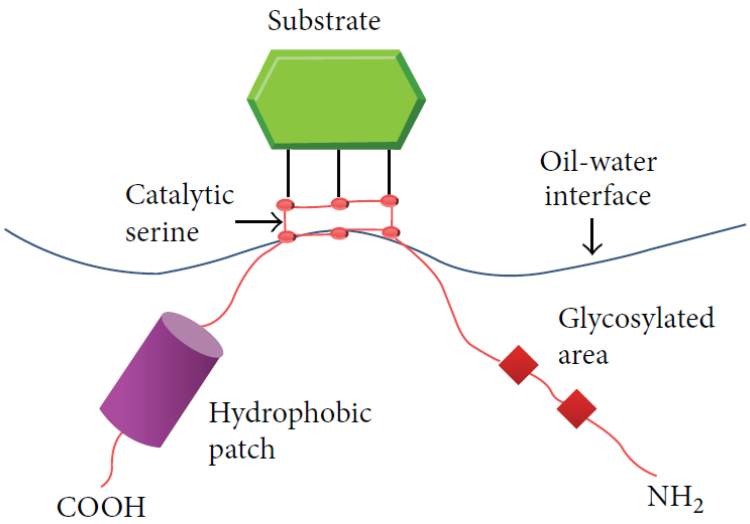


Figure 2.2. Hydrolysis of the triglyceride by lipolytic enzymes takes place at the interface of the insoluble substrate phase and the aqueous phase, the region where the enzyme is dissolved.

Reference: Lee *et al.*, 2015

Lipolytic enzymes were produced naturally in several species of animals, plants, bacteria, yeasts, and filamentous fungi with unique features that can be used for biotechnological applications. Among these, lipolytic enzymes produced by microbial are preferable due to their broad substrate specificity and higher stability under optimum pH at neutral or alkaline condition compared to the lipases from other sources (Gupta *et al.*, 2004). Lipolytic enzymes from microbial have gained special industrial attention due to their selectivity and short generation times. Besides, bacterial cultures were more readily scaled up for production and purification with lower production costs (Saxena *et al.*, 2003; Nagarajan, 2012). Moreover, genetic manipulations can be performed more easily on bacterial cells to increase the production of the lipolytic enzymes (Hasan *et al.*, 2006).

Presently, lipolytic enzymes (lipases/esterases) represent a significant segment with high growth potential in the World Industrial Enzymes Market. The report of The Microbial Lipase Market by Application and Region-Global Forecast they reported that the global market of lipase is projected to reach 590.5 Million dollars by 2023, at a Compound Annual Growth Rate (CAGR) of 6.5% between 2015 and 2020 in terms of value (http://www.researchandmarkets.com). The global esterase market is also expected to grow between 2018 to 2027 due to the growth in the production and demand for esterase enzymes in the multiple end-use industries across the globe. (https://www.factmr.com/report/3014/esterase-market). Due to the various range of utilities and applications in many industries, using lipolytic enzymes in industrial production processes is gaining the active interest of manufacturers around the world.

Lipases/esterases are employed in various processes of industrial relevance with applications in a variety of biotechnological fields such as food and feed (modification of fats to develop organoleptic and nutritional qualities), dairy, detergent, textiles, feminine hygiene products, agrochemical pulp, and paper processes (removal of the pitch from paper pulp), synthesis of biopolymers, biodiesel production, synthesis of optically pure compounds and fine chemicals of interest in the medical and pharmaceutical (antibiotics, anti-inflammatory drugs), cosmetic and aroma industry (flavor and fragrance compounds), oleochemical, agrochemical industries (herbicides,

insecticides), and bioremediation and waste treatment (Jaeger *et al.*, 1999; Pandey *et al.*, 1999; Houde *et al.*, 2004; Hasan *et al.*, 2006; López-López *et al.*, 2014).

2.2.2. Lipolytic enzymes from halophilic bacteria

One of the most interests of halophilic enzymes has been devoted to an enzyme that catalyzes the bioprocessing of lipid. Lipolytic enzymes isolated from thermophilic, halophilic, alkaline, and acidic sources tend to be thermostable, salt, alkaline, and acid-tolerant, respectively. In the case of salt tolerance, halophilic lipolytic activity is included in the list of the published properties. However, in many instances, the bacteria that able to hydrolyze Tween 20, 40, 60, or 80 (also called polysorbate) have been called lipolytic producing bacteria. Tween 20, 40, 60, and 80 are polyoxyethylene sorbitan ester of laurate, palmitate, stearate, and monooleate, respectively, whereas Tween 85 (polysorbate trioleate ester) is rarely evaluated. In addition, most in the taxonomic study, the API ZYM test kit was used to evaluate the hydrolysis potential between C4-esters (esterase activity), C8-ester (ester/lipase activity), and C14-ester (lipase activity). Although, in some cases, they only hydrolyze the C8-ester, they are still called lipolytic. Usually, hydrolysis of Tween 80 and 85 correlates with the presence of lipases able to hydrolyze glycerides with C18 fatty acids; however, sometimes, exceptions exist (Salameh & Wiegel, 2007).

Research in the area of lipolytic enzymes from halophilic bacterium has expanded quite a lot in recent years. Since industrial processes are commonly carried out under harsh conditions, it would be of great importance to obtain lipases which retain their optimal activity at extremes of temperature, pH, and different concentrations of salts and in the presence of organic compounds usually used in the industrial reactions as solvents (Mellado *et al.*, 2005). The search for the enzyme produced from halophiles seems to be particularly promising since they have particular adaptations to increased stability in adverse environments, and the microbial products are highly stable.

Lipolytic enzymes could be produced from a various genus of halophilic bacteria and extremely halophilic archaea such as *Bacillus* sp., *Chromohalobacter* sp., *Haloarcula* sp., *Halococcus* sp., *Halomonas* sp., *Idiomarina* sp., *Marinobacter* sp., *Micrococcus* sp., *Nesterenkonia* sp., *Oceanobacillus* sp., *Pseudoalteromonas* sp.,

Pseudomonas sp., *Salicola* sp., *Salinivibrio* sp., *Staphylococcus* sp., *Thalassobacillus* sp., and so on. The lipolytic enzymes produced from *Halobacillus* have been studied with a few reported; however, the production of lipolytic enzymes from *Lentibacillus* was also omitted. Some of the lipolytic enzyme produced from halophilic bacteria are summarized in Table 2.3

Table 2.8. Characteristics of lipolytic enzymes produced from halophilic bacteria

Organism	Lipase/ esterase	MW (kDa)	Enzyme properties	Enzyme stability	References
Staphylococcus warneri PB233	lipase		substrate: 10% olive oil, pH 7.0, Temp. 40°C, 2.5 M (15%) NaCl, used in fish sauce quality improvement	NR	Kanlayakrit & Boonpan, 2007
Salinivibrio sp. SA2	lipase		substrate: *p*-NPB, pH: 7.5, Temp. 50°C, 0.01 M $CaCl_2$	stable at a pH range of 7.5-8 and retained 90% of its activity at 80 °C for 30 min.	Amoozegar *et al.*, 2008
Bacillus pumilus LV01		77 KDa	pH 9, Temp. 30 °C, 1.7 M NaCl	NR	Guzmán *et al.*, 2008
Haloarcula marismortui	esterase	NR	substrate: *p*-NPV, pH: 7.5, Temp. 45 °C, 4 M NaCl	pH: NR, Loss of activity above 75 °C, 50% activity lost without salt	(Camacho *et al.*, 2009; Müller-Santos *et al.*, 2009)
Thalassobacillus sp. DF-E4	Carboxyl-esterase	45 kDa	substrate: *p*-NPB, pH 8.5, Temp. 40 °C, 0.5 M NaCl, K_m and V_{max} values of 0.69 mM and 0.84 µmol min^{-1} mg^{-1}	stable between pH 6.0-9.5, 45 °C for 1 h, Stable up to 4 M NaCl; retained 90 % activity after 12 h incubation	(Lv *et al.*, 2011)
Virgibacillus sp. IDS 20	lipase		pH 7.0, Temp. 40, 25% NaCl, Application in the Budu industry	NR	Bovornreungroj *et al.*, 2012
Halobacillus sp. LY5	esterase	96 kDa	substrate: *p*-NPB, highly active over broad ranges of temp. 30-90°C), pH 6.0-12.0, NaCl 0 to 20%, optimal at 50 °C, pH 10.0, 10% NaCl.	Complete inhibition by PMSF, PAO, DEPC SDS, Triton X-100.	Li *et al.*, 2012b
Chromohalobacter sp. LY7-8	lipase	44 kDa	substrate: *p*-NPM, pH 9.0, Temp. 60°C, 12.5% NaCl	significant inhibition by EDTA, PMSF, DEPC, SDS, Triton X-100.	Li et al., 2012a
Oceanobacillus. sp. PUMB02	lipase		substrate: 5% olive oil, pH 8.0, Temp. 30 °C, used to disrupt bacterial biofilms	NR	Kiran *et al.*, 2014
Oceanobacillus rekensis PT-11	lipase	23.5 kDa	pH 8.5, Temp. 30 °C	stable at pH 8.5, 10-35 °C, inhibited by Triton X-100, Cu^{2+}, enhanced by Na^{+}, Li^{+} and K^{+}	Jiewei *et al.*, 2014
Halobacillus trueperi whb27	esterase	35 kDa	pH 8.0,Temp. 42 °C, 2.5M NaCl	inhibited by PMSF, Ba^{2+}, Fe^{2+} ions	Yan *et al.*, 2014
Halobacillus trueperi RSK CAS9	lipase	44 kDa	substrate: *p*-NPP	enhanced by Mg^{2+}, Ca^{2+}, Mn^{2+}, inhibition by EDTA and suppressed with Co^{2+}, Hg^{2+}, Fe^{2+} at both 0.1 and 1 mM	Sathishkumar *et al.*, 2015
Bacillus cereus AGP-03	Dimeric esterase	41 kDa (25 kDa and 16 kDa)	substrate: *p*-NPB, V_{max} 52.46µM, K_m 1654 U mg protein $^{-1}$, pH 8.5, Temp. 55 °C, 4.5% NaCl	stable between pH 5.5 and 10, Temp 10-75 °C, 4.5-11% NaCl	Ghati & Paul, 2015
Halobacillus sp. AP-MSU 8	lipase	25 kDa	pH 9.0, Temp. 40 °C, 2.5 M NaCl	enhanced by $BaCl_2$, MgSO4, polyethylene glycol, Tween 20, inhibited by $ZnSO_4$, $ZnCl_2$, 20 % of all the tested solvents	Esakkiraj *et al.*, 2016

NR: not reported

2.3. Recombinant technology for lipolytic-producing moderately halophilic bacteria

Halophilic bacteria and archaea are genetically tractable; hence, they are an excellent model for genetic engineering. They possess unique valuable enzymes and many other substances with application potentials. Enzymes from halophilic microorganisms have evolved to function in a variety of extreme environments, and bio-prospecting for these microorganisms has led to the discovery of new enzymes with high tolerance to non-natural conditions (Liszka et al., 2012). The achievements in genetics and biotechnology would help us to comprehensively understand these halophiles and effectively utilize these unique microbial resources. The studied of recombinant technology for lipolytic-producing moderately halophilic bacteria was described below.

Five lipase genes (*lipab4*, *lipab8*, *lipab15*, *lipag18*, and *lipab18*) have been extracted from *Halomonas* and *Chromohalobacter* sp. (Asyari *et al.*, 2015). The recombinant plasmid was constructed by ligating with *pGEM-T* easy plasmid and transform into *E. coli* Top 10. Based on amino acid sequence analysis, all of the lipases were classified as a member of family IV (HSL, hormone-sensitive lipase), and the genes showed some unique motif of amino acid sequences. These lipases show high similarities of conserved regions with lipolytic of *Halomonas* and formed a distinct cluster with other types of HSL, such as esterase/lipase and carboxylesterase. All of the lipases contain more negative charges of amino acid residues compared to the mesophilic and thermophilic ones and tend to have similarity to lipases of moderate halophilic. The result of homology and phylogenetic analysis showed that these lipases were clustered into three groups. Group, I (*lipab8*, *lipab18,* and *lipag18*) closed to the lipolytic gene of *Halomonas elongata* DSM 2581, while groups II (*lipab4*) and III (*lipab15*) created new branches in the phylogenetic tree. In addition, analysis of GC, GC-AT, and GC-AT content on the codon usage of the genes revealed the unique profile compared to that the other lipase genes.

Pérez and co-workers (Pérez *et al.*, 2011) were cloned a lipolytic enzyme *LipBL* from the moderate halophile *M. lipolyticus* SM19 into *E. coli* and overexpressed and purified the recombinant lipase. Notably, this enzyme's maximal activity was found to

be in the absence of NaCl. The addition of 0.5 M NaCl inhibited activity by 80%, yet the enzyme showed a 20% activity at NaCl concentrations up to 4M. While enzymes that are not a genuinely halophilic enzyme, the protein showed remarkable stability in a wide variety of organic solvents, including DMSO, DMF, methanol, ethanol, toluene, diethyl ether, propanol, and acetone, all at 30% concentration. *LipBL* was immobilized on various solid supports and retained 92% of its activity against *p*-nitrophenol substrates when immobilized on CNBr support. Under these conditions, *LipBL* was assayed for its ability to produce both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil. The fish oil used in the experiment contained 18% of EPA and increased to 27% in the released fatty acids by the activity of the CNBr derivative. These polyunsaturated fatty acids are essential components in health food preparations and dietary supplements, so any improvement in their production would be of interest to the food and health industry. These results indicate that immobilized *LipBL* is the right candidate for the preparation of polyunsaturated fatty acids (PUFAs) on an industrial scale.

Pérez and colleagues (Pérez *et al.*, 2012) used a strain of *M. lipolyticus*, a moderate halophile isolated from Cádiz, Spain, to isolate and express lipase LipBL in Escherichia coli. This lipase, although not very stable in saline media, showed some interesting features when characterized. It was found to have an optimal temperature of 80 °C, the ability to hydrolyze olive oil and fish oil, and high stability in various organic solvents, such as DMSO (30%), N, N-dimethylformamide (30%), ethanol (30%), 2-propanol (30%), diethyl ether (30%), toluene (5%), and hexane (5%) when incubated for 30 min at room temperature.

An esterase PE10 (279 aa, 29.91 kDa) from the marine bacterium, *Pelagibacterium halotolerans* B2^{T}, was cloned and overexpressed in *Escherichia coli* Rosetta (DE3) in a soluble form. The phylogenetic analysis showed it represented a new family of lipolytic enzymes. The purified esterase PE10 exhibited maximum activity toward short-chain *p*-nitrophenyl esters, *p*-NPA and showed its optimal temperature and pH were 45 °C and pH 7.5, respectively. The enzyme was shown to be unstable at temperatures above 40 °C for more than 10 min, losing 60% of its activity. Esterase PE10 was classified as halotolerant esterase and still active under 4

M NaCl and was also stable in the presence of some organic solvents and detergent. They were maintaining more than 50% of its activity in individual reaction mixes containing either 15% DMSO, 15% methanol, or 1% Triton X-100. The three-dimensional structure of PE10 suggested that the high negative electrostatic potential on the surface may be relevant to its tolerance to a high salt environment. (Jiang *et al.*, 2012).

The 23.19 kDa recombinant alkaline esterase PE8 (219 aa) was extracted from *P. halotolerans* B2^{T}. They displayed an optimal at pH of 9.5 and a temperature of 45 ℃ toward *p*-NPA ester. PE8 exhibited activity and enantioselectivity in the synthesis of methyl (*R*)-3(4-fluorophenyl) glutarate ((*R*)-3-MFG), a pharmaceutically necessary precursor in the synthesis of the widely used antidepressant (-) paroxetine hydrochloride, from the prochiral dimethyl 3-(4-fluorophenyl) glutarate (3-DFG). (R)-3-MFG was obtained in 71.6% ee and 73.2% yield after 36 hr reaction under optimized conditions (0.6 M phosphate buffer pH 8.0 contained 17.5% 1,4-dioxane at 30 ℃) (Wei *et al.* 2013). In 2017, Huo, with co-workers, reported the crystal structure of esterase PE8 and this enzyme belonging to the Lysophospholipase carboxylesterase (LPCE) family (Huo *et al.* 2017).

A lipolytic enzyme encoded a protein of 404 amino acids (45.3 kDa) and high identity to class C β-lactamases, which isolated from *Marinobacter lipolyticus* SM19. This enzyme, designated LipBL, was cloned and expressed in *E. coli* DH5α. The recombinant enzyme showed maximal activity at 80 ℃ without NaCl, and the pH optimum at 25 ℃ was 7.0 while maintaining 20% activity in a wide range of NaCl concentrations. *LipBL* exhibited high activity against short-medium substrate ester, *p*-NPH, and tricaproin could be hydrolyzed olive oil and fish oil. The hydrolyzed fish oil using LipBL results in an enrichment of free eicosapentaenoic acid (EPA), but not for docosahexaenoic acid (DHA) when compared to its levels present in fish oil. *LipBL* was immobilized in CNBr-activated Sepharose for improving the stability and being used to release of EPA. The *LipBL* had remarkable activity and stability when exposed to buffer-solvent mixtures in all organic solvents tested (Pérez *et al.,* 2011). *LipB*L gene from *M. lipolyticus* SM19 was cloned in *E. coli* BL21 (DE3) contained a single active site (S72-M-T-K75) responsible for catalytic activity and comprised a loop (H310-S321) located

nearly the active site of *LipBL* and interacts with substrates during hydrolysis. The mutation at some sites affected the stability of *LipBL*, such as the presence of organic solvents (S321A and S321M), at high temperatures (S321D and S321A), and extreme pH (S321D). The results showed that the engineering strategy allowed to identify several LipBL variants with modifying catalytic properties that could be useful in various temperatures, pHs, and substrates. (Pérez *et al.,* 2012).

The *YpmR* enzyme (29 kDa) from moderate halophilic *B. licheniformis* was heterologously synthesized in *E. coli* (Mach1, BL21, or JM107 strains) cells have 7-8 fold higher hydrolytic activity on the *p*-NPL substrate. The enzyme showed high enzymatic activities at pH 8, 45 ℃ and 0.5 M NaCl. It was observed that the enzyme lost approximately 50% activity at 55 ℃ and 2.5 M NaCl concentration. The enzyme was honored to be more tolerant of an increase in NaCl and Triton X-100 (0.01 to 0.25%) and showed potential for application in the detergent industry (Touray *et al.*, 2020). Furthermore, a lipase/esterase from extremely halophilic bacteria and archaea were reported, such as; esterase from *Janibacter* sp. R02 (Castilla *et al.*, 2017) and *LipC* from *Haloarcula marismortui* were cloned and expressed in *E. coli* BL21 (DE3) (Müller-Santos *et al.*, 2009; Lv *et al.*, 2011).

So far, numerous recombinant halophilic lipases/esterases have been reported. *E. coli* was used as a host for recombinant protein production. However, the desired product was produced as an intracellular protein that required extensive downstream extraction and purification processes. The contamination of *E. coli* endotoxin (Lipopolysaccharide) may be concerned about the safe use of products. Hence, the salt-inducible heterologous gene expression system in *B. subtilis* is an attractive alternative system (Westers *et al.*, 2004).

In contrast to the familiar Gram-negative bacterium *E. coli*, the Gram-positive bacterium *B. subtilis* is well known for its contributions to agricultural, medical, food biotechnology, and the production of recombinant proteins. Naturally, *B. subtilis* secretes high levels of extracellular proteases, which degrades the secreted foreign proteins. It is well established that *B. subtilis* has six extracellular proteases, including neutral protease A, subtilisin (also known as alkaline protease), extracellular protease, metalloprotease, bacillopeptidase F, and neutral protease B. Since the protease

production is limited, protease deficient *B. subtilis* strains have been developed by genome engineering techniques. For example, *B. subtilis* WB800, deficient in eight extracellular proteases, can serve as an excellent host for the expression of heterologous proteins (Wu *et al.*, 1991; Kodama *et al.*, 2012).

B. subtilis established as a cell factory for heterologous protein production by providing high accessibility for genetic manipulations and has been developed as an attractive host because of several reasons. It is a non-pathogenic attractive host, and the status approved by GRAS organism of the U.S. Food and Drug Administration (FDA) (Promchai *et al.*, 2016); It has no significant bias in codon usage (Wang *et al.*, 2015b); It is capable of secreting functional extracellular proteins directly into the culture medium (at present, about 60% of the commercially available enzymes are produced by *Bacillus* species) (Wang *et al.*, 2015b). Additionally, a large body of information concerning transcription, translation, protein folding, and secretion mechanisms, genetic manipulation, and large-scale fermentation has been acquired (Wong, 1995).

Finally, exciting work regarding a more concrete industrial application for halophilic bacteria came from Promchai and co-workers (Promchai *et al.*, 2016). The system for protein expression has been employed to improve *B. subtilis* as a protein secretion factory. A novel salt-inducible shuttle vector, pSaltExSePR5 with *opuAA* (salt-inducible promoter), and signal peptide of subtilisin E (*SubE*) was developed. Sodium chloride was used as the inducer for the systems. The results demonstrated an alternative vector for the efficient and straightforward production of heterologous a protease gene from moderately halophilic bacteria, *Halobacillus* sp. SR5-3, in *B. subtilis* with a safer and more economic inducer. The highest protease activity of 9.1 U/mL was obtained after induction with 4% NaCl, while the non-induced culture exhibited an activity of 0.128 U/mL. Furthermore, a serine protease gene (*hproPRW1*) extracted from *H. salinarum* BCC 83492 isolated from fish sauce in Thailand was successfully constructed in pSaltExSePR5 vector, named pSaltExPR5-HProPRW1, and expressed *B. subtilis* WB800. The results showed that recombinant *B. subtilis* harboring pSaltExPR5-HProPRW1 produced serine protease within 22 hr comparing to wild type (96 hr). The recombinant enzyme displayed optimal activity at 60 °C, pH 9, and 12% NaCl and

stable at least one month at 4 ℃ (Promchai *et al.*, 2018). Therefore, the secretory production prototype using that vector incorporates with the selected lipolytic encoding gene in this study was then evaluated in *B. subtilis* as a host.

CHAPTER III

RESEARCH METHODOLOGY

3.1. Bacterial strains

All type strains of moderately and extremely halophilic bacteria used in the present study for DNA fingerprinting and comparative strain of novel species were obtained from JCM, KCTC, TISTR, and from Prof. Dr. Somboon Tanasupawat, Faculty of Pharmaceutical Sciences, Chulalongkorn University. For *Escherichia coli* DH5α and *B. subtilis* WB800 obtained from Dr. Plearnpis Luxananil, Microbial Cell Factory Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC). Media and temperature for growth can be found by searching under the organism name or strain number on the website are shown in Table 3.1.

Table 3.1. List of strains used in this study

No.	Name	Strain	Medium	Temp.(°C)	Purpose
1	*Bacillus marisflavi*	KCTC 3906^T	Marine agar 2216 (Difco)	30	DNA fingerprint
2	*Bacillus subtilis* subsp. *subtilis*	KCTC 3135^T	Marine agar 2216 (Difco)	30	DNA fingerprint
3	*Haloarcula amylolytica*	JCM 13557^T	Halobacteria medium 168	37	DNA fingerprint
4	*Halobacillus halophilus*	JCM 20832^T	Marine agar 2216 (Difco)	30	DNA fingerprint
5	*Halobacillus profundi*	JCM 14154^T	Halophile medium 464	30	DNA fingerprint
6	*Halobacillus salinus*	JCM 11546^T	Marine agar 2216 (Difco)	30	Comparative strain
7	*Halobacillus locisalis*	KCTC 3788^T	Marine agar 2216 (Difco)	30	Comparative strain
8	*Halobacillus yeomjeoni*	KCTC 3957^T	Marine agar 2216 (Difco)	37	Comparative strain
9	*Halobacterium halobium*	JCM 8980^T	Halobacteria medium 168	37	DNA fingerprint
10	*Natrinema altunense*	JCM 12890^T	Halobacteria medium 168	37	DNA fingerprint
11	*Natrinema gari*	BCC 24369^T	Halobacteria medium 168	37	DNA fingerprint
12	*Natrinema versiforme*	JCM 10478^T	Halobacteria medium 168	37	DNA fingerprint
13	*Paenibacillus cineris*	KCTC 3998^T	CASO Agar (Merck 105458)	30	DNA fingerprint
14	*Salinicoccus roseus*	JCM 14630^T	Moderate Halophile medium 599	37	DNA fingerprint
15	*Thalassobacillus devorans*	CCM 7282^T	Mueller Hinton broth	37	DNA fingerprint
16	*Virgibacillus carmonensis*	KCTC 3819^T	Marine agar 2216 (Difco)	30	DNA fingerprint
17	*Virgibacillus itoralis*	KCTC 13228^T	Marine agar 2216 (Difco)	30	DNA fingerprint
18	*Virgibacillus pantothenticus*	CCM 2049^T	Marine agar 2216 (Difco)	30	DNA fingerprint
19	*Oceanobacillus. picturae*	KCTC 3821^T	Marine agar 2216 (Difco)	37	DNA fingerprint
20	*Lentibacillus juripiscarius*	TISTR 1535^T	Lentibacillus medium 377	37	Comparative strain
21	*Lentiacills halophilus*	TISTR 1549^T	Halobacteria medium 168	37	Comparative strain
22	*Escherichia coli*	DH5α	LB Broth (Lennox)	37	Plasmid preparations
23	*Bacllus subtilis*	WB800	LB Broth (Lennox)	37	Expression host

3.2. Samples

Various types of fermented food samples in this study including fish sauce (*Nam-Pla*); fermented-fish (*Pla-ra and Pla-chom*); fermented shrimps (*Kung-chom* and *Kung-som*), shrimp paste (*Ka-pi*); salted crab (*Poo-Khem*); salted-fish (*Pla-khem*); fermented mussel (*Hoi-dong*); pickled vegetable (*Pak-dong*) and soil salt pan were collected for isolation of moderately halophilic bacteria. Samples were transported to the laboratory for bacteriological isolation within 24 hr or as soon as possible after collected and processed immediately.

3.3. Isolation of moderately halophilic bacteria

Twenty-five grams of each sample were mixed with 225 mL of 0.85% (w/v) NaCl and homogenized using stomacher for 2 min, then 0.1 mL portion of each serially dilution was spread onto *Lentibacillus* medium (JCM No. 377) (containing in g L^{-1}: NaCl 100.0, casamino acids 5.0, yeast extract 5.0, glutamic acid 1.0, trisodium citrate 3.0 , $MgSO_4 \cdot 7H_2O$ 20.0, $FeCl_2.4H_2O$ 36.0 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.36 mg L^{-1}, agar 20.0, distilled water 1 L, pH 7.2) (https://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD=377). After incubation at 37 ℃ for 3-5 days, colonies representing various morphology types detectable on the plates were selected and re-streaked them to purity on solid media, ensuring the recovery of a single morphology for each. All purify isolates were stored as stock cultures at 80 ℃ on the same medium supplemented with 5 % (v/v) dimethyl sulfoxide (DMSO) (Yim *et al.*, 2014) and 20% (v/v) glycerol as a cryoprotectant agent (Viver *et al.*, 2015), for long-term preservation the lyophilization would be performed. The isolate and type strains were routinely grown aerobically on the same medium except where indicated otherwise for phenotypically characterize.

3.3. Screening of lipolytic-prodableucing moderately halophilic bacteria on the agar plate

The purify isolates were subjected to screen for the production of extracellular lipolytic in a simple qualitative plate assay according to the modified method of Barrow and Feltham (Barrow & Feltham, 1993). The lipolytic activity of the isolated was checked using lipolytic medium (concentrations in g L^{-1}): NaCl 100, peptone 1, $CaCl_2 \cdot 2H_2O$ 0.01, agar 20, pH 7.0 supplemented with 1% (w/v) of each Tween 20, Tween 40, Tween 60, Tween 80 and glycerol butyrate (tributyrin) as substrates. The agar plates

were inoculated with eight isolates by line-inoculation technique and incubated at 37 °C for two days in plastic bags to avoid desiccation of the medium. The presence of lipase-producing strains was demonstrated by the formation of conspicuous halos due to the formation of precipitates of calcium laurate, palmitate, stearate, or oleate around the zones of bacterial growth (Gutierrez & Gonzalez, 1972).

3.4. Screening of lipolytic-producing moderately halophilic bacteria in the liquid medium

All of the isolates which were produced lipolytic enzymes on the agar plate from section 3.3 were tested for their ability to extracellular lipase production in the liquid medium. The inoculum was routinely grown in 5 mL portions in 16 x 25 cm glass tube with shaking at 200 RPM in an incubator shaker (Sartorius, Certomat® BS-1, Goettingen, Germany) at 37 °C. After two days incubation, 1% (v/v) of inoculum was added to the 73EP medium without any inducers and cultured in the same conditions. Cultures supernatant was harvested by centrifugation (Eppendorf 5810R, Hamburg, Germany) at 10,000 x g, at 4 °C for 5 min and kept refrigerated at 4 °C until the extracellular lipolytic activity was analyzed

Lipolytic activity was performed in 96 well plate via the colorimetric measurement of *p*-nitrophenol (*p*-NP) release from *p*-nitrophenyl ester (Sigma Chemical, Co, LTD, USA) with slight modification method as described by Bhatnagar *et al.* (2005). Two different carbon chain length substrates, *p*-NPB (C4:0) and *p*-NPP (C16:0), were used as the substrate for esterases and lipases assay, respectively. The substrate was dissolved in isopropanol as a stock solution and stored in the dark at -20 °C until use. Immediately before the assay, *p*-nitrophenyl ester was mixed with 50 mM Tris-HCl buffer pH 8.0 at a concentration of 5 mM. The 250 µL of the reaction mixture in each well contained 1 mM *p*-nitrophenyl ester (the final concentration of isopropanol not more than 1% (v/v) in a total volume of reaction), 50 mM Tris-HCl buffer pH 8.0, and NaCl 10%. After 10 min pre-incubation in thermo mixer (Eppendorf ThermoMixer®C, Hamburg, Germany), the appropriate dilution of the enzyme was added and mixed well. The absorbance was monitored immediately at $A_{410\ nm}$ after 10 min of incubation at 37 °C using a Microplate reader (Spectra Max Plus 384 Microplate Reader, CA, USA). The non-enzymatic hydrolysis was subtracted by using a blank with a denatured

enzyme (100 °C for 10 min). The enzyme assay was performed in triplicate. The amount of released *p*-NP was determined for the lipase activity when *p*-NP was used as a standard. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate one µmol of *p*-NP formed per min under specified conditions. Specific activity was determined by calculating enzyme activity per milligram of protein. Unless specially mentioned, all measurements of enzyme activity were conducted under standard conditions.

3.5. DNA fingerprinting of LPMHB

In this study, a repetitive sequence-based on polymerase chain reaction (rep-PCR) fingerprinting was used to study the diversity of LPMHB. Crude DNA was extracted from the pellet using the alkaline polyethylene glycol-based method (Alkaline-PEG) (Chomczynski & Rymaszewski, 2006). The oligonucleotide primers used in this study were: $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). PCR amplification was done in 25 µL reaction mixture consisting 2 µL of crude DNA, 2.5 µL of 10X PCR reaction buffer containing 20 mM $MgCl_2$, 1.25 µL of 20 mM each of the primers, 2 µL of 2.5 mM of the dNTPs mix, and 0.2 µL of Takara *Taq* DNA polymerase (5 U/µL) (Takara Bio Inc, Japan) and nuclease-free water up to 25 µL. The amplification program was performed in a Bio-Rad T100 PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA) consisted of an initial denaturation step at 95 °C for 5 min, which was followed by 30 cycles of 94 °C for 0.45 min, 40 °C for 1 min, 65 °C for 10 min, and a final extension at 65 °C for 20 min (Chokesajjawatee *et al.,* 2008)

Ten microliters of amplification products were separated in a 1% (w/v) agarose gels (15x25 cm) for 2.20 hr at a constant voltage of 150 volts in 0.5X TBE buffer at room temperature (25 °C) and stained with 0.5 µg/mL EtBr, and then the image was captured under UV light using Gel document™ XR+ imaging system (Bio-Rad, USA). The images were saved in TIFF format and imported into the computer software GelCompar II version 5.10 (Applied Maths, Kortrijk, Belgium). Each track on the gel was normalized by an alignment of the reference tracks to a standard reference for comparison of the various migration distances within and between gels. A similarity coefficient was performed with Pearson's correlation, and NJ was used to generate the dendrogram. The cophenetic correlation coefficient was used as a statistical method to estimate

the error associated with dendrogram branches (Sokal & Rohlf, 1962). The position tolerance was set at 1.00 (optimization 1.00%) for the gels cluster analysis and uncertain bands were ignored. The clustering value of 75% was mentioned as a significant grouping. For the identification of the representative strains of each bacterial group, the 16S rRNA gene was used as described below.

3.6. Identification of novel LPMHB

The polyphasic taxonomic studies were carried out with the recommendations according to the proposed minimum standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009). For strain SSKP1-9^T, *L. juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T, obtained from Thailand Institute of Scientific and Technology Research (TISTR) were used as reference strains for comparative study. *H. salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T, and *H. yeomjeoni* KCTC 3957^T were used as reference strains for comparative taxonomic characterization of strain SKP4-6^T

3.6.1. Phenotypic characteristics

The colonial appearance was assessed after routine cultivation as described previously. Gram-staining was performed following the method of Dussault (1955). Cell morphology, shape, and size were observed by scanning electron microscope (JSM-IT300, JEOL). Flagellation was determined as described by Forbes (1981). To determine the effect of temperature on growth, strains were cultivated in JCM No. 377 supplemented with 15% (w/v) NaCl at different temperatures (5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C). For the effect of pH ranges for growth, the pH values were adjusted before sterilization to pH 4.0-11.0 (in increments of 1.0 pH unit) using appropriate buffers at a concentration of 50 mM: citrate/Na_2HPO_4 buffer for pH 4.0-5.0, phosphate buffer for pH 6.0-7.0, Tris-HCl buffer for pH 8.0-9.0 and $NaHCO_3$/Na_2CO_3 buffer for pH 10.0-11.0 (Sun *et al.*, 2016). Tolerance of NaCl was tested by using NaCl-free JCM No. 377 supplemented with different NaCl concentrations in the range of 0-35% (w/v, in increments of 1.0%). Growth was monitored by measuring absorbance at 600 nm using a spectrophotometer after incubation at 37 °C, 200 RPM for two days. Growth under the anaerobic condition was determined after incubation for seven days in a Bactron anaerobic system (Sheldon) with a mixed gas phase of N_2:H_2:CO_2 (88:7:5%,

v/v). Catalase, oxidase, citrate utilization, methyl red-Voges-Proskauer (MR-VP), nitrate reduction, and hydrolysis of casein, skim milk, starch, lecithin, Tween 20, Tween 40, Tween 60, and Tween 80 were tested as recommended by Barrow & Feltham (1993) and Logan *et al.* (2009). The isolate and all reference strains were tested for their enzymatic characteristics, acid production, and other biochemical tests using API ZYM, API 50 CHB, and API 20NE strips (bioMérieux) following the manufacturers' instructions. For the strain SSKP1-9^T, the salinity in the API 50 CHB medium was adjusted to 15% (w/v), and the strips were read every 24 hr till 120 hr while being cultured at 37 °C. For determination of the range of carbohydrates used for acid production of strain SKP4-6^T, the classical medium (Leifson, 1963) supplemented with 15% NaCl (w/v) was used.

For susceptibility to antibiotics studies, all tested strains were routinely grown, centrifuged, and washed cells twice with 10% (w/v) normal saline before re-suspension in the same solution and use as an inoculant. A cell suspension was adjusted to McFarland standard no. 0.5, then swabbed on modified Difco marine agar 2216 supplemented with NaCl to 15% w/v to create a lawn. The Thermo Scientific Oxoid antimicrobial susceptibility disks (Thermo Fisher Scientific) were placed onto the medium surface (antibiotics are listed in Table 1). An inhibition zone was recorded after the plate was incubated at 37 °C for three days.

3.6.2. Chemotaxonomic characteristics

The chemotaxonomic studies were analyses using freeze-dried biomass. Two novel strains (SSKP1-9^T and SKP4-6^T) and their closely related type strains were cultivated in JCM No. 377 medium supplemented with 15% (w/v) NaCl 200 RPM shaking at 37 °C for two days. Cells were harvested by centrifuged at 10,000 RPM, 25 °C for 10 min, and then the culture broth was discarded and washed cell twice with 10% (w/v) NaCl before freeze-drying the cells.

3.6.2.1. Diaminopimelic (DAP) acid isomers analysis

The isomeric forms of *meso*-DAP of strain SSKP1-9^T were separated on cellulose sheet plates (Rhuland *et al.*, 1955). Briefly, dried cells (approx. 10 mg) were hydrolyzed with 1 mL of 6N HCl at 15 pounds pressure and 121 °C for 20 min. The hydrolysate solution was filtrated with a No 1 Whatman filter paper and evaporated to dryness, then dissolved with 0.3 mL of water. Each sample (3 µL) was spotted on a

cellulose TLC plate and developed twice with the solvent system methanol:water:6N HCl:pyridine (80:26:4:10, v/v). After the second developing, the TLC plate was dried in a fume hood and spraying with 0.2% (w/v) ninhydrin solution and heated at 100 °C for 5 min. For the strain SKP4-6^{T}, cell wall peptidoglycan was separated using the methods as previously described (Schleifer & Kandler, 1972).

3.6.2.2. Cellular fatty acids analysis

About 40 mg of the dried cell was suspended in 0.1 mL of reagent 1 (sodium hydroxide 15 g, methanol 50 mL, and distilled water 50 mL) in a screw cap tube (16 mm x 150 mm) and vigorously mixed with a vortex mixer for 10 sec. The solution was heated at 100 °C for 5 min, cooled down, mixed and heated again at 100 °C for 25 min and immediately cooled. After cooling, 2 mL of reagent 2 (6N HCl 65 ml, methanol 55 mL) was added to the tube, mixed and heated at 80 °C for 10 min. The reagent 3 (*n*-hexane 50 mL, methyl-*tert*-butyl ether 50 mL) 1.25 mL was added to the mixture and mixed for 10 min. The tube was centrifuged at 4,500 RPM for 10 minutes, and then the upper layer was transferred to a new tube. Three ml of reagent 4 (sodium hydroxide 1.2 g, milli-Q water 100 mL) was added and mixed for 5 min, then transferred 2/3 part of the sample to a GC vial. The fatty acids profile after saponified, methylated, extracted and analyzed using gas chromatography according to the standard protocol of the Sherlock MIDI Identification System (MIDI Sherlock version 6.2B; MIDI database RTSBA6) (Sasser, 1990).

3.6.2.3. Polar lipids analysis

The polar lipids were extracted according to the method of Minnikin and co-workers (Minnikin *et al.*, 1984). Briefly, about 100-150 mg of freeze-dried cells were suspended in a screw-capped tube with 3 mL of methanol 0.3% (w/v) NaCl and 3 mL of petroleum ether. The solutions were mixed well for 15 min and centrifuged at 3,000 RPM for 10 minutes, and then the upper layer was discarded. The lower layer was added with 1 mL of petroleum ether, mixed well for 15 min centrifuged, and removed the upper layer. After heated the lower layer at 100 °C for 5 minutes and immediately cooled, 2.3 mL of chloroform-methanol-water (90:100:30, v/v) was added and mixed well for 15 minutes, centrifuged, and transferred the upper layer into a new screw-capped tube. The lower layer was extracted twice with 2.3 mL of chloroform-

methanol-water (50:100:40, v/v), and the supernatants were transferred to the tube in the previous step. The upper layer was collected to the tube in the previous step. The combined supernatant of the extraction step was added with 1.3 mL of each chloroform and water, mixed well, centrifuged, and the upper layer was removed. The lower layer was dried with N_2 gas and re-suspended in 120 µL of chloroform-methanol (2:1, v/v).

The two-dimensional TLC technique was used to determined polar lipids, 10 µL of the samples was applied to the corner of the silica-gel TLC plate in the size of 10 x 10 cm and first developed on the solvent system chloroform-methanol-water (65:25:4, v/v) and the second dimension was developed on the second solvent system chloroform:acetic acid:methanol:water (40:7.5:6:2, v/v). To detect spots, each TLC plate was sprayed with specific reagents including phosphomolybdic acid solution, molybdenum blue solution, Dragendroff's reagent, α-naphthol sulfuric solution, and ninhydrin solution (used for total lipids, phospholipids, phosphatidylcholine, glycolipids, and amino lipids, respectively. After spraying with α-naphthol sulfuric solution and Ninhydrin solution, the TLC plate was heating at 110 ℃ for 10 min. Glycolipids were presented as green-yellow spots while PE and its derivatives were displayed as purple spots. For phospholipids detection, after spraying with Molybdenum blue solution, all phospholipids were presented as blue spots. After spraying with the Dragendroff's reagent, choline-containing phospholipids (phosphatidylcholine, PC) was shown as a brown spot. After spraying with phosphomolybdic acid and heating at 130 ℃ for 10 min, all polar lipids were exhibited as dark spots.

3.6.2.4. Analysis of quinone

Isoprenoid quinones were extracted using the method described by Komagata & Suzuki (1987). Approximately 100-300 mg of dried cells were extracted with 20 ml of chloroform-methanol (2:2, v/v) and shaking overnight in an incubator shaker at room temperature. The solution was filtrated with a Whatman No 1 filter paper, and the filtrate was evaporated and dissolved in a trace amount of acetone. The quinones extracted were spotted onto a silica-gel TLC plate (MERCK Kiesel-gel 60 F_{254}, 20 x 10 cm) and developed with benzene. The menaquinone spot was detected

under UV light at 254 nm, then scraped off and extracted with acetone. The acetone extract was filtrated through a 0.5 µm membrane and analyzed by reversed-phase HPLC using a cosmosil C_{18} column (Tamaoka, 1986)

3.6.3. Genotypic characteristics

3.6.3.1. Extraction of genomic DNA

The DNA was extracted using the *AccuPrep*® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's recommendations. For whole-genome sequencing (WGS), DNA purity was evaluated by measure the absorbance using a Nanodrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) at a ratio of A_{260}/A_{280} (around 1.8-2.0) and A_{260}/A_{230} nm (greater than 1.5 but not exceed 2.1) together with agarose gel electrophoresis.

3.6.3.2. Amplification of 16S rRNA gene

The 16S rRNA gene was amplified using 21F (5´-TTCCGGTTGATCCTGCCGGA-3´) (DeLong, 1992) and 1530R (5´-GTTACCTTGTTACGACTT-3´) primers (Suriyachadkun *et al.*, 2009). The 100 µL of reaction mixture contained 10 µL of 10x *Taq* buffer S, 10 µL of dNTP mix (2 mM), 10 µL of 10 µM each forward and reverse primers, 0.8 µL of 5 U/µL *Taq* DNA polymerase (Vivantis CA, USA), 2 µL of DNA template (approx. 50-100 ng/µL) and Dnase free water up to 100 µL. The amplification reaction was performed using the following program: one denaturing cycle at 94 °C (2 min) and 30 cycles of 94 °C (30 sec), 55 °C (30 sec), 72 °C (1.30 min); and a final extension step at 72 °C (7 min). To confirm the presence of PCR products, 1% (w/v) agarose electrophoresis was performed in 0.5X TBE buffer at 100 Volt for approximately 40 min, and visualization was carried out after staining with EtBr. PCR products (Approx. 1,500 bp) were purified with *AccuPrep*® PCR purification Kit (Bioneer, Daejeon, Korea) under the manufacturer's indications. The purified PCR products were sequenced by Bioneer (Republic of Korea) following the manufacturer's protocols using the primer sets: 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), respectively. The 16S rRNA gene sequencing was manually edited by removing unaligned nucleotides at the ends and aligned using MEGA 7 software (Kumar *et al.*,

2016). All of the processed sequences were deposited in the public website (DNA Data Bank of Japan, DDBJ) with accession numbers.

3.6.3.3. Phylogenetic tree and phylogenomic construction

For the determination of similarity values of the 16S rRNA gene sequence to related neighbors were performed on the EzBiocloud server (Yoon *et al.*, 2017). To ascertain the phylogenetic position of the novel strain, the Kimura two-parameter model was used to calculate evolutionary distances (Kimura, 1980). Multiple phylogenetic trees were constructed via the NJ method (Saitou & Nei, 1987), the ML method (Felsenstein, 1981), and the MP method (Fitch, 1971) using MEGA 7. In each case, bootstrap values were calculated based on 1000 replications (Felsenstein, 1985), only values >50% are displayed. A phylogenomic tree of novel strains and their closest type strains was constructed using the TYGS webserver (Meier-Kolthoff & Göker, 2019).

3.6.3.4. Draft Whole genome analysis

The draft genome of novel strains, SSKP1-9^T and SKP4-6^T, and two closest related type strains, *L. juripiscarius* TISTR 1535^T and *H. salinus* JCM 11546^T were sequenced (NovogeneAIT Genomics, Singapore) using an Illumina Hiseq platform (Illumina, Inc., San Diego, US-CA) with 2 x 150 bp paired-end reads. The raw reads were QC-filtered using FASTQC version 0.11.4 (Leggett *et al.*, 2013) and AfterQC version 0.9.6 (Chen *et al.*, 2017). The adapter sequence was trimmed using Trimmomatic version 0.36 (Bolger *et al.*, 2014), and the trimmed sequences were de novo assembled with SPAdes version 3.10.1 (Bankevich *et al.*, 2012). Genomes were annotated using PROKKA genome annotation version 1.13 (Seemann, 2014) following the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). A phylogenomic tree was constructed by using the TYGS web server (https://tygs.dsmz.de/) (Meier-Kolthoff & Göker, 2019). Average nucleotide identity (ANI) values of novel strains along with their closely related type strains were pairwise calculated using ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms (Richter *et al.*, 2009) using the JSpeciesWS software (Richter *et al.*, 2016). The *is*DDH values were calculated using Genome-to-Genome Distance Calculator (GGDC 2.1) with the BLAST$^+$ method (Meier-Kolthoff *et al.*, 2013). Results were determined using the recommended formula 2 (identities/HSP length), which is liberated of genome length and is thus prosperous when using incomplete draft

genomes. Gene prediction and functional annotation were performed by Rapid Annotation Server Technology (RAST) server (Aziz *et al.*, 2008). BLASTX analysis was used to blast the similarity of the protein sequence (Stephen et al., 1997). All software tools were run using default parameters.

The draft assembled genomes of strains SKP4-6^{T} and *H. salinus* JCM 11546^{T} were assessed by the bioinformatics tool CheckM Version 1.0.18 (Chun *et al.*, 2018; Parks *et al.*, 2015). The orthologous genes of selected *Halobacillus* genomes were aligned by Multiple Alignment using Fast Fourier Transform, MAFFT software (Katoh & Standley, 2013). All predicted protein-coding genes annotated from each available genome were compared using the all-versus-all BLAST search (Altschul *et al.*, 1990). The core phylogenetic tree was constructed via the FastTree method (Price *et al.*, 2009). The Species Tree Root Inference from Duplication Events (STRIDE) algorithm was applied to root the species tree. All steps in phylogenetic construction were performed in OrthoFinder software (Emms & Kelly, 2019). The distribution of unique proteins was displayed by the Venn diagram (Bardou *et al.*, 2014). All of the draft assembled genomes that were sequenced in this study were publicly available from GenBank® (http://www.ncbi.nlm.nih.gov) (Clark *et al.*, 2016). The DNA G+C content and full-length 16S rRNA were also extracted from the whole genome sequence and analyzed. The gene encoding halophilic lipolytic enzymes (Enzyme Class of 3.1.1.X) of novel strain, SSKP1-9^{T,} and SKP4-6^{T,} were searched from the incomplete whole-genome sequence for gene cloning.

3.6.3.5. DNA fingerprinting on novel strain

The genotypic differentiation was assessed by DNA fingerprinting using the oligonucleotide primers as previously described (Versalovic *et al.*, 1994). The primers used in this study were: BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3'); ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3'), $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'), and REP1R-I (5'-IIIICGICGICATCIGGC-3'). The optimal PCR program for each of the primer sets used was described by Versalovic *et al.* (1994). The purified DNA (50 ng/µL) was used as the template. PCR mixture and amplification conditions for ERIC, $(GTG)_5$, and REP primers were the same as in section 3.5, whereas the cycle for REP primer was up to 45 cycles. For Box primer, the amplification

condition was modified from Chokesajjawatee *et al.*, 2008 consisted of an initial denaturation step at 97 °C for 7 minutes, which was followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 56 °C for 4 min, and a final extension at 65 °C for 16 min. Gel electrophoresis and analysis methods were conducted as previously mentioned above.

3.7. Production and characterization of native extracellular halophilic lipolytic enzyme produced by *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T

The time course of native extracellular halophilic lipolytic enzyme production and cell growth of *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T were studied. The strains were grown in a modified 73EP medium containing 10% NaCl and incubated at 37 °C with 200 RPM shaking for three days. The culture supernatant was collected every 6 hr, and the number of cells was monitored by OD at 600 nm. The cell-free supernatant was used for the monitoring of halophilic lipolytic activity. Characterization of native lipolytic crude extract produced from *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T were performed as described below.

3.8. Cloning and expression of halophilic esterase in *B. subtilis*

3.8.1. Bacterial strains and growth conditions

Escherichia coli DH5α (New England BioLabs, MA, USA) was used for high-quality recombinant plasmid preparations, and *B. subtilis* WB800 (Wu *et al.*, 2002) were used as an expression host. Difco™ LB Broth, Lennox (Cat. No. 240230) (concentration in g L^{-1} NaCl 5, tryptone 10, yeast extract 5, Distilled water 1 L, pH 7.0±0.2) was used to grow bacterial cultures at 37 °C with shaking at 200 RPM. The media for colony selection was prepared by supplementing the medium with 15 g/L agar. The erythromycin (400 µg/mL and 20 µg/mL) antibiotic was added to the plate when required for the selection of *E. coli* and *B. subtilis* transformed with plasmid DNA, respectively. Cell growth was observed by optical density at A_{600} nm measurement of the cultures broth using a spectrophotometer and plate count on LB agar plates.

3.8.2. Preparation of plasmid

A shuttle vector with salt-inducible expression and secretion vector named pSaltExSePR5 was used for the expression of recombinant lipolytic enzymes in *B. subtilis* WB800 (Fig 3.1). pSaltExSePR5 having 5.4 kb in size and contained 12 recognition

sites of common restriction enzymes for the insertion of foreign genes. Besides, the plasmid having the multiple cloning sites (MCS), salt-inducible promoter (*opuAA*), a DNA fragment encoding a signal peptide of subtilisin E (*SubE*), start codon, and *EryR* represent erythromycin-resistant genes, respectively (Promchai *et al.*, 2016). First, *B. subtilis* WB800 containing pSaltExSePR5 plasmid was propagated in LB supplemented with 20 µg/mL erythromycin at 37 °C with shaking at 200 RPM for 17 hr. Then, cells were lysed with 10 mg/mL lysozyme solution, and the produced plasmids were extracted and purified using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA USA) following the recommendation's protocol of Kit. Finally, the *pSaltExSePR5* plasmid was diluted by TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5), and carefully divided and store at -80 °C until use. The pSaltExSePR5 plasmid was purified and evaluated by nanodrop and 1% (w/v) agarose gel electrophoresis.

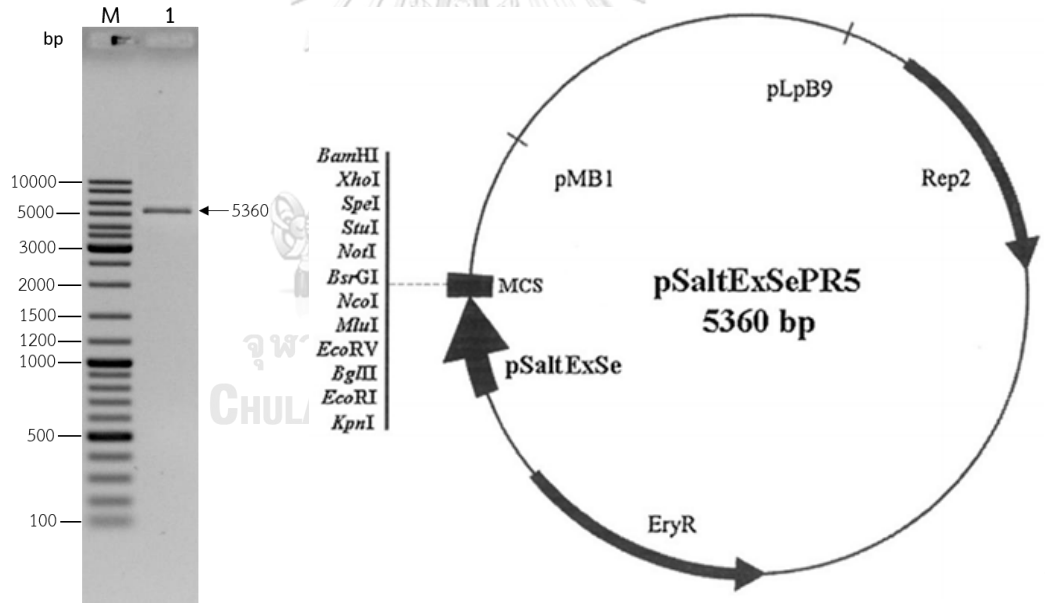


Figure 3.1. Plasmid maps of pSaltExSePR5 (5,360 bp). Panel M: DNA ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific), Panel 1: pSaltExSePR5.

Reference: (Promchai *et al.*, 2016).

3.8.3. Primer design

The gene sequences encoding the lipolytic gene of each strain derived through a genome survey were used to primers design for gene amplification. The name of the gene, similarity value, and other details of degenerate oligonucleotide primers are displayed in Table 3.1. The sequences in this study were also manipulated using NEB CUTTER V 2.0 (Vincze *et al.*, 2003) to include suitable restriction sites; *XbaI* (5'-T↓CTAGA-3' and 5'-AGATC↑T-3') in the red alphabet and *MluI* (5'-A↓CGCGT-3' and 5'-TGCGC↓A-3') in blue alphabet cutting sites for forward and reverse primer are underlined, respectively. A single G or C nucleotide at the 3' end helps to stabilize binding near the site of extension, which can enhance the priming specificity (Innis, 1990). Therefore, primers ending in an A or a T base could be omitted, and G or C nucleotide was added in the first strand nucleotide residues (5' GC content) and the last nucleotide residues (3' GC content). The designed primers were synthesized by Bioneer (Daejeon, Korea).

Table 3.2. Primers for amplification of the esterase gene of strain SSKP 1-9^{T}

No	Gene	Primer's Name	Sequence (5'-3')	TM (°C)	Annealing (°C)
1	>APNFEMBD_00261	LLL-00261 -F	GGGGTCTAGAATGCCGGAAGTACTTATTATC	48.5	46.0
		LLL-00261-R	CCCCACGCGTCTATGTCAATGATGTTCG	43.5	
2	>APNFEMBD_00267	LLL-00267-F	GGGGTCTAGAATGCGGTCTGTTTCATAC	45.8	44.7
		LLL-00267-R	CCCCACGCGTCTATACCGAAAGTTTAGC	43.5	
3	>APNFEMBD_00455	LLL-00455-F	GGGGTCTAGAATGAATGAAACAAGCTGGC	46.8	47.5
		LLL-00455-R	CCCCACGCGTTCAACTTTTGTTGGAATTTATCC	48.1	
4	>APNFEMBD_00457	LLL-00457-F	GGGGTCTAGAATGAAAATTAAATTACCGAAGCC	48.1	52.8
		LLL-00457-R	CCCCACGCGTTCATACCTGCCAGTCAAGTGACTC	57.4	
5	>APNFEMBD_00639	LLL-00639-F	GGGGTCTAGAGTGCCAAAACAGCCGC	48.5	47.6
		LLL-00639-R	CCCCACGCGTTTATTTTAAAAAATATGTTTGTTTTAGG	46.7	
6	>APNFEMBD_01756	LLL-01756-F	GGGGTCTAGAATGGGCGAACTATCGTTTATCC	53.0	52.1
		LLL-01756-R	CCCCACGCGTTTATTCACCCCCACGATGC	51.1	
7	>APNFEMBD_02564	LLL-02564-F	GGGGTCTAGAATGACAGGATGTTTGCTGATACACG	56.0	56.9
		LLL-02564-R	CCCCACGCGTTTACTTGGTATTTTTCTCCGTTAGAAACTGG	57.8	
8	>APNFEMBD_02578	LLL-02578 -F	GGGGTCTAGAATGACACATACACC	34.4	35.2
		LLL-02578-R	CCCCACGCGTTTACGCTTCACC	36.0	

Table 3.3. Primers for amplification of the esterase gene of strain SKP 4-6^{T}

No	Gene	Primer's Name	Sequence (5'-3')	TM (°C)	Annealing (°C)
1	>NKILIEJB_00202	HFL-00202-F	GGGGTCTAGAATGGGATCGGTACAAATTGTTACG	54.0	55.0
		HFL-00202-R	CCCCACGCGTTCACCGGGGTCCAACTTTGG	55.9	
2	>NKILIEJB_00139	HFL-00139-F	GGGGTCTAGAATGCCGAAAGTGTTGATC	45.8	42.5
		HFL-00139-R	CCCCACGCGTTTACTCTTTCGCTGC	39.2	
3	>NKILIEJB_00478	HFL-00478-F	GGGGTCTAGAATGGCTAAAACAATGATTTTCGC	49.9	50.2
		HFL-00478-R	CCCCACGCGTTTAGTCCTTCCTATGTTTTTCAGC	50.5	
4	>NKILIEJB_00719	HFL-00719-F	GGGGTCTAGAATGAAAGTGATCGAAACACC	47.7	48.3
		HFL-00719-R	CCCCACGCGTTTATTGTGGCTCCATAGCC	48.9	
5	>NKILIEJB_01195	HFL-01195-F	GGGGTCTAGAATGAGTCTTAAGGTTATGGAAGGG	54.0	51.6
		HFL-01195-R	CCCCACGCGTTTACTTTGTGTTCACATAACGC	49.2	

3.8.4. Amplificaion of halophilic esterase gene

The PCR amplifications were done using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA USA) via the standard reaction buffer as supplied by the manufacturer. The PCR reaction contained 0.5 µl (50 µM) of each of forward and reverse oligonucleotide primer (Table 3.1), 10 µL of 5X GC buffer, 4 µL of 2.5 mM dNTP mix, 1.5 µL of 100% DMSO, 0.5 µL of 2U/µL Phusion High-Fidelity DNA Polymerase, and 2 µL of 25 ng/µL of SSKP1-9^{T} or SKP4-6^{T} genomic DNA as a template in a total volume of 50 µL. The amplification of the lipolytic gene was performed in two steps. First, pre-denaturation was performed at 98 °C for 10 min followed by 2 PCR cycles of denaturation (98 °C, 30 sec), annealing (50 °C, 30 sec), and extension (72 °C, 2 min). The second amplification cycles consisted of 32 cycles under the following program: an initial denaturation cycle at 98 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 2 min, and final extension was done for 5 min at 72 °C and held at 12 °C. The PCR products were separated by agarose gel electrophoresis and purified by GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA USA), then the resulting fragment was inserted into pSaltExSePR5 plasmid to obtain recombinant plasmid as described below.

3.8.5. Construction of recombinant plasmid

3.8.5.1. Digestion of plasmid and gene insertion

Purified pSaltExSePR5 plasmid (from section 3.7.2) and purified DNA fragment (from section 3.7.4) were digested with *XbaI* and *MluI*, respectively. The

digestion reaction (30 µL) consisted of 1 µL restriction enzyme (10 U/µL), 2 µL 10X digestion buffer (Tango Buffer or Buffer R), 2 µL of plasmid DNA or purified PCR product from the previous step (concentration 100 ng/µL), and 15 µL nuclease-free water. The digestion buffer "Tango Buffer" for 100% *XbaI* and *NcoI* digestion were as following (1X): 33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate and 0.1 mg/mL BSA. The digestion buffer "Buffer R" for 100% *MluI* digestion was consist of (1X): 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mg/mL BSA (All products purchased from Thermo Fisher Scientific Inc, USA). The digestion was performed under 37 °C for 1 hr, and then the digestion result was investigated by agarose gel electrophoresis and purified by GeneJET PCR Purification Kit before used in a further step.

3.8.5.2. Ligation

The two resulting sticky fragment products previously digested with the same restriction enzymes (pSaltExSePR5 and digested PCR product) were ligated into the expression vector. The ligation mixture composed of the purified vector at a concentration of 1 ng/µL (2 µL) and purified gene insert (10 µL) (Approx of 1:5 molar ratio, respectively), 10X ligation buffer (2 µL), dATP (1 µL), 1U/µL T4 DNA ligase (1.5 µL) (Thermo Fisher Scientific, Waltham, MA USA) were added at final volume of 20 µL and incubated for 60 min at 22 °C in thermomixer. Each gene was inserted into pSaltExSePR5 between restriction sites *XbaI* and *MluI* to obtain the recombinant plasmid, followed by transformation into competent cells of *E. coli* DH5α.

3.8.5.3. Transformation

E. coli DH5α competent cells were prepared by DMSO treatment (Inoue *et al.*, 1990). The ligation reactions were performed using the heat shock method. Briefly, 20 µL ligation mixture was added to 200 µL competent cells solution. The mixture was placed on an ice bath for 30 min, then incubated at 42 °C in a thermomixer for 90 sec and cooled on ice for 5 min. Transferred the ligation mixture into a 15 mL centrifuge tube contained 2 mL of LB antibiotic-free liquid medium enhanced with 1% (w/v) D-glucose and activated at 37 °C, 200 RPM for 60 min. The activated transformant cells were kept by centrifuged at 10,000 x g at 4 °C for 5 min. The culture broth was discarded, and cells were re-suspended with 600 µL LB broth. One hundred microliters

of each transformant sample were spread on six plates of LB agar plate containing erythromycin (400 µg/mL) and incubated at 37 °C overnight until the colony appeared. The resulting positive colonies carrying plasmid harboring each of the halophilic lipolytic genes were confirmed by colony-PCR and double digestion with the same restriction enzymes as described previously.

3.8.5.4. Screening of E. coli DH5α harboring the halophilic esterase gene

To select the recombinant *E. coli* DH5α carrying the halophilic lipolytic gene, about 40 single colonies were randomized picked and re-suspended in 50 µL of sterile *Dnase* free water. One microlite was used as template DNA for PCR amplification as the condition described in section 3.7.4. All colonies showing prominent bands were cultivated in LB Broth supplemented with erythromycin (400 µg/mL) at 37 °C with shaking at 200 RPM for 17 hr. The pellet was kept by centrifugation and re-suspended in the same medium supplemented with 15% (w/v) glycerol and stored at -80 °C for stock cultured. Three positive colonies were randomized selected for plasmid extraction using GeneJET Plasmid Miniprep Kit for characterization. Finally, to verify the recombinant plasmid containing lipolytic gene insert, the plasmid was digested with the same restriction enzyme and visualized by agarose gel electrophoresis. The recombinant plasmid was then transformed into *B. subtilis* WB800 for the expression of lipolytic enzymes.

3.8.5.5. Transformation of recombinant plasmid harboring halophilic esterase gene in B. subtilis WB800

B. subtilis WB800 was grown on LB-antibiotic free medium agar plate at 37 °C for 24 hr. A single colony was transferred to 5 ml of LB broth and cultivated for 18 hr before used as an inoculum for transformation. Transfer the inoculum into 5 ml of freshly prepared synthetic Modified Competence (MC) medium (contained: 100 mM KH_2P0_4, 3 mM tri-Na-citrate, 3 mM $MgS0_4 \cdot 7H_20$, 2% (w/v) D-Glucose, 22 µg/mL ferric ammonium citrate, 0.1% (w/v) casein, 0.2% (w/v) potassium glutamate, 50 µg/mL L-tryptophan, pH 7.0 with KOH and sterilized by filtration) containing in 50 mL centrifuge tube with an optical density of 0.1 read at 600 nm. The cultures were incubated with shaking (about 200 RPM) at 37 °C until OD 600 nm reach to 0.5 (between 0.4-0.6) or

when the incubation period of about 2.5 hr. The transformation was started by the recombinant plasmid was added to 5 mL cultures broth with the final concentration 2 µg/mL. The plasmid pSaltExSePR5 without the lipolytic gene was used as a control for plasmid self-ligation checked. The culture broth was incubated at the same conditions for 3 hr, and cells were harvested by centrifugation at 4 °C and 10,000 x g for 5 min. After centrifugation, cell pellets were re-dissolved with 1 mL LB broth and then spread onto ten plates of LB agar plate containing erythromycin (20 µg/mL) and incubated at 37 °C overnight. The recombinant *B. subtilis* was chosen at random (40-50 colonies) for verified the positive clone by colony-PCR and kept as a stock culture at -80 °C.

3.8.5.6. Screening of B. subtilis WB800 harboring the halophilic esterase gene

The slight modifications protocol of Promchai and co-workers (Promchai *et al.*, 2016) was used to express the recombinant halophilic esterase. The PCR-positive recombinant *B. subtilis* WB800 carrying the plasmid was inoculated into 5 ml LB erythromycin (20 µg/mL). The cultures were cultivated for 18 hr with shaking (about 200 RPM) at 37 °C to an optical density of about 1.5 read at 600 nm. The cells were harvested and washed twice with LB before re-suspension in 5 mL of fresh LB erythromycin medium. An aliquot of inoculum (10% v/v) was inoculated into 3 mL LB erythromycin medium and incubated at 37 °C for 4 hr with 200 RPM shaking. The cells were then induced by adding 3 mL of LB supplemented with 8% (w/v) NaCl (final concentration of 4% (w/v)) and let grow at the same conditions for 3 hr. When the fermentation ended, the culture broth was centrifuged at 10,000 x g at 4 °C for 10 min, and the supernatant containing the recombinant halophilic esterase enzymes was recovered. The crude halophilic esterase enzymes of each culture were treated at 55 °C for 1 hr before analyzed the levels of enzyme activity. *B. subtilis* WB800 without recombinant plasmid was used as a control for comparison of the enzyme productivity. The highest esterase activity of the selected clone was studied for characterizations.

3.8.5.7. Expression and partial purification of recombinant halophilic esterase enzyme

The partial purification of the recombinant halophilic esterase was performed to concentrate the recombinant enzyme before the characterization was studied. Briefly, the selected recombinant was grown as the conditions described above (section 3.7.5.4). A 1000-mL flask containing 250 mL LB broth medium supplemented with 20 µg/mL erythromycin was inoculated with 10% (v/v) of pre-cultured cells. Recombinant cells were discarded by centrifugation at 10,000 x g at 4 ℃ for 10 min and recovered supernatant for concentration by ammonium sulfate precipitation. To partial purify, the supernatant (1,000 mL) was treated with ammonium sulfate to achieve 100% saturation, and the container was allowed to stir on-iced for 3 hr. The precipitate was collected by centrifugation at 15,000 x g for 10 min at 4 ℃ and diluted with 50 mL of 50 mM Tris-HCl buffer (pH 8.0). Un-dissolved substances were discarded by centrifugation. The resulting supernatant was dialyzed using SnakeSkin Dialysis Tubing, 3.5K MWCO, 35 mm (Thermo Scientific, USA) in 50 mM Tris-HCl buffer (pH 8.0) for overnight at 4 ℃ with six times buffer changes. For this purpose, about 20-fold concentrated recombinant halophilic esterase was achieved and used for further characterizations.

3.9. Biochemical Characterization of recombinant halophilic esterase

3.9.1. Characterization of the deduced amino acid sequences

Molecular weights and isoelectric points (pI) were calculated in ExPASy (Gasteiger *et al.,* 2005) Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The presence of signal peptide was detected with the bioinformatic tools: Signal P (Petersen *et al.,* 2011) and PrediSi (https:// www.predisi.de/home.html). BLASTX analysis was performed to blast the similarity of protein sequence on the NCBI Database platform (Stephen et al., 1997). Multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al., 2019).

3.9.2. Effect of NaCl concentration on esterase activity and stability

The effect of NaCl on enzyme activity was determined at 50 ℃ in 0.05 M Tris-HCl buffer (pH 8.0) with the different concentrations of NaCl (0-30% w/v) for 10 min. To study the salt stability on enzyme activity, the enzyme was incubated in 50 mM

Tris-HCl (pH 8.0) containing various concentrations of NaCl (0-30% w/v) at 4 °C and 50 °C for 24 hr and 1 hr, respectively. The percent residual activity was measured following standard assay conditions under the optimal salt concentration using *p*-NPB as a substrate by comparing with the salt-free state.

3.9.3. Effect of temperature on esterase activity and stability

The optimal temperature for enzyme activity was assayed over the range of temperatures from 5 to 80 °C and 37 °C was also included. The reaction mixtures containing *p*-NPB as a substrate in 50 mM Tris-HCl buffer supplemented with 15% (w/v) NaCl, pH 8.0. Thermostability was determined by pre-incubation of the enzyme in 50 mM Tris-HCl buffer pH 8.0 with or without NaCl (15% w/v) at 5-80 °C for 60 min, and then the solution was rapidly cooled to 4 °C. The residual activity was measured by the colorimetric method using *p*-NPB as the substrate in 50 mM Tris-HCl buffer pH 8.0. The results were expressed as the percentage of residual activity obtained at either the optimum Temperature or NaCl concentration by comparing with an untreated enzyme.

3.9.4. Effect of pH on esterase activity and stability

The effect of pH on enzyme activity was determined in the pH range of 4.0-10.0 with 1.0 increments. For this purpose, enzyme assays were conducted in 50 mM Britton and Robinson buffer (Britton & Robinson, 1931) at optimal Temperature and NaCl concentration. To determine the pH-stability of the recombinant enzyme, the enzyme solution was pre-incubated (in a ratio of 1:1) in the above-mentioned buffer systems for 1 hr under the optimum temperature, pH, and salt concentration. After incubation, the solution was suddenly cooled on iced, and the residual enzyme activities were subsequently measuring. The percentage of initial activity of enzymes without pre-incubation was set as 100%.

3.9.5. Substrate specificity

To investigate the substrate specificity of recombinant halophilic esterase, the activity was determined using the standard assay in the presence of 1 mM of the different *p*-NP esters with varying lengths of carbon chain: *p*-Nitrophenyl acetate (*p*-NPA, C2:0); *p*-Nitrophenyl butyrate (*p*-NPB, C4:0); *p*-Nitrophenyl caprylate (*p*-NPO, C8:0); *p*-Nitrophenyl decanoate (*p*-NPD, C10:0), *p*-Nitrophenyl laurate (*p*-NPL, C12:0), *p*-NP

myristate (*p*-NPM, C14:0) and *p*-NP palmitate: (*p*-NPP, C16:0). The experiments were carried out in triplicates under the optimum conditions.

3.9.6. Protein determination

Protein concentration was determined using the Lowry method (Lowry *et al.*, 1951). All assays were performed in triplicate and average. A standard curve was drawn using bovine serum albumin

3.10. Statistical studies

The independent measurements were carried out for the different values of each parameter by keeping the optimum values of the other parameters. For the differences of the enzyme activity results, ANOVA and student t-test were performed by using SPSS statistical program. Duncan's test was also used to detect the differences between the groups. The analyses were conducted with a significance level of 0.05.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1. Isolation of moderately halophilic bacteria

Halophiles have been recognized as a potential source of industrially useful enzymes provided with exceptional capabilities. This step of the work aimed to isolate moderately halophilic bacteria from samples collected in various parts of Thailand. The colonies were selected based on their distinctive morphology with multiple sizes, shapes, and colors that differently appeared on agar plates were picked and purified. After processing all the samples, a total of 831 moderately halophilic bacteria were isolated from diverse saline-based samples, and the isolates grow optimally in media with 10% (w/v) of NaCl at 37 °C pH 7. Thus, they can be classified as moderately halophilic bacteria, according to the classification proposed by Kushner (1985). Distributions among the isolates are summarized in Table 4.1.

An extensive collection of moderately halophilic bacterial (473 isolates) obtained from various fermentation time of two fish sauce (*Nam-pla*) samples collected from two factories in Chon Buri (79 isolates) and Samut Sakhon province (394 isolates), respectively. The 149 isolates were isolated from four shrimp paste (*Ka-pi*) samples. Seventy-eight strains were isolated from fifteen samples of fermented fish (*Pla-ra*) and 3 isolates from (*Pla-chom*). Thirty-three isolates were recovered from 7 pickled vegetables (*Phak-dong*) samples and twenty-six isolates from four salted crab (*Poo-khem*) samples, followed by 14 isolates from three samples of salted fish (*Pla-khem*), respectively. For three samples of fermented shrimp (*Kung-chom* and *Kung-som*), 5 and 11 isolated were obtained, respectively. Also, a total of 32 strains were obtained from two samples of saline soil of Sakhon Nakhon, and 6 isolates were taken from fermented mussel (*Hoi-dong*).

Table 4.1. Source of sample, location, and isolate code

Sample	Location	Sample code	Isolate no.	Total no.
Fish sauce	Samut Sakhon	MSQM1	MSQM1-1 to MSQM1-17	17
(Nam-pla)		MSQM2	MSQM2-1 to MSQM2-41	41
		MSQM3	MSQM3-1 to MSQM3-35	39
		MSQM6	MSQM6-1 to MSQM6-41	41
		MSQM9	MSQM9-1 to MSQM9-59	64
		MSQM12	MSQM12-1 to MSQM12-33	33
		MSQC	MSQC-1 to MSQC-54	55
		MSQL	MSQL-1 to MSQL-37	37
		MSQW	MSQW-1 to MSQW-66	67
	Chon Buri	MPCM0	MPCM0-1 to MPCM0-2	2
		MPCM1	MPCM1-1 to MPCM1-12	12
		MPCM2	MPCM2-1 to MPCM2-4	4
		MPCM3	MPCM3-1 to MPCM3-10	10
		MPCM4	MPCM4-1 to MPCM4-10	10
		MPCM5	MPCM5-1 to MPCM5-4	4
		MPCM6	MPCM6-1 to MPCM6-6	6
		MPCM7	MPCM7-1 to MPCM7-6	6
		MPCM8	MPCM8-1 to MPCM8-6	6
		MPCM9	MPCM9-1 to MPCM9-4	4
		MPCM10	MPCM10-1 to MPCM10-3	3
		MPCM11	MPCM11-1 to MPCM11-5	5
		MPCM12	MPCM12-1 to MPCM12-7	7
Soil salt pan	Sakon Nakhon	SSP1	SSP1-1 to SSP1-17	17
		SSP2	SSP2-1 to SSP2-15	15
Fermented fish	Pathum Thani	MPR1	MPR1-1 to MPR1-2	2
(Pla-ra)	Pathum Thani	MPR2	MPR2-1 to MPR2-6	6
	Pathum Thani	MPR3	MPR3-1 to MPR3-4	4
	Pathum Thani	MPR4	MPR4-1 to MPR4-4	4
	Chachoengsao	MPR5	MPR5-1 to MPR5-3	3
	Nakhon Sawan	MPR6	MPR6-1 to MPR6-10	10
	Nakhon Sawan	MPR7	MPR7-1 to MPR7-2	2
	Nakhon Sawan	MPR8	MPR8-1 to MPR8-3	3
	Bangkok	MPR9	MPR9-1 to MPR9-5	5
	Roi Et	MPR10	MPR10-1 to MPR10-13	13
	Roi Et	MPR11	MPR11-1 to MPR11-8	8
	Nakhon Phanom	MPR12	MPR12-1 to MPR12-5	5
	Nakhon Phanom	MPR13	MPR13-1 to MPR13-4	4
	Surin	MPR14	MPR14-1 to MPR14-4	4
	Surin	MPR15	MPR15-1 to MPR15-5	5

Table 4.2. (Cont.) Source of sample, location, and isolate code

Sample	Location	Sample code	Isolate code	Total no.
Shrimp paste (*Ka-pi)*	Pathum Thani	MKP1	MKP1-1 to MKP1-6	6
	Chachoengsao	MKP2	MKP2-1 to MKP2-6	6
	Krabi	MKP3	MKP3-1 to MKP3-11	11
	Rayong	MKP4	MKP4-1 to MKP4-127	130
	Samut Sakhon	SKP	SSKP1-9	1
	Samut Sakhon	SSKP	SKP4-6	1
Fermented Shrimps *(Kung-chom)*	Chachoengsao	MKC1	MKC1-1 to MKC 1-5	5
Fermented Shrimps (*Kung-som)*	Surat Thani	MKS1	MKS1-1 to MKC 1-6	6
	Bangkok	MKS2	MKS2-1 to MK2 1-5	5
Salted fish *(Pla-khem)*	Pathum Thani	MPT	MPT1	1
	Phetchaburi	MPLK1	MPLK1-1 to MPLK1-5	5
	Nakhon Phanom	MPLK2	MPLK2-1 to MPLK2-8	8
Salted crab *(Poo-khem)*	Surat Thani	MPK1	MPK1-1 to MPK1-7	7
	Pathum Thani	MPK2	MPK2-1 to MPK2-3	3
	Chachoengsao	MPK3	MPK3-1 to MPK3-7	7
	Chachoengsao	MPK4	MPK4-1 to MPK4-9	9
Fermented fish *(Pla-chom)*	Chachoengsao	MPC	MPC1-1 to MPC 1-3	3
Pickled Vegetable (*Luk-rieng-dong*)	Surat Thani	MLR1	MLR1-1 to MLR1-2	2
Pickled bamboo shoots *(Nhor-Mai-Dong)*	Surat Thani	MNM1	MNM1-1 to MNM1-2	2
Pickled Vegetable (*Phak-siaen-dong)*	Bangkok	MPV1	MPV1-1 to MPV1-6	6
Pickled green mustard *(Phak-gard-dong)*	Bangkok	MPV2	MPV2-1 to MPV2-7	7
	Surin	MPV3	MPV3-1 to MPV3-4	4
Pickled spring onion *(Ton-Hom-dong)*	Nakhon Phanom	MPV4	MPV4-1 to MPV4-4	4
Pickled Thai Eggplant with chilies *(Ma-khuae-dong)*	Nakhon Phanom	MPV5	MPV5-1 to MPV5-8	8
Fermented mussel (*Hoi-dong*)	Chachoengsao	MHD1	MHD1-1 to MHD1-6	6

4.2. Screening of LPMHB on the agar plate

The first goal of this work included the screening of moderately halophilic bacteria that able to catalyze the hydrolysis of short (lower than 10 carbon atoms) and long-chain triglycerides (higher than 10 carbon atoms). To observe halophilic lipolytic activities, the strains were cultured on agar plates containing the different substrates at the level of 1% (w/v) of Tween 20, Tween 40, Tween 60, Tween 80, and tributyrin under moderate salinity pressure (10% w/v NaCl). Among the 831 isolates, 349 moderately halophilic bacteria (42%) that display lipolytic activity have been detected as opaque halos of calcium salt on the tested medium. From this point, it could be assumed 349 isolates as halophilic lipolytic producers.

Significant differences in the hydrolytic activities with various types of inducers were observed. It was found that the isolated of moderately halophilic bacteria could be used the different kinds of induction substances when considering the presence of lipolytic activity that appeared on the agar plate. Among them, 135 isolates had conspicuous halos around colonies when Tween 20 was used as an inducer, and 295 isolates hydrolyzed Tween 40. Besides, 285 isolates could be hydrolyzed Tween 60, while 87 isolates showed lipolytic activity when Tween 80 contained in the medium. One hundred eighty-three isolates presented the hydrolytic activities against tributyrin. Only 58 isolates of them yielded positive results of lipolytic activity against all induction substances. Hence, 349 isolates that produced lipolytic activity were subsequently for the second screening of their extracellular halophilic lipolytic activities in a liquid medium and genotypic grouping of strains by using DNA fingerprinting

4.3. Screening of LPMHB in the liquid medium

The ability to produce extracellular halophilic lipolytic was assayed qualitatively. The lipolytic activity was tested using synthetic substrates with various types of carbon chain lengths, including *p*-nitrophenyl butyrate (*p*-NPB, C4:0) and *p*-nitrophenyl palmitate (*p*-NPP, C16:0). A total of 349 isolates were able to produce lipolytic enzymes after 48 hr of growth in 73EP medium without any inducers. When *p*-NPB was used as a substrate for enzyme assays, the results show that 322 isolates from 349 isolates exhibited esterase activity in the ranged of 0.01±0.42 to 65.22±1.45

U/mL while the remaining isolates were not (Fig. 4.3-4.10). The isolate MSQM3-33 showed highest esterase activity (65.22±1.45 U/mL) and followed by (MPCM12-7 (61.08±1.73 U/mL), MSQM6-12 (54.93±0.58 U/mL), MKP 3-9 (53.96±2.35 U/mL) and MSQC-44 (50.47±0.30 U/mL), respectively. For lipases activities, 176 isolates were detected ranged from (0.01±0.13 to 6.95±0.07 U/mL) when *p*-NPP was used as substrate MKP4-36 exhibited the highest activity (6.95±0.07 U/mL), followed by MKP3-11 (6.79±0.17 U/mL), MKP2-6 (6.79±0.17 U/mL), MKP4-68 (6.79±0.17 U/mL) and MKP2-4 (5.70±0.78 U/mL), respectively. The highest lipase activity in the first five orders was produced by isolate recovered from shrimp paste.

The results of this study indicated that moderately halophilic bacteria had the highest lipolytic activity when the short-chain synthetic substrates (*p*-NPB) were used. On the other hand, some of the isolates revealed the lipolytic activity against long carbon chain length (*p*-NPP). Although most of the isolates are produced in both esterases and lipases, they can produce esterases better than lipases, as can be seen from the specificity with the short-chain substrate (*p*-NPB) in the enzyme assay. Therefore, the analysis of enzyme activity in the subsequent studies was used only one substrate, *p*-NPB, for further investigation of esterase activity. Of these, 322 isolates displayed lipolytic activity on both agar and liquid medium were selected for grouping using DNA-fingerprinting.

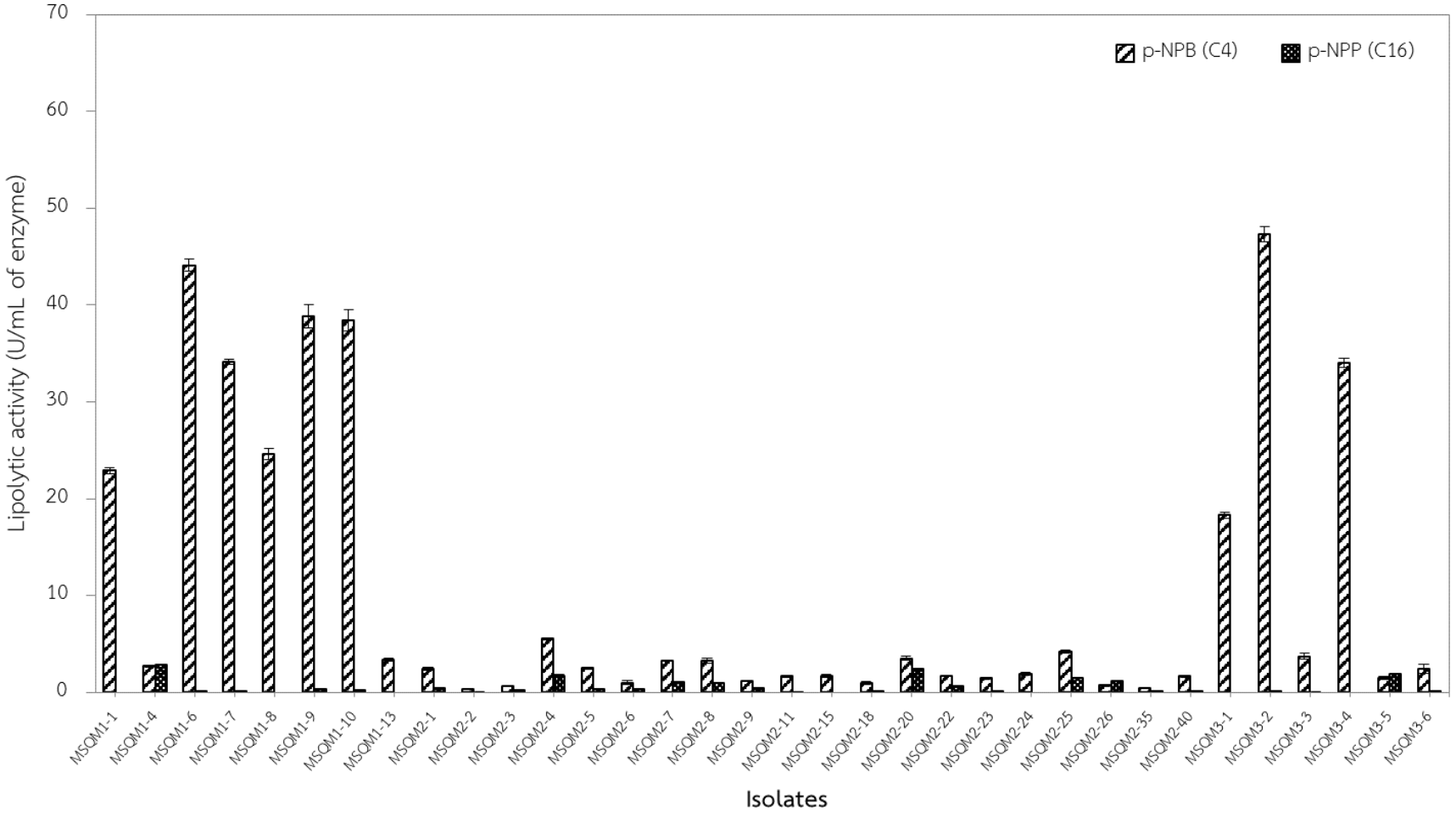


Figure 4.1. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

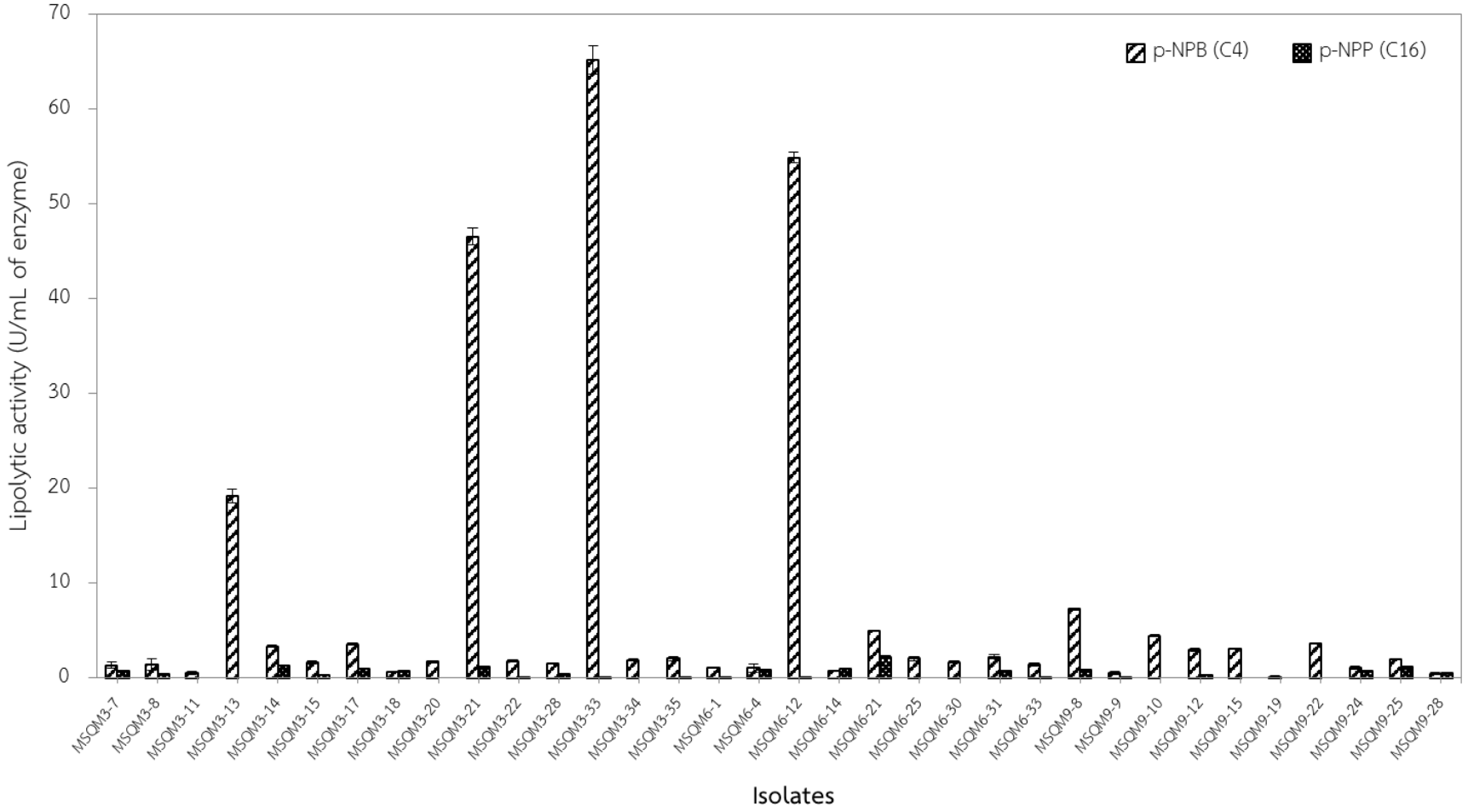


Figure 4.2. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

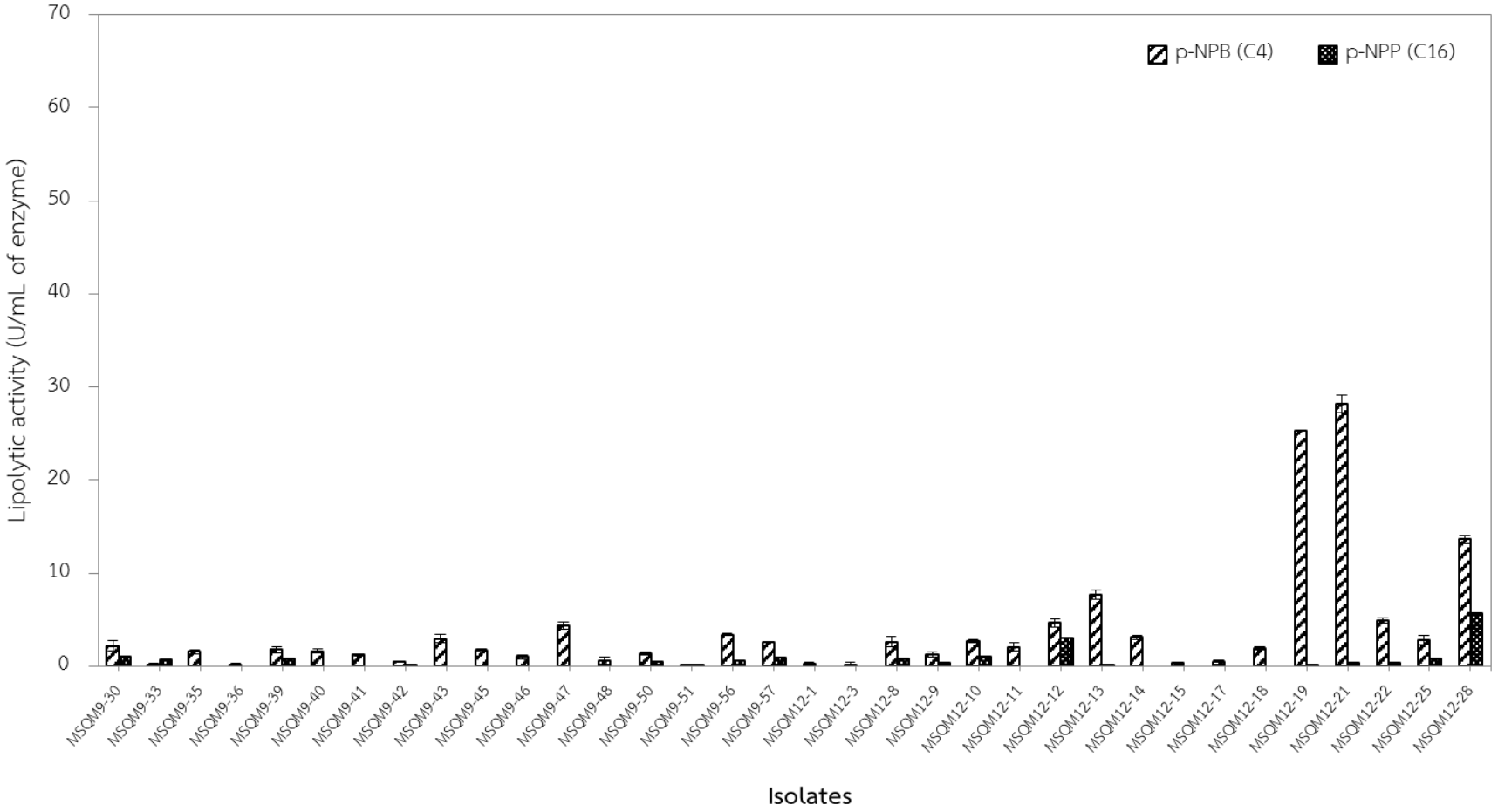


Figure 4.3. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

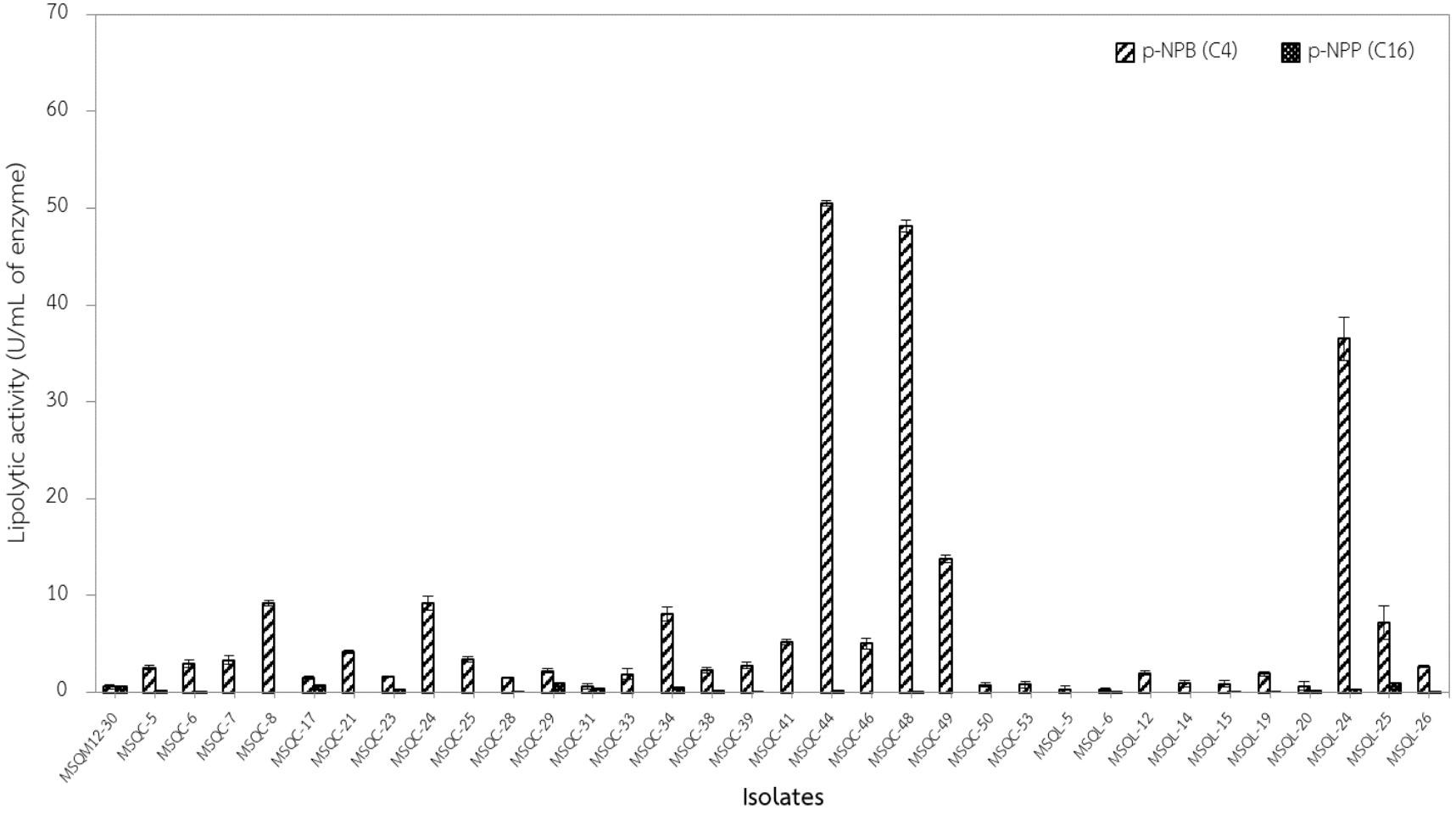


Figure 4.4. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

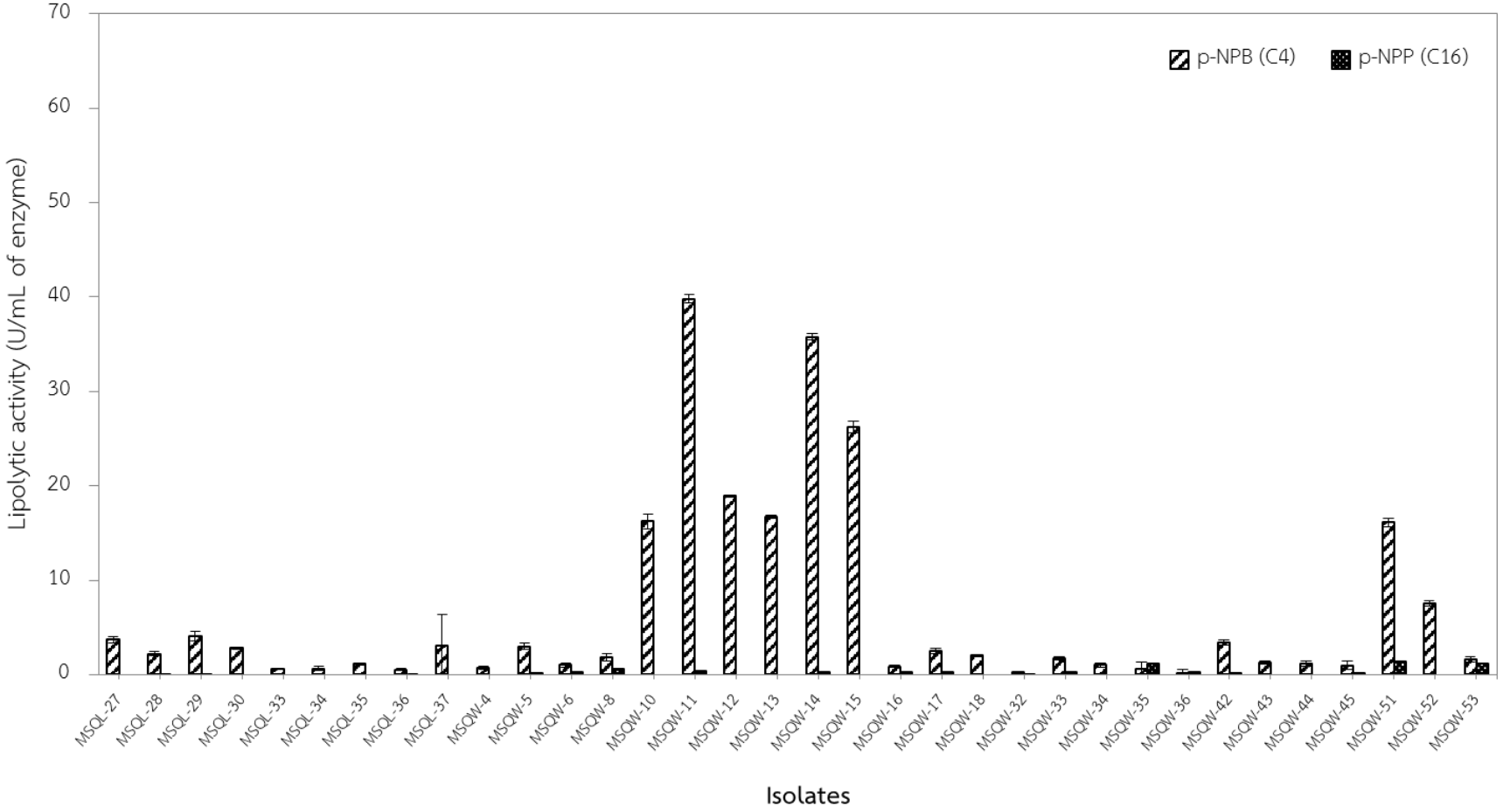


Figure 4.5. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

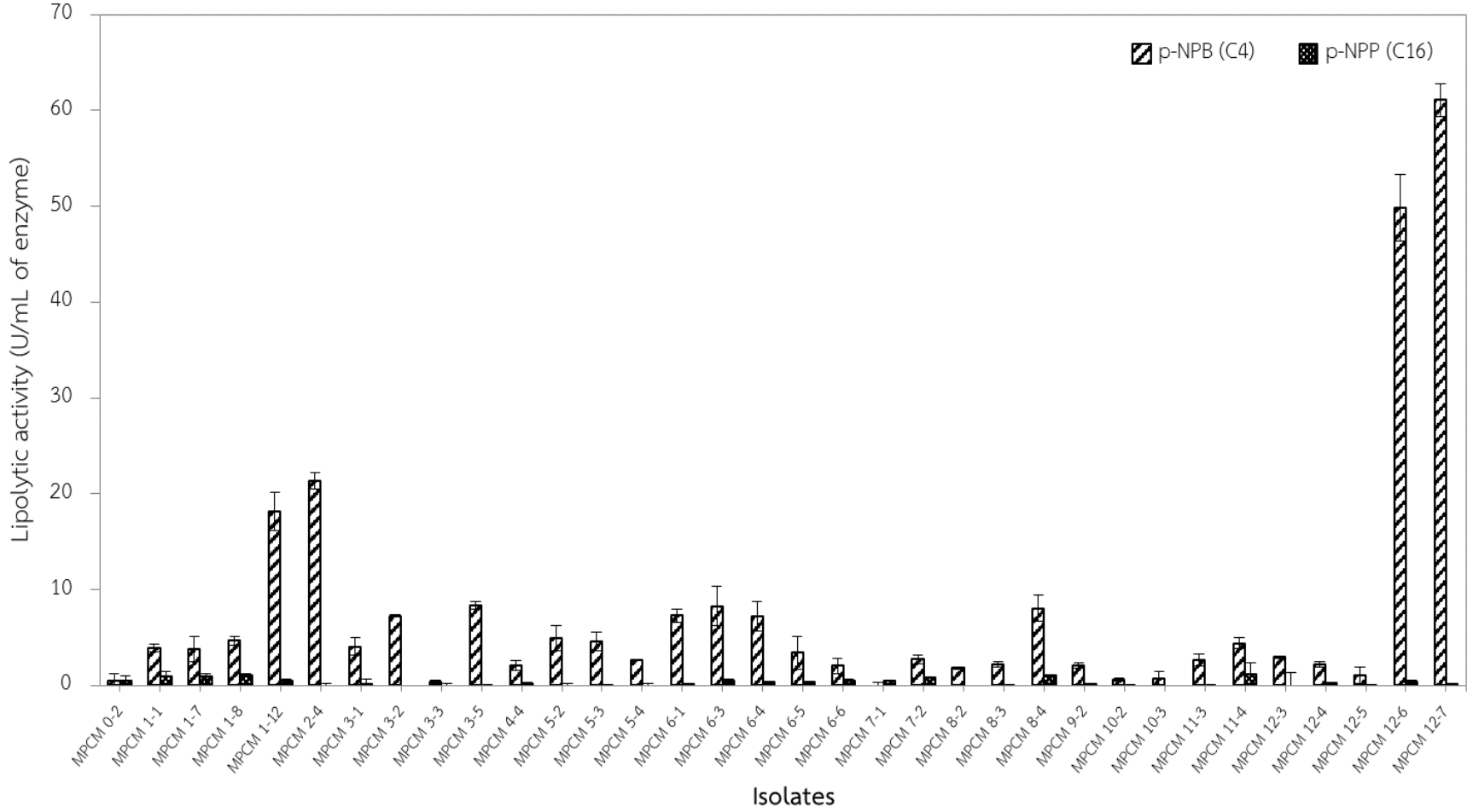


Figure 4.6. Lipolytic activity of moderately halophilic bacteria isolated from fish sauce.

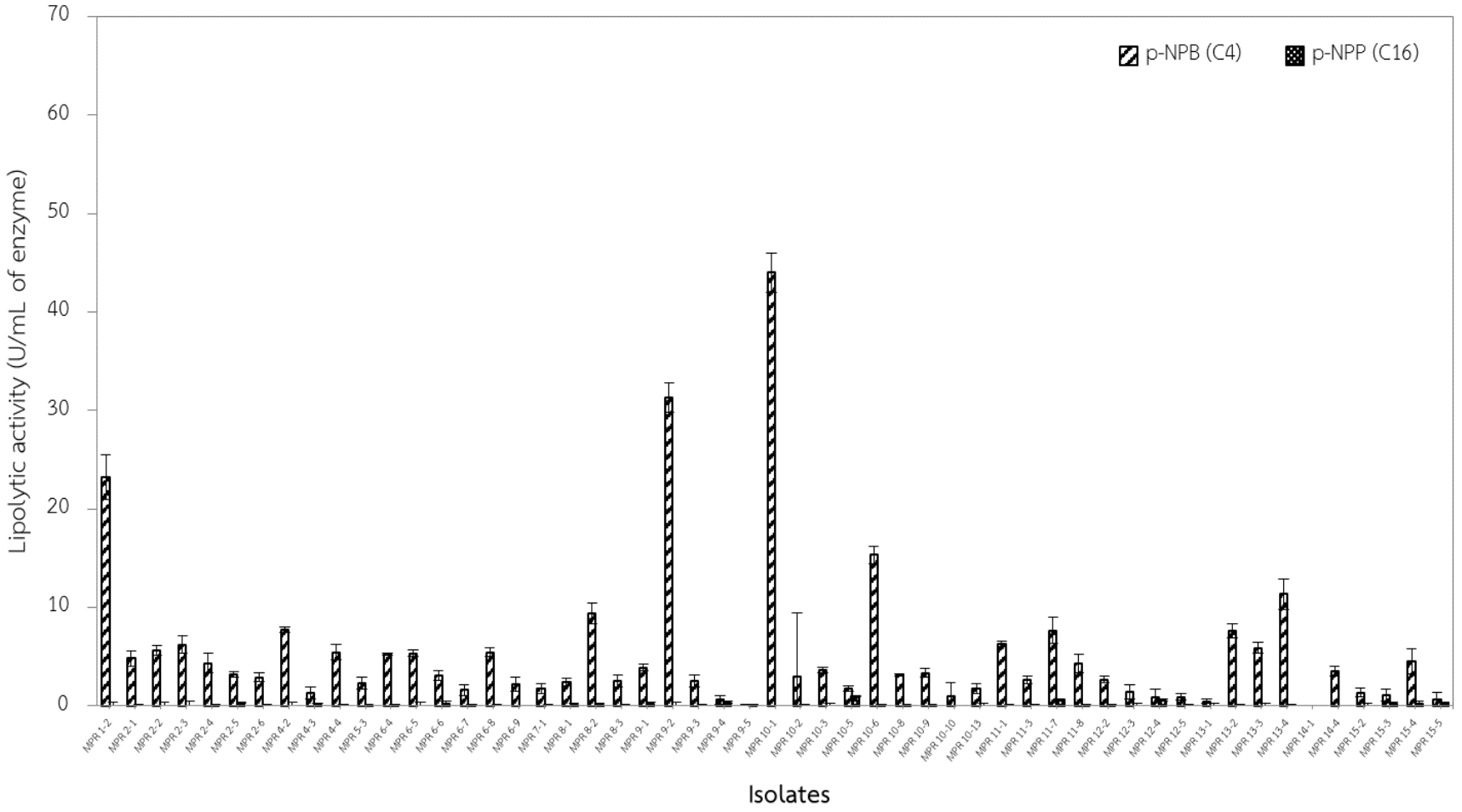


Figure 4.7. Lipolytic activity of moderately halophilic bacteria isolated from *Pla-ra.*

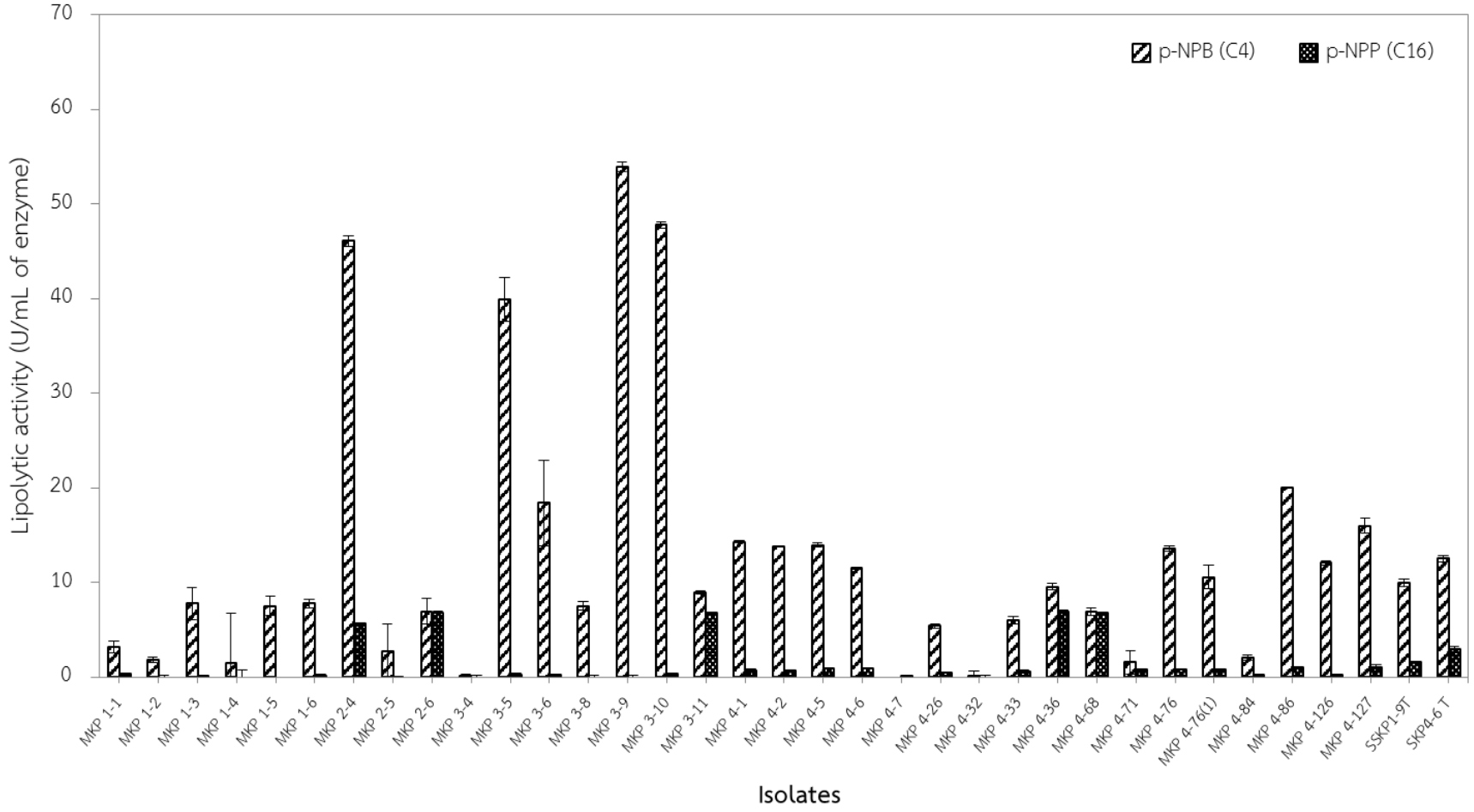


Figure 4.8. Lipolytic activity of moderately halophilic bacteria isolated from *Ka-pi*.

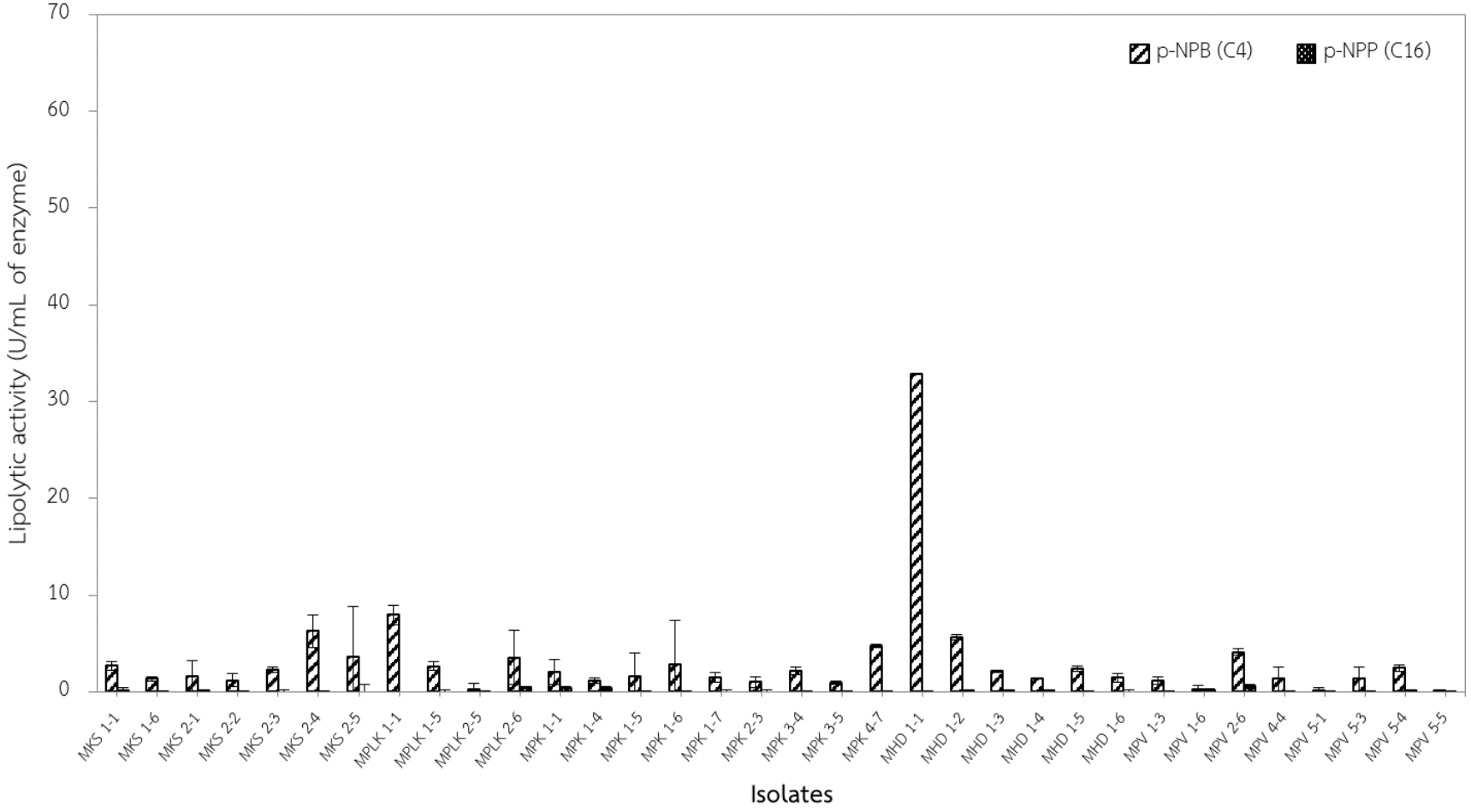


Figure 4.9. Lipolytic activity of moderately halophilic bacteria isolated from *Kung som, Pla-khem*, *Poo-khem, Phak-dong, Hoi-dong.*

4.4. DNA fingerprint of LPMHB and Identification of selected isolated

4.4.1. DNA-fingerprint of the type strain

The 18 reference strains of the moderately and extremely halophilic bacteria and also archaea (Table 3.1) were amplification, compared, and digitally processed. The different sets of primers: ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3', ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3', $(GTG)_5$ 5'-GTGGTGGTGGTGGTG-3', and multiplex reactions with the combined ERIC1R+$(GTG)_5$, ERIC2+$(GTG)_5$, and ERIC1R+ERIC2+$(GTG)_5$ were tested to assess which was the most suitable for analysis of LPMHB isolates. The result found that using the combination primers between $(GTG)_5$ and ERIC2 showed distinguished of clearly DNA polymorphism about 10-20 DNA band, and the size of the DNA band was spread in the range of 200-10,000 bp comparing with the standard DNA (Fig. 4.10). The neighbor-joining hierarchical clustering dendrogram of the tested halophilic type strain is reliable for determining relationships and provide a good description of the measured Pearson coefficients. The cophenetic correlation values were all very high, where *r*=0.9-1 (90%-100%) indicates a very good fit; *r*=0.8-0.9 (80%-90%) indicates a good fit; *r*=0.7=0.8 (70%-80%) indicates a poor fit. The resulted dendrogram grouped the isolates into six main clusters (yellow-line) with a similarity level of 30-75% (red-line) and was able to discriminate 18 different biotypes. Therefore we defined threshold value of 75% profile similarity for the definition of distinguishes isolate. The dendrogram generated a cophenetic correlation coefficient of 80%-100%, indicating an acceptable clustering consistency. From this preliminary study, it can be concluded that DNA fingerprinting was able to differentiate the type strain of moderately and extremely halophilic bacteria.

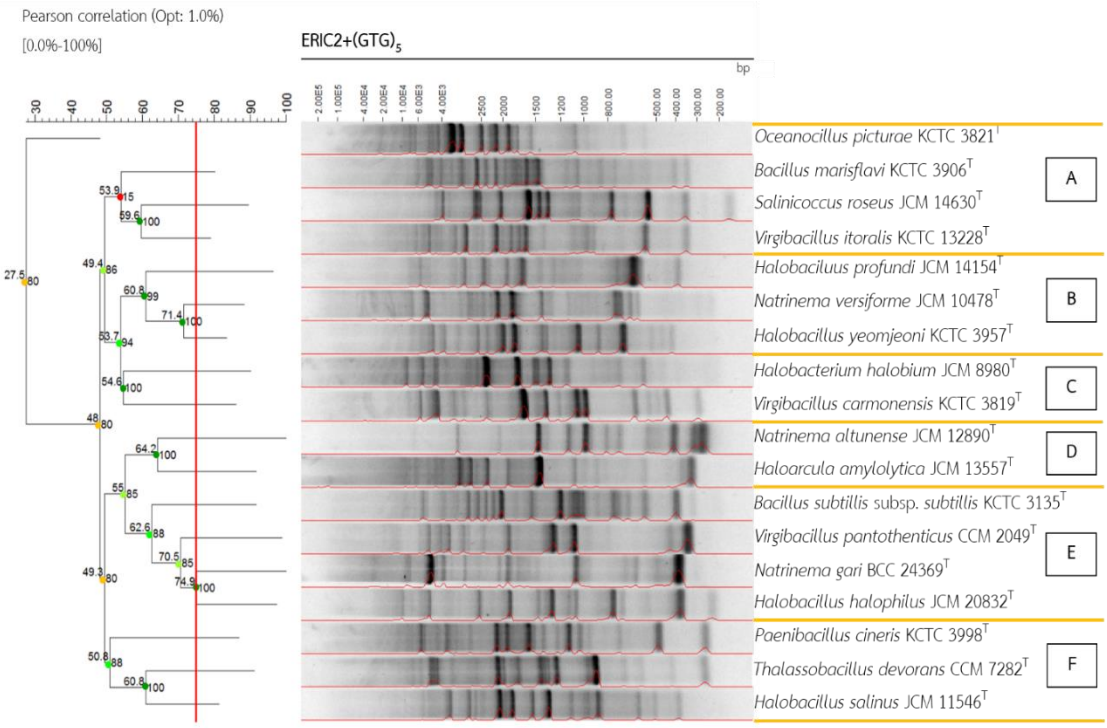


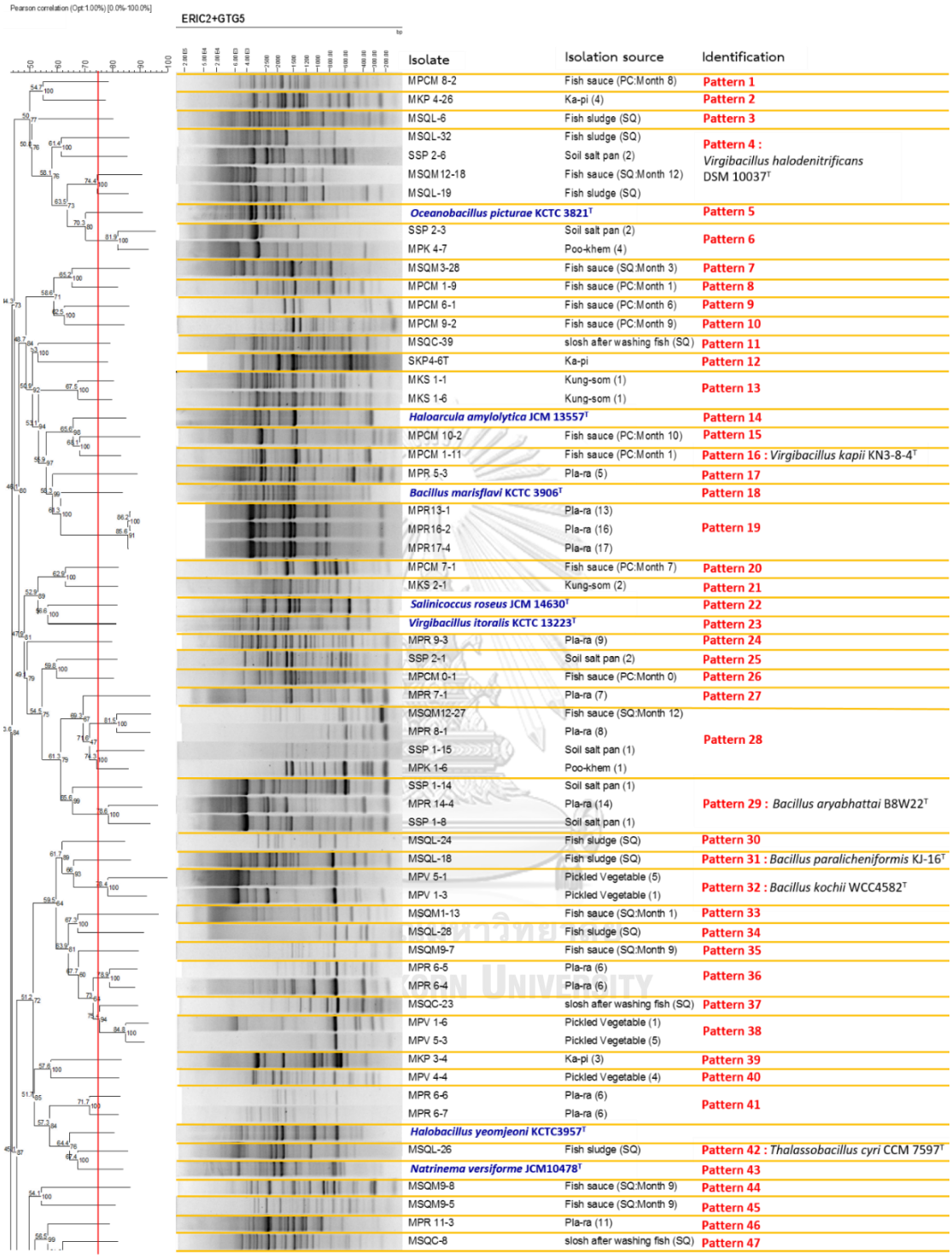
Figure 4.10. The banding pattern of type strain of halophilic bacteria. Cophenetic correlation coefficient values are shown at each branch by numbers and colored dots, ranging between green-lighting, green-yellow, red, according to decreasing values.

All primers showed high discrimination performance when comparing the discriminatory indices obtained from the primers used. Although other primers used in this study could discriminate against the type strains, they generated complex profiles with a faint band on the agarose gel electrophoresis. When $(GTG)_5$+ERIC1 was used for amplification, some strain could not be amplified. For $(GTG)_5$ amplification, the PCR fragments were stack with the large size on the migration gel. Moreover, the cophenetic correlation in the rest of the primers displayed the low fit values (data not shown). Therefore, we propose that the DNA fingerprinting methods using the combination of primers, $(GTG)_5$+ERIC2 could be used for discrimination among moderately halophilic bacteria isolates) and classify all-new isolated in this study.

4.4.2. DNA-fingerprint of LPMHB

To evaluate the applicability of primer for identification of unknown isolates, LPMHB 322 isolates were subjected to $(GTG)_5$+ERIC2 fingerprinting. The numbers of recognizable bands were between 5 and 25 for each strain. From the cluster analysis with the fingerprint data, 180 patterns could be recognized. The analysis was performed using Pearson's correlation with Neighbor-joining clustering. The red line indicates the similarity value (approx. 75%) for the separation of biotypes. Each unique pattern was shown with a yellow stripe. Reference strains were highlighted with a bold blue alphabet. For the unidentified pattern, each pattern containing 1 isolate to 23 isolates as displayed in Figure 4.11.

The largest of them was pattern 54 with unidentified of 23 isolates including MPCM7-2, MSQM1-4, MSQM2-22, MSQM6-4, MSQM6-24, MSQM6-26, MSQM6-31, MSQM6-32, MSQM9-14, MSQM9-21, MSQM9-24, MSQM9-39, MSQM9-49, MSQM9-50, MSQM9-51, MSQM9-57, MSQM12-8, MSQM12-9, MSQM12-10, MSQM12-12, MSQM12-30, MSQC-33 and MSQC-38. For the second set, pattern 51 contained 12 isolates, MSQM3-6, MSQM3-17, MSQM3-18, MSQM3-22, MSQM6-38, MSQM9-9, MSQM9-22, MSQM9-25, MSQM9-41, MSQC-25, MSQL-20, and SSP2-4 were isolated from fish sauce fermentation and soil salt pan, respectively. Following by pattern 117 (MSQM1-8, MSQM2-20, MSQM2-24, MSQM3-2, MSQM3-3, MSQM12-21, MSQW-12, MKP4-1) and pattern 152 (MPR4-4, MPR6-9, MPR8-3, MPR15-2, MPR15-4 MPLK2-5, MPLK2-6 SSP1-16) composed of 8 isolates in each pattern which were isolated from fish sauce fermentation. *Ka-pi*, soil salt pan, *Pla-ra,* and *Pla-khem.* The other patterns waiting for identification step contained one to six isolates in each pattern are pattern 1-3, pattern 6-12, pattern13-15, pattern 17, pattern 20-21, pattern 24-28, pattern 30, pattern 33-41, pattern 44-47, pattern 49, pattern 52, pattern 55-56, pattern 59-61, pattern 64-66, pattern 71-72, pattern 76-79, pattern 81, pattern 83-88, pattern 90, pattern 92, pattern 93-96, pattern 98, pattern 100-101, pattern 103-116, pattern 122-125, pattern 127, pattern 130-136, pattern 138-139, pattern 141-150, pattern 153-167 and pattern 170-178.



Isolate	Isolation source	Identification
MPCM 8-2	Fish sauce (PC:Month 8)	Pattern 1
MKP 4-26	Ka-pi (4)	Pattern 2
MSQL-6	Fish sludge (SQ)	Pattern 3
MSQL-32	Fish sludge (SQ)	Pattern 4 : *Virgibacillus halodenitrificans* DSM 10037^T
SSP 2-6	Soil salt pan (2)	
MSQM12-18	Fish sauce (SQ:Month 12)	
MSQL-19	Fish sludge (SQ)	
***Oceanobacillus picturae* KCTC 3821^T**		Pattern 5
SSP 2-3	Soil salt pan (2)	Pattern 6
MPK 4-7	Poo-khem (4)	
MSQM3-28	Fish sauce (SQ:Month 3)	Pattern 7
MPCM 1-9	Fish sauce (PC:Month 1)	Pattern 8
MPCM 6-1	Fish sauce (PC:Month 6)	Pattern 9
MPCM 9-2	Fish sauce (PC:Month 9)	Pattern 10
MSQC-39	slosh after washing fish (SQ)	Pattern 11
SKP4-6T	Ka-pi	Pattern 12
MKS 1-1	Kung-som (1)	Pattern 13
MKS 1-6	Kung-som (1)	
***Haloarcula amylolytica* JCM 13557^T**		Pattern 14
MPCM 10-2	Fish sauce (PC:Month 10)	Pattern 15
MPCM 1-11	Fish sauce (PC:Month 1)	Pattern 16 : *Virgibacillus kapii* KN3-8-4^T
MPR 5-3	Pla-ra (5)	Pattern 17
***Bacillus marisflavi* KCTC 3906^T**		Pattern 18
MPR13-1	Pla-ra (13)	Pattern 19
MPR16-2	Pla-ra (16)	
MPR17-4	Pla-ra (17)	
MPCM 7-1	Fish sauce (PC:Month 7)	Pattern 20
MKS 2-1	Kung-som (2)	Pattern 21
***Salinicoccus roseus* JCM 14630^T**		Pattern 22
***Virgibacillus itoralis* KCTC 13223^T**		Pattern 23
MPR 9-3	Pla-ra (9)	Pattern 24
SSP 2-1	Soil salt pan (2)	Pattern 25
MPCM 0-1	Fish sauce (PC:Month 0)	Pattern 26
MPR 7-1	Pla-ra (7)	Pattern 27
MSQM12-27	Fish sauce (SQ:Month 12)	Pattern 28
MPR 8-1	Pla-ra (8)	
SSP 1-15	Soil salt pan (1)	
MPK 1-6	Poo-khem (1)	
SSP 1-14	Soil salt pan (1)	Pattern 29 : *Bacillus aryabhattai* B8W22^T
MPR 14-4	Pla-ra (14)	
SSP 1-8	Soil salt pan (1)	
MSQL-24	Fish sludge (SQ)	Pattern 30
MSQL-18	Fish sludge (SQ)	Pattern 31 : *Bacillus paralicheniformis* KJ-16^T
MPV 5-1	Pickled Vegetable (5)	Pattern 32 : *Bacillus kochii* WCC4582^T
MPV 1-3	Pickled Vegetable (1)	
MSQM1-13	Fish sauce (SQ:Month 1)	Pattern 33
MSQL-28	Fish sludge (SQ)	Pattern 34
MSQM9-7	Fish sauce (SQ:Month 9)	Pattern 35
MPR 6-5	Pla-ra (6)	Pattern 36
MPR 6-4	Pla-ra (6)	
MSQC-23	slosh after washing fish (SQ)	Pattern 37
MPV 1-6	Pickled Vegetable (1)	Pattern 38
MPV 5-3	Pickled Vegetable (5)	
MKP 3-4	Ka-pi (3)	Pattern 39
MPV 4-4	Pickled Vegetable (4)	Pattern 40
MPR 6-6	Pla-ra (6)	Pattern 41
MPR 6-7	Pla-ra (6)	
***Halobacillus yeomjeoni* KCTC3957^T**		
MSQL-26	Fish sludge (SQ)	Pattern 42 : *Thalassobacillus cyri* CCM 7597^T
***Natrinema versiforme* JCM10478^T**		Pattern 43
MSQM9-8	Fish sauce (SQ:Month 9)	Pattern 44
MSQM9-5	Fish sauce (SQ:Month 9)	Pattern 45
MPR 11-3	Pla-ra (11)	Pattern 46
MSQC-8	slosh after washing fish (SQ)	Pattern 47

Figure 4.11. Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

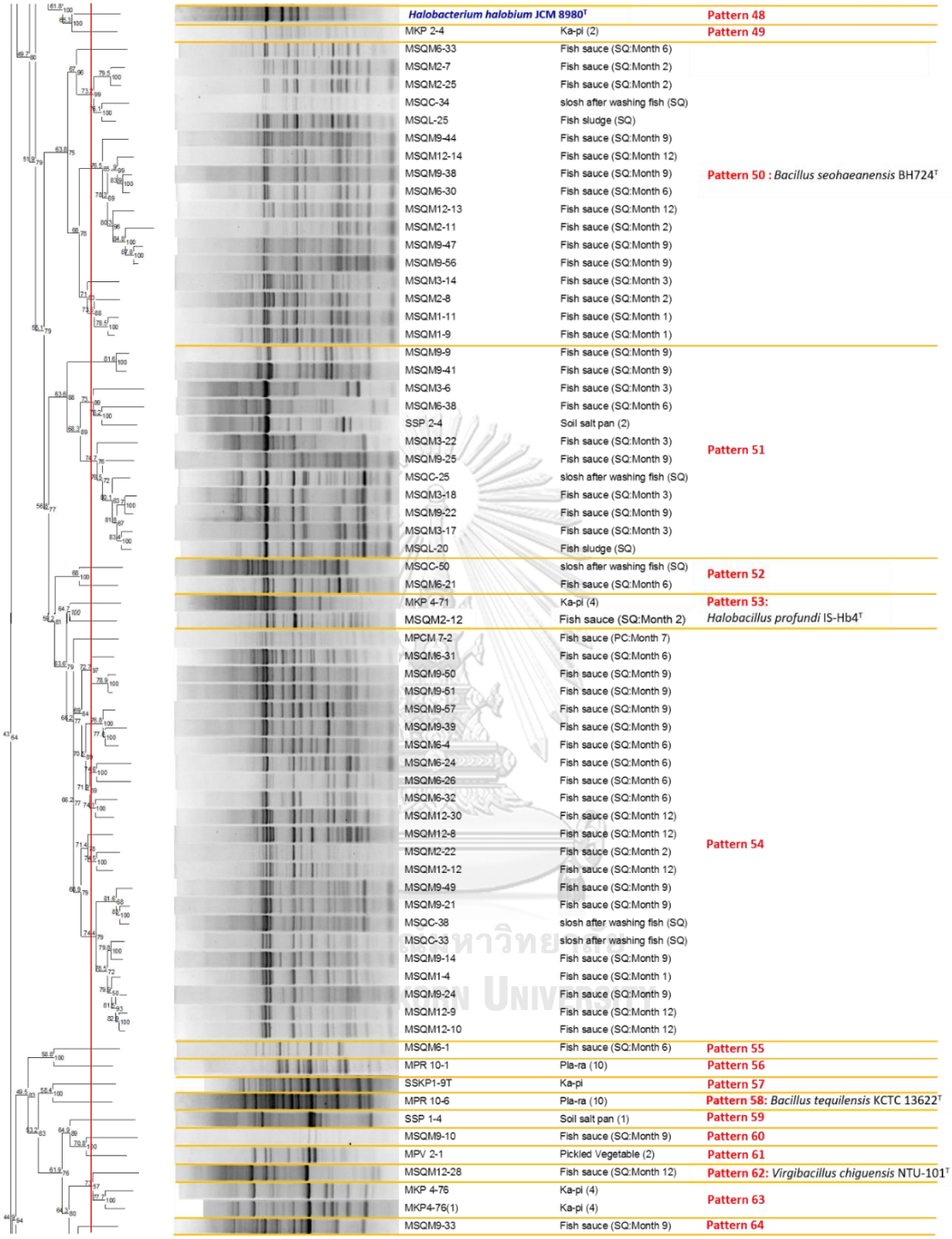


Figure 4.11. (Cont.) Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

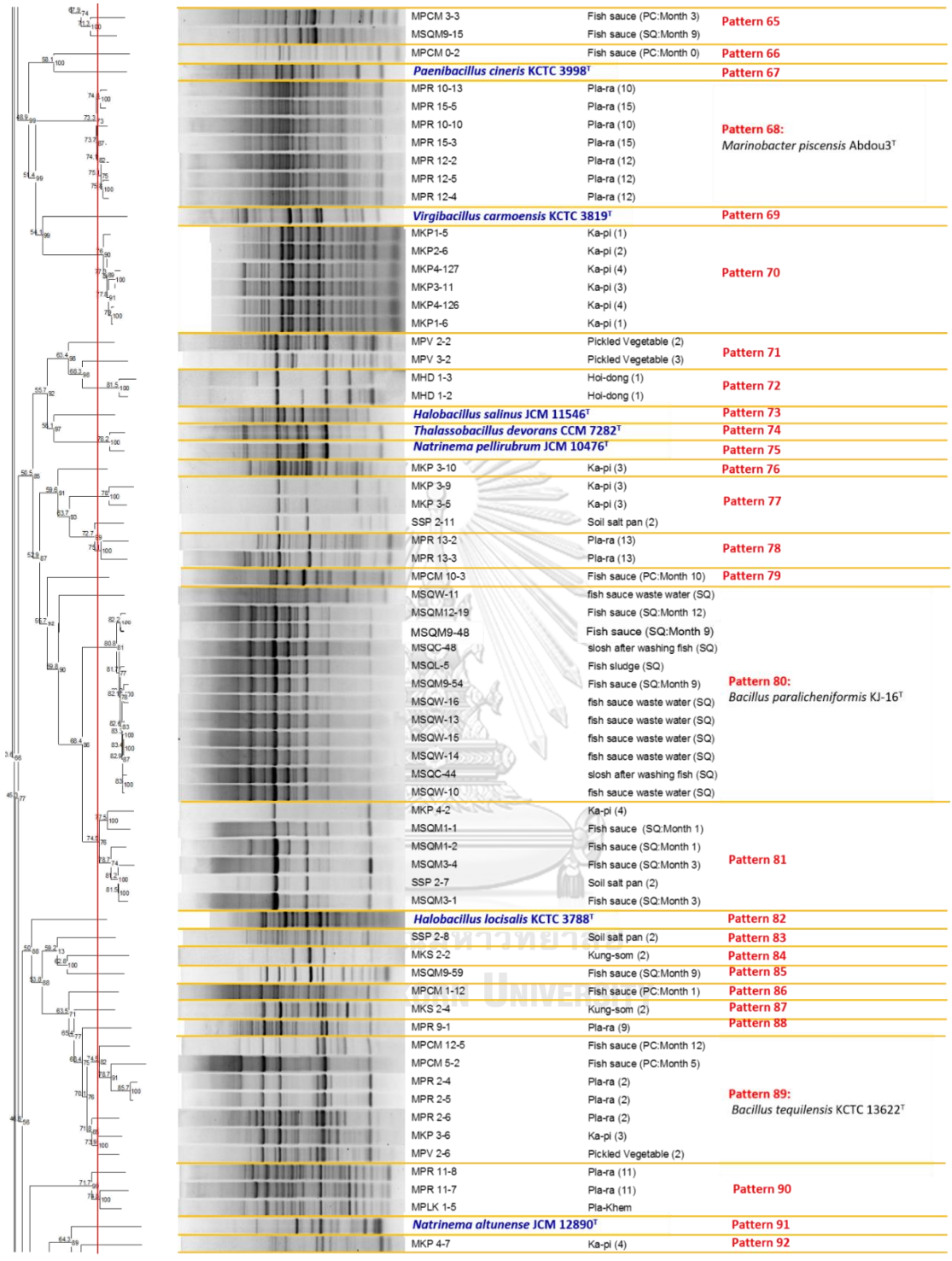


Figure 4.11. (Cont.) Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

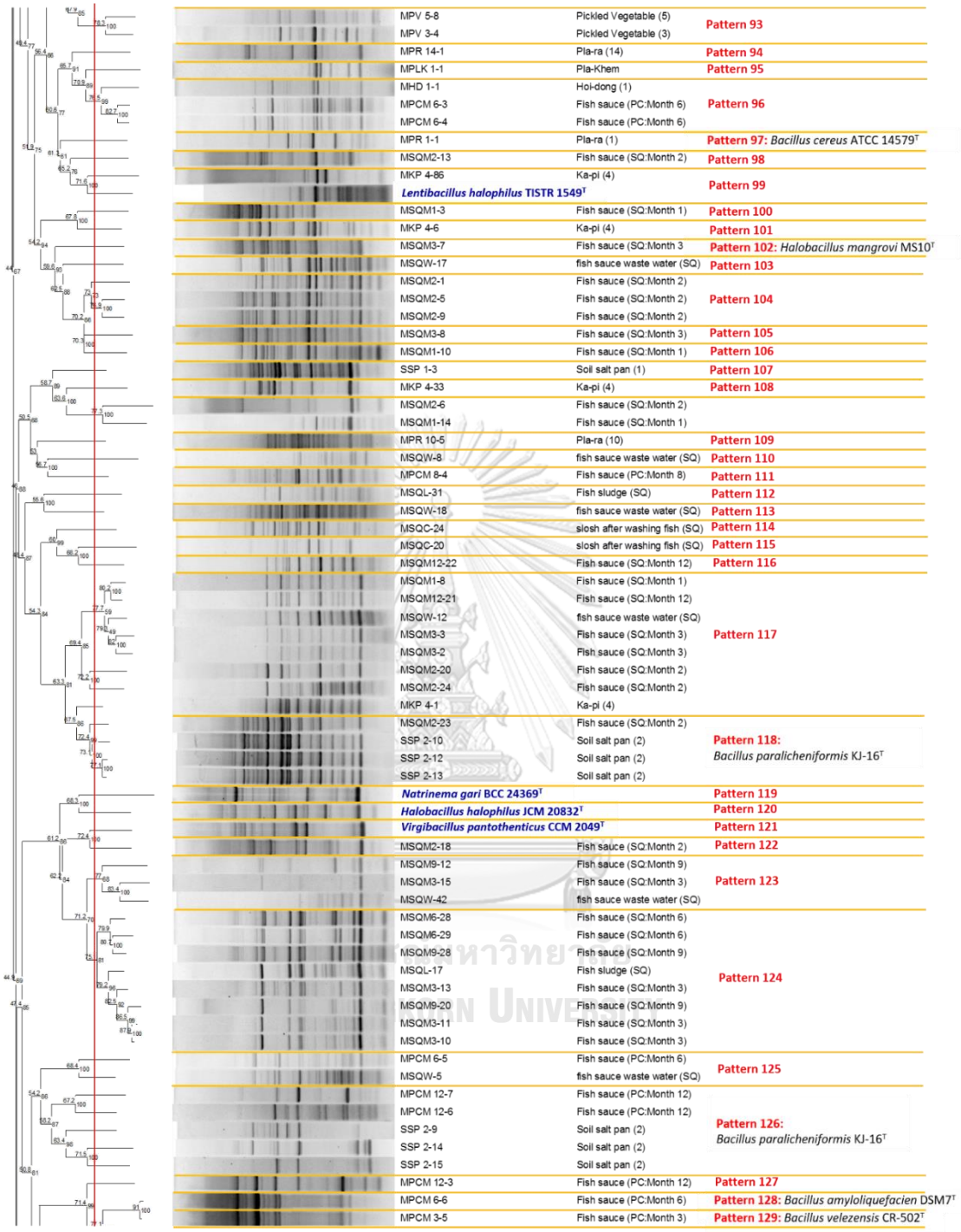


Figure 4.11. (Cont.) Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

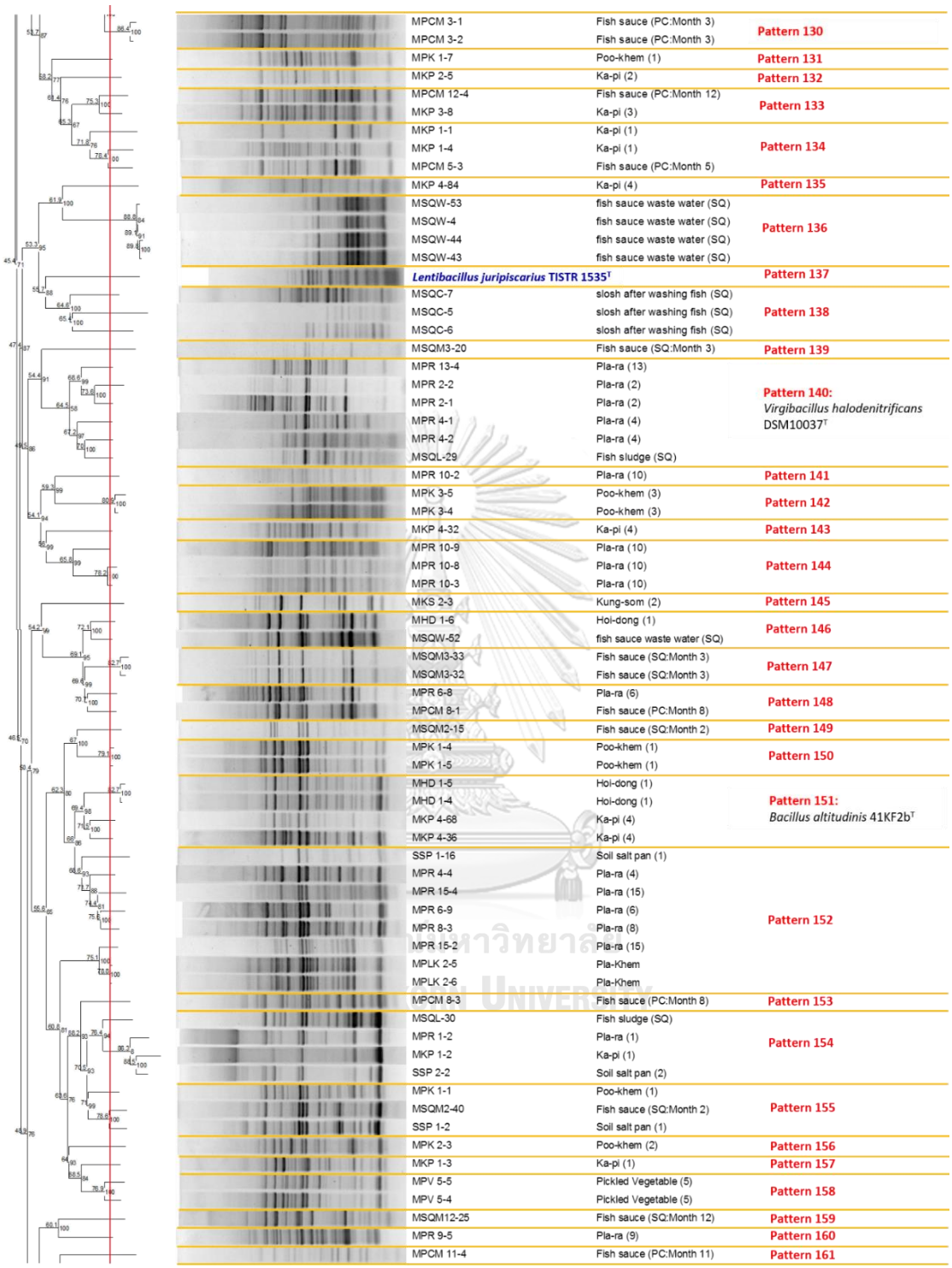


Figure 4.11. (Cont.) Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

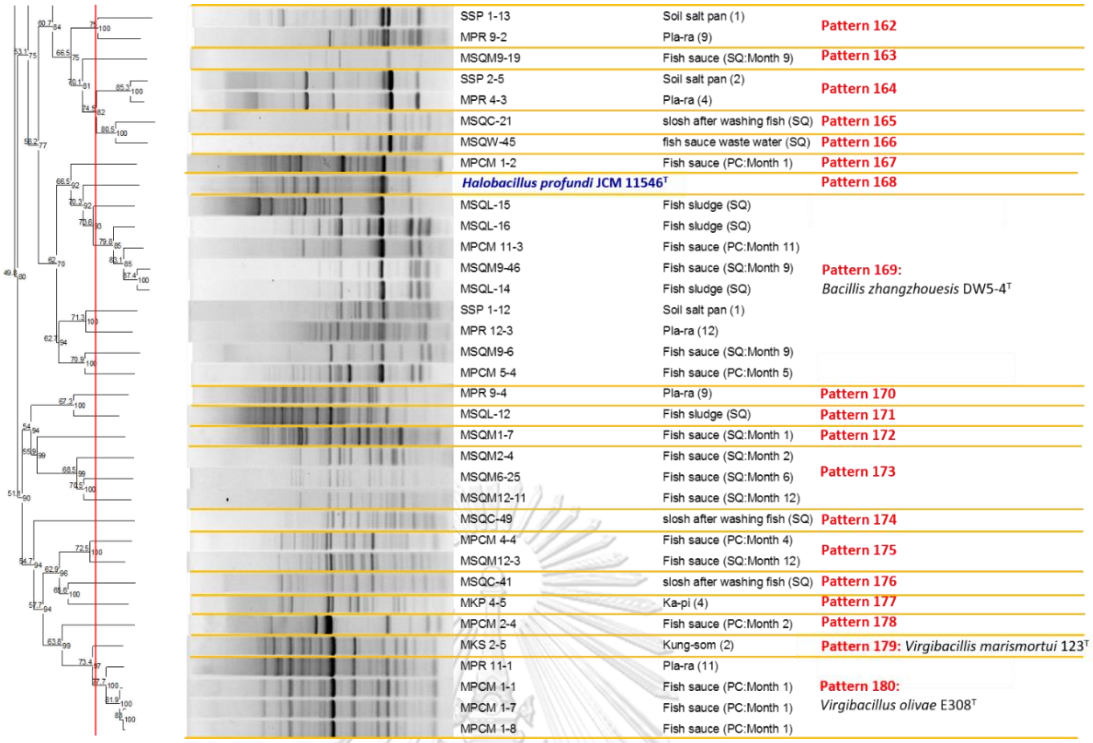


Figure 4.11. (Cont.) Dendrogram based on $(GTG)_5$+ERIC2 fingerprint profile similarities of type strains and LPMHB 322 isolates.

Based on variation in banding pattern, some of the representative isolate in each pattern was randomized selected for identified taxonomically using the 16S rRNA gene sequence. The 16S rRNA gene sequence of each strain was analyzed, and phylogenetic trees were constructed to determine their phylogenetic position. Phylogenetic analysis indicated that the representative of isolate belonged to genera: *Bacillus*, *Halobacillus*, *Lentibacillus*, *Marinobacter, Thallossobacillus*, and *Virgibacillus*.

4.4.2.1. DNA-fingerprint of LPMHB in genus Bacillus

Pattern 29 contained three isolates; MPR14-4, SSP1-14, and SSP1-8 isolated from *pla-ra* and soil salt pan, respectively. It was identified as *B. aryabhattai* based on the 16S rRNA gene sequence of 1,390 bp from its 99.93% sequence similarity to *B. aryabhattai* B8W22^T (Shivaji *et al.*, 2009). The phylogenetic tree analysis is shown in Figure 4.12. A few studies reported about *B. aryabhattai* strain SE3-PB isolated from lipid-rich wastewater samples collected from an oil mill industry in South Africa on production and optimization of lipase by its strain using response surface methodology

(Adetunji & Olaniran 2018a). The lipase produced by strain SE3-PB was immobilized and the properties were studies (Adetunji & Olaniran 2018b). *B. aryabhattai* strain C26-1 was screened from sludge and had reported ability to produced lipase, which could be used for selectively hydrolyze (R, S)-ethyl indoline-2-carboxylate in the aqueous phase to produce an optically pure (S)-indoline-2-carboxylic acid (Zhang *et al.*, 2019).

Pattern 50 encompass with 17 isolates: MSQM1-9, MSQM1-11, MSQM2-7, MSQM2-8, MSQM2-11, MSQM2-25, MSQM3-14, MSQM6-30, MSQM6-33, MSQM9-38, MSQM9-44, MSQM9-47, MSQM9-56, MSQM12-13, MSQM12-14, MSQC-34 and MSQL-25 were isolated from during fermentation time of fish sauce. Based on the 16S rRNA gene sequence (1,399 bp) of represented isolated (MSQM6-33), it was identified as *B. seohaeanensis* from its 100% sequence similarity with 0/1398 variation nucleotide ratio to *B. seohaeanensis* BH724^{T,} which was isolated from solar saltern at Taean in Korea (Lee *et al.*, 2006). The NJ tree analysis is shown in Figure 4.12. No report about the production of esterases and lipases by *B. seohaeanensis* but has been reported for used in the methods for cleaning water dispensers systems (Khanh, 2019).

The differentiate of DNA fingerprinting as can be seen in pattern 31, pattern 80, pattern 118 and pattern 126 which compose of various isolates included: MSQL-18 (pattern 31); MSQM9-48, MSQM9-54, MSQM12-19, MSQC-48, MSQC-44, MSQL-5, MSQW-10, MSQW11, MSQW-13, MSQW-14, MSQW-15, MSQW-16 (pattern 80); MSMQ2-23, SSP2-10, SSP2-12, SSP2-13 (pattern 118) and MPCM12-6, MPCM12-7, SSP2-9, SSP2-14, SSP2-15 (pattern 126). The BLAST analysis of 16S rDNA sequences (1,470 bp) of these isolates has shown the similarities with the *Bacillus paralicheniformis* KJ-16^{T} (Dunlap *et al.*, 2015) and the phylogenetic tree, NJ, analysis are shown in Figure 4.12. Obeidat (2017) reports that *B. paralicheniformis* isolated from Dead Sea black mud was classified as an extremely halotolerant and exhibited esterase activity on API ZYM tested. Another study concluded that *Bacillus paralicheniformis* isolated from the dunes of the Thar Desert in India as a moderately thermophilic strain and showed an ability to produce extracellular lipase (Neelam *et al.*, 2018).

Pattern 32, having two isolates (MPV5-1, MPV1-3) and the 16S rRNA gene (1,468 bp), showed 100% sequence identity to *Bacillus kochii* WCC4582^{T} (Seiler *et al.*, 2012), which was reclassified as *Cytobacillus kochii* based on 1172 core *Bacillaceae*

proteins, 87 proteins conserved within the phylum Firmicutes, GyrA-GyrB-RpoB-RpoC proteins, and UvrD-PolA proteins (Patel & Gupta 2020). The phylogenetic tree analysis has supported the results, as shown in Figure 4.12.

Isolate, MPR10-6 of pattern 58 and isolate MPCM5-2, MPCM12-5, MPR2-4, MPR2-5, MPR2-6, MKP3-6, and MPV2-6 of pattern 89 were isolates from *Pla-ra*, fish sauce, *Ka-pi,* and pickled vegetable. The 16S rRNA gene sequence and NJ tree analysis (Fig. 4.12) revealed that these isolates exhibited the highest 16S rRNA gene sequence similarity value of 100% with *B. tequilensis* KCTC 13622^{T} (Gatson *et al.*, 2006). This species had been reported as lipolytic-producing that was isolated from oil mill waste (Bonala & Mangamoori, 2012; 2013). The reported of (Daroonpunt *et al.*, 2019a) showed that *Bacillus tequilensis* KP1-09 was isolated from *Ka-pi* and can be produced lipase when cultivated in modified NB medium supplemented with 1% (v/v) Tween 80 and used *p*-NPP as a substrate.

Pattern 97 consisted of a single isolate, MPR1-1, based on the analysis of the 16S rRNA gene sequence and phylogenetic tree (Fig. 4.12), isolate MPR1-1 was phylogenetically closest to *Bacillus cereus* ATCC 14579^{T} (Frankland & Frankland 1887) with 99.92% similarity. Several studies have shown that strain *Bacillus cereus* could be a lipolytic producer. This strain can be isolated from various source, i.e., soil (El-Shafei & Rezkallah, 1997; Mazhar *et al.*, 2018); spoiled coconut (Dutta & Ray, 2009); dairy products (Kumari & Sarkar, 2014), and hot spring (Ghati & Paul (2015).

Pattern 128 comprised one isolate, MPCM6-6, it was identified as *Bacillus amyloliquefaciens* DSM7^{T} (Priest *et al.*, 1987) based on its 99.86% 16S rRNA gene sequence (1,429 bp) similarity. The phylogenetic tree analysis is shown in Figure 4.12. Previous studies have been remarkable reported in lipase production and also cloning and expression of the lipase gene from *B. amyloliquefaciens* in *E. coli* DH5α cells. An active strain for lipase production, *B. amyloliquefaciens*, was isolated from Rock Lobster (*Panlirus homarus*) (Selvamohan *et al.*, 2012). *B. amyloliquefaciens* isolated from industrial effluents and show high lipase activity (6.46 to 7.29 U/ml) when the medium was composed of starch, casein, and groundnut oil (Prasad & Sethi, 2013). Strain Nsic-8 isolated from a stinky tofu brine encoded a novel alkaline thermostable lipase gene (lipBA) (Cai *et al.*, 2014). A *p*-nitrobenzyl esterase cloned from *B.*

amyloliquefaciens (BAE) was employed to use as a critical chiral intermediate to hydrolyze 1-(3',4'-methylenedioxyphenyl) ethyl ester for the production of (R)-1-(3',4'-methylenedioxyphenyl)ethanol (Liu *et al.*, 2014). Kanmani and fellow workers (Kanmani *et al.*, 2015) cloned and heterologously expressed a novel lipase gene from strain PS-35, which isolated from poultry slaughterhouse. A strain KUB29 isolated from soil produced thermostable lipase and is effectively used in transesterification to produce methyl ester. (Devaraj *et al.*, 2018). *B. amyloliquefaciens* AIKK2 isolated from dry salted fish samples and used coconut fiber was the best supporting substrate for thermostable halophilic lipase production, as reported by Musa *et al.* (2018a).

Pattern 129 contained one isolate, MPCM3-5, based on the 16S rRNA gene sequence (1,285 bp), it was identified as *Bacillus velezensis* from its 99.88% sequence similarity to *Bacillus velezensis* CR-502^{T} (Figure 4.12), and this species was reported as halotolerant (Ruiz-García *et al.*, 2005). (Huang *et al.*, 2019) reported that a gene carboxylesterase (BaCEs04) encoding the novel PAEs hydrolase was screened and cloned from the genome of *B. velezensis* SYBC H47, then expressed in *E. coli* BL21 (DE3).

Pattern 151 involved with four isolates, MHD1-4, MHD1-5, MKP4-36, MKP4-68, were isolated from *Hoi-dong* and *Ka-pi*, respectively. It was identified as *Bacillus altitudinis* 41KF2b^{T} (Shivaji *et al.*, 2006) from its 99.93% sequence similarity based on the 16S rRNA gene sequence (1,372 bp) and phylogenetic tree analysis (Fig. 4.12). Esakkiraj *et al.* (2012) also reported the production of a novel halophilic esterase by *B. altitudinis* AP-MSU isolated from the gut of marine fish (*Sardinella longiceps*) using tuna wastes. Similarly, the production of esterase has been reported from the same strain, novel esterase gene (*EstBAS*) was expressed in *E. coli* as EstBASΔSP. Then, EstBASΔSP was immobilized onto a novel epoxy resin (Lx-105s) named Lx-EstBASΔSP and used as a biocatalyst to synthesize chloramphenicol palmitate by regioselective modification at the primary hydroxyl group (Dong *et al.*, (2019).

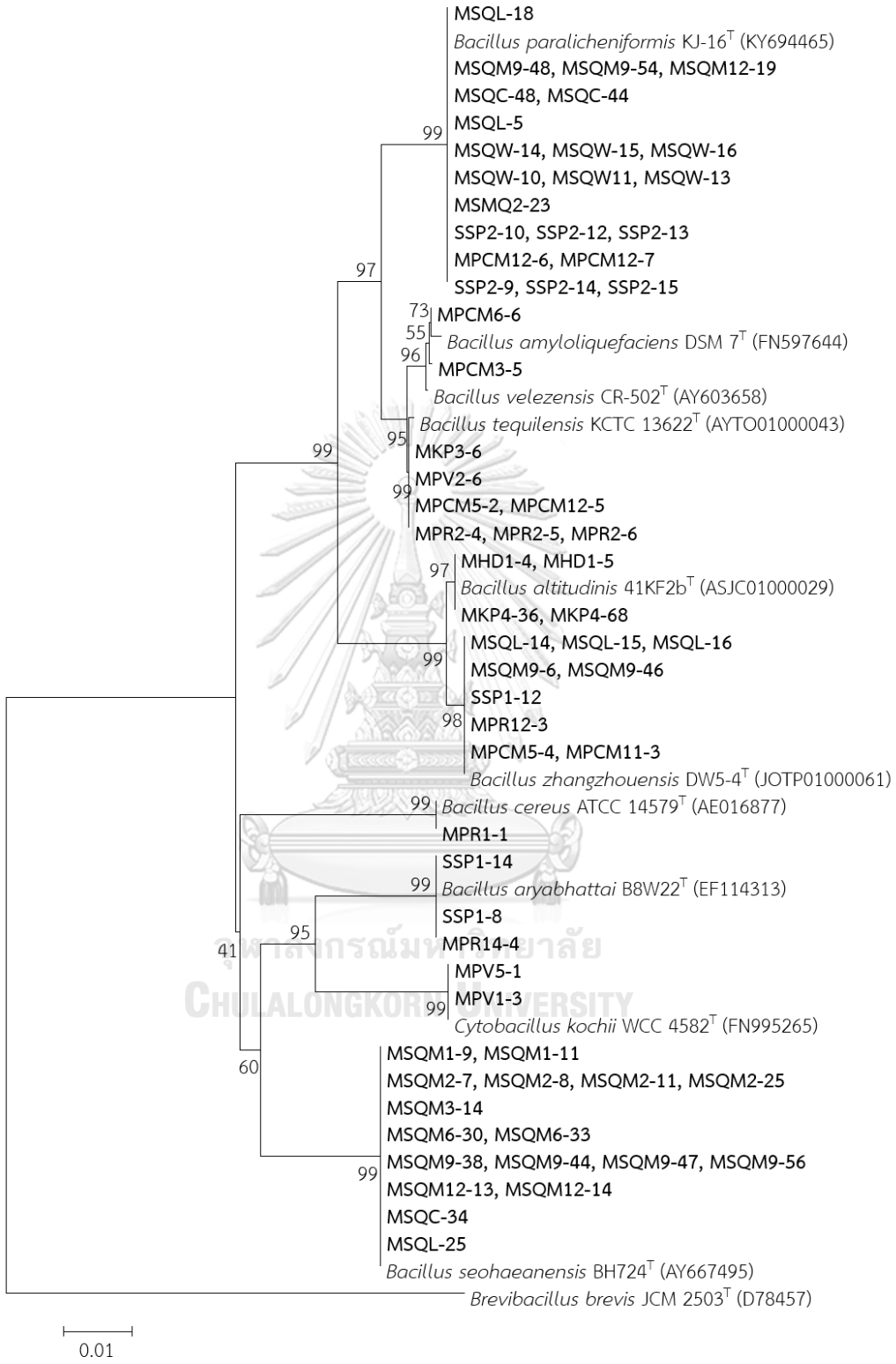


Figure 4.12. Neighbor-joining tree based on the 16S rRNA gene sequences showing relationships among *Bacillus* isolates and related species.

Nine isolates of pattern 169 (MPCM5-4, MPCM11-3, MSQM9-6, MSQM9-46, MSQL-14, MSQL-15, MSQL-16, MPR12-3, and SSP1-12) was designed into the genus *Bacillus* belonged to the family *Bacillaceae*. The 16S rRNA gene sequence and NJ tree of these isolates exhibited the highest similarity (100%) to *B. zhangzhouensis* DW5-4^T in that order on the EzTaxon server. The phylogenetic tree analysis of these isolates is shown in Figure 4.12. Strain DW5-4^T was isolated from aquaculture water of a shrimp farm in China and demonstrated positive results for esterase (C4) and esterase lipase (C8) on the API ZYM test kit (Liu, 2016).

4.4.2.2. DNA-fingerprint of LPMHB in genus Halobacillus

Pattern 53 comprised of two isolates, MKP4-71 and MSQM2-12. The 16S rRNA gene sequence analysis revealed that the isolate exhibited the highest 16S rRNA gene sequence similarity value of 99.11% with *Halobacillus profundi* IS-Hb4^T (Hua *et al.*, 2005) and formed a cluster with those validly published *Halobacillus* species in the phylogenetic tree (Fig. 4.13). Strain IS-Hb4^T was isolated from a deep-sea carbonate rock at cold methane seep in Japan and was classified as moderately halophilic bacteria. (Hidri *et al.*, 2013) reported that strain *H. profundi* isolated from Chott el Djerid of southern Tunisia and could be produced protease DNase and amylase but not for lipase.

For pattern 102 consisted of a single isolate (MSQM3-7), which was identified as *Halobacillus mangrovi* MS10^T (Soto-Ramírez *et al.*, 2008) using the 16S rRNA gene (1,453 bp) sequence similarity value of 100% (Fig. 4.13). No studies in the field of lipase and esterase production, and the result on API ZYM was negative for lipase (C14).

4.4.2.3. DNA-fingerprint of LPMHB in genus Lentibacillus

Based on the 16S rRNA gene sequence (1,410 bp) and phylogenetic analysis, isolate MKP4-86 of pattern 99 was closely related to *L. halophilus* TISTR 1549^T (Tanasupawat *et al.*, 2006) with 99.80% sequence similarity. Therefore, isolate MKP4-86 was identified as *L. halophilus* (Fig. 4.13).

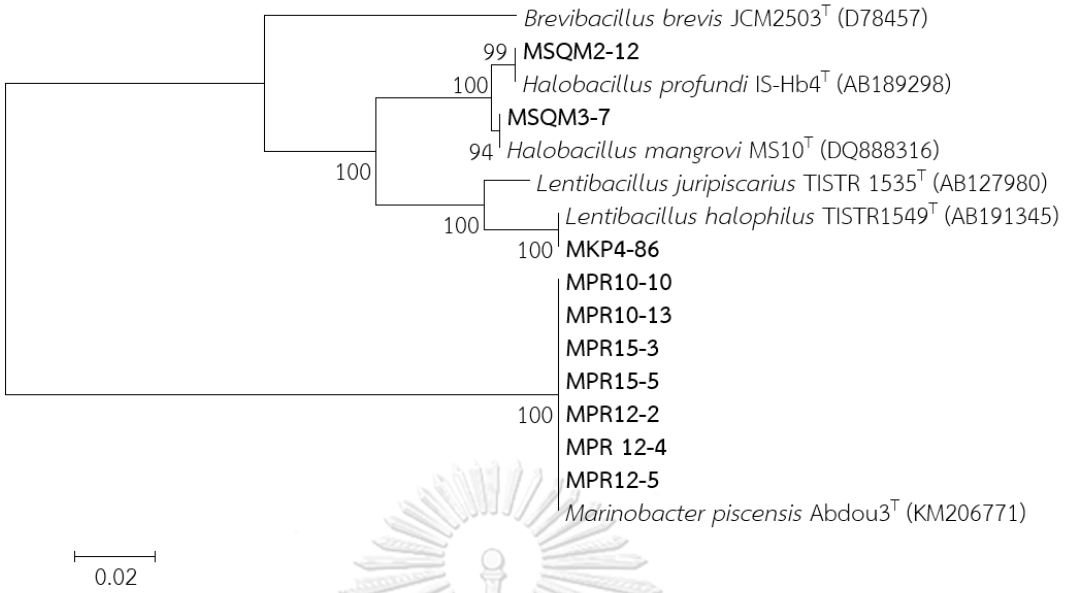


Figure 4.13. Neighbor-joining tree based on the 16S rRNA gene sequences showing relationships among *Halobacillus*, *Lentibacillus* and *Marinobacter* isolates and related species.

4.4.2.4. DNA-fingerprint of LPMHB in genus Marinobacter

Pattern 68 contained seven isolates, including MPR10-10, MPR10-13, MPR15-3, MPR15-5, MPR12-2, MPR 12-4, and MPR12-5. All isolates were screened from various locations of fermented fish (*pla-ra*) samples. According to the 16S rRNA gene sequence analysis, all isolates showed the highest similarities of the 16S rRNA gene sequence with the type strains of the genus *Marinobacter* and was identified as *Marinobacter piscensis* Abdou3^T (Hedi *et al.*, 2015). The NJ phylogeny highlighted that all these isolates formed a clade within the genus *Marinobacter,* as shown in Figure 4.13. Genus *Marinobacter* has been reported as a lipolytic producer. *M. lipolyticus* have previously been isolated from saline soil in Spain (Martín *et al.*, 2003), and recombinant *LipBL* from this strain was studied Pérez *et al.* (2012). A lipolytic gene, designated *LipBL*, from *M. lipolyticus* SM19, was expressed in *E. coli LipBL* and encoded a protein of 404 amino acids with a molecular mass of 45.3 kDa for used in the production of eicosapentaenoic acid (EPA) (Pérez *et al.*, 2011). *M. flavimaris* SWJ2

exhibited the highest extracellular lipase production was isolated from seawater in Malaysia (Musa *et al.*, 2018b). A solvent-tolerant halophilic lipase producing strain, *M. litoralis* SW-45, was isolated from seawater with the promising industrial potential to be utilized as a biocatalyst for the esterification and transesterification (Musa *et al.*, 2018c) which has been confirmed by the presence of lipase-encoding genes as well as genes coding for esterase (Musa *et al.*, 2019).

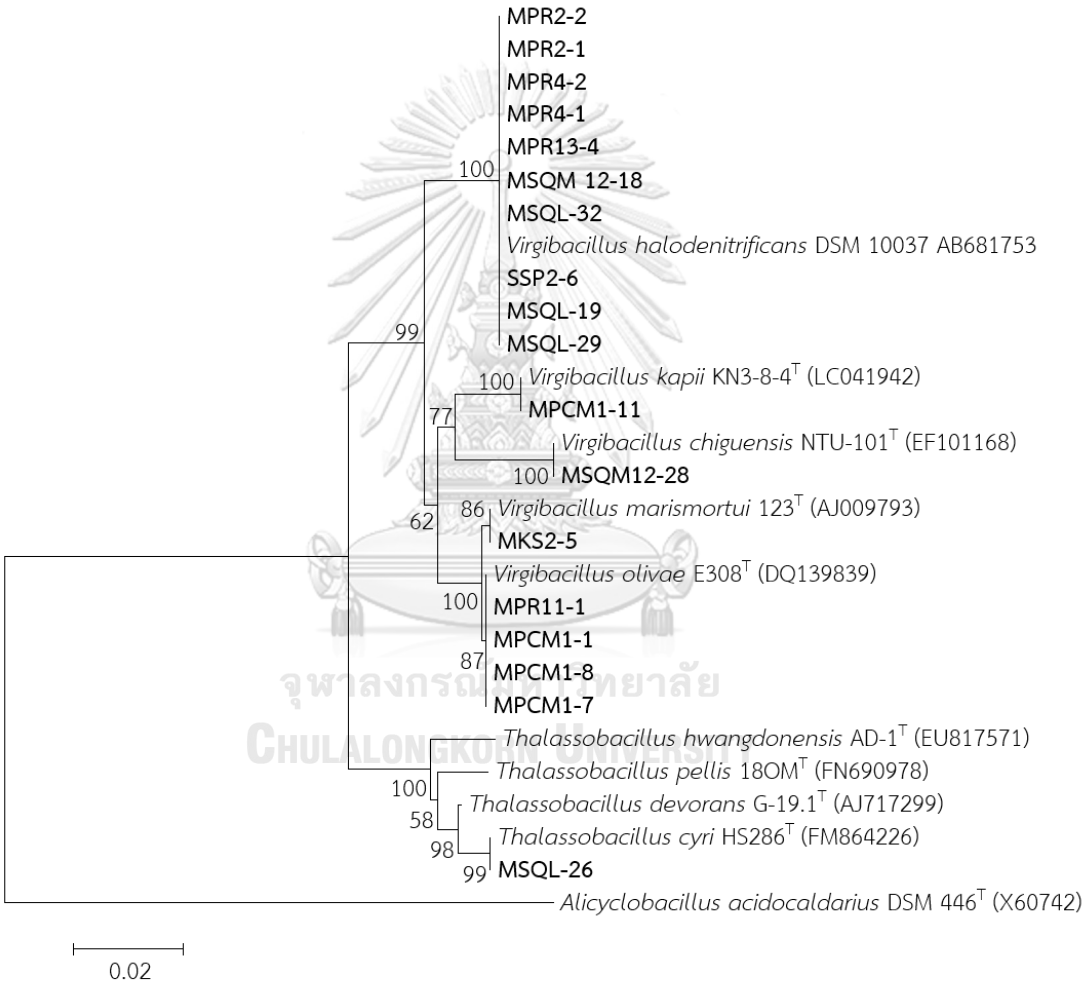


Figure 4.14. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences showing relationships among *Thalassobacillus* and *Virgibacillus* isolates and related species.

4.4.2.5. DNA-fingerprint of LPMHB in genus Thalassobacillus

Isolate MSQL-26 (pattern 42) showed 99.48% of the 16S rRNA gene sequence similarity with *T. cyri* CCM 7597^T (Sánchez-Porro *et al.*, 2009). The 16S rRNA gene sequence analysis showed the isolate MSQL-26 formed a cluster with other species in the phylogenetic tree (Fig. 4.16). Genus *Thalassobacillus* was first reported by García *et al.* (2005) with the type species of *T. devorans* G-19.1^T and was classified as moderately halophilic with the potential of phenol-degrading bacteria. Lv *et al.* (2011) reported that an extracellular halophilic carboxylesterase from *Thalassobacillus* sp. strain DF-E4 was active and stable in neutral to alkaline pH range with a molecular mass of 45 kDa.

4.4.2.6. DNA-fingerprint of LPMHB in genus Virgibacillus

For Pattern 4 (MSQM 12-18, MSQL-19 MSQL-32, SSP2-6) and pattern 140 (MPR2-1, MPR2-2, MPR4-1, MPR4-2, MPR13-4, MSQL-29), based on 16S rRNA gene sequence and phylogenetic tree analysis (Fig. 4.16) it was identified theses isolates as *V. halodenitrificans* from its 99.95% sequence similarity to *V. halodenitrificans* DSM 10037^T (Yoon *et al.*, 2004b) *V. halodenitrificans* had been reported as moderately halophilic bacteria isolated from various sauce such as shrimp paste (Tanasupawa *et al.*, 2011), Fermented crab, *Pla-ra* (Taprig *et al.*, 2013) and fish sauce (Montriwong *et al.*, 2012; Kanlayakrit *et al.*, 2013).

The isolate MPCM1-11 of pattern 16 was identified as a member of the genus *Virgibacillus* from its 100% sequence similarity (Fig. 4.16). The 16S rRNA gene sequence (1441 bp) indicated isolate MPCM1-11 was identified as *V. kapii* KN3-8-4^T, which was previously isolated from a traditional shrimp paste (*Ka-pi*) in Thailand by Daroonpunt with co-workers (Daroonpunt *et al.*, 2016a).

Pattern62 consisted of one isolate, MSQM12-28. The 16S rRNA gene sequence of this isolate showed 100% similarity to *V. chiguensis* NTU-101T (Wang *et al.*, 2008) as shown in figure 4.16.

One isolate named MKS2-5 from pattern 179 had a 99.5% pairwise 16S rRNA gene sequence similar to that of type strain *V. marismortui* 123^T. According to phylogenic tree analysis, isolate MKS2-5 was located within the cluster of *V.*

marismortui. Therefore, these isolates were identified as *V. marismortui*. (Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003)

The isolates MPR11-1, MPCM1-1, MPCM1-7, and MPCM1-8 from pattern 180 was closely related to *V. olivae* E308^{T} (Quesada *et al.*, 2007). It was identified as *Virgibacillus olivae* with 99.78-99% similarity of the 16S rRNA gene sequence (1390-1411 bp) (Fig. 4.16).

V. halodenitrificans SF-121 was isolated from a marine solar saltern in Korea displayed lipase and esterase activity as documented by (Yoon *et al.*, 2004b). As reported by Dueramae and a fellow worker (Dueramae *et al.*, 2017). *V. alimentarius* LBU20907 was isolated from fermented fish (*Bu-du*) and found to be an efficient producer of extracellular halophilic lipase. The enzyme was purified 5.99-fold with a 0.15% final yield, and molecular weight was estimated at 100 kDa. Three isolated (TPPN1-1, J4, and CC7-1) were identified as *V. marismortui,* which are shown protease activities (Taprig *et al.*, 2013). Some strain of *V. halodenitrificans* was also reported as several enzymes producer, i.e., extracellular proteases (Tanasupawat *et al.*, 2011; Kanlayakrit *et al.*, 2013), novel fibrinolytic enzymes (Montriwong *et al.*, 2012), and NaCl-activated detergent-stable proteinases (Montriwong *et al.*, 2015). Another reported shown that the peptides hydrolysate prepared by *V. halodenitrificans* SK1-3-7 proteinases have shown angiotensin I-converting enzyme (ACE) inhibitory activity (Toopcham *et al.*, 2015) and anti-inflammatory activity (Toopcham *et al.*, 2017).

In conclusion, the DNA fingerprinting technique known as rep-PCR is a rapid, easy to conduct, and reproducible tool for discrimination at the species, subspecies, and potentially up to the strain level of halophilic bacteria, with a single-performance protocol. The majority of the strains analyzed had different DNA-fingerprinting patterns, confirming the high discriminative power of the $(GTG)_5$+Eric2 primer and reporting high diversity of halophilic bacteria in various saline environment of Thailand. Most of the strains shared more than 99% identity with their closest phylogenetic relatives. However, the phylogenetic analyses showed that few strains could further be studied taxonomically to delineate as novel species.

4.5. Taxonomic studies of novel LPMHB

Two LPMHB isolates that exhibited the 16S rRNA gene sequence similarities lower than 99% using the EzBioCloud database comprised of SKP4-6 (pattern 12) and SSKP1-9 (pattern 57) from DNA-fingerprinting were recruited for taxonomic study. Strains, SSKP1-9^T and SKP4-6^T isolated from *Ka-pi* collected from Samut Sakhon Province, Thailand (13° 32′ 42.7122″ N, 100° 16′ 342336″ E) were characterized based on a polyphasic approach.

4.5.1. *Lentibacillus* strain SSKP1-9^T

4.5.1.1. Phenotypic characteristics

Colonies were white to cream, low-convex or raised, smooth and circular (0.1-1.08 mm in diameter) after growth for two days on JCM no.377 medium (Figure 4.15a). Cells of strain SSKP1-9^T were Gram-stain-positive, aerobic, motile, and moderately halophilic rods (0.4-0.6 μm wide and 1.6-2.7 μm long). Oval-shaped endospores were observed at the terminal position of the swollen sporangia (Figure 4.15b). Peritrichous flagella were observed. Oxidase and catalase were positive, but urease, citrate utilization, and MR-VP were negative. No growth occurred on *Lentibacillus* agar under the anaerobic condition. Growth was observed at 20-50 °C, 4-35% (w/v) NaCl, pH ranged from 5.0 to 8.0, but no growth at 15 °C and over 55 °C. Optimal growth was observed at 37-40 °C, pH 7.0 and in 8-16% (w/v) NaCl. Hydrolysis of Tween 20, Tween 80, tributyrin, starch, and lecithin were negative (Table 4.2).

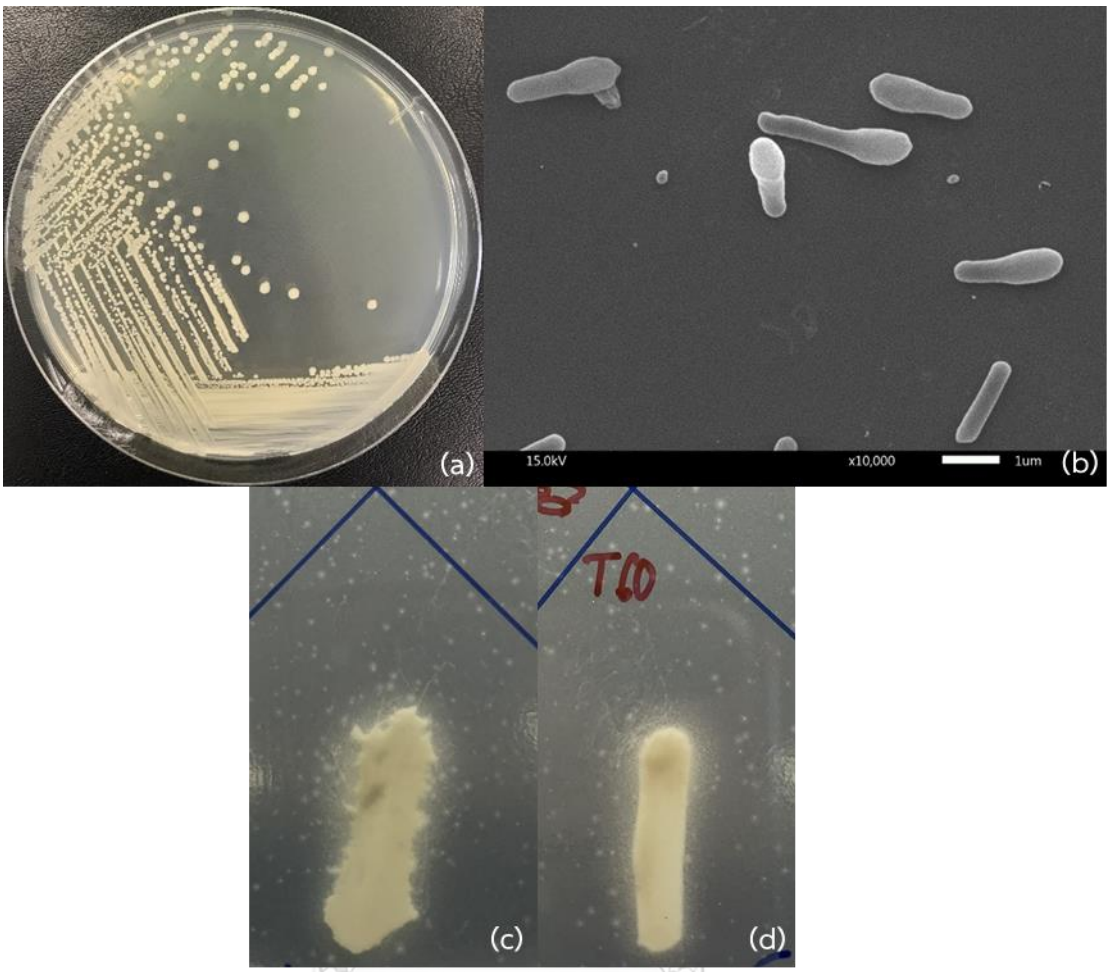


Figure 4.15. (a) Morphology of colonies formed and (b) scanning electron micrographs during the spore's formation of strain SSKP1-9^{T} growing on JCM No. 377 agar plate at 37 °C for two days. Bar, 1 µm. Hydrolysis of (c) Tween 40 and (d) Tween 60 on agar plate.

In the API ZYM tests, the strain was found to be positive for alkaline phosphatase, esterase (C4), leucine arylamidase, α-chymotrypsin, and naphthol-AS-BI-phosphohydrolase whilst esterase lipase (C8) and valine arylamidase were weakly positive. The strain was found to be negative for lipase (C14), cystine arylamidase, trypsin, acid phosphatase, α-galactosidase, β-galactosidase (OPNG), β-glucuronidase, α-glucosidase, b-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, indole production, lysine decarboxylase, and urease.

In the API 20NE strip, nitrate, oxidase, and gelatin hydrolysis were positive, whilst acid from D-glucose was weak. D-Glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid were not found to be assimilated as sole carbon sources. In the API 50 CHB assay, acid was produced from potassium 5-ketogluconate while glycerol, D-ribose, D-glucose, *N*-acetylglucosamine, aesculin, and D-tagatose were weakly positive. No acid was produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, and potassium 2-ketogluconate.

Table 4.3. Phenotypic characteristics between strain SSKP1-9^T and related type strains.

Characteristics	SSKP1-9^T	*L. juripiscarius* TISTR 1535^T	*L. halophilus* TISTR 1549^T
Temperature (°C) for growth			
Range	20-50	20-50	20-45
Optimal	37-40	37-40	37-40
pH for growth			
Range	5.0-8.0	5.0-10.0	5.0-8.0
Optimal	7.0	6.0	7.0
Optimal NaCl for growth (% w/v)	8-16	9-18	10-14
Hydrolysis of:			
Casein	+	-	-
Gelatin	+	-	-
Skim milk	+	-	-
Tween 40	+	+	-
Tween 60	+	-	-
Acid production from (API 50 CHB)			
Adonitol	-	w	-
Glycerol	w	-	-
D-Glucose	w	+	-
D-Ribose	w	+	-
D-Xylose	-	+	-
N-acetylglucosamine	w	+	-
Enzyme activities (API ZYM)			
Acid phosphatase	-	-	w
Alkaline phosphatase	+	w	-
α-Chymotrypsin	+	w	-
Esterase/Lipase (C 8)	w	w	-
Valine arylamidase	w	w	-

All tests were performed under the same conditions from this study.

+, Positive reaction; **w**, weak positive reaction; **-**, negative reaction.

Strain SSKP1-9^T was susceptible to (µg per disc unless otherwise stated): amoxicillin (30), amoxycillin/clavulanic acid (30), ampicillin (10), bacitracin (10 U), cefepime (30), cefotaxime (30), cefoxitin (30), ceftazidime (30), ceftriaxone (30), chloramphenicol (30), clindamycin (2), ertapenem (10), erythromycin (15), levofloxacin (5), meropenem (10), netilmicin (30), novobiocin (30), piperacillin/tazobactam (110), rifampicin (30), tetracycline (30) and vancomycin (30), whilst amikacin (30), nystatin (10) and polymyxin B (300 U) were weakly susceptible. The strain was resistant to nalidixic acid (30), neomycin (30), and sulphamethoxazole/ trimethoprim (25). For gentamicin (10), imipenem (10), kanamycin (30), nitrofurantoin (300), and streptomycin (10), only the novel strain was susceptible while the related type strains were resistant.

4.5.1.2. Chemotaxonomic characteristics

The major cellular fatty acids (>10% of the total fatty acids) of strain SSKP1-9^T were anteiso$_{C15:0}$ (51.4%), anteiso$_{C17:0}$ (17.8%), and iso-C$_{16:0}$ (11.0%), which were similar to those of the reference strains (Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006), however, there were some differences in the amount of fatty acid of the strains, as shown in Table 4.3. Although present in low amounts, the novel strain contained C$_{12:0}$ (0.3%), C$_{15:0}$2OH (0.4%), iso-C$_{18:0}$ (0.5%), iso-C$_{17:0}$3OH (0.4%), anteiso-C$_{14:0}$ (4.0%), isoC$_{17:1\omega5c}$ (1.1%), C$_{18:3\ \omega6,9,12c}$ (0.1%), C$_{16:1\ \omega7c}$ alcohol (0.7%) and C$_{16:1\ \omega11c}$ (0.6%), which are absent in all the closely related type strains. Strain SSKP1-9^T contained *meso*-DAP in the cell-wall peptidoglycan (Fig. 4.16). Major polar lipids were phosphatidylglycerol and diphosphatidylglycerol, and there were minor amounts of unidentified lipids (L1-L4), an unidentified phospholipid (PL1), and glycolipid (Fig. 4.17). The predominant isoprenoid quinone was MK-7, the same as in *L. juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T (Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006).

Table 4.4. Cellular fatty acids composition (%) of strain SSKP1-9^T and related type strains. Major components are highlighted with bold text.

Fatty acids	SSKP1-9^T	*L. juripiscarius* TISTR 1535^T	*L. halophilus* TISTR 1549^T
Saturated fatty acids			
$C_{12:0}$	0.3	-	-
$C_{14:0}$	1.3	-	2.4
$C_{16:0}$	5.1	1.9	6.1
$C_{17:0}$	-	1.6	1.3
$C_{18:0}$	-	-	0.7
$C_{19:0}$	-	-	0.2
Unsaturated fatty acids			
$C_{15:0}$2OH	0.4	-	-
Branched fatty acids			
iso-$C_{10:0}$	-	3.3	2.6
iso-$C_{14:0}$	-	6.5	4.6
iso-$C_{15:0}$	3.3	5.9	5.9
iso-$C_{16:0}$	**11.0**	**10.4**	**7.6**
iso-$C_{17:0}$	0.7	0.8	0.8
iso-$C_{18:0}$	0.5	-	-
iso-$C_{17:0}$3OH	0.4	-	0.7
anteiso-$C_{13:0}$	0.2	1.2	0.8
anteiso-$C_{14:0}$	4.0	-	-
anteiso-$C_{15:0}$	**51.4**	**52.8**	**53.3**
anteiso-$C_{17:0}$	**17.8**	**15.6**	**12.6**
anteiso-$C_{19:0}$	0.32	-	0.4
Unsaturated branched fatty acids			
iso-$C_{17:1\omega 5c}$	1.1	-	-
$C_{18:3\omega 6,9,12c}$	0.1	-	-
$C_{20:1\omega 6,9c}$	-	-	0.4
$C_{16:1\omega 7c\ alcohol}$	0.7	-	-
$C_{18:1\omega 9c}$	-	-	0.7
$C_{16:1\omega 11c}$	0.6	-	-
10-Methyl fatty acids			
Summed Feature 3[a]	0.2	-	1.0

[a]Summed feature 3 contained $C_{16:1\omega 6}c$ or $C_{16:1\omega 7}$

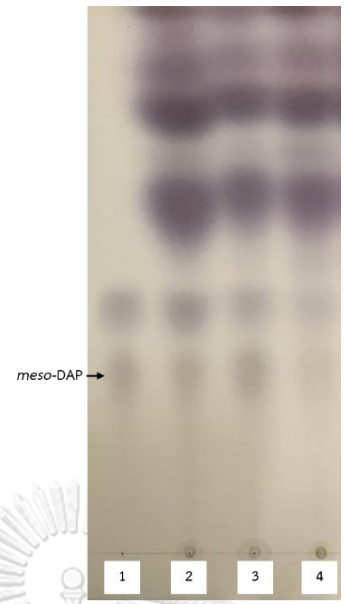


Figure 4.16. *meso*-DAP of strain SSKP1-9^T and related type strains. Panel M, Standard *meso*-DAP; Panel 1, SSKP1-9^T; Panel 2, *L. juripiscarius* TISTR 1535^T and Panel 3, *L. halophilus* TISTR 1549^T

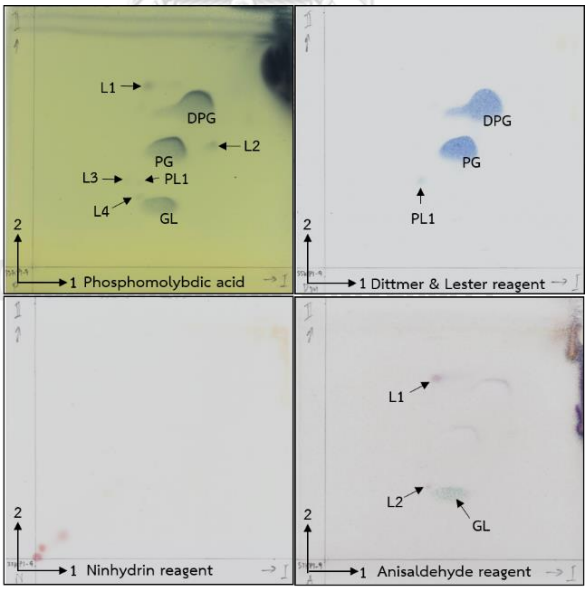


Figure 4.17. Polar lipids profile of strain SSKP1-9^T based on a 2-dimensional thin-layer chromatogram.

Abbreviation: DPG, Diphosphatidylglycerol; PG, Phosphatidylglycerol; PL1, Unidentified phospholipids; GL, Unidentified glycolipid; L1-L4, Unidentified lipid

4.5.1.3. Genotypic characteristics

4.5.1.3.1. Phylogenetic and phylogenomic analysis

The results of phylogenetic analysis based on the NJ algorithm demonstrated that strain SSKP1-9^T formed a cluster with members of the genus *Lentibacillus* (Fig. 4.18) based on 16S rRNA gene sequence (1,446 bp) similarity. The topologies of phylogenetic trees built using the ML and MP also supported these results and formed a stable clade. Strain SSKP1-9^T was located in a phylogenetic clade together with all recognized species and was most closely related to *L. juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T with 98.7% and 97.2% similarity, respectively.

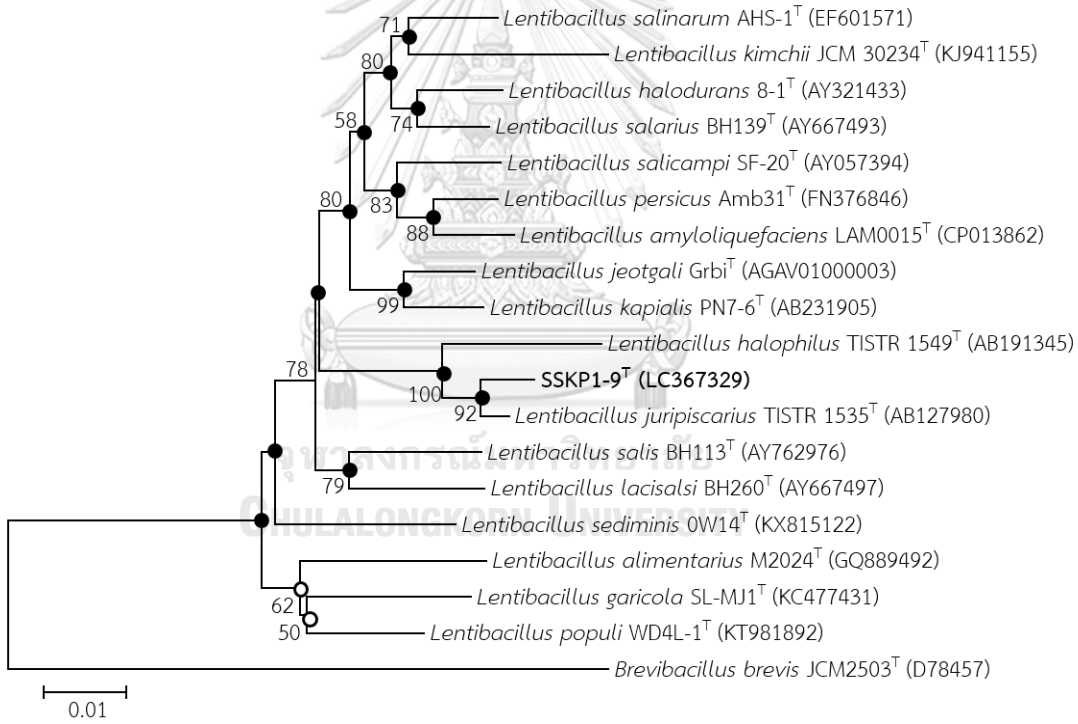


Figure 4.18. Neighbor-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences, displaying the relationships between strain SSKP1-9^T and related type strains. Filled circles indicate that the corresponding branches were recovered in the NJ, ML, and MP tree; open circles indicate that the related branches were retrieved in the NJ and ML trees.

To strengthen the phylogenetic signal and better characterize the relationship of strain SSKP1-9^{T}, the phylogenomic analysis based on a draft whole-genome sequence was performed. The phylogeny analysis (Fig. 4.19) may suggest that the strain SSKP1-9^{T} is evolutionarily closer to *L. juripiscarius* TISTR 1535^{T} than other reference *Lentibacillus* strains. According to the phylogenomic analysis, the strain SSKP1-9^{T} also had more diverse and distinct metabolic pathways. Thus, the overall genomic comparison indicated that strains SSKP1-9^{T} could be unique from each other and other *Lentiacillus* species on the basis of different genomic features. As mentioned above, the similarity of the 16S rRNA gene sequence between SSKP1-9^{T} and *L. halophilus* TISTR 1549^{T} (97.2%) is below the previously accepted cut off of 98.7-99% proposed by Stackebrandt & Ebers, 2006. Therefore, we only selected *L. juripiscarius* TISTR 1535^{T} for comparing the genomic data in the next step.

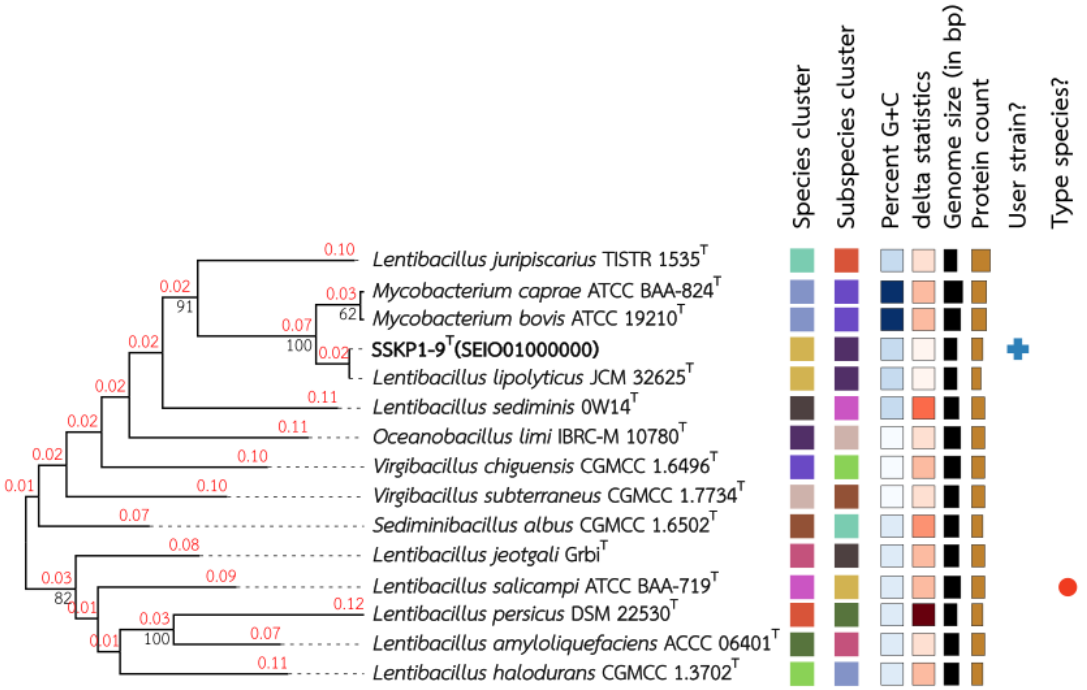


Figure 4.19. Phylogenomic analysis of the novel strain, SSKP1-9^{T} with closely related *Lentibacillus* type and reference strains. The red numbers represent the branch length values.

4.5.1.3.2. DNA fingerprinting analysis

The intraspecies diversity among the strains SSKP1-9^T and the related type strains was shown by using the genomic DNA fingerprinting with BOX, ERIC, $(GTG)_5$, and REP primers. The analysis based on UPGMA cluster analysis of Pearson correlation coefficient values of novel strains is significantly different from their closest phylogenetic neighbors. Thus, the differences in the DNA fingerprinting confirmed that strains SSKP1-9^T are genetically differentiation between intraspecies and also encouraged their distinct lineage to a novel species (Figure 4.20).

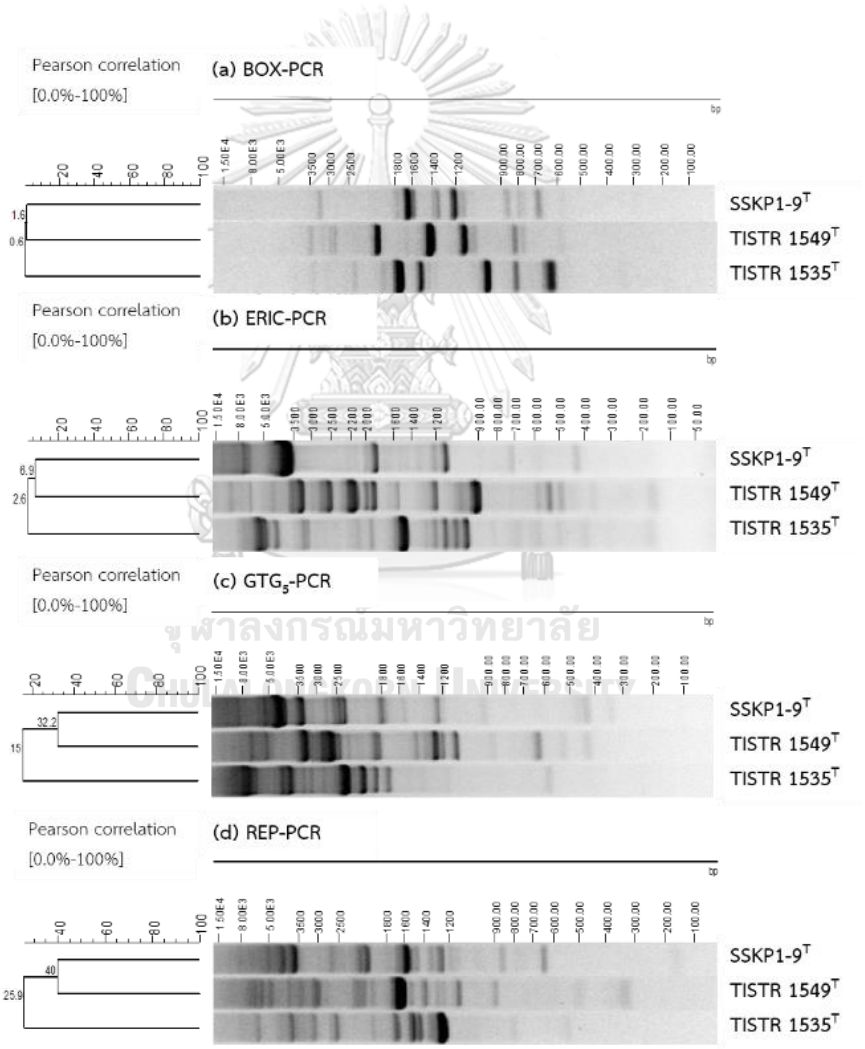


Figure 4.20. The dendrogram of (a) BOX-PCR, (b) ERIC-PCR, (c) $(GTG)_5$-PCR, and (d) REP-PCR fingerprints patterns of strain SSKP1-9^T, *L. juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T.

4.5.1.3.3. General genomic features

Strain SSKP1-9^T (SEIO00000000) had 3,516,313 bp genome length with 44.6 mol% G+C content. The genome sequence of *L. juripiscarius* TISTR 1535^T (BBCA00000000) was obtained from GenBank and was 3,208,301 bp in size (Hattori *et al.*, 2014) with 44.1 mol% G+C content. The detailed general genomic features are presented in Table 4.4. The ANIb and ANIm values of the strain SSKP1-9^T genomes and its closest species, *L. juripiscarius* TISTR 1535^T, was 82-85.1%. The findings of *is*DDH revealed 26.3% (C.I. model 24.0-28.8%) DNA-DNA relatedness for strain SSKP1-9^T and *L. juripiscarius* TISTR 1535^T. The calculated similarity values were below the ANI, and *is*DDH cut-off values proposed for genus and species delineation (Stackebrandt & Ebers, 1994; Konstantinidis & Tiedje, 2004, 2005; Stackebrandt & Ebers, 2006; Goris *et al.*, 2007; Ritcher & Rosselló-Móra, 2009; Meier-Kolthoff *et al.*, 2013; Kim *et al.*, 2014; Lee *et al.*, 2016; Chun *et al.*, 2018; Parks *et al.*, 2018), hence confirming the different taxonomic status of newly isolated strains associated with genus *Lentibacillus.*

Table 4.5 Genome statistics of strain SSKP1-9^T and *L. juripiscarius* TISTR 1535^{T*}.

Feature	SSKP1-9^T	TISTR 1535^T
Accession no.	SEIO00000000	BBCA00000000*
Genome size (bp)	3,516,313	3,208,301**
G+C content (%)	44.61	44.1**
No. of contigs	625	139**
Largest contig	358,170	145,858**
N50	65,147	49,927**
Total genes	3,462	5,112**
Protein coding genes	3,318	5,041**
RNA genes	17	9**
tRNA	92	62**
CRISPR arrays	1	ND

*The genome of *L. juripiscarius* TISTR 1535^T received from Hattori *et al.*, 2014.

**The data were predicted by the same program used with our genome of novel strain. ND, No report.

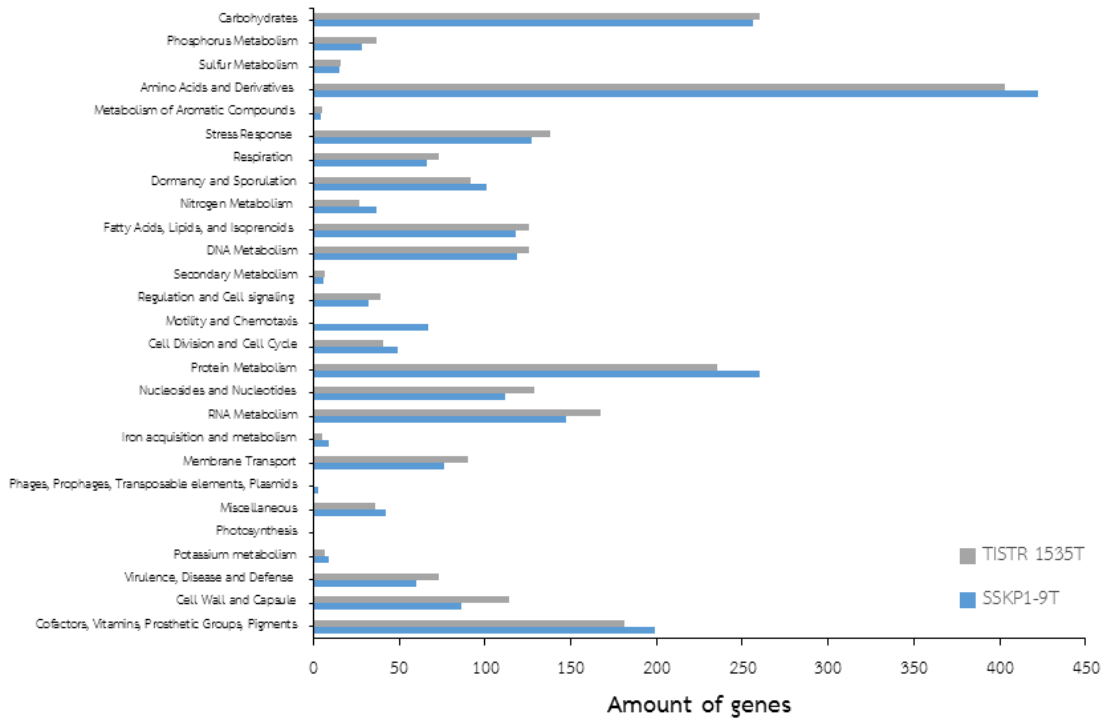


Figure 4.21. The functional gene annotations of strains SSKP1-9^{T} (green bar) and *L. juripiscarius* TISTR 1535^{T} (blue bar) using the RAST database.

The SSKP1-9T genome was predicted by RAST to have 3,462 genes encoding 3,318 proteins (48% were hypothetical), two pairs of 5S, 16S, and 23S rRNA genes (6 rRNA genes in total), and 92 tRNA genes. However, 52% of the predicted genes could not be assigned to a subsystem. The most annotated proteins were assigned to the relevant category: amino acid metabolism (422), protein metabolism (260), carbohydrate metabolism (256), cofactors-vitamins-prosthetic group's pigments metabolisms (199), RNA metabolism (147), DNA metabolism (119), and fatty acids-lipids-Isoprenoids (118).

For the RAST analysis of the genome of strain *L. juripiscarius* TISTR 1535^{T}, about 5,041 protein-coding genes were founded, and 42% were hypothetical proteins, whereas 52% of the predicted genes could not be assigned to a subsystem. The most annotated proteins were: amino acid metabolism (403), carbohydrate metabolism (260), protein metabolism (235), cofactors, vitamins, prosthetic groups,

pigments metabolisms (181), RNA metabolism (167), DNA metabolism (126), and fatty acids-lipids-Isoprenoids (126). Another comparable annotated protein is depicted in Figure 4.21.

Since strain SSKP1-9^T was a moderately halophilic bacterium, we focused on the analysis of genes contributing to survival and tolerance to stress response, 127 genes were detected. These genes were divided into several groups based on their function: osmotic stress (39), oxidative stress (40), cold shock (4), heat shock (19), and other stress response with no subcategory (25). A total of 39 genes associated with the survival in high salt conditions or osmotic stress were identified. Next, we searched for gene features that could enable strain SSKP1-9^T to survive in high salt environments. The genome contained one gene coding for osmoregulation (*glpF*), four gene coding for ectoine biosynthesis and regulation (*EctA, EctB, EctC, EctD*), and 34 genes coding for choline and betaine uptake and betaine biosynthesis included: *BetA* (one), *BetB* (one), *BetT* (three), *OpuD* (four), *OpuA* (nine), *ProU* (four), *OpuC* (nine) and *SOX* (three), which have been previously found to play an essential role in osmotic stress responses (Saum & Müller, 2007; Wargo, 2013; Diken *et al.*, 2015). All of the genes contributing to survival and tolerance to stress response (138) also presented the type strain, *L. juripiscarius* TISTR 1535^T. Furthermore, strain SSKP1-9^T also contained a CRISPR spacer in the genome, which the sequence about 35 nt (GCTCAAAAGAAATCGAACTGGACGAGAATGGCAAG).

Strain SSKP1-9^T showed typical chemotaxonomic characteristics and significant fatty acids found in members of the genus *Lentibacillus*. Nevertheless, strain SSKP1-9^T could be differentiated from phylogenetically related members of the genus *Lentibacillus* by several characteristics, as shown in Table 4.2. According to the above phylogenetic analysis, strains SSKP1-9^T are closely associated with the other *Lentibacillus* species, especially *L. juripiscarius* TISTR 1535^T. On the basis of phenotypic, chemotaxonomic, and genotypic characteristics, draft genomic data, and also antibiotic susceptibility, strain SSKP1-9^T was distinct from other valid type strains. These results suggested that strains SSKP1-9^T may represent novel species in the genus *Lentibacillus*, for which the name *Lentibacillus lipolyticus* (li.po.ly'ti.cus. Gr. neut. n. *lipos* fat; N.L. adj. *lyticus* from Gr. adj. *lytikos* dissolving; N.L. masc. adj. *lipolyticus*, fat-

dissolving) sp. nov. is proposed. The type strain, SSKP1-9^T (=JCM 32625^T=TISTR 2597^T), was isolated from *Ka-pi*, a traditional salted shrimp paste of Thailand. The GenBank/EMBL/DDBJ accession number for the nearest 16S rRNA gene sequences of *L. lipolyticus* SSKP1-9^T are LC367329, and that of the genome is SEIO00000000 (Booncharoen *et al.*, 2019).

4.5.2. *Halobacillus* strain SKP4-6^T

4.5.2.1. Phenotypic characteristics

Phenotypic properties of strain SKP4-6^T were consistent for assignment to the genus *Halobacillus*. An isolated strain is moderately halophilic, Gram-stain-positive, motile with peritrichous flagella. Colonies are circular, convex with entire margins, yellowish, and 1.0-3.0 mm in diameter on JCM No. 377 supplemented with 15 % NaCl (w/v) after two days of incubation at 37 °C (Fig. 4.22a). Cells were rods with a width of 0.6-0.8 μm, and a length of 1.8-2.6 μm and ellipsoidal shape endospores are located at the sub-terminal position of the swollen sporangia (Fig. 4.22b).

Growth occurs at 20-50 °C (optimally at 37-45 °C), but weakly at 5-15 °C and no growth at temperatures above 50 °C and below 5 °C. The optimal pH for growth is pH 7.0 (pH range 5.0-10.0), and no growth is observed at pH 4.0 and over pH 10.0. Strain SKP 4-6^T grows optimally in the presence of 10-15% NaCl (range 2-23 % w/v), and growth is deficient in the absence of Mg_2SO_4 and the NaCl concentration ranging from 23-35% (w/v) under static conditions. The strain is aerobic, and growth does not occur under anaerobic conditions on JCM No. 377 supplemented with 15% NaCl (w/v) after incubation at 37 °C for seven days.

Catalase positive. Tests of indole production, nitrate reduction, citrate utilization, and urease give negative results. The methyl red test was positive, but the Voges-Proskauer test was negative. Positive for hydrolysis of aesculin, casein, skim milk, starch, and Tween 20, Tween 40, Tween 60 but not for Tween 80, tributyrin and egg yolk. Resistant to (μg per disc unless otherwise stated) gentamicin (10), nystatin (10), and streptomycin (10). Other phenotypic characterizations are included in the species description, and the characteristics that differentiate strain SKP4-6^T from related type strains of species in the genus *Halobacillus* are concluded in Table 4.5.

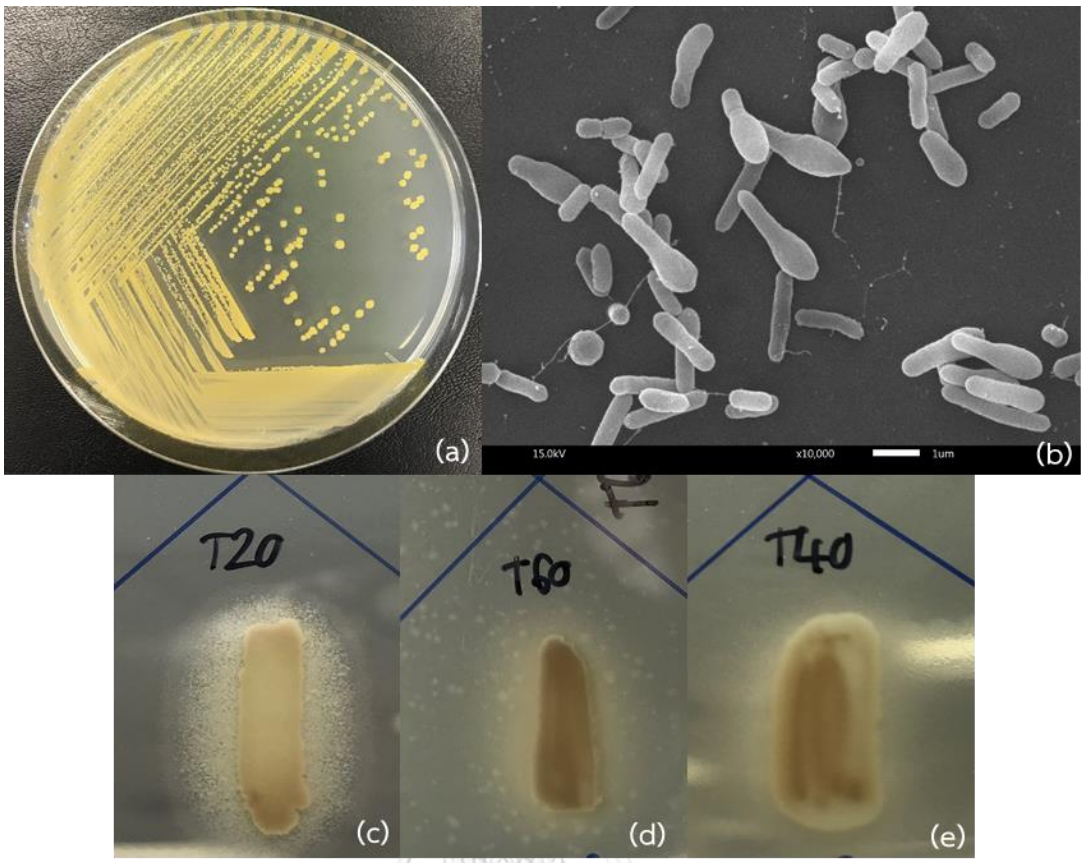


Figure 4.22. (a) Morphology of colonies formed and (b) scanning electron micrographs during the spore's formation of strain SKP4-6^{T} growing on JCM No. 377 agar plate at 37 °C for two days. Bar, 1 µm. Hydrolysis of (c) Tween 20 (d) Tween 60 and (e) Tween 40 on agar plate.

Table 4.6. Phenotypic characteristics of strain SKP4-6^T and other type strains of the genus *Halobacillus.*

Characteristics	SKP4-6^T	*H. salinus* JCM 11546^T	*H. locisalis* KCTC 3788^T	*H. yeomjeoni* KCTC 3957^T
Growth temperature (°C)				
Range	5-50	5-50	5-50	10-55
Optimal	37-45	30-45	30-37	30-45
Growth pH				
Range	5.0-10.0	5.0-10.0	5.0-10.0	5.0-9.0
Optimal	7.0	7.0	7.0	7.0
NaCl (%, w/v) for growth				
Range	2-33	1-32	4-24	0-35
Optimal	10-15	1-24	5-18	0-23
Hydrolysis of :				
Tween 20	+	+	-	-
Tween 40	+	+	-	-
Tween 60	+	+	-	+
Skim milk	+	+	-	-
Starch	+	-	+	-
Egg yolk	-	+	-	-
Casein	+	+	-	-
API 50 CHB :				
Aesculin	+	+	+	-
Adonitol	-	-	-	+
D(-) Arabinose	-	+	+	-
L(+) Arabinose	+	+	+	-
D(+) Arabitol	-	+	-	-
D(+) Cellobiose	+	+	+	-
Dextran	-	-	-	+
meso-Erythritol	-	-	+	-
D(+) Galactose	+	+	-	-
D(+) Galacturonic acid	+	-	-	-
Inulin	-	-	+(w)	+(w)
D(-) Lactose	-	-	+	-
D(-) Mannitol	-	+	-	+
D(+) Melibiose	+	-	+(w)	-
API ZYM :				
Acid phosphatase	+	+	-	-
Cystine arylamidase	-	-	+	+
α-Galactosidase	+	+	-	-
β-Galactosidase	+	+	-	-
β-Glucosidase	-	+	+	-
Trypsin	+	-	-	-
API 20NE:				
Gelatin hydrolysis	+	+	-	-
Nitrate reduction to nitrites	-	-	-	+
p-nitrophenyl-β-D-galactopyranoside	+	+	+	-

+, Positive reaction; w, weakly positive reaction; -, negative reaction.

4.5.2.2. Chemotaxonomic characteristics

Primary fatty acids (>10% of the total fatty acids) in strain SKP4-6^T were anteiso-$C_{15:0}$ (38.3%), iso-$C_{15:0}$ (20.8 %), anteiso-$C_{17:0}$ (15.5%), which is similar to those of its closely related type strains. Nevertheless, there are some differences between iso-$C_{19:0}$ and $C_{18:02}$OH only found in strain SKP4-6^T and *H. yeomjeoni* KCTC 3957^T, respectively (Table 4.6). Further chemotaxonomic analyses revealed the cell wall peptidoglycan of strain SKP4-6^T was of the type A4β based on the presence of L-Orn-D-Asp, which was similar to the type strain in the genus *Halobacillus* as previously stated by Spring *et al.*, 1996. The predominant menaquinone found in strain SKP4-6^T was menaquinone-7 (MK-7). Strain SKP4-6^T contained phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) as major polar lipids which found in the members of the genus *Halobacillus* (Kim *et al.*, 2006; Wang *et al.*, 2015a; Romano *et al.*, 2008) while unidentified lipids (L1), two unidentified phospholipids (PL1-PL2) and unidentified glycolipid (GL) were presented as minor components (Fig. 4.23).

Table 4.7. Fatty acid contents (%) of strain SKP4-6^T and closely related species. Major components are highlighted with bold type, and fatty acid lower than 0.5 % was omitted.

Fatty acid	SKP4-6^T	*H. salinus* JCM 11546^T	*H. locisalis* KCTC 3788^T	*H. yeomjeoni* KCTC 3957^T
Saturated fatty acids				
$C_{12:0}$	-	2.5	1.2	0.6
$C_{14:0}$	-	-	1.3	0.5
$C_{16:0}$	4.1	-	6.3	2.8
$C_{16:0}$ *N*-alcohol	-	1.5	1.4	2.3
$C_{17:0}$	-	**11.2**	-	-
$C_{18:0}$	1.0	1.6	-	-
$C_{19:0}$	-	3.4	-	-
Unsaturated fatty acids				
$C_{18:0}$2OH	-	-	-	0.9
Branched fatty acids				
iso-$C_{10:0}$	-	**20.2**	0.5	1.1
iso-$C_{14:0}$	1.2	-	5.9	1.8
iso-$C_{15:0}$	**20.8**	**7.0**	**4.8**	**4.6**
iso-$C_{16:0}$	**4.8**	**2.0**	**14.6**	**8.2**
iso-$C_{17:0}$	**9.0**	**2.1**	**1.4**	**1.6**
iso-$C_{19:0}$	0.5	-	-	-
iso-$C_{11:0}$3OH	-	2.5	-	-
iso-$C_{17:0}$3OH	-	9.5	-	-
anteiso-$C_{13:0}$	-	-	0.6	-
anteiso-$C_{14:0}$	-	-	-	0.73
anteiso-$C_{15:0}$	**38.3**	**10.0**	**43.78**	**57.6**
anteiso-$C_{16:0}$	-	-	-	-
anteiso-$C_{17:0}$	**15.5**	**5.0**	**12.9**	**15.2**
anteiso-$C_{19:0}$	0.6	7.3	0.5	-
Unsaturated branched fatty acids				
iso-$C_{17:1}\omega_{5c}$	-	6.5	0.5	0.8
antiso-$C_{17:1}\omega_{9c}$	-	2.1	-	0.7
$C_{18:3}\omega_{6,9,12c}$	-	-	1.1	-
$C_{20:2}\omega_{6,9c}$	-	-	0.5	-
$C_{18:1}\omega_{9c}$	0.7	-	0.9	-
Summed Feature 3[a]	-	-	0.5	-
Summed Feature 3[b]	-	-	0.5	-
Summed Feature 4[c]	-	-	-	-
Summed Feature 8[d]	-	5.7	-	1.0

[a]Summed feature 3 contained $C_{16:1}\omega_{6c}$ or $C_{16:1}\omega_{7c}$

[b]Summed feature 3 contained $C_{16:1}\omega_{7c}$ or $C_{16:1}\omega_{6c}$

[c]Summed feature 4 contained anteiso-$C_{17:1}$ B/so I

[d]Summed feature 8 included $C_{18:1}\omega_{7c}$

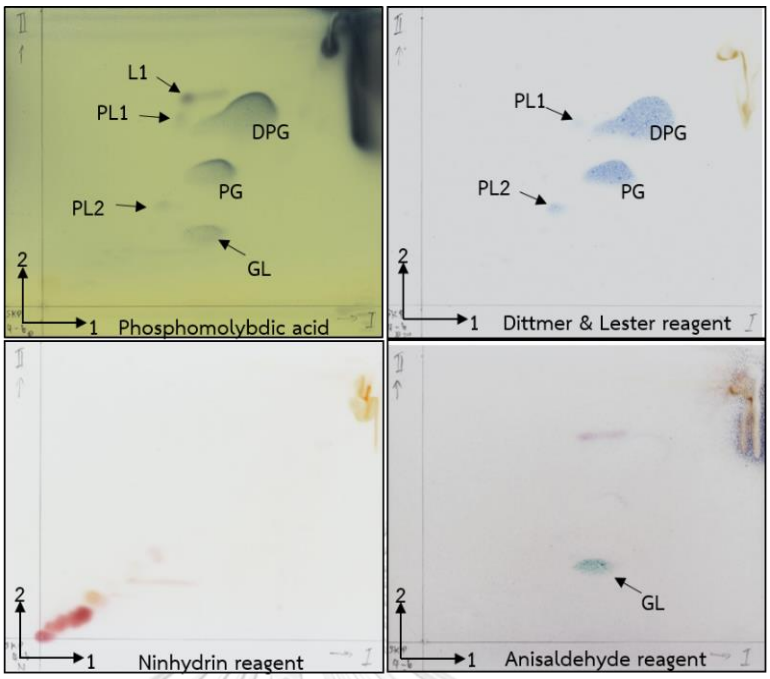


Figure 4.23. Polar lipids profile of strain SKP4-6^T based on a 2-dimensional thin-layer chromatogram.

Abbreviation: DPG, Diphosphatidylglycerol; PG, Phosphatidylglycerol; PL1, Unidentified phospholipids; GL, Unidentified glycolipid; L1-L4, Unidentified lipid

4.5.2.3. Genotypic characteristics

The EzBioCloud tool was used for the nearest full-length 16S rRNA genes sequence comparative analysis and showed that strain SKP4-6^T (1,435 bp) shared the highest sequence similarities with the type strains *H. salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T and *H. yeomjeoni* KCTC 3957^T (98.6% 16S rRNA gene sequence similarity) with strain SKP4-6^T with the difference nucleotides of 22/1435 nt, followed by *H. trueperi* DSM 10404^T (98.40%), *H. alkaliphilus* FP5^T (98.40%), H. faecis IGA7-4^T (98.39%), *H. dabanensis* D-8^T (98.33%), *H. litoralis* SL-4^T (98.33%), *H. campisalis* ASL-17^T (98.26%), *H. salicampi* TGS-15^T (98.26%), *H. andaensis* NEAU-ST10-40^T (98.25%), *H. halophilus* DSM 2266^T (98.19%) and *H. profundi* IS-Hb4^T (98.05%), with percentages of 16S rRNA gene sequence similarity in all cases lower than 98.4%.

4.5.2.3.1. Phylogenetic and phylogenomic analysis

Phylogenetic analysis based on the NJ, MP, and ML methods placed this strain in a cluster consisting of members of the genus *Halobacillus*. Strain SKP4-6^T formed a stable clade only with *H. salinus* JCM 11546^T (Fig. 4.24). This was further supported by draft whole-genome phylogenomically trees, in which the novel strain was clustered clearly separated from the most closely related type strain *H. salinus* JCM 11546^T (Fig. 4.25). Despite the analysis of 16S rRNA genes between strain SKP4-6^T and related type strains displayed the same value whereas the phylogenetic analysis of novel strain was separated from *H. locisalis* KCTC 3788^T and *H. yeomjeoni* KCTC 3957^T. As mentioned above, *H. salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T, and *H. yeomjeoni* KCTC 3957^T were chosen for phenotypic and chemotaxonomic studies, and we only selected *H. salinus* JCM 11546^T for comparing the genomes with the novel strain.

4.5.2.3.2. DNA fingerprinting analysis

The genetic diversity of the five isolates was examined by repetitive extragenic palindromic DNA-PCR fingerprinting using the BOX, ERIC, $(GTG)_5$, and REP primers. Differences between the DNA fingerprints of all isolates were observed. The cluster analysis based on Pearson correlation coefficient values of novel strain is exceptionally different from their closely related type strains (Fig. 4.26). Consequently, the results of DNA fingerprinting strongly supported the classification of strain SKP4-6^{T,} representing a novel species within the genus *Halobacillus*.

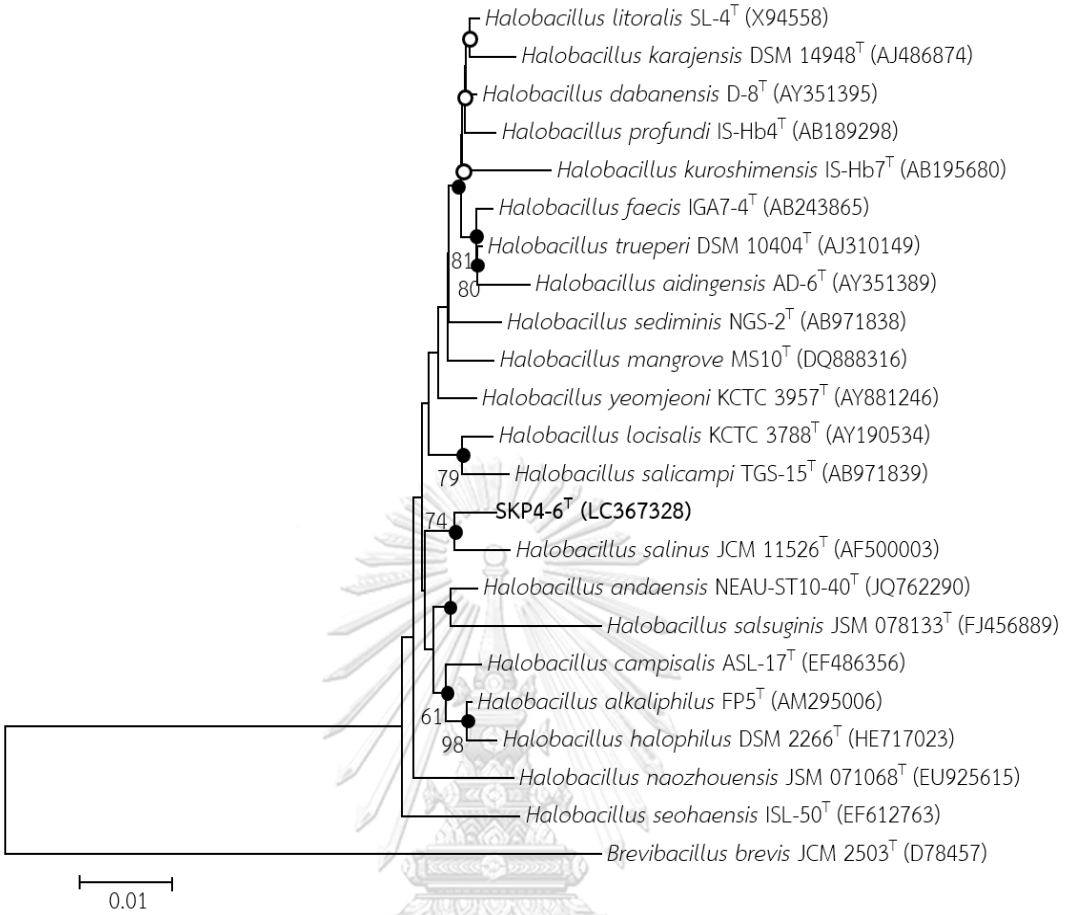


Figure 4.24. NJ phylogenetic tree based on the nearest full-length 16S rRNA gene sequences of strain SKP4-6^T (LC367328) and related members of the genus *Halobacillus*. Filled circles indicate nodes that were also supported by MP and ML trees based on the same sequences.

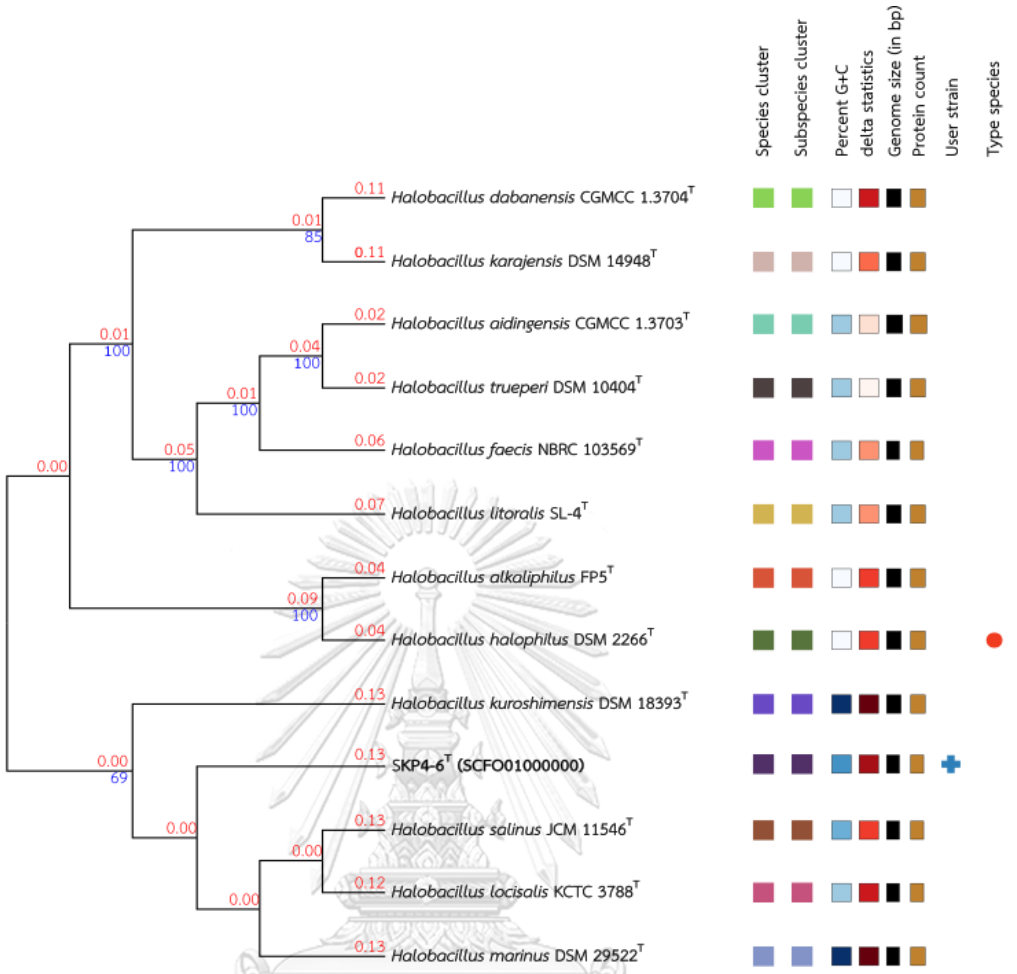


Figure 4.25. Phylogenomic of strain SKP 4-6^{T} and related members of the genus *Halobacillus.* The red numbers represent the branch length values.

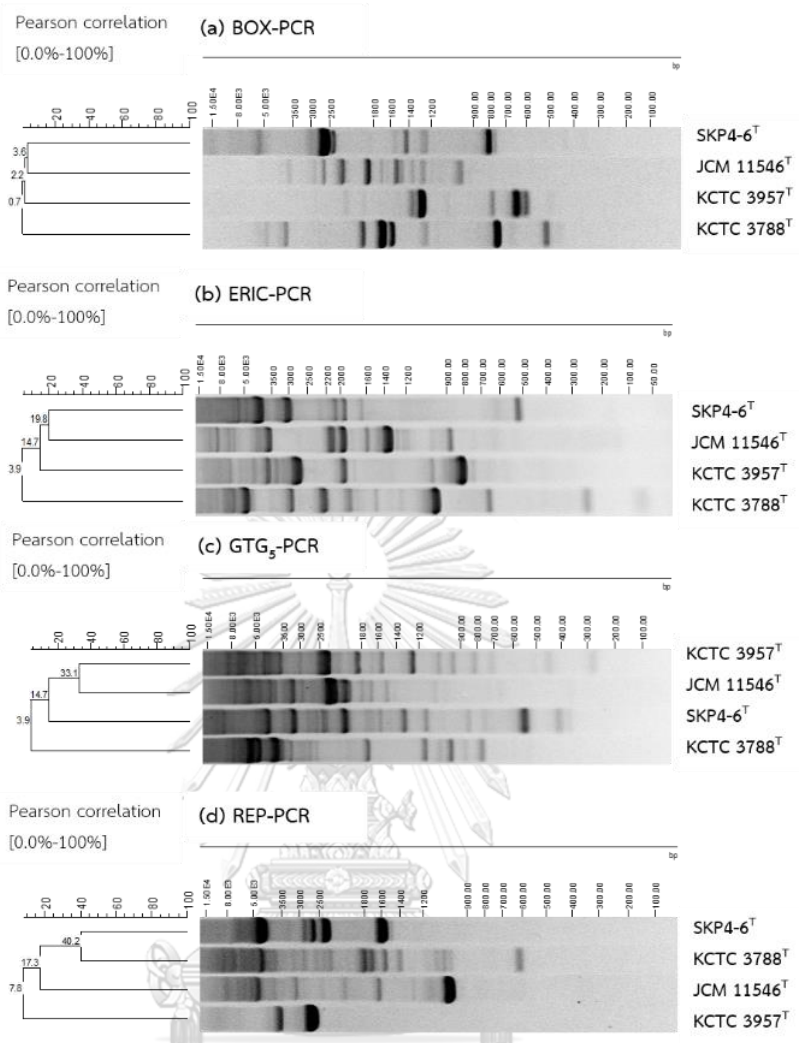


Figure 4.26. The dendrogram of (a) BOX-PCR, (b) ERIC-PCR, (c) $(GTG)_5$-PCR, and (d) REP-PCR fingerprints patterns of strain SKP4-6^T, *H. salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T, and *H. yeomjeoni* KCTC 3957^T.

Table 4.8. Genome statistics of strain SKP4-6^T and *H. salinus* JCM 11546^T.

Feature	SKP4-6^T	JCM 11546^T
Accession no.	SCFO00000000	SRJC00000000
Genome size (bp)	3,518,635	3,742,428
G+C content (%)	44.8	44.4
No. of scaffold	35	15
No. of contigs	35	16
Largest contig	1,151,678	1,968,592
N50	659,560	1,968,592
Completeness (%)	99.33	98.67
Contamination (%)	0.56	1.22
Total genes	3,629	3,921
Protein coding genes	3,663	3,926
RNA genes	80	78
tRNA	66	66
Pseudo Genes	22	27

4.5.2.3.3. General genomic features

The genome of the novel strain and *H. salinus* JCM 11546^T were sequenced with coverage of 150X in this study. Draft genome sequence of strain SKP4-6^T yielded a genome of 3.5 Mb in length after assembly. The genome was producing 32 contigs in which the largest contig was 1.1 Mb. The sequenced genome of strain SKP4-6^{T}have quality enough with an N50 value of 659,560 bp, 99.33% completeness, and 0.56% contamination (estimated using several markers as implemented in CheckM software v1.0.18). The draft genome sequencing of the type strain, *H. salinus* JCM 11546^T, was producing 16 contigs in which the largest contig was around 1,968,592 bp and yielded a genome size of 3.7 Mb in length after assembly. The genome sequencing of *H. salinus* JCM 11546^T had 98.67% completeness with 1.22% contamination. The DNA G+C mol% contents of strain SKP4-6^T and *H. salinus* JCM 11546^T 44.8 and 44.4 mol %, respectively. Other genome information is summarized in Table 4.7.

The ANIb among the strain SKP4-6^{T} and closest related type strain, *H. salinus* JCM 11546^{T,} were 72.1-72.2 %, and the ANIm value was 84.1%, which lower than the recommended value for the species delineation (Chun *et al.*, 2018, Ritcher & Rosselló-Móra, 2009). The *is*DDH value (Formula 2*) revealed 18.8 % (C.I. model 16.6-21.2 %) DNA-DNA relatedness by strain SKP4-6^{T} and *H. salinus* JCM 11546^{T} *H. yeomjeoni* KCTC 3957^{T} *and H. locisalis* KCTC 3788^{T}. The *is*DDH value of strain SKP4-6^{T} with the type strain are well below the recommended values (70%) proposed for species classification (Stackebrandt & Ebers, 1994; Konstantinidis & Tiedje, 2004, 2005; Stackebrandt & Ebers, 2006; Goris *et al.*, 2007; Ritcher & Rosselló-Móra, 2009; Meier-Kolthoff *et al.*, 2013; Kim *et al.*, 2014; Lee *et al.*, 2016; Chun *et al.*, 2018; Parks *et al.*, 2018). Hence, the *is*DDH value also supported the novelty of strain SKP4-6^{T} at the species level.

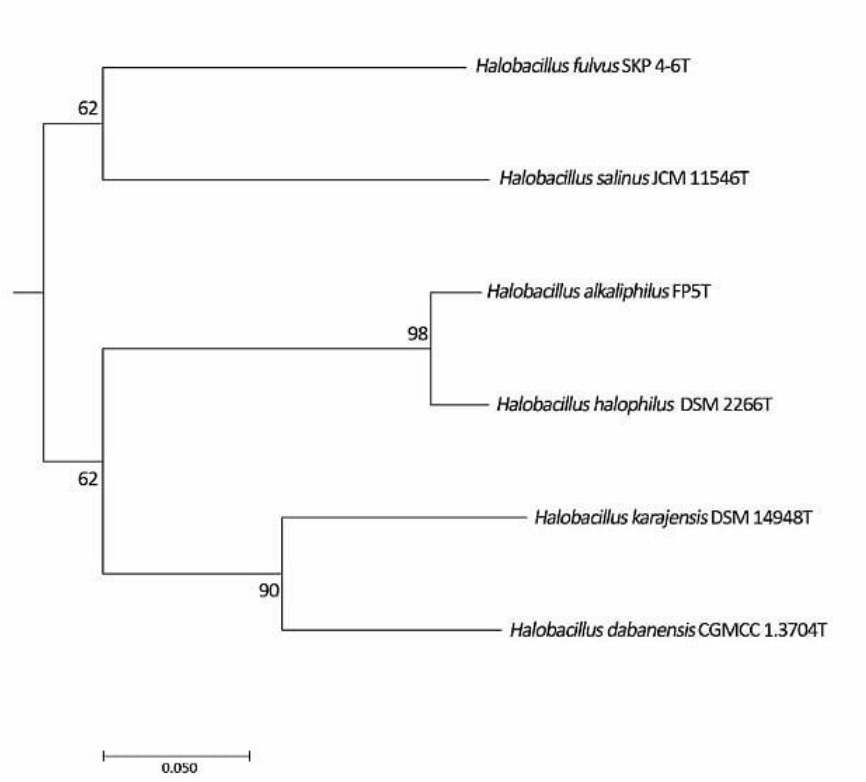


Figure 4.27. Core phylogenomic tree of strain SKP4-6^{T} and five closest related species in the genus *Halobacillus.*

An all-versus-all BLAST protein-coding gene comparison indicated that the pan-genome of novel strains and the closely related species of the genus *Halobacillus* comprised of 2,298 multi-copy and 1,897 single-copy orthologous genes of 6 *Halobacillus* genomes. The aligned protein-coding gene was displayed as a core phylogenetic tree (Fig 4.27).

Genomes of the novel strain and *H. halophilus* DSM 2266^{T}, *H. salinus* JCM 11546^{T,} and *H. trueperi* DSM 10404^{T} contained 3,625, 3,957, 3917, 4,026, and they sharing 2,454 of core orthologous proteins. When considering only the fourth selected type strain of the genus *Halobacillus*, *H. trueperi* DSM 10404^{T} possessed a higher number of unique proteins (418), followed by *H. halophilus* DSM 2266^{T} (411) and *H. salinus* JCM 11546^{T} (386), respectively. The new purpose species, SKP4-6^{T}, also contained a considerable number of exclusive proteins (255) as shown in the Venn diagram (Fig 4.28) and also confirmed that the novel strain distinguished from other closest related type strain in the genus *Halobacillus*

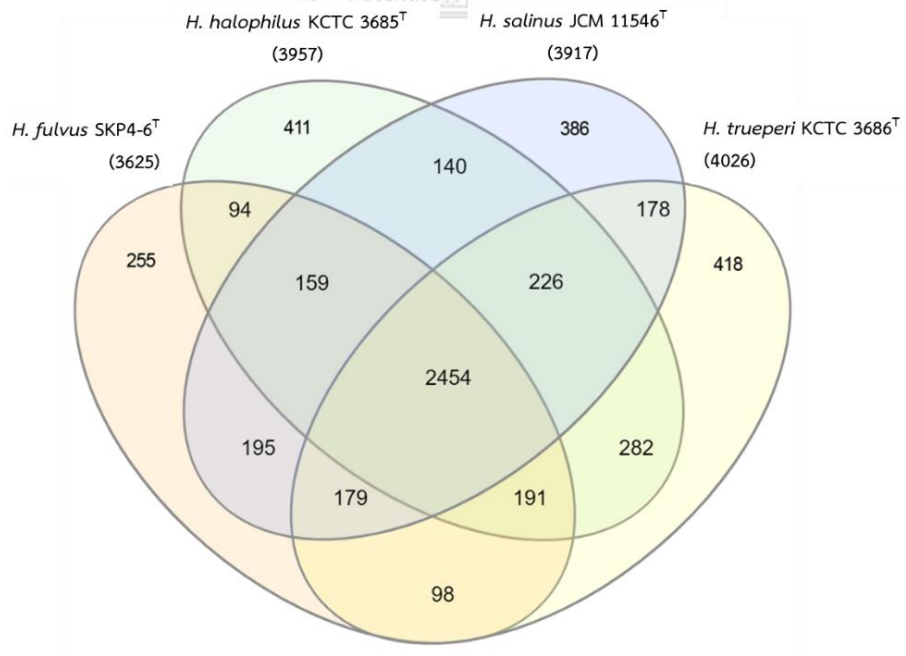


Figure 4.28. Venn diagram showing the number of core orthologous shared between the genome of strain SKP4-6^{T} and closest related species in the genus *Halobacillus*.

The hypothetical gene of strain SKP4-6^T (46%) was predicted from 3,629 genes encoding 3,514 proteins of the draft genome by RAST. Strain SKP4-6^T contained seven rRNA genes in total, which composted of two pairs of 5S, 23S, and three of 16S rRNA genes, and 66 tRNA genes, while 52% of the predicted genes could not be assigned to a subsystem. The predicted functional genes based on the RAST database of strain SKP4-6^T mainly belong to amino acid and derivatives (366 orthologs genes), carbohydrates (301), protein metabolism (214), cofactors, vitamins, prosthetic groups, pigments (182), RNA metabolism (144), fatty acids, lipids, and isoprenoids (135), DNA metabolism (119), cell wall and capsule (109), stress response (109) and nucleosides and nucleotides (106). These predicted genes also the same as discovered in the other type strains. Another comparable annotated proteins of strain SKP4-6^T and closest related type strains are depicted in Figure 4.29.

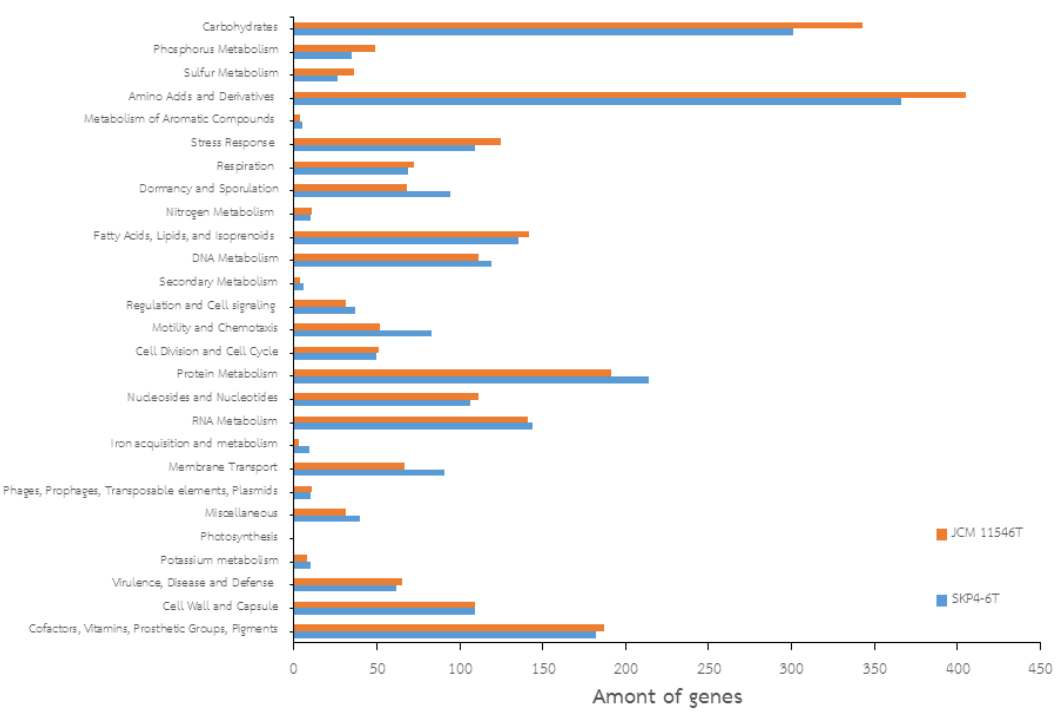


Figure 4.29. The functional gene annotations of strains SKP4-6^T (blue bar) and *H. salinus* JCM 11546^T (red bar) using the RAST database.

Owing to the strain SKP4-6^{T}and *H. salinus* JCM 11546^{T}were moderately halophilic bacteria, we analyzed the genes that contribute to survival and tolerance to stress response environments. A total of 13 genes and 21 genes from a novel strain and *H. salinus* JCM 11546^{T} were linked with the survival in osmotic stress, respectively. The genome of two strains contained betaine aldehyde dehydrogenase (*BetB*), choline dehydrogenase (*BetA*), glycine betaine transporter (*OpuD*), high-affinity choline uptake protein (*BetT*), L-proline glycine betaine ABC transport system permease protein (*ProV*), L-proline glycine betaine binding ABC transporter protein (*ProX*) and glycerol uptake facilitator protein (*glpF*), which are known previously to play a significant role in osmotic stress responses for surviving in high salt environments (Wargo, 2013; Booncharoen *et al.*, 2019). Also, these strains were composed of 6 carotenoid genes that could be associated with the pigment production and protect the cells from oxidative stress under saline growth as previously reported in *H. halophilus* DSM 2266^{T} (Köcher *et al*., 2009).

The strains SKP4-6^{T} clustered together very closely to *H. salinus* JCM 11546^{T} and clearly separate to the clusters containing type and reference strains in the phylogenomic tree (Fig. xx). Consequently, our phylogenetic data suggested that strain SKP4-6^{T} represents a novel species of the genus *Halobacillus*. Based on the data presented above, strain SKP4-6^{T} shared similar chemotaxonomic characteristics with members of the genus *Halobacillus*. However, strain SKP4-6^{T} could be distinguished from other closely related species by some chemotaxonomic, phenotypic, and genotypic features. Therefore, we propose strain SKP4-6^{T} represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus fulvus* sp. nov. (ful'vus. L. masc. adj. *fulvus*, deep yellow, yellowish-brown, gold-colored, pertaining to the color of the colonies on solid growth medium) is proposed, with the type strain SKP4-6^{T} (=JCM 32624^{T}=TISTR 2595^{T}). The GenBank/EMBL/DDBJ accession number for the nearest 16S rRNA and full-length 16S rRNA gene sequences of *Halobacillus fulvus* SKP4-6^{T} are LC367328, and MN585909, respectively, and that of the genome is SCFO00000000.

4.6 Extracellular halophilic esterase produced by *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T

The halophilic esterase activities produced by *L. lipolyticus* SSKP1-9^T could be observed after 12 hr while the bacterial cell goes to the lag phase; after 30 hr, the bacterial growth reached to the stationary phase. These results showed that the enzyme production corresponded to incubation time, and cell growth revealed that both bacterial cell growth and lipase production reached a maximum (4.24±0.14 to 4.37±0.26 U/ml) between 42 to 54 hr of incubation time. However, the activity started to decrease gradually after 54 hr, although the cell density was changed a bit (Fig. 4.30).

Esterase activity produced by strain, *H. fulvus* SKP4-6^{T,} was first detected at 6 hr, reaching the maximum of enzyme activity at 54 hr (12.49±0.87 U/ml), and this level was maintained until 72 hr of growth. Besides, the esterase production and cell growth were not changed during prolonged cultivation (Fig. 4.33).

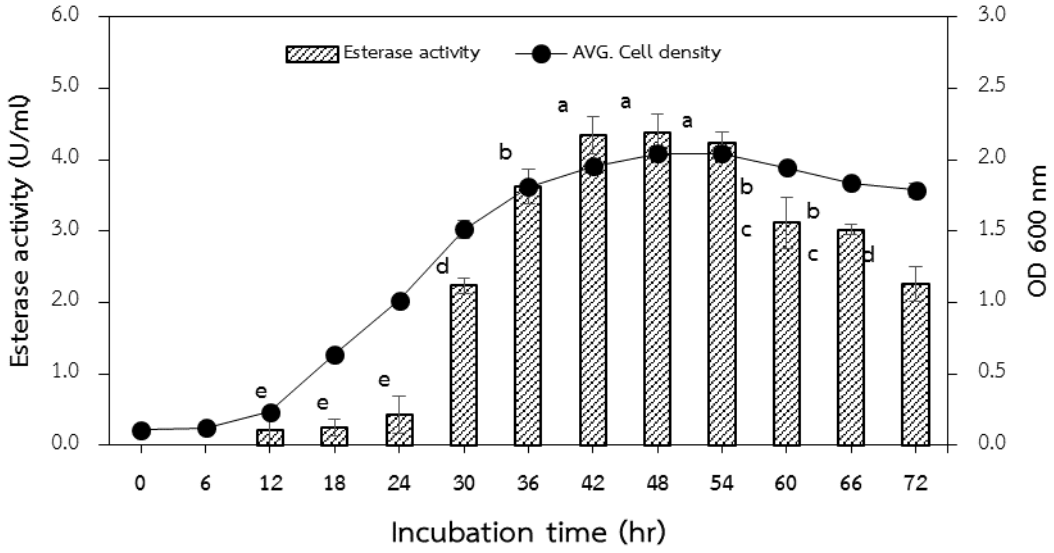


Figure 4.30. Kinetics of bacterial cell growth and native halophilic esterase produced by *L. lipolyticus* SSKP1-9^T.

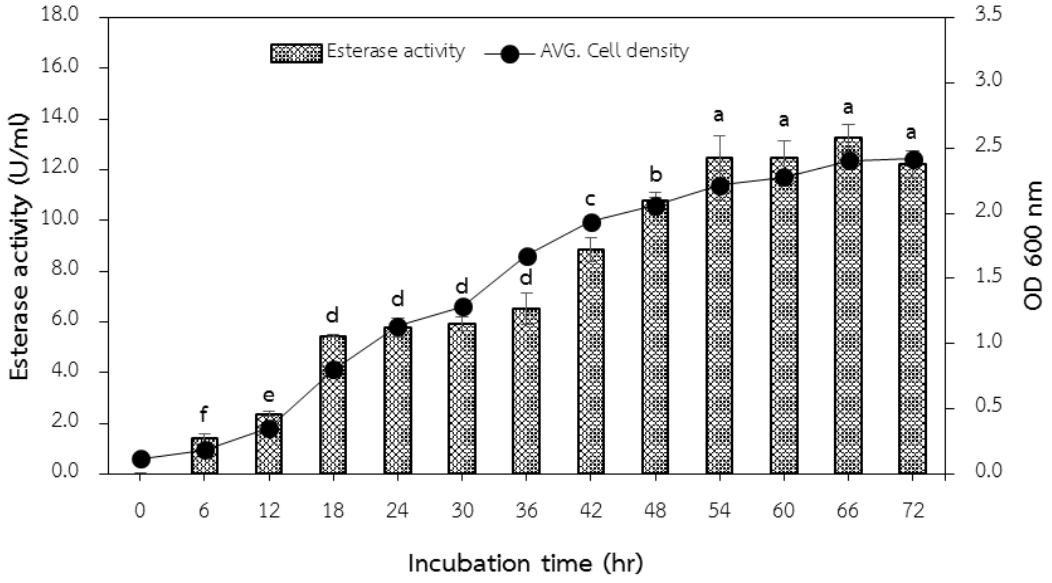


Figure 4.30. Kinetics of bacterial cell growth and native halophilic esterase produced by *H. fulvus* SKP4-6^T.

4.7. Characterization of native esterase crude extract produced from *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T

The effect of salt on *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T native esterase activity was studied, and enzymatic activity was optimal around 5-15% NaCl (Fig. 4.34a, Fig. 4.35a); the activity was also decreased at above 17% NaCl. According to the results shown in Fig. 4.34b, the optimal activity *L. lipolyticus* SSKP1-9^T esterase was observed over a wild range (25 to 60 °C) and rapidly decreased at a temperature higher than 60 °C. At the same time, the *H. fulvus* SKP4-6^T esterase was active in a broad range of 45-75 °C, and the activity decrease above 80 °C (Fig. 4.35b). The halophilic esterase activity of *L. lipolyticus* SSKP1-9^T and *H. fulvus* SKP4-6^T preferred neutral-alkaline pH, having the highest activity over a wide range of pH values from 7 to 9 with an optimal activity at pH 7 (Fig. 4.34c, Fig. 4.35c). The substrate specificity showed a broad substrate specificity of the *L. lipolyticus* SSKP1-9^T esterase on short to medium-chain fatty-acid esters (*p*-NPB (C4:0), *p*-NP) (C8:0), and *p*-NPD (C10:0), although the highest activities were found with *p*-NPB (Fig. 4.34d). Substrate specificity studies showed that the *H. fulvus* SKP4-6^T esterase (Fig. 4.35d) was specific with short-chain fatty-acid esters, *p*-NPB (C4:0).

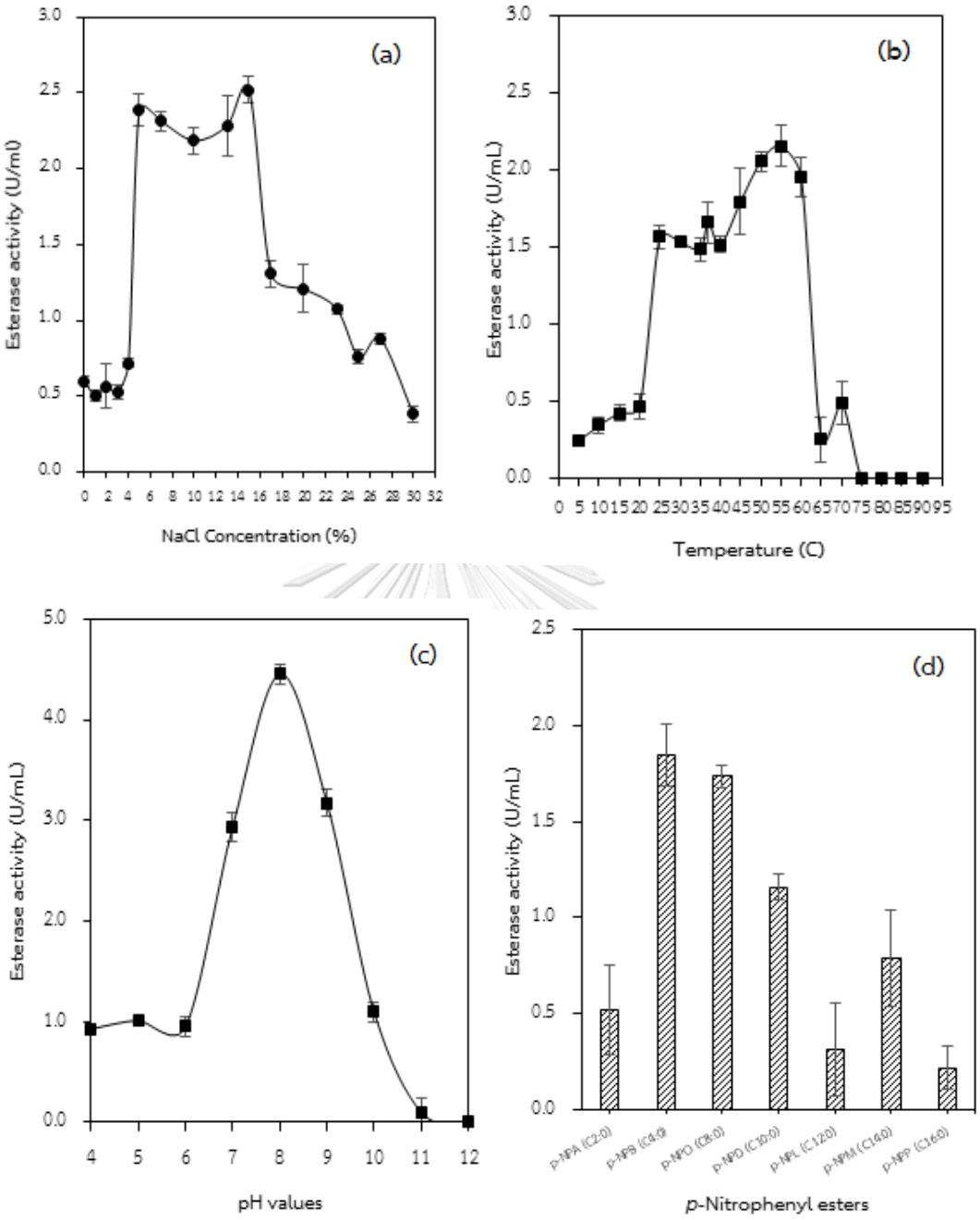


Figure 4.31. (a) Effect of NaCl concentration, (b) temperature, (c) pH, and (d) substrate specificity on native esterase crude extract produced from *L. lipolyticus* SSKP1-9^{T}.

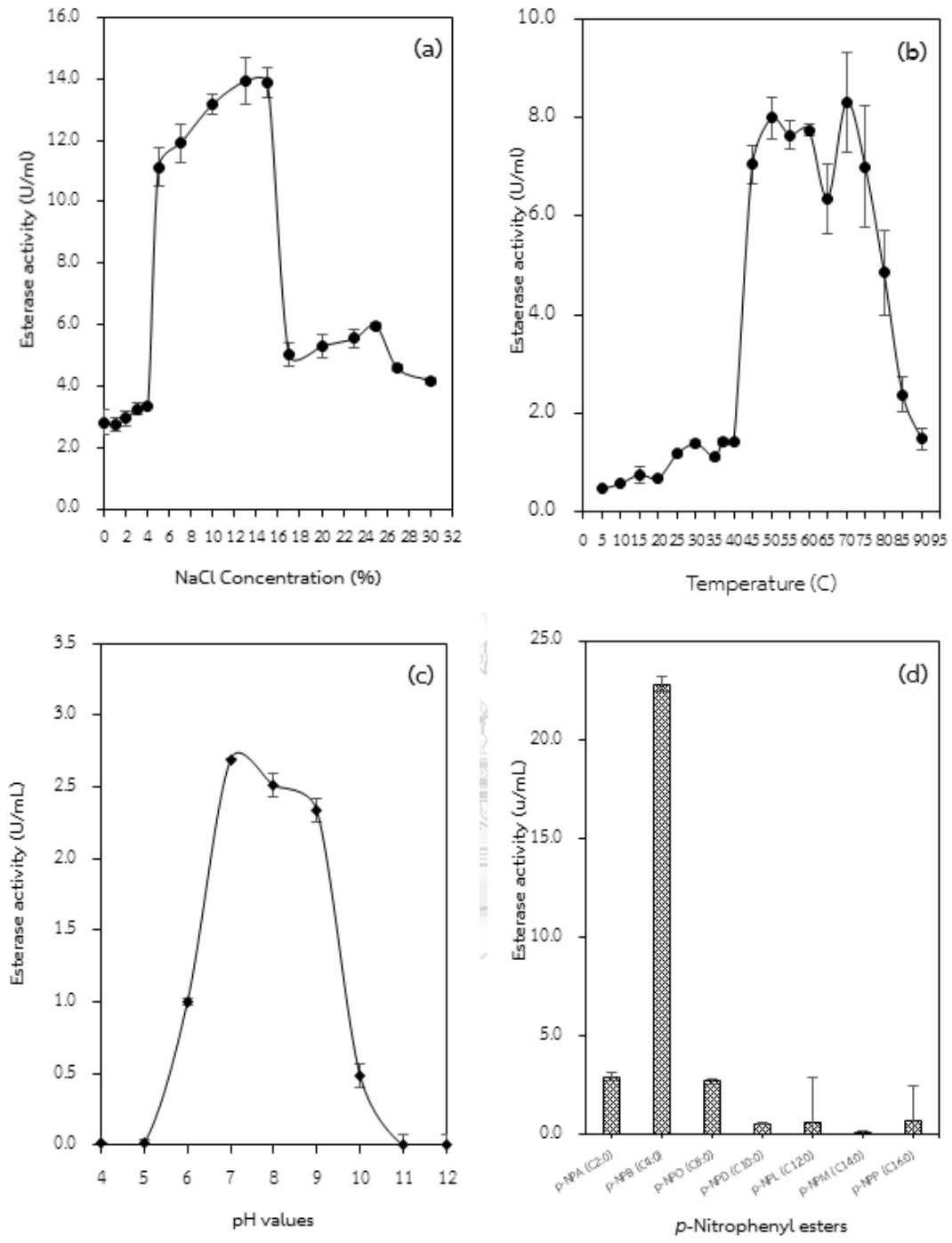


Figure 4.5. (a) Effect of NaCl concentration, (b) temperature, (c) pH, and (d) substrate specificity on native esterase crude extract produced from *H. fulvus* SKP4-6^{T}.

From the finding results, the native esterase crude extract produced from two novel strains possesses typical characteristics of a thermophilic and halophilic enzyme, which has great potential use in several industries. In addition, the crude enzyme could be classified as an esterase on the basis of substrate specificity since more than one putative extracellular halophilic esterase gene was predicted from the draft whole-genome sequences of two novel strains. Therefore, the cloning and expression process may be required to extract the target enzyme from other co-secreted halophilic esterases.

4.7. Cloning and expression of halophilic esterase enzymes in *B. subtilis*

4.7.1. Analysis of putative esterase sequence

The characterization of sequence similarity in outermost related proteins has proved gainful for understanding the evolution of gene families. Nowadays, knowledge of bacterial lipolytic enzymes is increasing at a rapid and exciting rate. As such, the number of putative lipolytic genes is rapidly rising with the progress of microbial genome research. Based on protein sequence analyses and comparison, many of the genes found in microbial genome databases have already been classified. Consequently, the current study analyzed some of them from bacterial genome databases of novel strains, SSKP1-9^T, and SKP4-6^T, using the RAST annotation.

The length of the gene varied from 420 to 1,230 bp of strain SSKP1-9^T and from 423 to 942 bp for strain SKP4-6^T, respectively. Almost genes founded in the strain SSKP1-9^T (Table 4.8) and SKP 4-6^T (Table 4.9) were matched with a gene coding for a lipolytic protein from genus *Lentibacillus* and *Halobacillus*, with around 49-89.52% identity. However, gene >APNFEMBD_00455 from strain SSKP1-9^T was found to match with alpha/beta fold hydrolase from *Virgibacillus salinus* (WP_092492042.1) with 59.2% identity. In the strain SKP4-6^T, the genes NKILIEJB_00202 shared 48.99% amino acid identity with alpha/beta fold hydrolase from *Jeotgalibacillus* sp. R-1-5s-1 (WP_134374132.1). The features encoded proteins, the results of the BLASTX search of GenBank of the identified putative lipolytic genes are presented in Table 4.8-4.9.

Table 4.9. Best matches obtained using BLASTN for the esterase genes identified in *L. lipolyticus* SSKP1-9^{T}.

No.	Location	length (bp)	Amino acid (aa)	Similarity (%)	Identities	Name of halophilic lipolytic gene	Origin	E-value	GenBank protein-id
1	>APNFEMBD_00261	513	170	83.53	142/170	YfcE family phosphodiesterase, metallophosphoesterase	*Lentibacillus* sp. NKC851-2, *Lentibacillus* sp. NKC220-2	5e-107, 5e-107	WP_142791798.1, WP_138601043.1
2	>APNFEMBD_00267	441	146	72.22	104/144	acyl-CoA thioesterase	*Lentibacillus halodurans*	4e-78	WP_090239087.1
3	>APNFEMBD_00455	915	304	59.20	177/299	alpha/beta fold hydrolase	*Virgibacillus salinus*	1e-133	WP_092492042.1
4	>APNFEMBD_00457	747	248	89.52	222/248	alpha/beta fold hydrolase	*Lentibacillus* sp. NKC220-2, *Lentibacillus* sp. NKC851-2	5e-156, 7e-156	WP_138601571.1, WP_142791971.1
5	>APNFEMBD_00639	1167	418	71.91	279/388	alpha/beta fold hydrolase	*Lentibacillus jeotgali*,	0.0	WP_138919713.1
6	>APNFEMBD_01756	1230	409	71.71	294/410	Metallophosphoesterase,	*Lentibacillus* sp. NKC851-2	0.0	WP_142790899.1,
7	>APNFEMBD_02564	699	232	77.06	178/231	alpha/beta fold hydrolase	*Lentibacillus* sp. NKC851-2	4e-134	WP_142790501.1
8	>APNFEMBD_02578	420	139	87.68	121/138	YbgC/FadM family acyl-CoA thioesterase	*Lentibacillus* sp. NKC851-2	4e-88	WP_142791462.1

Table 4.10. Best matches obtained using BLASTN for the esterase genes identified in *H. fulvus* SKP4-6^{T}

No.	Location	length (bp)	Amino acid	Similarity (%)	Identities	Name of halophilic lipolytic gene	Origin	E-value	GenBank protein-id
1	>NKILIEJB_00202	942	313	48.99	146/298	alpha/beta fold hydrolase	*Jeotgalibacillus* sp. R-1-5s-1	2e-97	WP_134374132.1
2	>NKILIEJB_00139	528	175	77.14	135/175	metallophosphoesterase	*Halobacillus* sp. BBL2006	4e-100	WP_035552944.1
3	>NKILIEJB_00478	750	249	78.78	193/245	Glycerophosphodiester phosphodiesterase	*Halobacillus* sp.	4e-143	WP_035508974.1
4	>NKILIEJB_00719	423	140	84.78	117/138	Acyl-CoA thioesterase	*Halobacillus salinus*	1e-85	WP_135327018.1
5	**>NKILIEJB_01195**	**744**	**250**	**73.14**	**177/242**	**alpha/beta fold hydrolase**	***Halobacillus alkaliphilus***	**7e-128**	**WP_089753193.1**

In this study, *B. subtilis* WB800, an eight-extracellular-protease-deficient originated from *B. subtilis* strain 168, was used as an expression host. After the consideration of the *B. subtilis* 168 genome (accession no. AL009126), it became clear that strain 168 contained eight genes encoding the lipolytic gene, which are different from the gene founded in the selected strain.

4.7.2. Amplification of the halophilic esterase gene

After purification of the genomic DNA of strain SSKP1-9^{T} and SKP4-6^{T}, the lipolytic encoding gene was amplified by PCR using specific primers as described in materials and methods. After separation, the DNA fragment through 1% agarose gel electrophoresis, the expected size of the PCR products were obtained. For strain SSKP1-9^{T}, eight lipolytic genes included >APNFEMBD_00261, >APNFEMBD_00267, >APNFEMBD_00455, >APNFEMBD_00457, >APNFEMBD_00639, >APNFEMBD_01756, >APNFEMBD_02564, and >APNFEMBD_02578, were detected on agarose gel (Figure 4.36). All of the lipolytic encoding genes from strain SKP4-6^{T} (>NKILIEJB_00202, >NKILIEJB_00139, >NKILIEJB_00478, >NKILIEJB_00719, >NKILIEJB_01195) could be amplified, as depicted in Figure 4.37.

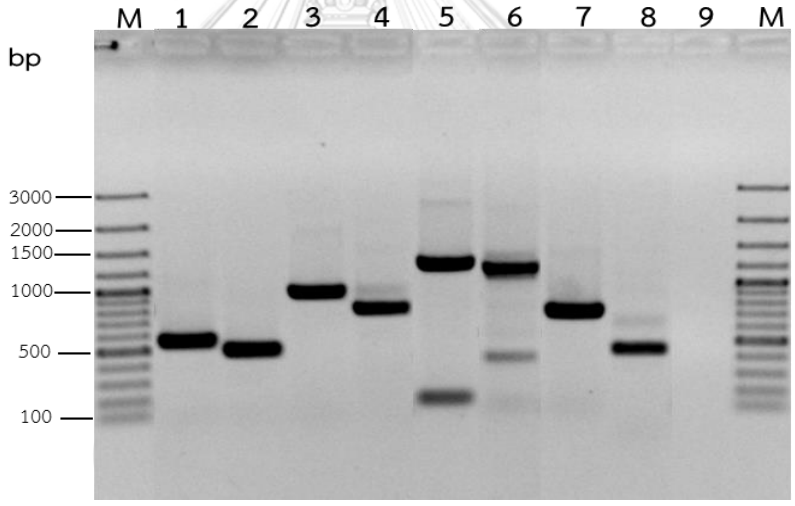


Figure 4.32. Amplification of esterase genes from strain SSKP1-9^{T}.
Panel M: DNA ladder (GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific), Panel 1: >APNFEMBD_00261, Panel 2: >APNFEMBD_00267, Panel 3: >APNFEMBD_00455, Panel 4: >APNFEMBD_00457, Panel 5: >APNFEMBD_00639, Panel 6: >APNFEMBD_01756, Panel 7: >APNFEMBD_02564, Panel 8: >APNFEMBD_02578, Panel 9: Negative control

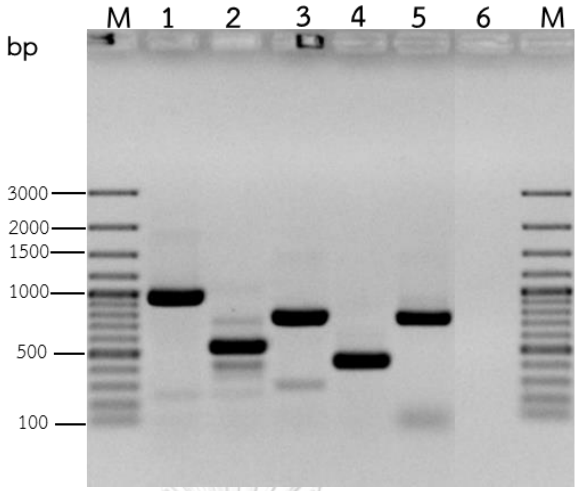


Figure 4.33. Amplification of esterase genes from strain SKP4-6^{T}.
Panel M: DNA ladder (GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific), Panel 1: >NKILIEJB_00202, Panel 2: >NKILIEJB_00139, Panel 3: >NKILIEJB_00478, Panel 4: >NKILIEJB_00719, Panel 5: >NKILIEJB_01195, Panel 6: Negative control

4.7.3. Construction of recombinant plasmid

In the present study, we prefer a salt inducible, pSaltExSePR5 (Promchai *et al.*, 2016), as a cloning and expression vector. The gene fragments were ligated into the plasmid, and then the recombinant plasmid was transformed into *E. coli* DH5α by heat shock method for propagation. Approximately at least 30 colonies of each transformant gene were randomized picked and used as a template for colony PCR detection of the recombinant clones. The results indicated that all of the amplification genes of strain SSKP1-9^{T} and SKP4-6^{T} were successfully inserted to the *pSaltExSePR5* plasmid as evaluated by PCR. About three positive colonies of each gene from colony PCR screening were selected for grown, extracted the recombinant plasmid, and verified by double digestion with restriction enzymes, as mentioned previously. Then, the selected plasmid containing the selected gene was used for transformed into *B. subtilis* WB800 for expression step.

4.7.4. Transformation of recombinant plasmid harboring halophilic esterase gene in *B. subtilis* WB800

For the study of the expression of the halophilic lipolytic gene, the protein-coding sequence of the selected gene was cloned into expression vectors pSaltExSePR5 under the control of the *OpuAA* promoter (Promchai *et al.*, 2016). This expression system allows the transcription of the cloned genes to be activated after induction with NaCl. Then, the plasmid carrying halophilic lipolytic gene was introduced into a compatible host, *B. subtilis* WB800.

The pSaltExSePR5 harboring the lipolytic gene from strain SSKP1-9^T and SKP4-6^T were successfully transformed into a host, *B. subtilis* WB800. Cell-free supernatant was used to determine the lipolytic activity with *p*-NPB as the substrate after induction with 4% w/v of NaCl (Figure 4.38-4.47). Cultures of the *B. subtilis* WB800 harboring *pSaltExSePR5* strains were used as control. The recombinant clone >APNFEMBD_02578-C112, which contains the lipolytic gene, had the highest level of lipolytic activity comparable to the control and among those genes from strain SSKP 1-9^T. The production of lipolytic enzyme by clone >NKILIEJB_01195-C9, which contains the lipolytic gene from strain SKP 4-6^T, was even higher, accounting for about a 10-fold increase. Two recombinant clones exhibited the highest lipolytic activity not only in non-treated at 50 C for 1 hr but also in treated conditions. Therefore, recombinant clone >APNFEMBD_02578-C112-40 from strain SSKP1-9^T and >NKILIEJB_01195-C9-35 from strain SKP4-6^T were chosen for expression of recombinant protein.

4.7.5. Expression, partial purification of recombinant halophilic esterase enzyme

The recombinant clone >APNFEMBD_02578-C112-40 from strain SSKP1-9^T and >NKILIEJB_01195-C9-35 from strain SKP4-6^T were cultured in LB medium containing 20 µg/ml erythromycin for expression recombinant protein. The expression was done within 1 hour after induction by 4% NaCl, and the active protein can be isolated from 1.0 liter of bacterial culture.

Table 4.11. Partial purification of two recombinant esterase enzymes produced from the selected clone.

Clone	Step	Total Vol. (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg^{-1})	Yield (%)	Purification fold
>APNFEMBD_02578-C112-40	Supernatant	1000	928.06	2,555.55	0.36	100	1
	$(NH_4)_2SO_4$ precipitation	50	167.38	75.87	2.21	18.04	6.14
>NKILIEJB_01195-C9-35	Supernatant	1000	1,524.93	2,610.75	0.58	100	1
	$(NH_4)_2SO_4$ precipitation	50	135.61	77.04	1.76	8.90	3.04

In the present study, partial purified of two recombinant enzymes was done by one step using 100% ammonium sulfate precipitation to obtain the concentrated enzyme for characterization. The enzyme produced from clone >APNFEMBD_02578-C112-40 showed 2.21 U/mg with 18.04% final yield and 6.14-fold specific activity while the enzyme from >NKILIEJB_01195-C9-35 showed specific activity 1.76U/mg, 8.90% yield with 3.04 purification fold (Table 4.10).

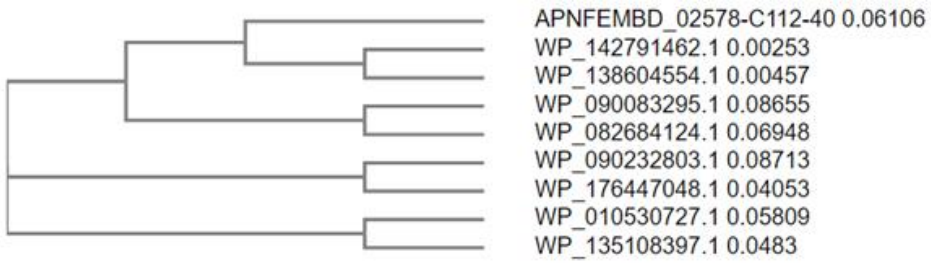
4.7.6. Sequences Analysis

The sequence of recombinant clone >APNFEMBD_02578-C112-40 of strain SSKP1-9^T was 420 bp in length. The nucleotide sequencing displayed the presence of a putative ATG start codon exhibits a *G+C* at 42.9%. This sequence contained no potential ribosome-binding site Shine-Dalgarno sequence (5′-GGAGG-3′), no signal peptide at the N-terminus, and no the GxSxG motif in the sequence. The deduced protein encoded by the clone >NKILIEJB_01195-C9-35) from strain SKP4-6^T composed of 247 amino acids and stop codon, with a predicted molecular mass of 16,223.34 Da (16.23 kDa) and a pI of 6.20.

Sequence analysis of amino acid indicated high identity to the sequences of the family acyl-CoA thioesterase founded in the genus *Lentibacillus*. Based on these identities, the sequence was compared to in NCBI BLASTX database. The highest identity (87.68%) was obtained to *YbgC/FadM* family acyl-CoA thioesterase from *Lentibacillus* sp. NKC851-2 (WP_142791462.1) (Figure 4.34a) followed by 86.96% of acyl-CoA thioesterase from *Lentibacillus* sp. (WP_138604554.1), 84.06% of acyl-CoA thioesterase from *L. salicampi* (WP_135108397.1), 84.06% of *YbgC/FadM* family acyl-CoA thioesterase from *Lentibacillus* sp. CBA3610 (WP_176447048.1), 82.61% of acyl-

CoA thioesterase from *L. amyloliquefaciens* (WP_082684124.1) 81.88% of acyl-CoA thioesterase from *L. jeotgali* (WP_010530727.1), 80.43% of acyl-CoA thioesterase from *L. halodurans* (WP_090232803.1), and 80.29% of acyl-CoA thioesterase from *L. persicus* (WP_090083295.1), respectively. Multiple sequence alignment of the most homologous regions of these proteins is depicted in Figure 4.34b.

(a)



(b)

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APNFEMBD_02578-C112-40  --MTHTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLTYMDMEKNNIVSPVVDA  58
WP_142791462.1          MHKTHTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLSYMDMENSHIVSPVVDA  60
WP_138604554.1          MHKTHTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLSYMDMENSHIVSPVVDA  60
WP_090083295.1          MHKINTPVEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGFTYTDMEKNDIVSPVVDA  60
WP_082684124.1          MHKTNTPVEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLTYADMEKNNIVSPVVDA  60
WP_090232803.1          MHRTKTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGFTYTDMEKNNIVSPVTDA  60
WP_010530727.1          MHKTHTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLIYTDMEKNNIVSPVVDA  60
WP_135108397.1          MHKTHTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLTYTDMEKNKVVSPVVDA  60
WP_176447048.1          MHKTNTPIEVRYQETDQMGVVYHANYLVWFEIGRTKYIESLGLTYTDMEKHNIVSPVVDA  60
                           :**:**********************************: * ***: .:****.**

APNFEMBD_02578-C112-40  QVNFRKPVRYGEKATVETWLDTYDGIRTVYAYNIKKENGEVAVSGTTKHVIVHKETFRPL  118
WP_142791462.1          QISFRKPVHYGEKATVETWLDTYDGLRTVYGYNIKKENGELAVSGTTKHVIVHKETFRPM  120
WP_138604554.1          QISFRKPVHYGEKATVETWLDTYDGLRTVYGYNIKKENGELAVSGTTKHVIVHKETFRPM  120
WP_090083295.1          QINFNKPVHYGEQAFVETWIESYDGIRTVYANNIFREDGDVAVSGTTKHVIVNKKSFRPL  120
WP_082684124.1          QINFKKPVRYGEKAFVETWLDSYDGIRTVYGYDILREDGVMAASGTTKHVIVDKESFRPL  120
WP_090232803.1          QINFKKPVHYGEKAAVETWIEQYDGIRTVYGYHILKEGAEVAVSGTTTHVIVHKDSFRPL  120
WP_010530727.1          QISFKNPVHYGEKAIVETWIDEYDGLRTVYAYKIIKENGELAVSGTTKHVIVNKDSFRPL  120
WP_135108397.1          QISFKKPVLYGEKAAVETWIETYDGLRTVYAYHIVKENGEVAVSGTTKHVIVHKESFRPL  120
WP_176447048.1          QINFKKPVHYGEKAAVETWIDTYDGIRTVYGYHIFKENGEIAVSGTTKHVIVHKESFRPM  120
                        *:.*.:** ***:* ****:: ***:****. .* :*.. :*.****.****.*.:***:

APNFEMBD_02578-C112-40  SLRKSFPEWHEAYSRQLDGEA  139
WP_142791462.1          SLRKAFPDWHEAYLQQLNGDA  141
WP_138604554.1          SLRKAFPDWHEVYLQQLNGDA  141
WP_090083295.1          SLRKAFPEWHRAYLRQVSGDA  141
WP_082684124.1          ALRKAFPEWHQAYLQQVSGEA  141
WP_090232803.1          SLRKVYPEWHQTYLRQVNGES  141
WP_010530727.1          SLRKAFPEWHQIYLKQVNGES  141
WP_135108397.1          SLRKAFPEWHKKYLKQVNGES  141
WP_176447048.1          SLRKAFPEWHQVYLQQVNGES  141
                        :*** :*:**. * :*:.*::
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Figure 4.34. (a) The dendrogram (guide tree) and (b) multiple amino acid sequence alignment obtained by the ClustalW program of the esterase gene >APNFEMBD_02578-C112-40 from strain SSKP1-9^{T}.

Nucleotide sequencing of >NKILIEJB_01195-C9-35 revealed the presence of putative ATG start codon and being 744-bp long. The nucleotide sequence exhibits a low *G+C* content (41.7%). Preceding the ATG start codon (Approx. 6 bp upstream), a potential ribosome-binding site Shine-Dalgarno sequence (5′-GGAGG-3′) was found. The deduced protein encoded by the lipolytic gene (>NKILIEJB_01195-C9-35) from strain SKP4-6^{T} is composed of 247 residues and stop codon, with a predicted molecular mass of 27669.48 Da (27.7 kDa) and a pI of 4.56. The N-terminus of this protein contains no signal peptide (SignalP V3 program). The deduced amino acid of this sequence had the typical GXSXG motif (where x represents any amino acid) found in most lipases and esterases (Cygler *et al.,* 1993), which was identified as GLSMG.

The encoded 247 aa fragment of had the highest identity to the amino acid sequence of the α/β hydrolase fold in the genus *Halobacillus*. Sequence alignment of amino acid had the highest similarity to α/β hydrolase fold from *H. alkaliphilus* (WP_089753193.1) 73.14% (Figure 4.35), followed by *H. halophilus* (WP_014642947.1) 71.90%, *H. dabanensis* (WP_139207326.1) 70.08%, *H. mangrovi* (WP_157130905.1) 69.96%, *Halobacillus* sp. BBL2006 (WP_035544386.1) 69.26%, *H. faecis* (WP_146813470.1) 68.85%, *H. trueperi* (WP_115822267.1) 68.85%, *H. aidingensis* (WP_089651133.1) 68.85%, and *H. litoralis* (WP_160914582.1) 68.44%, respectively. All genes were compared, and total nucleotide differences were observed (Figure 4.36).

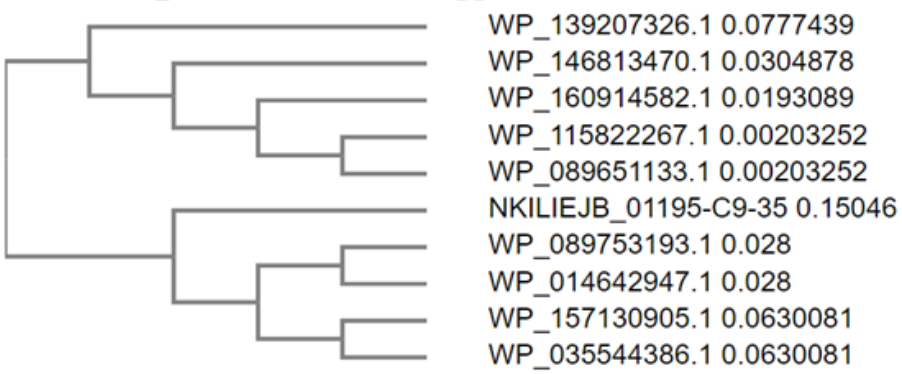

Figure 4.35. The dendrogram (guide tree) of the esterase gene >NKILIEJB_01195-C9-35 from strain SKP4-6^{T}.

Figure 4.36. Multiple amino acid sequence alignment was obtained by the ClustalW program of the esterase gene >NKILIEJB_01195-C9-35 from strain SKP4-6^{T}. The conserved motif G-x-S-x-G motif site is boxed.

4.8. Biochemical Characterization of recombinant halophilic esterase enzyme

The partial purified recombinant esterase enzymes have been characterized in terms of the effect of NaCl and stability, the effect of pH and stability, the effect of temperature and stability, and substrate specificity.

4.8.1. Effect of NaCl concentration on recombinant halophilic esterase activity and stability

The effect of salt on enzyme activity was studied by adding NaCl into the reaction mixture at the concentrations ranging from 0 to 30 % (w/v) (Figure 4.37). The activity of the enzyme was determined at pH 8.0 and 50°C using *p*-NPB as a substrate. Two recombinant enzymes, >APNFEMBD_02578-C112-40 (Figure 4.37A) and >NKILIEJB_01195-C9-35 (Figure 4.37B), showed activity at relatively higher NaCl concentration compared to the reaction mixture without NaCl. A gradual increase in enzyme activity was observed with increasing NaCl concentration from 0 to 15 % (w/v), and the enzyme showed the highest activity at 22 % (w/v) NaCl.

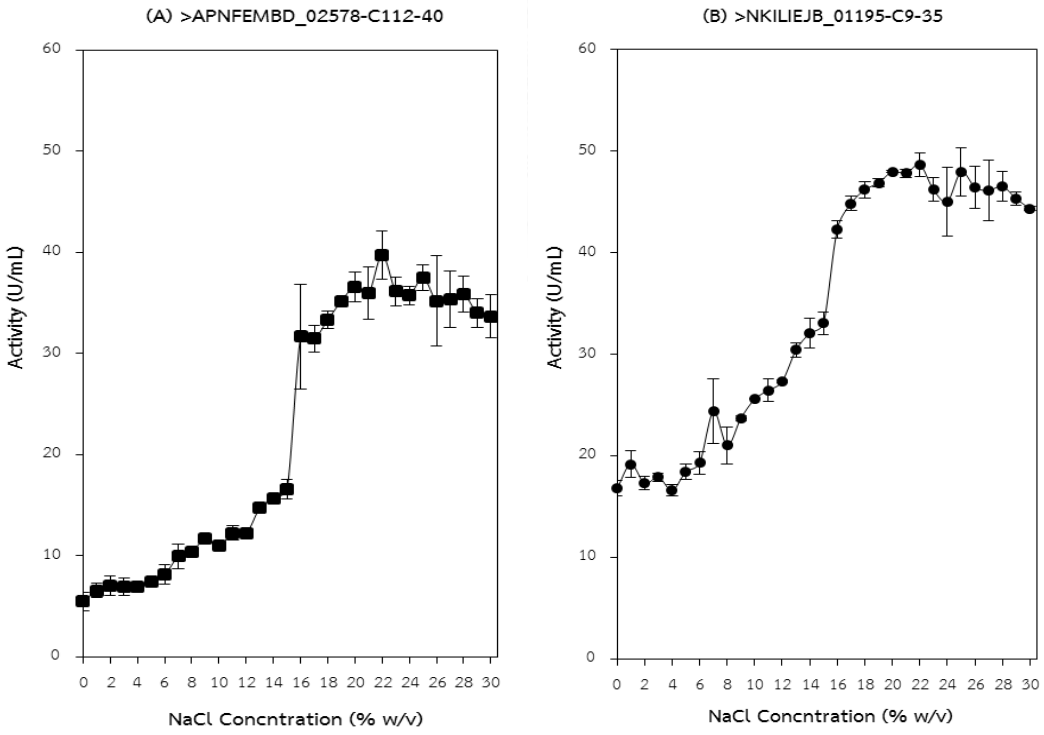

Figure 4.37. Effect of NaCl profile of recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35 on esterase activity.

Interestingly, the addition of NaCl resulted in an activation of the enzyme, and the activities sharply increased with NaCl and reached the maximum at 15-30% NaCl (w/v). This suggested that two recombinant enzymes were not salt-tolerant lipase, but halophilic lipolytic enzyme, and its activity depended on the presence of salt (NaCl). Therefore, 22% of NaCl concentration was used in subsequent determinations in the current study.

These are in agreement with other halophilic lipases. Lipase LipBL from *Staphylococcus warneri* PB233 was active at 2.5 M (15%) NaCl (Kanlayakrit & Boonpan, 2007), but that lipolytic enzyme from *B. pumilus* LV01 exhibited its maximum activity at 1.7 M NaCl (Guzmán *et al.*, 2008). Lv et al. (2011) reported that *Thalassobacillus* sp. strain DF-E4 was optimal at 0.5 M NaCl. The best activity of esterase from *Halobacillus* sp. strain LY5 is at 10% NaCl (w/v) and had enzyme activity up to 20% salinity (Li *et al.*, 2012). Lipase from *V. alimentarius* LBU20907 (Dueramae *et al.*, 2017) and *Virgibacillus* sp. IDS 20 (Bovornreungroj *et al.*, 2012) showed the highest activity at 20-25% NaCl, respectively. The lipase produced by *Chromohalobacter* sp. LY7-8 had the optimum activity at 12.5% NaCl (Li *et al.*, 2012a). Previously, the purified lipase from a similar strain, *Halobacillus* sp. AP-MSU 8 (Esakkiraj *et al.*, 2014) and esterase from *H. trueperi* whb27 (Yan *et al.*, 2014) were optimally active in the presence of 2.5 M NaCl. Ghati and Paul (2015) and Xin & Ying (2013) stated that esterase activities from *B. cereus* AGP-03 and *Salimicrobium* sp. LY19 were optimum at 4.5-5.0% NaCl.

These results indicate that NaCl was very effective in improving the activity of the enzyme. In a biotechnological view, enzymes excreted into the media, such as the lipolytic enzyme in this studied, also must work at salt concentrations at which other enzymes would lose their properties. These properties make them valuable because halophilic enzymes are not only able to deal with high ionic strength in their environment but also need it to maintain function and structure (Dym *et al.*, 1995). Also, it is well established that two recombinant enzymes are NaCl-dependent enzymes and was halophilic.

The salt stability of clone >APNFEMBD_02578-C112-40 and >NKILIEJB_01195-C9-35 was assayed by measuring the residual activity after incubation at various NaCl concentrations at 50 °C for 1 hr. After the NaCl was removed, the enzyme stability was tested at the optimum salt concentration (22% NaCl), pH 8.0, and 50 °C for 10 min using *p*-NPB as a substrate. The salt stability profile (Fig 4.38) shows that two recombinant enzymes remained active at a wide range of salt concentrations. The recombinant enzyme from clone >APNFEMBD_02578-C112-40 (Fig 4.38A) was found to be stable in the presence of salt as it retained about 60-80% of its activity after one hr incubation at 50 °C. Interestingly, the enzyme was stable in the presence of NaCl 16-23% and retained activity at 90% at 16% NaCl. In comparison, the activity was enhanced by up to 118% at 17% NaCl and slightly decrease when the concentration of NaCl above 18%.

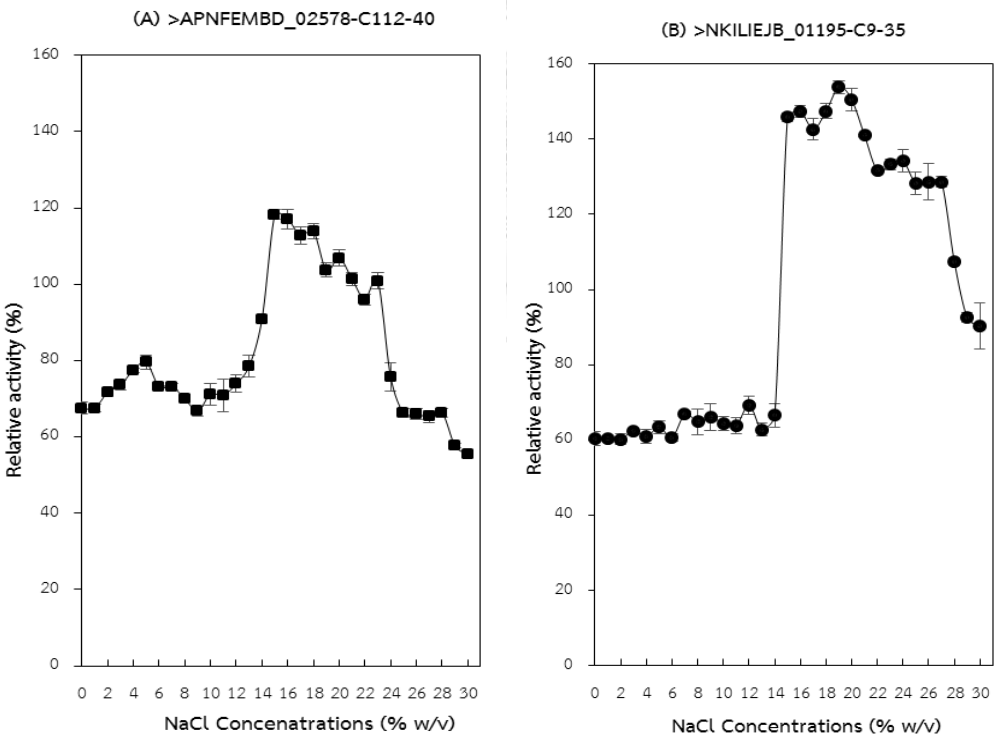

Figure 4.38. The NaCl stability of the recombinant esterase enzyme (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35.

For the recombinant clone >NKILIEJB_01195-C9-35 (Fig 4.38B), the enzyme has maintained the activity around 60-66% at the concentration of NaCl 0-14%. The enzyme activity reached 140-154% after incubation in NaCl above 17% and then decreased. The residual activity of *Natronococcus* sp. retain their lipase activity around 50% in 76 min when incubated at 80 C with 4 M NaCl (Boutaiba *et al.*, 2006). An extracellular lipase from *Salinivibrio* sp. strain SA-2 remained active in the presence of 17% NaCl (Amoozegar *et al.*, 2008). Such results are in line with those of carboxylesterase from *Thalassobacillus* sp. strain DF-E4. Lv and colleagues (Lv *et al.*, 2011) reported that recombinant carboxylesterase *LIP4* retained 90 % activity after 12 h incubation in 4 M NaCl at 25℃ for 1 hr. When the salinity up to 20%, the esterase activity of *Halobacillus* sp. LY5 was decreasing to 60%. Meanwhile, in the absence of NaCl, the enzyme still retained more than 70% of its activity (Li *et al.*, 2012). Xin & Ying (2013) showed that esterase activities from *Salimicrobium* sp. LY19 was stable with a wide range of NaCl concentrations (2.5%-25%). A similar result was shown by esterase from *B. cereus* AGP-03, which showed stability at a NaCl concentration range of 4.5-11% NaCl (Ghati & Paul, 2015).

As known that extracellular enzymes produced by halophilic bacteria usually require high-salt concentration for stability and optimal activity, and their activity was lost by such salinity (Camacho et al., 2009; Müller-Santos et al., 2009). At the same time, the activity of enzymes from non-halophilic bacteria was inhibited to varying degrees (Lv et al., 2011). It is worth noting that two recombinant enzymes detected in this work showed high activities and stability in high salt conditions revealed that it is a halophilic enzyme. This finding makes them interesting enzymes for future investigations at low water activity applications. These characters are unique and make it a very special enzyme in biotechnological implication.

4.8.2. Effect of temperature on recombinant halophilic esterase activity and stability

In order to determine the effect of temperature on enzyme activity, the activities at different temperatures ranging from 5 to 80 ℃ were measured, as shown in Figure 4.39. The results of these measurements indicated that the recombinant enzyme, >APNFEMBD_02578-C112-40 (Fig. 4.39A), showed the highest activity around 25-60 ℃, and the maximum activity was observed at 40-55 ℃ whereas >NKILIEJB_01195-C9-35 (Fig. 4.39B) had optimal activity at 55 ℃. The two enzymes have shown the same trend in terms of the temperature effect. The enzyme lost its activity rapidly above 65℃ of the remaining activity. The result indicates that >APNFEMBD_02578-C112-40 has activity at a wide temperature range but not for >NKILIEJB_01195-C9-35

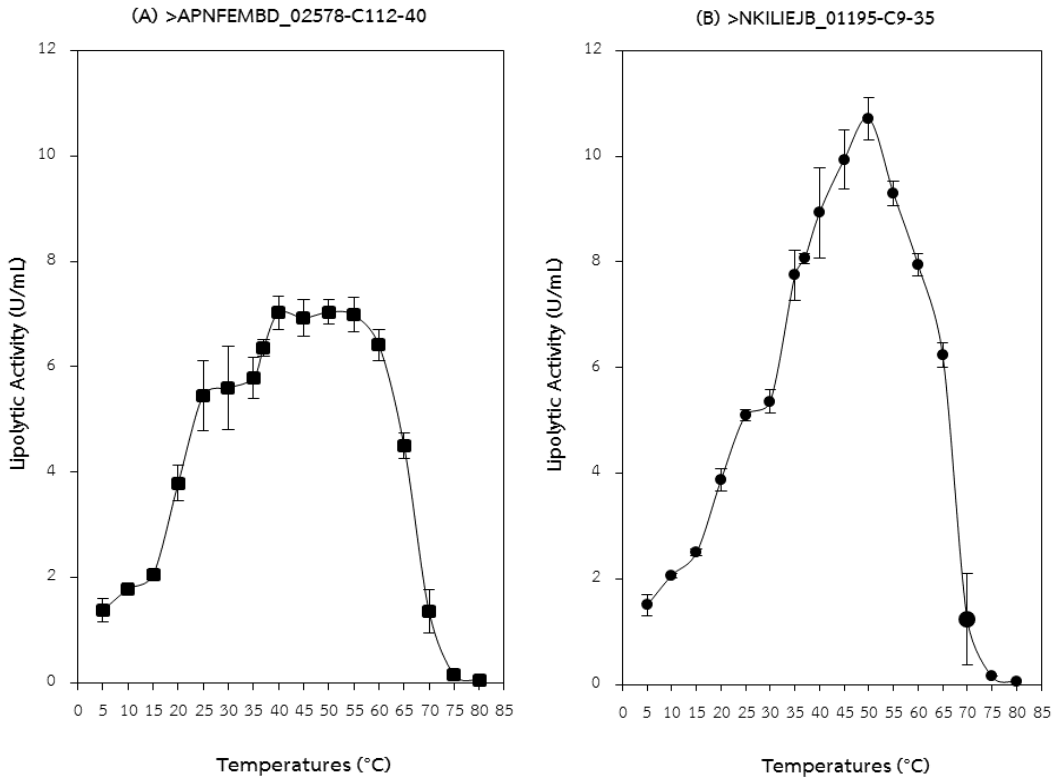

Figure 4.39. Temperature profile of recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35 on esterase activity.

They were supporting the present study, lipase produced by *Natronococcus* sp. (Boutaiba *et al.* 2006), *Salinivibrio* sp. SA2 (Amoozegar *et al.*, 2008) and esterase from *Salimicrobium* sp. LY19 (Xin & Ying, 2013) exhibited its maximum activity at 50 ℃, respectively. At the same time, lipase from *S. warneri* PB233 (Kanlayakrit & Boonpan, 2007), esterase from *H. marismortui* (Camacho *et al.*, 2009; Müller-Santos *et al.*, 2009), carboxyl-esterase from *Thalassobacillus* sp. DF-E4 (Lv *et al.*, 2011), lipase from *Virgibacillus* sp. IDS 20 (Bovornreungroj *et al.*, 2012), purified lipase from *Halobacillus* sp. AP-MSU 8 (Esakkiraj *et al.,* 2014) and lipase from *V. alimentarius* LBU20907 (Dueramae *et al.,* 2017) displayed optimum activity at 40 ℃. Similar results have been shown with esterase enzyme from *Halobacillus* sp. strain LY5 was highly active over broad ranges of temperature (30 to 90℃) and showing optimal activity at 50 ℃ (Li *et al.,* 2012). The esterase produced by the same strain, *H. trueperi* whb27, highly active at Temperature 42 ℃ (Yan *et al.*, 2014). In comparison, esterase from *B.s cereus* AGP-03 had an optimal temperature at 55 ℃ (Ghati & Paul, 2015). An extracellular lipase was characterized by *Chromohalobacter* sp. strain LY7-8, in which maximum activity of the enzyme was reported at 60 ℃ (Li *et al.*, 2012a). However, Guzmán *et al.* (2008) and Kiran et al. (2014) reported that the lipolytic enzyme from *B. pumilus* LV01 and a marine bacterium *Oceanobacillus* sp. PUMB02 had an optimum temperature of 30 ℃, respectively.

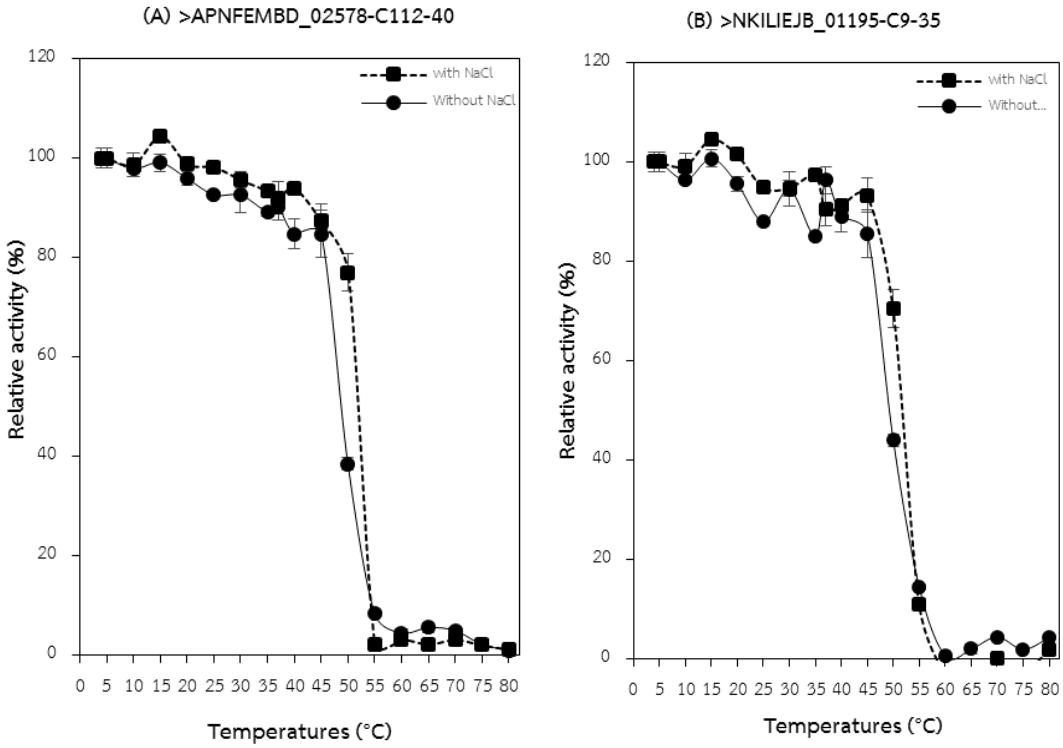

Figure 4.40. Thermostability profile of recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35 on esterase activity.

The thermal stability of the enzyme was also examined by determining the residual activity of the enzyme after incubation at various temperatures (5-80℃) for one hr (Fig. 4.40). It must be noted that the residual activity was determined at the temperature at which the enzyme showed maximum activity (50 ℃). The heat stability profile of the recombinant enzyme produced from >APNFEMBD_02578-C112-40 (Fig. 4.40A) remained activity about 80% at a broad range of temperatures from 5 to 45 ℃ after incubation at 45 ℃ with or without NaCl. However, the activity rapidly decreased as the reaction temperature increased above 45 ℃ in both conditions and lost activity when temperatures above 55 ℃. The enzyme produced from clone >NKILIEJB_01195-C9-35 (Fig. 4.40B) also displayed the thermal stability in the same pattern. They were stable at a temperature below 45℃ and lost its activity when incubated for 60 min at 50 ℃. The enzyme was inactivated entirely at 60℃. These results clearly show that

the stability of the enzyme activity to temperature was high and did not increase by NaCl addition.

The thermal stability of the recombinant found in this study corresponded with the stability of other lipolytic enzymes previously reported. Carboxylesterase from *Thalassobacillus sp.* strain DF-E4 was stable at 45 ℃ after heated for 1 hr and retained 90 % activity after 12 hr (Lv *et al.,* 2011). Such stability was also observed for esterase from *H. marismortui* at 40-50 ℃ for 2 hr. Still, an above 75 ℃ the activity was decreased to 50% and 100% loss of activity following incubation at 60 ℃ within 5 min (Camacho *et al.*, 2009; Müller-Santos *et al.*, 2009). Lipase from *V. alimentarius* LBU20907 was stable at the temperature of 30-50 ℃ and maintained more than 80% activity for 16 hr (Dueramae *et al.,* 2017). The results obtained are in agreement with esterase from esterase, which was found to be stable around 10-75 ℃ (Ghati & Paul, 2015). In contrast with our study, Amoozegar and co-workers (2008) reported that lipase from *Salinivibrio* sp. SA2 retained 90% of its lipase activity at high temperature (80 ℃) for 30 min. Xin & Ying (2013) informed that esterase from *Salimicrobium* sp. LY19 was stable over a broad temperature (20℃-70℃) ranges, and about 40% activity was still retained after incubation at 80℃ for 2 hr. However, *Oc. rekensis* PT-11 lipase was stable at 10-35 ℃ (Jiewei *et al.*, 2014).

In this study, we also noted that the lipolytic activity of our recombinant enzyme is sort of thermo-dependent until about 50℃, after which activities dropped. The maximum activities of two recombinant enzymes are generally exhibited at 40-55℃ or higher temperatures. Therefore, it could be concluded that this recombinant enzyme can be defined as both salt-dependent and thermo-dependent.

4.8.3. Effect of pH on activity and stability

The effect of pH on the two recombinant enzymes activity was measured using *p*-NPB as a substrate and was examined at various pHs (pH 5.0 to 10.0) at 50 ℃, 22% NaCl in the same buffers and same ionic concentrations. It was observed that the pH was powerfully effective on both recombinant enzyme activities. Data presented in Fig.4.41 indicate that the two recombinant enzymes were active over a wide range of pH from 6.0 to 9.0, recording maximal activity at pH 7.0-8.0 compared to that noted at other pH values. Also, lipase activity showed a linear increase between pH 6.0-7.0,

whereas at higher alkaline pH values (below pH 8.0) and then activity decreased rapidly. In the acidic (pH 6.0) and alkaline (pH9.0) range, a significant reduction in enzyme activity was observed. Enzyme activity was wholly lost activity under pH below 6.0 and above 9.0. So, activity was most active at neutral pH, with a pH optimum of 7.0-8.0. It was speculated that the two recombinant enzymes were classified as the neutral lipolytic enzyme.

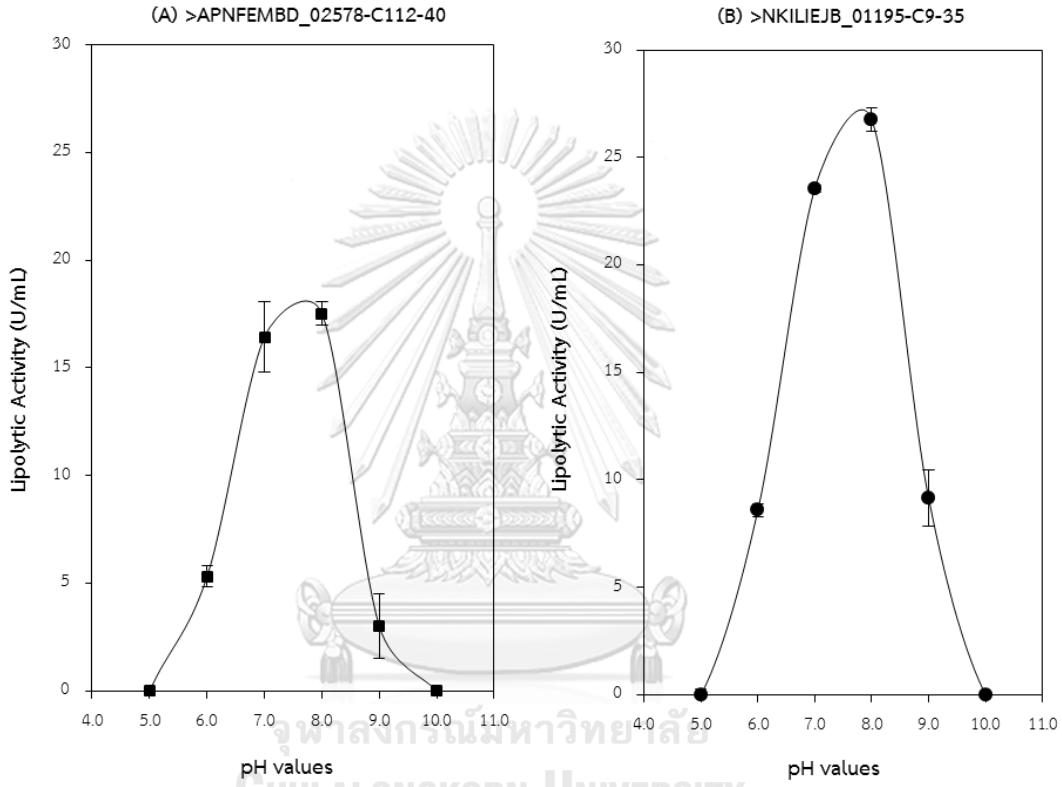

Figure 4.41. Effect of pH on esterase activity of recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35.

It is known that lipases appear in their activity at alkaline pH (Fojan *et al.*, 2000). In consistence with the present study, the lipase/esterase produced by *Natronococcus* sp., *S. warneri* PB233, *Virgibacillus* sp. IDS 20, and *Salimicrobium* sp. LY19 were reported maximum activity at pH 7.0. (Boutaiba *et al.*, 2006; Kanlayakrit & Boonpan, 2007; Bovornreungroj *et al.*, 2012; Xin & Ying, 2013). An extracellular lipase from moderately halophilic *Salinivibrio* sp. strain SA-2 displayed the maximum activity of the enzyme at pH 7.5 (Amoozegar *et al.*, 2008). The maximum activity of the lipase from and *H. marismortui* (Camacho *et al.,* 2009; Müller-Santos *et al.,* 2009) reported at pH 7.5-8.0 (Amoozegar *et al.*, 2008). Ozcan and co-workers reported that extracellular esterase and lipase activities from five halophilic archaeal strains were optimum at pH 8-8.5 (Ozcan *et al.*, 2009). The lipase/esterase production by *Thalassobacillus* sp. DF-E4 (Lv *et al.*, 2011), *H. trueperi* whb27 (Yan *et al.*, 2014), *Oc. rekensis* PT-11 (Jiewei *et al.*, 2014), *Oceanobacillus*. sp. PUMB02 (Kiran *et al.*, 2014) and *B. cereus* AGP-03 (Ghati & Paul, 2015) have been reported to reach a maximum level at pH around 8.0-8.5, respectively. The *V. alimentarius* LBU20907 purified lipase revealed maximum activity at pH 7.0 (Dueramae *et al.,* 2017). The results in this study do not agree with earlier reports showing pH optima at alkaline condition (pH 10.0) for esterase from *Halobacillus* sp. strain LY5 (Li *et al.,* 2012). Previously lipase from *B. pumilus* LV01 (Guzmán *et al.*, 2008), *Chromohalobacter* sp. LY7-8 (Li et al., 2012a), and *Halobacillus* sp. AP-MSU 8 (Esakkiraj *et al.*, 2016) was reported as alkaline with optimum activity at pH 9.0.

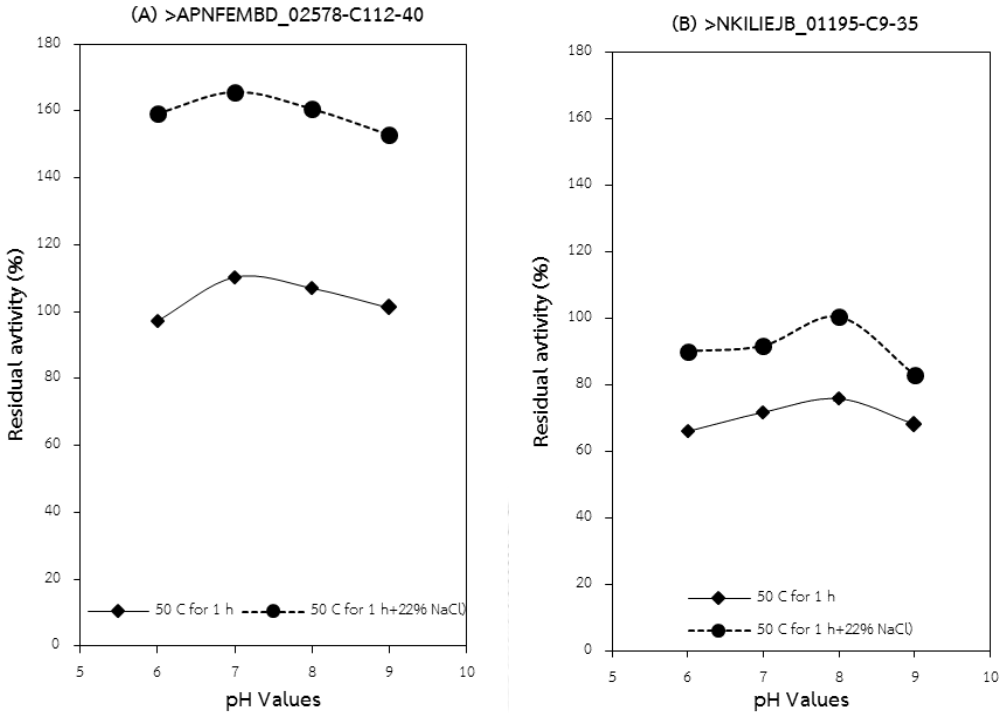

Figure 4.42. pH stability profiles of recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35.

The pH stability of the enzyme was assayed by measuring the residual activity at only pHs (pH 6.0 to 9.0) after 1 hr of incubation at 50 °C with or without optimal NaCl concentration (Fig 4.42). The recombinant (A) >APNFEMBD_02578-C112-40 and (B) >NKILIEJB_01195-C9-35 was stable over a wide range of acidic pH (pH 6.0 to 9.0) in with or without NaCl. The enzymes from clone >APNFEMBD_02578-C112-40 exhibited maximum stability at pH range 7.0. They retained almost 100% of its maximum activity in the condition that absent NaCl. Astonishingly, when NaCl was added, the enzyme was enhanced the activity more than 100% at all ranges of pH tested (Fig. 4.42A).

For the clone (B) >NKILIEJB_01195-C9-35, the enzyme displayed maximum stability at pH range 8.0, retaining 100% activity incubation at 50 °C for 1 hr on both conditions (Fig. 4.42B). The results obtained from the pH stability assay showed that the enzyme was stable (>65%) between pH 6.0 and 9.0 after 1 hr incubation without NaCl addition. In addition, the recombinant enzyme is highly durable at pH 8.0, with the activity remained 100% after incubation with when compared with initial activity.

Similar results were shown by some collagenases described previously. The pH stability of finding results was identical to that of *Salinivibrio* sp. SA2 lipase when the enzyme was remarkably stable w pH range of 7.5-8 (Amoozegar *et al.*, 2008). Carboxyl-esterase from *Thalassobacillus* sp. DF-E4 had a stable between pH 6.0-9.5 (Lv *et al.*, 2011). Extracellular esterase from *Salimicrobium* sp. LY19 was stable at pH 7.0-10.0 (Xin & Ying, 2013). The results obtained are in agreement with lipase from *Oc. rekensis* PT-11, which was found to be stable at pH 8.5 (Jiewei *et al.*, 2014). The esterase from *B. cereus* AGP-03 is stable over a broad range of pH 5.5 and 10 (Ghati & Paul, 2015). The *V. alimentarius* LBU20907 lipase was also highly stable in a pH range of 6.0-7.0 and retained more than 90% activity for 24 h (Dueramae *et al.,* 2017).

4.8.4. Substrate specificity

The hydrolysis ability of two recombinant enzymes from the selected clone, (A) >APNFEMBD_02578-C112-40 (Fig. 4.43A), and (B) >NKILIEJB_01195-C9-35 (Fig. 4.43B), toward the acyl chains length from C2:0 to C16:0 was initially studied using several *p*-NP esters. For both recombinant enzymes, *p*-NPB (C4:0) was the most suitable substrate among the *p*-nitrophenyl esters examined. Both enzymes exhibited the activity around 45% toward the short-chain substrates (C2:0). The activity declined along with longer chain-length, reaching around 50-55% activity with p-NPO (C8:0), 20% with *p*-NPD (C10:0) and 10-12% with *p*-NPL (C12:0) and *p*-NPM (C14:0), respectively. A substrate profile test of an enzyme from >NKILIEJB_01195-C9-35 showed relative activity below 10% toward substrates with long acyl chain lengths (C16:0). At the same time, the enzyme activity produced from clone >APNFEMBD_02578-C112-40 does not detect when *p*-NPP was used as a substrate.

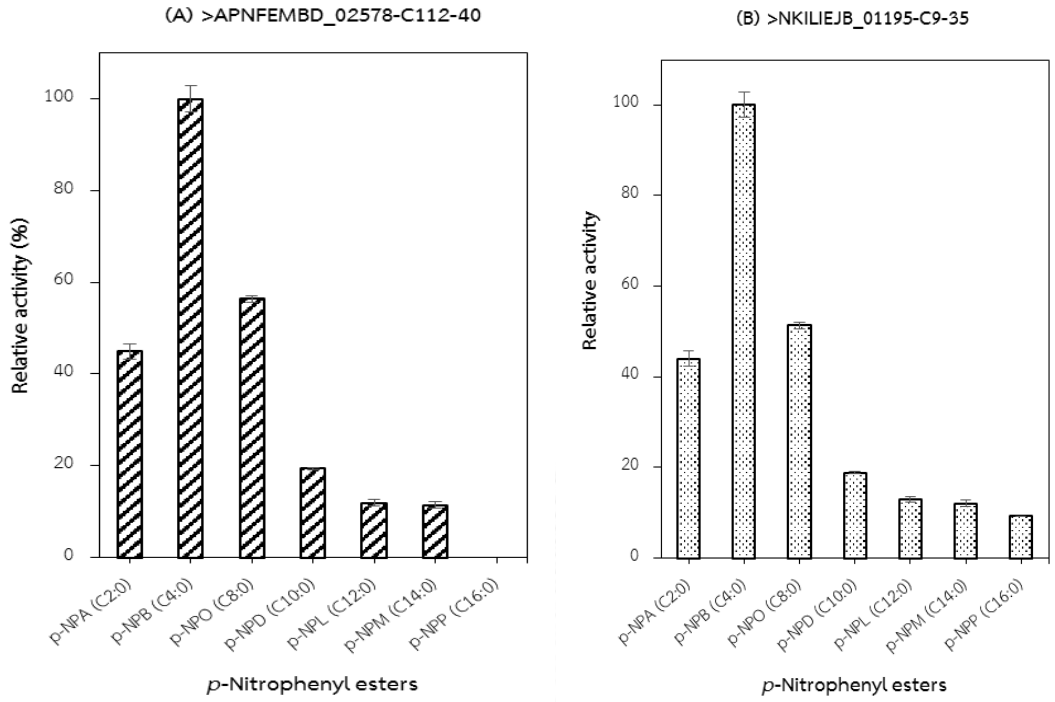

Figure 4.43. Enzyme activity values toward p-Nitrophenyl esters containing the different number of carbon chain lengths of Recombinant enzyme from the selected clone.

Similar results have been demonstrated with the highest activity towards C2-C10 of esterase produced by *Halobacillus* sp. LY5 (Li et al., 2012b). The optimum substrate of two recombinant enzymes obtained from this study was similar substrate specificity had been reported in the case of lipase/esterase from *Salinivibrio* sp. SA2 (Amoozegar et al., 2008), *Thalassobacillus* sp. DF-E4 (Lv et al., 2011), *Halobacillus* sp. LY5 (Li et al., 2012b), *Salimicrobium* sp. LY19 (Xin & Ying, 2013), *B. cereus* AGP-03 (Ghati & Paul, 2015) were able to specify with a short-chain substrate, *p*-NPB (C4:0). The lipase of *Salicola* sp. IC10 (Moreno *et al.*, 2009), *LipBL* from *M. lipolyticus* (Pérez *et al.*, 2011), and *Marinobacter* sp. EMB5 (Hemamalini & Khare, 2016) exhibited broad substrate specificity with maximum activity towards *p*-NPO and *p*-NPD substrates. The esterase from *H. marismortui* (Camacho et al., 2009; Müller-Santos et al., 2009) and lipases from *Chromohalobacter* sp. LY7-8 (Li et al., 2012a) were able to hydrolyze *p*-NPV and *p*-

NPM, respectively. A contrary result was obtained from previous work, lipase from *H. trueperi* RSK CAS9 (Sathishkumar *et al.*, 2015) and *V. alimentarius* LBU20907 (Dueramae *et al.*, 2017) reported that enzyme activity was highest when *p*-NPP used as substrate.

This characteristic of the recombinant enzyme in this study, which has relatively high activity against short to medium chain ester substrates while the acyl chain length of the substrate was increased starting with C10, there was a decrease in the enzyme activity. This could be assumed that theses recombinant enzyme is a typical esterase with broad substrate specificity (C2-C10). These results were not surprising because esterases use short-chain fatty acids as a substrate for their catalytic activity and indicate that *p*-NP esters with acyl chains longer than C10 are not suitable substrates for enzyme assays against the recombinant enzyme. Therefore, the recombinant enzyme in this study can be classified as an esterase based on these substrates specificity toward short to medium-chain acyl chains length (Augustinsson, 1961; Fojan *et al.*, 2000; Kademi *et al.*, 2000; Xin & Ying, 2013).

4.8.5. Determination of Molecular Mass

The molecular mass of the recombinant enzyme was calculated by the ProtParam tool (ExPASy) based on the amino acid sequence. The recombinant enzyme produced by clone >APNFEMBD_02578-C112-40 having a molecular mass of 16 kDa (16,223.34 Dalton). The enzyme produced by clone >NKILIEJB_01195-C9-35 was estimated to be 28 kDa (27,669.48 Dalton).

Similar to the present study, Jiewei et al. (2014) purified 23.5-kDa lipase from *Oc. rekensis* PT-11 with 2.51-fold purity and 14.80% enzyme recovery. Esakkiraj et al. (2016) purified 25-kDa recombinant lipase with 10.6-fold purity from *Halobacillus* sp. AP-MSU 8. Supporting to the present study, Zhang et al. (2005) purified 23-kDa lipase from *Bacillus* sp. So, dimeric esterase from *B. cereus* AGP-03 with 41 kDa (25 kDa and 16 kDa) agreed with this knowledge (Ghati & Paul, 2015). In previous studies, the molecular weight of lipase/esterase from halophilic bacteria was variable from 35 to 58 kDa, such as *H. trueperi* whb27 (35 kDa), *H. trueperi* RSK CAS9 (44 kDa), *Thalassobacillus* sp. DF-E4 (45 kDa), *M. lipolyticus* SM19 (45.3 kDa), *Salimicrobium* sp. LY19 (57 kDa) and *Chromohalobacter* sp. LY7-8 (58 kDa) (Yan *et al.*, 2014; Sathishkumar *et al.*, 2015; Lv *et al.*, 2011; Pérez *et al.*, 2011; Xin & Ying, 2013; Li *et al.*, 2012a).

Nevertheless, in comparison with our studied, the lipolytic enzyme and purified lipase from *B. pumilus* LV01 and *V. alimentarius* LBU20907 had higher molecular weight, which was determined to be 77 and 100 kDa by SDS-PAGE, respectively (Guzmán et al., 2008; Dueramae *et al.*, 2017). The lipase with a molecular mass of 96 kDa was purified from *Halobacillus* sp. LY5 with 8.7-fold purity and 10.3 % enzyme recovery (Li et al., 2012b).

CHAPTER V
CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

Halophilic enzymes, especially esterase and lipases, have a wide and varied industrial & biotechnological application in the present era. The use of lipases/esterases for a variety of biotechnological applications is rapidly and steadily increasing. Many novel lipolytic encoding genes are still to be identified, and enzymes with new and unique properties will be discovered. The ability of the halophilic bacteria to grow and produce enzymes over a vast range of salinities makes them very attractive for research and the isolation of novel enzymes with unique properties. Screening of novel halophilic microorganisms is essential for the production of the novel halophilic enzymes. Two strains of novel lipolytic-producing moderately halophilic bacteria, *Lentibacillus lipolyticus* SSKP1-9^{T} and *Halobacillus fulvus* SKP4-6^{T}, used for the present study was isolated in our laboratory. This study was carried out to amplify the lipolytic encoding gene from those strain, partially purified, and also characterization.

Significant findings in this dissertation can be concluded as follows:

In this study, the main goal was isolation and identification of lipolytic-producing moderately halophilic bacteria (LPMHB) from various sources of Thailand. A total of 831 moderately halophilic bacteria were isolated fish sauce (*Nam-pla*) (473 isolates), shrimp paste (*Ka-pi*) (149 isolates) fermented fish (*Pla-ra*) (78 isolates), fermented fish (*Pla-chom*) (3 isolates), pickled vegetable (*Phak-dong*) (33 isolates), salted crab (*Poo-khem*) (26 isolates), salted fish (*Pla-khem*) (14 isolates), fermented shrimp (*Kung-chom* and *Kung-som*), (16 isolates), fermented mussel (*Hoi-dong*) (6 isolates) and saline soil (32 isolates),

All moderately halophilic bacterial strain was first screened for the presence of extracellular lipolytic enzyme activities. These two enzymes were lipases and esterases. For enzyme screening, Tween 20, Tween 40, Tween 60, Tween 80, and tributyrin were used as substrates on the agar plate. Of these, 349 of 831 isolates

exhibited lipolytic activity as opaque halos of calcium salt on tested medium. Among them, 135 isolates hydrolyzed Tween 20, 295 isolates hydrolyzed Tween 40, 285 isolates hydrolyzed Tween 60, and 87 isolates hydrolyzed Tween 80 while 183 isolates presented the hydrolytic activities against tributyrin. Surprisingly, 58 isolates of them yielded positive results of lipolytic activity against all induction substances.

In the second screening, a total of 349 isolates were able to produce lipolytic enzymes in a liquid medium without inducers. The 322 isolates from 349 isolates exhibited esterase activity (0.01±0.42 to 65.22±1.45 U/ml) when *p*-NPB (C4) was used as a substrate for enzyme assays. The isolate MSQM3-33 showed highest esterase activity (65.22±1.45 U/ml) and followed by (MPCM 12-7 (61.08±1.73 U/ml), MSQM6-12 (54.93±0.58 U/ml), MKP 3-9 (53.96±2.35 U/ml) and MSQC-44 (50.47±0.30 U/ml), respectively. For lipases activities, 176 isolates were detected (0.01±0.13 to 6.95±0.07 U/ml) when *p*-NPP (C16) was used as substrate MKP 4-36 exhibited the highest lipases activity (6.95±0.07 U/ml), followed by MKP 3-11 (6.79±0.17 U/ml), MKP 2-6 (6.79±0.17 U/ml), MKP 4-68 (6.79±0.17 U/ml) and MKP 2-4 (5.70±0.78 U/ml), respectively. Hence, 322 isolates displayed lipolytic activity on both agar and liquid medium were selected for groping using DNA-fingerprinting.

As the second step, DNA-fingerprinting using $(GTG)_5$+ERIC2 primer was used to grouping unknown 322 LPMHB isolates. They generated the amplification band between 5 and 25 from each strain. From the cluster analysis, 180 DNA patterns could be developed. Based on the unique pattern, some of the representative isolate in each pattern was selected for identified using 16S rRNA gene sequence. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the representative of isolate belonged to genera: *Bacillus*, *Halobacillus*, *Lentibacillus*, *Marinobacter, Oceanobacillus, Ornithinibacillus, Thallossobacillus*, and *Virgibacillus*.

In the course of an investigation of novel LPMHB, pattern 19 was involved with three isolates, MPR13-1, MPR16-2, MPR17-4, isolated from *Pla-ra*. On the basis of 16S rRNA gene sequence analyses, these isolates showed the highest level of 16S rRNA gene sequence similarity to *Oc. caeni* strain S-11^T (98.79 % with 18/1488 different nucleotide ratio). The phylogenetic tree based on the NJ method showed that isolates MPR13-1, MPR16-2, and MPR17-4 would be another novel member of this genus

Oceanobacillus. In pattern 70, they consisted of six isolates included MKP1-5, MKP1-6, MKP2-6, MKP3-11, MKP4-126, and MKP4-127 were isolated from *Ka-pi.* All isolates shared <98.5% identity with their closest phylogenetic species, *Or. scapharcae* TW25^T (98.47%) with the nucleotide difference ratio around 22/1440 bp. In the NJ phylogenetic tree analysis, all isolates are located to a unique branch within the *Oceanobacillus* group. These fundamental results confirmed that these isolates belong to new species of the genus *Ornithinibacillus*.

Two novel species of anaerobic, Gram-staining-positive, endospore-forming, rod-shaped moderately halophilic bacterium, *Lentibacillus* sp. SSKP1-9^T (pattern 57) and *Haloacillus* sp. SKP4-6^T (pattern 12) were isolated from shrimp paste (*Ka-pi*) collected from Samut Sakhon Province, Thailand. Strain SSKP1-9^T grew optimally at 37-40 °C, pH 7.0 and 8-16% (w/v) NaCl. The 16S rRNA gene sequence analyses indicated that strain SSKP1-9^T highest similarity with *L. juripiscarius* TISTR 1535^T and *L. halophilus* TISTR 1549^T were 98.7 and 97.2%, respectively. The major isoprenoid quinone was MK-7. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. Polar lipid analysis was composed of phosphatidylglycerol, diphosphatidylglycerol, four unidentified lipids, an unidentified phospholipid, and an unidentified glycolipid. The major cellular fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0,}$ and iso-$C_{16:0}$. The genomic DNA G+C content was 44.6 mol%. The results of phenotypic, chemotaxonomic, genotypic characteristics, and whole-genome analysis support that strain SSKP1-9T represents a novel species of genus *Lentibacillus*, for which the name *Lentibacillus lipolyticus* sp. nov. is proposed.

Strain SKP4-6^T grew at 20-50 °C (optimum 37-45 °C), pH 5.0-10.0 (optimum 7.0) and in 2-23% (w/v) NaCl (optimum 10-15% w/v NaCl). The nearest full-length 16S rRNA genes sequence analysis showed that strain SKP4-6^T (1,435 bp) shared the highest sequence similarities, 98.6% with *H. salinus* JCM 11546^T, *H. locisalis* KCTC 3788^T and *H. yeomjeoni* KCTC 3957^T. Genomic analyses, DNA fingerprinting, and whole-cell protein analyses clearly separated strain SKP4-6^T from other species with values below the thresholds for species delineation. It contained L-Orn-D-Asp in cell wall peptidoglycan. DNA G+C content was 44.8 mol%. The major fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$. The predominant isoprenoid quinone was menaquinone with seven

isoprene units (MK-7). Phosphatidylglycerol and diphosphatidylglycerol were presented as major polar lipids. On the basis of finding results, strain SKP4-6^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus fulvus* sp. nov. is proposed.

Based on the whole-genome analysis, strain SSKP1-9T and SKP4-6T contained protein-encoding esterase gene for 8 and 5 genes, respectively. The genomic DNA was isolated, oligonucleotide primers specific to the selected gene of two strains were designed, and its PCR amplification was done. By using the pSaltExSePR5 cloning and expression vector, all genes of strain SSKP1-9^T and SKP4-6^T were successfully cloned and expressed into *Bacillus subtilis* WB800. The recombinant expression of >APNFEMBD_02578-C112-40 from strain SSKP1-9^T and >NKILIEJB_01195-C9-35 from strain SKP4-6^T studied by NaCl inducible expression and displayed the highest activity. Gene sequencing of the insert from two selected recombinant clones showed it to be 420 and 744 bp gene encoding a protein of 139 and 247 amino acids, respectively. The sequence was blasted and aligned with known lipolytic genes on the NCBI database for comparison with their nucleotide and amino acid sequences. These two genes identified major matches with closely related to a member of family acyl-CoA thioesterase and the alpha/beta hydrolase fold founded in genus *Lentibacillus* and *Halobacillus*, respectively. Of the two recombinant enzymes, the first one has a molecular weight of 16.2 kDa, and the other was 27.7 kDa, identical to the predicted protein size from the amino acid sequence.

Two recombinant enzymes reached the maximum activity at 15-30% NaCl (w/v), and the highest activity was shown at 22 % (w/v) NaCl. They stable in NaCl concentration above 17% (w/v) after incubated at 55 ℃ for 1 hr. The recombinant >APNFEMBD_02578-C112-40 displayed the activity around 25-60 ℃, and the maximum activity was observed at 40-55 ℃ while >NKILIEJB_01195-C9-35 had optimal activity at 55 ℃ and lost its activity rapidly above 65 ℃ of the remaining activity. Being thermophilic, two enzymes showed good stability and remained activity about 80% in a temperature range of 5-45 ℃ after 1 hr of incubation at 50 ℃ with or without NaCl. Theses enzymes reached hight activity over a wide range of pH from 6.0 to 9.0, maximal recording activity at pH 7.0-8.0, and displayed excellent stable in with or without NaCl

at various pH ranges (pH 6.0 to 9.0). Besides, the two recombinant enzymes were found to have higher esterase activity against *p*-nitrophenyl butyrate (C4:0) comparing to other esters substrate based on substrate specificity results. However, it would be more informative if recombinant lipolytic enzymes are purified and then characterized, which is the next step of the current work.

5.2 Future perspectives

Future perspectives in this dissertation can be divided into three parts, as follows:

5.2.1 Taxonomy studies

For this part, the unidentified isolate should be identified. All candidates of moderately halophilic bacteria with the low similarity of the 16S rRNA gene sequence should be studied for taxonomy characterization and publication for new species discovered in Thailand.

5.2.2 Molecular studies

The complete genome of novel species discovered in this study should be further studied to better understand the function of the protein produced by the new strain. The host for expression of an interesting halophilic protein, lipolytic enzyme, should be created. The lipolytic encoding gene founded in the host strain, *B. subtilis* WB800, should be knocked out, or the new host strain should be selected for facilitated the overproduction of the enzyme for industrials uses in the future. The optimization for recombinant enzyme production should be performed. Besides, other protein-encoding genes which are founded in the genome of new species in this study, such as proteases or other enzymes, should be further studied

5.2.3 Purification of the enzyme

In this study, the recombinant enzyme was only concentrated for characterization so, the purification steps of the recombinant enzyme produced by the selected clone by using FPLC or other techniques should be considered. Furthermore, other characteristics such as the effect of different organics solvents, effects of various metal ions, the impact of several detergents, the impact of inhibitors, kinetic parameters, and activity staining should be performed.

APPENDICES

APPENDIX A

Culture media

All components of media were mixed and dissolved in distilled water, and then the pH was adjusted and bring volume to 1.0 L. Sterilized in an autoclave for 20 min at 15 pounds pressure and 121 °C, except where indicated otherwise.

1. Lentibacillus medium (JCM medium no. 377)

Casamino acids (BD-Difco)	5	g
Yeast extract (BD-Difco)	5	g
Sodium glutamate	1	g
Trisodium citrate (Carlo erba)	3	g
$MgSO_4 \cdot 7H_2O$ (Carlo erba)	20	g
KCl (MERCK)	2	g
NaCl (Carlo erba)	100	g
$FeCl_2 \cdot 4H_2O$ (WAKO)	36	mg
$MnCl_2 \cdot 4H_2O$ (Carlo erba)	0.36	mg
Agar (BD-Difco)	20	g
Distilled water	1	L
pH	7.0-7.2	

2. Lipolytic medium

NaCl	100	g
Peptone	1	g
$CaCl_2 \cdot 2H_2O$	0.01	g
Agar	20	g
pH	7.0	
Distilled water	1	L
Substrate	10	g

(Substrate: Tween 20, 40, 60, 80 or glycerol butyrate)

APPENDIX B

Reagents for chemotaxonomic characteristic

1. Reagents for fatty acids extraction

Saponification reagent

NaOH	15	g
Methanol (HPLC grade)	50	mL
Milli-Q water	50	mL

Dissolve NaOH in Milli-Q water and add methanol.

Methylation reagent

6N HCl	65	mL
Methanol (HPLC grade)	55	mL

Adjust pH to below 1.5.

Extraction reagent

n-Hexane (LC grade)	50	mL
Methyl-3-butyl ether (LC grade)	50	mL

Base washing reagent

NaOH	1.2	g
Milli-Q water	100	mL

Saturated NaCl solution

NaCl saturated in Milli-Q water

2. Reagents for the analysis of polar lipids

Anisaldehyde reagent

Ethanol	90	mL
Conc. H_2SO_4	5.0	mL
p-Anisaldehyde	5.0	mL
Acetic acid	1.0	mL

Dragendorff's reagent

Solution A

Basic bismuth nitrate	1.7	g
Acetic acid	20	mL
Distilled water	80	mL

Solution B

KI	40	g
Distilled water	100	mL

Before detection, mix each 10 ml of solution A, solution B, and acetic acid.

Phosphomolybdic acid reagent

Phosphomolybdic acid	5.0	g
Absolute ethanol	100	mL

Molybdenum blue reagent

Solution A

MoO_3	4.01	g
25N H_2SO_4	100	mL

Dissolve MoO_3 with 25N H_2SO_4 and heat until completely dissolved.

Solution B

Molybdenum powder	0.178	g
Solution A	50	mL

Add the molybdenum powder into solution A and heat at 100 °C for 15 min. After cooling, remove the precipitation by decantation. Mix each 50 mL of solution A, solution B, and distilled water before spraying.

Saline-EDTA buffer (pH 8.0)

0.15 M NaCl	8.77	g
0.1 M EDTA (EDTA·2Na·$2H_2O$)	37.22	g
Distilled water	1	L
pH	8.0	

Tris-SDS buffer (pH 9.0)

0.1 M Trizma base	12.11	g
1% (w/v) Sodium dodecyl sulphate	10.0	g
Distilled water	1	L
pH	9.0	

10x NaCl sodium citrate solution

1.5 M NaCl	87.66	g
0.15 M Trisodium citrate	44.12	g
Distilled water	1	L
pH	7.0±0.2	

Reagents and buffers for DNA-DNA hybridization

Pre-hybridization solution (10 ml)

20X SSC	1	mL
10 mg/mL Salmon sperm DNA	1	mL
Formamide	5	mL
Sonicated 10 mg/mL salmon sperm DNA	0.1	mL
Distilled water	2.9	mL

Hybridization solution (10 mL)

Prehybridization solution	100	mL
Dextran sulphate	5	g

Solution 1 (10 mL)

BSA (Bovine serum albumin)	0.05	g
Triton X	10	µL
20X PBS	0.5	mL
Distilled water	9.5	mL

Solution 2

Strepavidin-POD conjugate	1	µL
Solution 2	4	mL

Solution 3

10 mg/mL 3,3',5,5' Tetramenthylbenzidine (TMB)*	100	mL

0.3% (v/v) H_2O_2	100	mL

*3,3',5,5' Tetramenthylbenzidine was dissolved in DMSO

0.1 M citric acid + 0.2 M Na_2HPO_4 buffer pH 6.2 in 5 mL of 10% DMSO (Freshly prepare)

20X Phosphate buffered saline (PBS)

Na_2HPO_4	28.8	g
NaCl	160	g
KH_2PO_4	4	g
KCl	4	g
Distilled water	1	L
pH	7.2-7.4	

100X Denhardt solution

Bovine serum albumin	2	g
Polyvinylpyrrolidone	2	g
Ficollc 400	2	mL

Dissolve in 100 mL Nanopure water and store at 4 ℃ until used

10 mg/mL Salmon sperm DNA

Salmon sperm DNA	10	mg
TE buffer	1	mL

Dissolve salmon sperm DNA in TE buffer, boil the solution for 10 min, immediately cool in ice and sonicate for 3 min (obtional).

20X Saline sodium citrate (SSC)

NaCl	175.3	g
Sodium citrate	88.2	g
Distilled water	1	L
pH	7.0	

1X Saline sodium citrate

20X SSC	50	mL
Sterile distilled water	950	mL

2. Polar lipids

2.1 Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	mL

2.2 Dittmer&Lester reagent

Reagent A

MoO_3	4.011	g
25 N H_2SO_4	100	mL

Dissolve 4.011 g of MoO_3 in 100 mL of 25 N H_2SO_4 by heating

Reagent B

Molybdenum powder	0.178	g
Solution A	59	mL

Add 0.178 g of Molybdenum powder to 50 mL of solution A, and boil for 15 min. After cooling, remove the precipitate by decantation. Before spraying, mix solution A (50 mL), solution B (50 mL) and water (100 mL).

2.3 Anisaldehyde reagent

Ethanol	90	mL
p-Anisaldehyde	5.0	mL
H_2SO_4	5	mL
Acetic acid	1	mL

3. Cellular fatty acid analysis

3.1 Reagent 1 (Saponification reagent)

Sodium hydroxide	15	g
MeOH (HPLC grade)	50	mL
Milli-Q water	50	mL

Dissolve NaOH in Milli-Q water and add MeOH

3.2 Reagent 2 (Methylation reagent)

6 N HCl	65	mL
MeOH (HPLC grade)	55	mL
pH must be below	1.5	

3.3 Reagent 3 (Extraction solvent)

n-Hexane (HPLC grade)	50	mL
Methyl-tert-Butyl ether	50	mL

3.4 Reagent 4 (base wash reagent)

Sodium hydroxide	1.2	g
Milli-Q water	100	mL

3.5 Reagent 5 (Saturated NaCl)

NaCl saturated in Milli-Q water

4. RNase A solution

RNase A	20	mg
0.15 M NaCl, pH 5.0	10	mL

Dissolve *RNase A* in 0.15 M NaCl, pH 5.0, and heat at 95 ℃ for 5-10 min. Keep the solution at -20 ℃ until use.

5. RNase T solution

RNase T	800	U
0.1 M Tris-HCl (pH 7.2)	1	mL

Mix *RNase T* in 0.1M Tris-HCl (pH 7.2) and heat at 95 ℃ for 5 min. Keep the solution at -20 ℃ until use.

APPENDIX C

Reagents and buffers

Buffers and reagents for lipolytic enzyme determination and purification

1. Universal buffer (Britton-Robinson buffer, pH 2.0-12.0)

0.04 M H_3BO_3	2.47	g
0.04 M CH_3COOH	2.07	mL
0.04 M H_3PO_4	2.29	mL

Adjust pH with 0.2 N NaOH and made up the volume to 1 L with Milli-Q water

APPENDIX D

Partial of 16S ribosomal RNA gene sequence of strains

1. *Halobacillus fulvus* SKP4-6^T (LC367328) (1,435 bp)

CCTATACATGCAAGTCGAGCGCGGGAAGCGAGCTGATCCCTTCGGGGTGACGCTCGTGGAACG
AGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGAATAACTCCGGGAAA
CCGGAGCTAATGCCGGGTAACACCTTCTTTCGCATGAAGGAAGGTTAAAAGATGGCTTCTCGCT
ATCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG
AACGATGAAGGTTTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTGCGAATAG
AGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAA
GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGAC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAG
TGGCGAAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGCTTCCACCC
CTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCTTCGGACCACCCTAGAGATAGGGTCTTCCCTTCGGGGACCG
AATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCCTAATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTGTAGCAAATCCCATAAAACCATTC
TCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGC
AACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCC

2. *Lentibacillus lipolyticus* SSKP1-9^T (LC367329) (1,445 bp)

CTATACATGCAGTCGAGCGCGGGAAGCAGGCAAACACCCTTCGGGGTGTGCGCCTGTGGAACG
AGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTCGCGGAAA
CGTGAGCTAATACCGGATAATGTCTTTCCTCGCATGAGGGAGGGCTGAAAGACGGCCTTTGTGC
TGTCACTTACAAATGGGCCCGCGGCGCATTAGTTAGTTGGTGAGGTAAGAGCTCACCAAGGCGA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGCGTGGTTCGAATA
GGGCCATGCCTTGACGGTACCTGACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTA
AGTCTGATGTGAAATCTTGCGGCTTAACCGCAAGTGGTCATTGGAAACTGGGAGGCTTGAGTAC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAG
TGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGTAGCGAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGAGTGCTAGGTGTTAGGGGGTTTCCGCCC
CTTTGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTC
AAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAGCGGCAGAGATGCCGTGTTCCCTTCGGGGACAG
AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAAC
GAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGATGGAACAAAGGGAAGCGAAGCCGTGAGGTGTAGCAAATCCCACAAAACCATTC
TCAGTTCGGATTGCAGGCTGCAACTCGCCTGTATGAAGCCGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGC
AACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAA

>Full-PCR-LLL02578

TGGGGTCTAGAATGACACATACACCAATTGAAGTAAGGTATCAGGAAACCGACCAGATGGGCGT
TGTTTATCATGCGAATTACCTAGTCTGGTTTGAAATTGGAAGAACGAAATACATAGAAAGCTTG
GGACTCACCTATATGGATATGGAGAAAAATAATATTGTCTCACCAGTGGTAGACGCCCAAGTCA
ATTTCAGAAAGCCCGTTCGTTATGGGGAAAAGGCCACGGTGGAAACTTGGCTCGATACATATGA
TGGCATTCGTACTGTCTACGCATATAATATTAAAAAAGAAAATGGAGAAGTTGCTGTCAGTGGA
ACAACGAAGCATGTAATTGTCCACAAAGAAACATTTCGTCCCTTGTCACTGCGCAAAAGCTTCC
CGGAAtGGCATGAAGCGTATTCCCGGCaaTTAGATGGTGAAGCGTAAACGCGTGGGGAAGA

>Full-PCR-HFL01195

ATGAGTCTTAAGGTTATGGAAGGGGCCGAAGCTTTAACTTTCGTGGGAAATGAAGTGGGAATTC
TCATTTCCCACGGTTTTACGGGAACGACCCAAAGTATTCGTCCATTAGCAGAAGCCTATGCAAA
AGCAGGGTATACAGTTTGCGCGCCAAGACTGAAAGGTCACGGGACAACGCCTGAAGAAATGGA
AGCAACCCAATATGAAGACTGGATTGAATCAGTGGAAGAAGGGCTGGAATGGCTCCGTGGCAG
GTGCGACATTCTATTTGTGACGGGCCTATCGATGGGAGGAACGCTCACGTTATACCTGGCAGAA
AAATATCAAGATATTCAAGGCGTAATCCCGATTAATGCTGCGATTGATATTCCAGCCATGGCTG
ATTTAGATGGTGTAACAGAGAGGTTTCAAGATGCCATCGGATCCGACATCAAGCAAGAAGGCGT
GCGTGAATTGACATACGACCGTACACCTGTCCTTTCAATGAAGGAATTGAATAAGCTAATGACG
ATCGTTCAAGAAAAATTAACGGGCATTCGCTGTCCGATTCTCGTATTTGTCTCTGATGAAGATC
ATGTCGTCCCACCGAACAACTCGGAGGTTATTTTTGAGAAAGTTTTCTCCGAACAGAAAGAAAT
CGTCCGCTTGGAAAACAGTTATCACGTCGCTACTCTCGATCAGGATCAGGATTTGATCATCGAA
CGCAGCTTGGAATTCTTTGAGCGTTATGTGAACACAAAGAACGCCCGTGGGGGAAG

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