อนุกรมวิธานของแบคทีเรียชอบเค็มปานกลางที่ผลิตไลเปส

นางสาวรังสิมา ดรุณพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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TAXONOMY OF LIPASE PRODUCING MODERATELY HALOPHILIC BACTERIA

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รังสิมา ดรุณพันธ์ : อนุกรมวิธานของแบคทีเรียชอบเค็มปานกลางที่ผลิตไลเปส (TAXONOMY OF LIPASE PRODUCING MODERATELY HALOPHILIC BACTERIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.วรรณพ วิเศษสงวน, 147 หน้า.

ในการคัดแยกสายพันธุ์แบคทีเรียชอบเค็มปานกลางจำนวน 138 ไอโซเลต ที่สร้างไลเปสจากตัวอย่างน้ำปลา, ผลิตภัณฑ์ปลาหมัก ปูเค็ม และกะปิ ที่เก็บจากโรงงานและตลาด พบว่าแบคทีเรียชอบเค็ม 70 ไอโซเลตมีกิจกรรมของเอนไซม์ไลเปส บนอาหารแข็งที่มี Tween 20, 40, 60 หรือ 80 เป็นซับสเตรท เมื่อนำมาศึกษาในอาหารเหลว (complex medium) คัดเลือกแบคทีเรียชอบเค็มจำนวน 36 ไอโซเลตที่แสดงผลของกิจกรรมของเอนไซม์ไลเปสสูงกว่า 1 ยูนิตต่อมิลลิลิตรในอาหารเหลวเพื่อพิสูจน์เอกลักษณ์โดยอาศัยการศึกษาลักษณะทางฟีโนไทป์และการวิเคราะห์ลำดับในช่วง 16S rRNA สามารถแบ่งแบคทีเรียออกเป็น 24 กลุ่มและพิสูจน์เอกลักษณ์ได้เป็น *Virgibacillus dokdonensis* (3 สายพันธุ์) *Virgibacillus alimentarius* (1 สายพันธุ์) *Virgibacillus halodenitrificans* (2 สายพันธุ์) *Lentibacillus juripiscarius* (1 สายพันธุ์) *Oceanobacillus iheyensis* (1 สายพันธุ์) *Alkalibacillus almallahensis* (1 สายพันธุ์) *Halobacillus trueperi* (1 สายพันธุ์) *Bacillus amyloliquefaciens* subsp. *plantarum* (1 สายพันธุ์) *Bacillus altitudinis* (1 สายพันธุ์) *Bacillus seohaeanensis* (1 สายพันธุ์) *Bacillus zhangzhouensis* (1 สายพันธุ์) *Corynebacterium falsenii* (2 สายพันธุ์) *Corynebacterium variabile* (2 สายพันธุ์) *Brevibacterium sediminis* (2 สายพันธุ์) *Proteus penneri* (1 สายพันธุ์) *Staphylococcus saprophyticus* subsp. *bovis* (2 สายพันธุ์) *Staphylococcus saprophyticus* subsp. *saprophyticus* (1 สายพันธุ์) *Staphylococcus nepalensis* (1 สายพันธุ์) *Salinicoccus salsiraiae* (1 สายพันธุ์) *Salinicoccus siamensis* (3 สายพันธุ์) *Vibrio alginolyticus* (4 สายพันธุ์) *Shewanella indica* (1 สายพันธุ์) *Bacillus* สายพันธุ์ NR1-3-2 จากน้ำปลาและ *Virgibacillus* สายพันธุ์ KN3-8-4 จากกะปิเป็นแบคทีเรียสปีชีส์ใหม่จากผลการศึกษาลักษณะอนุกรมวิธานแบบโพลีฟาสิก สายพันธุ์ NR1-3-2 มีกรดไขมันเป็น anteiso-$C_{15:0}$, iso-$C_{15:0}$ และ anteiso-$C_{17:0}$ และมี polar lipid เป็น diphosphatidyl glycerol, phosphatidyl glycerol และ glycolipid มีปริมาณ G+C ของ DNA เป็น 44.2 โมลเปอร์เซ็นต์ ผลการวิเคราะห์ลำดับเบสในช่วง 16S rDNA ใกล้เคียงกับ *Bacillus iranensis* DSM 23995^{T} (97.4 เปอร์เซ็นต์) และมีความคล้ายคลึงของ DNA ต่ำเทียบกับ *Bacillus iranensis* DSM 23995^{T} (39.8%) จึงเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Bacillus piscicola* ส่วนสายพันธุ์ KN3-8-4 มีกรดไขมันเป็น anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ และ iso-$C_{15:0}$ และมี polar lipid เป็น phosphatidyl glycerol, diphosphatidyl glycerol, unknown phospholipids และ glycolipid ปริมาณ G+C ของ DNA เป็น 43.58 โมลเปอร์เซ็นต์ ผลการวิเคราะห์ลำดับเบสในช่วง 16S rDNA ใกล้เคียงกับ *Virgibacillus olivae* JCM 30551^{T} (97.85 เปอร์เซ็นต์) และมีความคล้ายคลึงของ DNA ต่ำเทียบกับ *Virgibacillus olivae* JCM 30551^{T} (28.8%) จึงเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Virgibacillus kapii*

การศึกษาไลเปสจากสายพันธุ์ KN3-8-4 พบว่าสามารถสร้างไลเปสสูงสุดในระยะ stationary phase เมื่อเลี้ยงในอาหารดัดแปลง JCM no. 377ที่ความเข้มข้นเกลือ 1 เปอร์เซ็นต์ โดยแทนที่ casamino acid ด้วยน้ำมันปาล์ม 0.5 เปอร์เซ็นต์ พีเอช 8.5 และอุณหภูมิ 40 องศาเซลเซียส ทำให้เอนไซม์ไลเปสบริสุทธิ์โดยเทคนิค acetone precipitation, gel filtration และ anion exchange chromatography พบว่าไลเปสที่ได้ความบริสุทธิ์สูงขึ้น 18.66 เท่า และลักษณะสมบัติของไลเปสบริสุทธิ์ที่แยกได้คำนวณจาก gel filtration และ SDS-PAGE พบว่ามีน้ำหนักโมเลกุล 19.5 และ 19 kDa ตามลำดับ และทำปฏิกิริยาได้ดีที่สุดในสภาวะที่มีความเข้มข้นเกลือ 7 เปอร์เซ็นต์ พีเอช 8.0 และที่อุณหภูมิ 40 องศาเซลเซียส นอกจากนี้เอนไซม์ยังมีความจำเพาะเจาะจงต่อสารตั้งต้นของ *p*-nitrophenyl esters หลายตัว โดยเฉพาะอย่างยิ่ง *p*-nitrophenyl butyrate ความจำเพาะเจาะจงสูงสุด

ภาควิชา	อาหารและเภสัชเคมี	ลายมือชื่อนิสิต ---
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RUNGSIMA DAROONPUNT: TAXONOMY OF LIPASE PRODUCING MODERATELY HALOPHILIC BACTERIA. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: WONNOP VISSESSANGUAN, Ph.D., 147 pp.

One hundred and thirty eight of lipase-producing halophilic bacteria from fish sauce, fermented fish, shrimp paste and *Poo-chem* collected from the factories and the markets were isolated. Seventy isolates showed lipolytic activity on lipolytic agar using Tween (20, 40, 60 or 80) as substrates. Thirty-six isolates that showed more than 1 unit/ml of lipase activity in liquid medium were collected for identification. On the basis of phenotypic characteristics and 16S rRNA sequences, thirty strains were identified as *Virgibacillus dokdonensis* (3 isolates), *Virgibacillus alimentarius* (1 isolate), *Virgibacillus halodenitrificans* (2 isolates), *Lentibacillus juripiscarius* (1 isolate), *Oceanobacillus iheyensis* (1 isolate), *Alkalibacillus almallahensis* (1 isolate), *Halobacillus trueperi* (1 isolate), *Bacillus amyloliquefaciens* subsp. *plantarum* (1 isolate), *Bacillus altitudinis* (1 isolate), *Bacillus seohaeanensis* (1 isolate), *Bacillus zhangzhouensis* (1 isolate), *Corynebacterium falsenii* (2 isolates), *Corynebacterium variabile* (2 isolates), *Brevibacterium sediminis* (2 isolate), *Proteus penneri* (1 isolate), *Staphylococcus saprophyticus* subsp. *bovis* (2 isolates), *Staphylococcus saprophyticus* subsp. *saprophyticus* (1 isolate), *Staphylococcus nepalensis* (1 isolate), *Salinicoccus salsiraiae* (1 isolate), *Salinicoccus siamensis* (3 isolates), *Vibrio alginolyticus* (4 isolates), *Shewanella indica* (1 isolate). *Bacillus* strain NR1-3-2 isolated from fish sauce and *Virgibacillus* strain KN3-8-4 isolated from shrimp paste (*ka-pi*) were novel species based on polyphasic taxonomy. Strain NR1-3-2 contained anteiso-$C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{17:0}$ as major cellular fatty acids and had diphosphatidyl glycerol (DPG), phosphatidyl glycerol (PG) and one glycolipid as polar lipids. DNA G+C content was 44.2 mol%. The 16S rDNA sequence analyses indicated that strain NR1-3-2 highest similarity with *Bacillus iranensis* DSM 23995^T (97.4%). Strain NR1-3-2^T exhibited low DNA -DNA relatedness (39.8%) with *Bacillus iranensis* DSM 23995^T and was proposed as *Bacillus piscicola* sp. nov. Strain KN3-8-4 contained anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$ as major cellular fatty acids and had PG, DPG, two unknown phospholipids and one glycolipid as polar lipids. The DNA G+C content was 43.58 mol%.The 16S rDNA sequence analyses indicated that strain KN3-8-4 closely related to *Virgibacillus olivae* JCM 30551^T (97.85%). This strain showed low DNA -DNA relatedness with *Virgibacillus olivae* JCM 30551^T (28.8%) and was proposed as *Virgibacillus kapii* sp. nov.

Strain KN3-8-4 selected for lipase purification produced the maximum lipase at stationary phase and could be achieved when casamino acids was replaced by 0.5% palm oil (w/v) in a modified JCM no. 377 medium with 1% NaCl (w/v), pH 8.5 and incubated at 40°C for 36 h. The KN3-8-4 lipase was purified by cold acetone precipitation, gel filtration and anion exchange chromatography with 18.66-fold purification. The purified lipase from KN3-8-4 was monomeric protein with the molecular mass of about 19.5 kDa by gel filtration and 19 kDa by SDS-PAGE. The enzyme had a maximal activity in the presence of 7% w/v NaCl, pH 8.0 at 40°C. The enzyme exhibited a variable specificity activity towards various *p*-nitrophenyl esters especially *p*-nitrophenyl butyrate (C4).

Department:	Food and Pharmaceutical Chemistry	Student's Signature ---
Field of Study:	Pharmaceutical Chemistry and Natural Products	Advisor's Signature ---
		Co-Advisor's Signature ---
Academic Year:	2016	

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	=	American Type Culture Collection
bp	=	Base pairs
$^{\circ}C$	=	Degree Celsius
DAP	=	Diaminopimelic acid
DPG	=	Diphosphatidylglycerol
FPLC	=	Fast protein liquid chromatography
G	=	Gram
G+C	=	Guanine-plus-cytosine
HCl	=	Hydrochloric acid
H_2O	=	Water
hr	=	Hour
JCM	=	Japan Collection of Microorganisms
KCl	=	Potassium chloride
kDa	=	Kilo Dalton
L	=	Liter
Log	=	logarithm
mM	=	Millimolar
M	=	Molar
MEGA	=	Molecular Evolutionary Genetics Analysis
meso-DAP	=	meso- Diaminopimelic acid
Min	=	Minute
mg	=	Milligram
MK	=	Menaquinone
ml	=	Milliliter
µg	=	Microgram

µl	=	Microliter
MW	=	Molecular weight
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
nm	=	Nanometer
nov.	=	Novel
4-NPB	=	para-Nitrophenyl butyrate
4-NPP	=	para-Nitrophenyl palmitate
OD	=	Optical density
p-NP	=	p-Nitrophenol
PAGE	=	Polyacrylamide gel electrophoresis
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PL	=	Phospholipids
%	=	Percentage
PCR	=	Polymerase chain reaction
rDNA	=	Ribosomal deoxynucleic acid
rpm	=	Round per minute
SEM	=	Scanning electron microscope
SDS	=	Sodium dodecyl sulfate
sp.	=	Species
SSC	=	Standard sodium citrate
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
v/v	=	volume/volume
w/v	=	weight/volume

CHAPTER 1

INTRODUCTION

Halophiles are salt-loving organisms that flourish in saline environments and can be classified as slightly, moderately or extremely halophilic, depending on their requirement for sodium chloride, whereas those capable of growth in the absence of salt but tolerant of varying concentrations, are considered to be halotolerant. The moderately halophilic bacteria grow best with 0.85-3.4 M (5-20%) NaCl and there were found in salt lake, soda lake, salterns, hypersaline soils, and salted fish, meat, and other foods. In the hypersaline environments, moderate halophiles accumulate in the cytoplasm high amounts of specific organic osmolytes called compatible solutes such as glycine betaine, ectoine, simple sugars, and many others, which function as osmoprotectants, providing osmotic balance without interfering with the normal metabolism of the cell.

The moderately halophilic bacteria have adapted to live in wide range of salt concentration and they able to produce extracellular enzymes (such as lipases, proteases, amylases, cellulases, xylanases, pullulanases and pectinases) will provide the possibility to have optimal activities at different salt concentrations. In generally, industrial processes are carried out under specific physico-chemical conditions which may not be definitively adjusted to the optimal points required for the activity of the available enzymes. So, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations, pH and temperature. Moderately halophilic bacteria are best source of such enzymes especially lipases which are not only salt tolerant, but also may be active at high pH values and temperature.

Currently lipases of moderately halophilic bacteria are interested and much of demands because of these lipases show high selectivity and no requirement for added cofactors. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and

free fatty acids including low molecular weight volatile fatty acids such as acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid. These free fatty acids are associated with the aroma and flavor in food products such as fish sauce (*Nam-pla*), shrimp paste (*Ka-pi*), fermented fish entrails (such as *Tai-pla*, *Kee-dee* and *Koey-pla*). Therefore, the moderately halophilic bacteria are interesting group of microorganisms that could be used as a source of salt-adapted enzyme in fermented foods for enhancement of aroma and flavor. In addition, Lipases are among the most important hydrolytic enzymes with potential applications in a variety of biotechnological fields such as food and dairy, detergent, cosmetic, pulp and paper, synthesis of biodiesel and pharmaceutical However, the lipase-producing moderately halophilic bacteria are rarely studied.

In this study, the main objectives are focused on the isolation and identification of moderately halophilic bacteria, screening of their lipase, purification and characterization of lipase from selected moderately halophilic bacteria.

The main objectives of this research study are as follows:

1. To isolate and screen lipase-producing moderately halophilic bacteria
2. To identify the selected moderately halophilic bacteria based on phenotypic, chemotaxonomic and genotypic characteristics
3. To purify and characterize of lipase from the selected isolates

CHAPTER 2

LITERATURE REVIEW

2.1 Halophilic bacteria

Halophiles are salt-loving organisms that flourish in saline environments and can be classified as slightly, moderately or extremely halophilic, depending on their requirement for sodium chloride, whereas those capable of growth in the absence of salt but tolerant of varying concentrations, are considered to be halotolerant (Ventosa 1995). Halophilic bacteria classified according to the salt concentration (Kushner 1985) that was optimal for growth, non-halophiles are those that grow best in media containing < 1% (w/v) NaCl (the halotelerant can tolerate higher concentrations); slight halophiles (marine bacteria) grow best with 1-5% (w/v) NaCl; moderate halophiles grow best with 5-15% (w/v) NaCl which moderately halophilic bacteria constitute a heterogeneous physiological group of microorganisms which belong to different genera such as Gram positive bacteria; *Lentibacillus, Virgibacillus, Piscibacillus, Bacillus, Tetragenococcus, Halococcus, Halobacillus, Salinicoccus, Planococcus, Sporosarcina, Arthrobacter, Kocuria* and *Marinococcus,* Gram negative bacteria; *Halomonas, Deleya, Volcaniella, Flavobacterium, Paracoccus, Pseudomonas, Halovibrio, Marinobacter* and *Chromobacterium*; extreme halophiles show optimal growth in media containing 20-30% (w/v) NaCl which contains genera, namely, *Halobacterium, Halococcus, Haloarcula, Haloferax, Halorubrum, Halobaculum, Natrialba, Natronomonas, Natronobacterium* and *Natronocuccus* (Kamekura 1998).

In recent years, the number of biotechnological uses of halophilic microorganisms has increased, and additional applications are under development. The halophiles were used in biotechnology which can be divided into a number of categories. First, the halotolerant of many enzymes derived from halophilic

microorganisms can be exploited wherever enzymatic transformations are required to function at low water activities, such as in the presence of high salt concentrations. Second, some organic osmotic stabilizers produced by halophiles have found interest applications. Third, some halophilic microorganisms may produce valuable compounds that can also be found in non - halophiles, often without any direct connection with their halophilic properties, but halophiles may present distinct advantages for the development of biotechnological production processes (Oren 2002).

At high osmolarity in saline conditions, most halophilic bacteria accumulate intracellular concentrations of organic compounds called "compatible solutes" within the cytoplasm because they are solutes responsible for the osmotic balance of the cell and are also compatible with the cellular metabolism. These compatible solutes are generally amino acid, sugar and polyols, which do not interfere with intracellular processes and have no net charge at physiological pH (DasSarma 2012).

2.2 Sources of moderately halophilic bacteria

Moderately halophilic bacteria were generally found in saline environments such as brine lakes, ocean, salts marshes, saltern, deep-sea sediment, saline soil and saline mud which including *Halobacillus* sp., *Salinicoccus* sp., and *Marinococcus* sp. (Ventosa 1998); *B. iranensis* was found in saline mud of the hypersaline lake Aran-Bidgol in Iran (Bagheri 2012); *Idiomarina sp.* W33 was found in saline soil of Yuncheng Salt Lake, China (Li 2014); *V. dokdonensis* isolated from sea water at Dokdo, an island in the East Sea, Korea (Yoon 2005); *Halobacillus trueperi* isolated from hypersaline sediments of the Great Salt Lake in Utah (Spring et al 1996); *Oceanobacillus iheyensis* isolated from deep-sea sediment collected at a depth of 1050 m on the Iheya Ridge (Lu et al 2001); *Marinobacter lipolyticus* was found in saline soil in Cadiz, Spain (Martin 2003); *V. halodenitrificans* was found in a marine solar saltern of the

Yellow Sea in Korea (Yoon et al 2004); *B. zhangzhouensis* and *B. australimari* were found in the aquaculture water of a shrimp farm of Zhangzhou city in China and surface sediment of the South China Sea, China (Liu et al 2016).

In addition, some moderately halophilic bacteria were found in fermented products. These moderately halophilic bacteria are important role in the quality of fermented products and it has been claimed to be important in aroma production, particularly fish sauce, shrimp paste and fermented fish products. Some moderately halophilic bacteria that was found in fish sauce such as *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Tetragenococcus halophilus*, *T. muriaticus*, *Halobacterium salinarum*, *Halococcus thailandensis* (Thongthai 1992; Tanasupawat 2001; Udomsil 2010), *Lentibacillus salicampi* and *L. juripiscarius* (Namwong 2005), *B. piscicola* (Daroonpunt et al 2016a); in *Pla-ra* such as *Salinivibrio siamensis*, *Gracilibacillus thailandensis* (Chamroensaksri 2009; Chamroensaksri 2010), *Piscibacillus salipiscarius*, *V. siamensis*, *V. dokdonensis*, *V. halodenitrificans*, *V. marismortui*, *B. vietnamnensis*, *Chromohalobacter salexigens* (Tanasupawat 2007; Tanasupawat 2010); in shrimp paste (*Ka-pi*) such as *Salinicoccus siamensis*, *L. kapialis* (Pakdeeto 2007a; Pakdeeto 2007b) *Oceanobacillus kapialis* (Namwong 2009), *V. kapii* (Daroonpunt et al 2016b), *T. muriaticus* (Kobayashi 2003), *Bacillus*, *Pseudomonas*, *Micrococcus*, *Kurthia* and *Sporolactobacillus* (Surono 1994).

2.3 Lipase and applications

Lipases (Triacylglycerol acylhydrolase, EC 3.1.1.3) are water soluble enzymes which catalyze a wide range of reactions, including hydrolysis, interesterification, alcoholysis, acidolysis, esterification and aminolysis. The lipases have the ability to hydrolyse triacylglycerols to release free fatty acids and glycerol (Berg 2002). The lipase-catalyzed reactions are:

- Hydrolysis

Triacylglycerol   Glycerol   Fatty acid

- Synthesis

1. Esterification

RCOOH + R'OH $\longrightarrow$ RCOOR' + H_2O

2. Interesterification

RCOOR' + R"COOR* $\longrightarrow$ RCOOR* + R"COOR'

3. Alcoholysis

RCOOR' + R"OH $\longrightarrow$ RCOOR" + R'OH

4. Acidolysis

RCOOR' + R"COOH $\longrightarrow$ R"COOR' + RCOOH
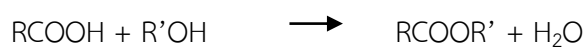
Lipases and esterases can be distinguished on the basis of their substrate spectra, as esterases catalyze the hydrolysis of carboxylic ester bonds of short chain fatty acids (<10 carbon atoms) and Esterase activity is found to be highest towards more water soluble substrates while true lipases have marked preference for long chain fatty acids (>10 carbon atoms) as substrates (Kumar et al 2012). Thus the lipase has to be capable of identifying an insoluble or heavily aggregated substrate and lipase activity is directly correlated with the total substrate area, and not with the substrate concentration (Verger 1997).

Lipases are ubiquitous in nature and are widely distributed in plants, animals and microorganisms such as bacteria, yeasts and fungi (Ghasemi 2010). Of all these, bacterial lipases are more economical and stable (Snellman 2002). Bacterial lipases

are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavor enhancement and lipolysis of butter fat and cream (Falch 1991). Lipase are also used in detergent industry as additive in washing powder (Fuji 1986), textile industry to increase fabric absorbency (Sharma 2001), for synthesis of biodegradable polymers or compounds (Linko 1998) and different transesterification reaction (Hasan 2006). In addition, the enzyme is used in cosmetic industry (Eugene 1974), in pulp and paper industry (Bajpai 1999), in synthesis of biodiesel (Noureddini 2005), degreasing of leather (Nakamura 1990) and in pharmaceutical industry (Higaki 2003). These applications of lipases are summarized in Table 2.1.

Bacterial lipases are mostly extracellular and are produced by submerged fermentation (Gupta 2004). The natural substrates of lipase are long-chain triacrylglycerols, which have very low solubility in water; and the reaction is catalyzed at the lipid-water interface. Most bacterial lipases are greatly influenced by nutritional and physic-chemical factors, for example temperature, pH, carbon and nitrogen sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Brune 1992; Aires-Barros 1994; Jaeger 1994; Kim 1996b). The carbon sources are the major factor for expression of lipase activity, such as an oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts and glycerol (Ghosh 1996; Dharmsthiti 1998; Shirazi 1998; Bradoo 1999; Rathi 2001). However, their production is significantly influenced by other carbon sources, such as sugar, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources (Gilbert 1991b; Lotrakul 1997; Dharmsthiti and Kuhasuntisuk 1998; Ghanem 2000; Rashid 2001).

Besides carbon source, the type of nitrogen source in the medium also influences the lipase activity in production broth (Ghosh et al. 1996). The organic nitrogen is preferred, such as peptone, yeast extract and tryptone (Gupta et al. 2004). In addition, Inorganic nitrogen sources have been reported to be effective for lipase

production, such as ammonium chloride and diammonium hydrogen phosphate (Gilbert 1991a; Bradoo et al. 1999; Dong 1999; Rathi et al. 2001).

Moreover, the physiological parameters such as pH, temperature, agitation, aeration and incubation period also play an important role in influencing production by different microorganisms. Most lipase can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common (Gupta et al. 2004).

Table 2.1 Industrial applications of microbial lipases (Vulfson 1994)

Industry	Action	Product or application
Detergent	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy food	Hydrolysis of milk fat, cheese ripening, modification of butter fat	development of flavoring agents in milk, cheese and butter
Beverages	Improved aroma	Beverages
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, hydrolysis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipid, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Cleaning	Hydrolysis	Remove of fats

2.4 Bacterial lipase production

Lipases are produced by many microorganisms and higher eukaryotes. Their production varies according to the strains, the composition of the growth medium, cultivation conditions, pH, temperature and the kind of carbon and nitrogen sources (Sharma 2014). Most commercially useful lipases are of microbial origin because of they have the shortest generation time, high yield of conversion of substrate into product, great versatility to adapt to environmental conditions and simplicity in genetic manipulation as well as in cultivation conditions (Ribeiro 2011). The bacterial lipases are diversified in their enzymatic properties and substrate specificity. Some of the lipase-producing bacteria are listed in Table 2.2.

Some halophilic bacteria are produced lipase such as *Idiomarina* sp. W33 (Li et al. 2014), *Marinobacter lipolyticus* (Martin et al. 2003), *Natronococcus* sp. TC6 (Boutaiba 2006) and many of *Bacillus genera* that have been isolated from different saline habitats such as salt lakes, salty foods, salterns and sea ice were showed lipase activity, such as *Bacillus* sp. MPTK 912 , *B. vallismortis* BCCS 007, *B. cereus* C71, *B. thermoleovorans* ID1, *B. coagulans* BTS3, *Geobacillus* sp. TW1, *Bacillus* sp. strain L2, *B. sphaericus* 205y, *B. bogoriensis* sp. nov., *B. salarius* sp. nov., *B.pumilus* and *B. sphaericus* JS1 (Mukesh Kumar 2012; Ghasemi 2011; Ertugrul 2007; Sulong 2006; Cho 2000; Li 2005; Kumar 2005; Shaoxin 2007; Lee 2001; Ruiz 2003; Heravi 2008; Shariff 2007; Vargas 2005; Lim 2006; Guzman 2008; Singh 2004). However, the lipase-producing moderately halophilic bacteria are rarely studied about their production and characterization.

Table 2.2 Lipase-producing bacteria (Sharma et al. 2001)

Gram positive bacteria	Species
Bacillus	*B. megaterium, B. cereus, B. stearothermophilus, B. subtilis, Recombinant B. subtilis 168, B. brevis, B. thermocatenulatus, B. coagulans, B. acidocaldarius, B. thermoleovorans* ID-1
Staphylococcus	*S.* canosus, *S. aureus, S. hyicus, S. epidermidis, S. warneri*
Lactobacillus	*Lactobacillus delbruckii sub sp. bulgaric*
Streptococcus	*Streptococcus lactis*
Micrococcus	*Micrococcus freudenreichii, M. luteus*
Propionibacterium	*Propionibacterium acne, Pr. granulosum*
Burkholderia	*Bu. glumae*
Gram negative bacteria	
Pseudomonas	*P. aeruginosa, P. fragi, P. mendocina, P. putida* 3SK, *P. glumae, P. cepacia, P. fluorescens, P. pseudoalcaligenes* F-111
Chromobacterium	*Ch. viscosum*
Acinetobacter	*Aci. Pseudoalcaligenes, Aci. radioresistens*
Aeromonas	*A. hydrophila, A. sorbia* LP004

2.5 Purification of lipase of halophilic bacteria

Purification methods used have generally depended on nonspecific techniques such as precipitation, Affinity chromatography, hydrophobic interaction chromatography, gel filtration and ion exchange chromatography (Sharma et al. 2001). Some of purification techniques are showed in Table 2.3.

- Precipitation

Ammonium sulfate precipitation

Ammonium sulfate has been one of the classical methods of protein separation. At higher salt concentrations, protein solubility usually decreases and leading to precipitation, this effect is termed "salting-out". Salts that reduce the solubility of proteins also tend to enhance the stability of the native conformation (Wingfield 2001). Ammonium sulfate, $(NH_4)_2SO_4$, is often used for salting out because of its high solubility, which allows for solutions of very high ionic strength, low price, and availability of pure material (Duong-Ly 2014). Salting out removes proteins that easily aggregate from those that are very soluble making it a good initial purification step for small soluble proteins (Englard 1990). Many reports of lipase purification used Ammonium sulfate precipitation in first step (Handelsman 1994; Nawani et al. 2000; Lee et al. 2001; Borkar et al 2009; Li and Yu 2012; Sharma and Kanwar 2012; Kumar 2012; Li et al. 2014; Sivaramakrishnan 2016).

Cold Acetone precipitation

Acetone precipitation is a common method for precipitation and concentration of proteins. This method can remove many lipid soluble contaminants. Proteins are insoluble in acetone (particularly at low temperatures) whilst many small molecules which could interfere with downstream protein work are soluble. One advantage of organic solvent fractionation is that it can be carried out at subzero temperatures. The miscible solvents form mixtures with water that freeze well below 0°C. It most important that the temperature is kept low because at temperature above +10°C denaturation effects become substantial. The reason for denaturation concerns the intramolecular hydrophobic interactions that help maintain protein structure. At low temperatures, the lack of conformational flexibility means that organic solvent molecules are unlikely to penetrate the internal structure and cause destabilization. But at higher temperatures small organic molecules enter

"cracks" in the surface and effect to loss of the internal hydrophobic forces in the protein molecule quickly results in autocatalytic denaturation in Figure 2.1 (Scopes 1987). In many researches were selected acetone precipitation for lipase purification (Shabtai 1992; Handelsman 1994; Imamura and Kitaura 2000; Peng et al 2010; Patel 2014)
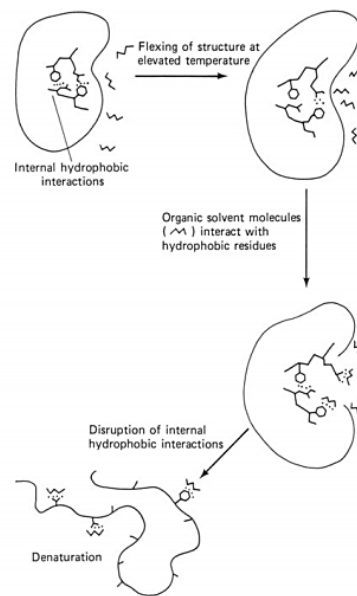
Figure 2.1 Effects of organic solvents in causing protein denaturation (Scopes 1987).

- **Hydrophobic interaction chromatography**

Hydrophobic interaction chromatography (HIC) is separated on the basis of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins and this interaction is depending on the salt concentration of the solution (Queiroz 2001). The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent which the decrease in salt concentration will lead to the elution of bound molecules. The surface of the beads was modified by hydrophobic alkyl or aryl groups. Such media include the polysaccharide-based Butyl-Sepharose,

Octyl-Sepharose and Phenyl-Sepharose materials (Roe 1989) that are derived from polymers that had proven to be suitable for chromatographic separation of proteins.

- **Gel filtration chromatography**

Size exclusion chromatography (SEC, also known as gel-filtration chromatography) is a technique for separating protein on the basis of molecular size. In this method, large proteins elute through the column sooner than small ones. The solid phase consists of beads with pores of a particular size. Large proteins cannot enter the pores, and so take a short path through the column, whereas small ones enter the pores, and migrate through the column more slowly (Cutler 1998). In addition to obtaining molecular parameters, SEC is useful also for preparative fractionation of polymers and for separating small molecules from complex polymeric or biogenic matrixes as an aid to sample cleanup (Barth 1996).

Advances in SEC packings include the use of mixed-bed columns, allowing a wide molecular weight separation range with fairly linear calibration. For protein separations, composite packings, such as the Superdex series, are increasing in popularity because of their high pore volume, inertness, and small particle size availability example, Superdex 75 (separation range 3 kDa - 70 kDa) and Superdex 200 (separation range 10 kDa – 600 kDa); Seperose series, are SEC media with high physical and chemical stability based on highly cross-linked porous agarose particles and fractionation range for molecular weight between 5 kDa to 5000 kDa example, Superose 6 (separation range 5 kDa - 500 kDa) and Superose (separation range 1 kDa - 300 kDa) (Meyer 1998; Podzimek 2011).

- **Ion exchange chromatography**

Ion-exchange chromatography is one of the most widely used forms of column chromatography. It is used in research, analysis, and process-scale purification of proteins (Selkirk 2004). Ion exchange chromatography separates proteins with difference in their charge. The separation is based on the interaction

between a charged protein and an oppositely charged chromatographic medium. The elution is done with gradient in order the change elution conditions. This elution is usually performed by increases in salt concentration. The charged molecules in a buffer solution come from the buffer components (e.g., salts). The charged groups on a protein are provided by the different amino acids in the protein. Lysine, arginine, and histidine have a positive charge at physiological pH, whereas aspartic acid and glutamic acid have a negative charge at physiological pH (Selkirk 2004). Ion-exchange matrices are divided into two major types according to the charge on the ion-exchange ligands (Jungbauer 2009; Meyer 2006).

- Cation Exchange: Cation-exchange resins have negatively charged groups on the surface. These are used to bind proteins that have an overall positive charge. Proteins will have an overall positive charge at a pH below their isoelectric point. Therefore, cation exchange is used at a pH below the isoelectric point of the protein(s) to be bound.

- Anion Exchange: Anion-exchange resins have positively charged groups on the surface. These are used to bind proteins that have an overall negative charge. Proteins will have an overall negative charge at a pH above their isoelectric point. Therefore, anion exchange is used at a pH above the isoelectric point of the protein(s) to be bound.

Ion exchangers are also divided into strong and weak ion exchangers. Strong ion-exchange ligands maintain their charge characteristics, and therefore ion-exchange capacity, over a wide pH range, whereas weak ion-exchange ligands show a more pronounced change in their exchange capacity with changes in pH (Gorbunoff 1985). DEAE–Sepharose Fast Flow (weak anion) has a working pH range of 2.0–9.0, whereas Q–Sepharose Fast Flow (strong anion) has a working pH range of 2.0–12.0. CM–Sepharose Fast Flow (weak cation) has a working pH range of 6.0–10.0 and SP–Sepharose Fast Flow (strong cation) has a working pH range of 4.0–13.0 (Selkirk 2004).

- **Affinity chromatography**

Affinity chromatography is separates proteins based on the binding affinity and the most selective and versatile form of liquid chromatography relying on the reversible and specific binding between a protein and its cognate ligand, e.g. binding of a hormone with its receptor, interaction of an enzyme with its substrate, and binding of an antibody with its target antigen (Arora 2017). The technique can be used whenever a suitable ligand is available for the protein of interest. Affinity chromatography has high selectivity and resolution, also give specific results for protein interests. This specificity of the interaction is used in affinity chromatography by immobilizing one of the interacting agents, called "affinity ligand" on to the column as a stationary phase support. The specific ligand is covalently immobilized onto a solid support, commonly agarose or derivatives (Roque 2007). This method can be applied for the large-scale purification of antibodies to achieve desired yield and purity of the product (Cuatrecasas 1971; Ayyar 2012).

Table 2.3 Purification techniques and molecular weight of some halophilic bacteria

Species	Purification techniques	Mw	References
Idiomarina sp. W33	1. 75% ammonium sulphate precipitation 2. DEAE-Sepharose anion exchange 3. Sephacryl S-200 gel filtration	67 kDa	Li et al. 2014
Bacillus sp. MPTK 912	1. Sephadex G-100 column	66 kDa	Mukesh Kumar et al. 2012
B. thermoleovoran ID-1	1. Ammonium sulphate precipitation 2. DEAE-Sephacel ion exchange 3. Sephacryl S-200 gel filtration	34 kDa	Lee et al. 2001
Pseudomonas aeruginosa MB5001	1. Ultrafiltration 2. Ion exchange chromatography 3. Gel filtration chromatography	29 kDa	Chartrain 1993
Proteus vulgaris	1. Ion exchange chromatography	31 kDa	Kim 1996a
Bacillus sp. J33	1. Ammonium sulphate 2. phenyl Sepharose column	45 kDa	Nawani 2000

Table 2.3 Purification techniques and molecular weight of some halophilic bacteria (Cont.)

Species	Purification techniques	Mw	References
Chromohalobacter sp. LY7-8	1. ammonium sulphate 2. dialysis 3. anion exchange chromatography 4. cation exchange chromatography	44 kDa	Li and Yu 2012
Bacillus licheniformis MTCC-10498	1. Ammonium sulphate 2. dialysis 3. hydrophobic interaction chromatography (Octyl sepharose)	19 kDa	Sharma and Kanwar 2012
Bacillus pumilus RK31	1. ammonium sulphate 2. Dialysis 3. Sephadex G-200 gel filtration 4. DEAE cellulose ion exchange	62.2 kDa	Kumar 2012
Geobacillus thermodenitrificans IBRL-nra	1.Ultrafiltration 2. HiTrap Heparin column affinity chromatography 3. Sephadex G-100 gel filtration	30 kDa	Balan 2012
Pseudomonas aeruginosa SRT 9	1. Ammonium sulphate 2. dialysis 3. phenyl Sepharose CL-4B 4. Mono Q HR5/5 column	29 kDa	Borkar et al 2009

Table 2.3 Purification techniques and molecular weight of some halophilic bacteria (Cont.)

Species	Purification techniques	Mw	References
Vibrio fischeri	1. Ammonium sulphate 2. dialysis 3.DEAE cellulose Ion exchange	57 kDa	Ranjitha et al. 2009
Pseudomonas aeruginosa Ps-x	1. Ammonium sulphate 2. dialysis 3. Quaternary amino methyl Sepharose fast flow (Q-sepharose)	15.5, 54.97 kDa	Saeed 2005
Microbacterium sp.	1. Ammonium sulphate 2. dialysis 3. Saphadex G-100	40 kDa	Tripathi et al. 2014
Bacillus coagulans BTS-3	1.ammonium sulphate 2.dialysis 3. DEAE-Sepharose column	31 kDa	Kumar et al 2005
Bacillus thermoleovorans CCR11	1. polyethersulfone ultrafiltration membrane (Cut off 500 kDa) 2. Rotofor® preparative electro focusing cell) 3 polyethersulfone ultrafiltration membrane (Cut off 10 kDa)	11 kDa	Castro-Ochoa 2005

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

- **Bacterial culture**

    1. Incubator shaker, Certomat$^{®}$ BS-1, Sartorius , Germany

    2. Incubator, Model: BE600, Memmert, Germany

    3. Microplate reader (Microplate Reader Wallac 102, PerkinElmer^{TM})

    4. Autoclave, Model: HA-3D, Hirayama, Japan

    5. Laminar flow hood, Model: BV-126, ISSCO, Thailand

    6. PCR Authorized Thermal Cycler, Eppendorf, Germany

    7. Mini-Run Gel Electrophoresis System, Model: GE-100 , Hangzhou Bioer technology, China

    8. Casamino acid, Difco, USA

    9. Yeast extract, Difco, USA

    10. Sodium chloride (NaCl), Carlo erba

    11. L(+)-glutamic acid monosodium salt monohydrate 99%, ACROS Organic, Belgium

    12. Potassium chloride (KCl), Carlo erba

    13. Magnesium sulfate (MgSO$_4$. 7H$_2$O), Carlo erba

    14. Manganese (II) chloride tetrahydrate (MnCl$_2$.4H$_2$O), Carlo erba

    15. Trisodium citrate dehydrate (C$_6$H$_5$Na$_3$O$_7$.2H$_2$O), Merck, Germany

- **Lipase production and determination**

    1. Ultraviolet-visible (UV-Vis) spectrophotometer, Helios alpha, Thermo Scientific™, USA

    2. Incubator shaker, Certomat$^{®}$ BS-1, Sartorius, Germany

3. Incubator, Model: BE600, Memmert, Germany

4. Thermo mixer C, Eppendorf, Germany

5. Vortex mixer, Model: Vortex-Genie 2, Scientific Industries, United States

6. pH meter, Model: SevenEasy™, METTLER TOLEDO, Italy

7. Shaking water bath, Model WNB22, Memmert, Germany

8. Electronic analytical balance, Model: AB204-S, METTLER TOLEDO, Italy

9. Stirrer, Model: IKA$^®$ C-MAG HS-7

10. *p*-Nitrophenyl-butyrate, *p*-Nitrophenyl-palmitate, Sigma-Aldrich

11. Beef extract, Difco, USA

12. Urea ($CO(NH_2)_2$), Merck, Germany

13. Triton$^®$X-100, Sigma-Aldrich, USA

14. Acetic acid, Merck, Germany

15. Ortho-phosphoric acid (85%), Merck, Germany

16. Boric acid, Carlo erba

17. Peptone, Difco, USA

18. Ammonium sulfate (Na_2SO_4), Merck, Germany

19. Gum arabic from acacia tree, Sigma-Aldrich, USA

- **Purification**

1. ÄKTA explorer 100, GE Healthcare

2. Fraction collector Frac-901, GE Healthcare

3. Sonicator

4. Microplate reader, Model: SpectraMAX 190, Molecular Devices

5. Thermo mixer C, Eppendorf, Germany

6. Centrifuge 5810 R, Eppendorf, Germany

7. Incubator, Model BE600, Memmert, Germany

8. Centrifuge, Model: Avanti$^®$ J-E, Beckman Coulter

9. Mini-slab size electrophoresis system , Model: AE-6530, ATTO, Japan

10. Stirrer, Model: IKA$^®$ C-MAG HS-7

11. pH meter, Model: SevenEasy^{TM}, METTLER TOLEDO, Italy

12. Concentrator plus, Eppendorf, Germany

13. ChemiDoc™ MP Imaging System, BIO-RAD, USA

14. Rocker, Model: VSR-50, PRO Scientific, USA

15. PowerPac™ Basic Power Supply, BIO-RAD

16. Trizma$^®$ base, Sigma-Aldrich, USA

17. Sodium dodecyl sulfate electrophoresis purity reagent, BIO-RAD,
    United States

18. Sodium hydroxide (NaOH), Merck, Germany

19. TEMED, OmniPur$^®$, Merck, Germany

20. Acetone, Carlo erba

21. Ethanol absolute for analysis, Merck, Germany

22. Ethanol gradient grade for liquid chromatography, Merck, Germany

23. Ammonium persulfate (($NH_4)_2S_2O_8$), Sigma-Aldrich, USA

24. Bovine serum albumin (BSA), Sigma-Aldrich, USA

25. Gel filtration standard, BIO-RAD, USA

3.2 Methods

3.2.1 Sample collection and bacterial isolation

Four fermented fish samples, comprised of one *Kee-dee* [Fermented three spot gouramifish (*Trichopodus trichopterus*) entrails], one *Tai-pla* (Fermented fish entrails) sample collected from Mueang district, Nakhon Si Thammarat province and two *Koey-pla* (Fish paste) samples collected from Mueang and Cha-uat district, Nakhon Si Thammarat province. One samples of *Ka-pi* (shrimp paste) and *Poo-khem* (salting crab) were collected from various places in Mueang district, Nakhon Si

Thammarat province and three samples of fish sauce (*nam-pla*) were collected from the factory in Chonburi) and Nakhon Si Thammarat provinces (two samples). All of Samples were stored in sterile plastic containers, except the samples of fish sauce were stored in sterile glass bottles. All of these samples were preserved in cold room at 4°C. One gram of samples was suspended in 9 ml of sterile JCM medium No. 377 (Appendix A-1) and prepared serial dilution by the 10-fold dilution method. 0.1 ml of 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} suspensions were spread on JCM medium No. 377 (10% NaCl) and JCM medium No. 168 (20% NaCl) and incubated at 37°C for 1-2 weeks. A single colony of the halophilic bacteria was transferred to a JCM medium No. 377 and incubated at 37 °C, 3-7 days. The pure culture was preserved in a JCM medium No. 377 slant for a short period and a freezed dry tube for long term preservation.

3.2.2 Screening of lipase activity

All of isolated halophilic bacteria were screened on lipolytic agar (Barrow 1993) composed of peptone 1%, $CaCl_2.2H_2O$ 0.01%, agar 2% and 1% of Tween 20, Tween 40, Tween 60 or Tween 80 supplemented with 10% NaCl and were incubated at 37 °C, 3-7 days. Isolated halophilic bacteria that show clear zone or turbidity zone around colony were selected for further study.

The selected isolates were cultivated in the complex medium (CM) (Appendix A-4) containing (g/l): casein peptone 7.5; yeast extract 10.0; sodium citrate 3.0; $MgSO_4.7H_2O$ 20.0; KCl 2.0; $FeSO_4.7H_2O$ 0.01; NaCl 5% and pH 7.0 for their lipase production. Lipase activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) as substrate according to Li et al. (2014), with some modifications. The substrate *p*-NPP was dissolved in 2-propanol and mixed with 9 ml of Tris-HCl buffer (10 mM, pH 8.0) to give a final concentration of 1 mM. After pre-incubation for 5 min, the reaction was initiated by adding 0.5 ml of appropriately diluted enzyme solution to 0.5 ml of substrate solution, and incubation was carried out at 37°C for 1 h. Following the

addition of 1 ml of 20% Sodium dodecyl sulfate (SDS) to stop the reaction, the amount of *p*-nitrophenol (*p*-NP) released was measured at 405 nm against a blank. One unit (U) was defined as the amount of enzyme liberating 1 nanomole of *p*-NP per minute under the standard assay conditions. The halophilic isolates that show highest lipase activity was selected for further study.

3.2.3 Identification methods

3.2.3.1 Phenotypic characteristics

The morphological and cultural characteristics will be determined as described by Barrow and Feltham 1993, Leifson 1963 and Namwong et al 2005. The isolates will be cultivated on JCM no. 377 containing 5% (w/v) NaCl at 37°C for 2-3 days and will be examined for their characteristics. Gram staining was examined by modification method of Hucker-Conn (Hucker 1923) which colonial appearances were examined after incubated for 3 days. The growth of isolates was determined after 5 days culture. The isolates were cultured on JCM medium no.377 with various incubation temperatures (25°C, 30°C, 37°C, 40°C, 45°C and 50°C) and pH (5.0-9.5, with an interval of 0.5). Effects of growth at various NaCl concentrations were investigated in modified JCM medium no. 377 broth omitting the MgSO$_4$.7H$_2$O with different concentrations of NaCl (0, 1, 3, 5, 7, 10, 15 and 20%, w/v). Oxidase test was examined using a few drops of 1% tetramethyl-*p*-phenylenediamine on the moist paper disc that it smeared the tested culture. The appearance of dark-purple color on filter paper disc within 30 sec, denoted a positive reaction. Catalase test was examined by transferring one colony of pure culture onto the slide and then placed 3% of hydrogen peroxide onto the slide. The evolution of gas bubbles indicating a positive test was observed. L-Arginine hydrolysis was examined using arginine agar slant (Appendix A-6). The positive reaction is shown by a color change of the indicator to red after incubated for 5-7 days. Starch hydrolysis was examined using

JCM medium no. 377 containing 1% of soluble starch. The clear colorless zones around colonies after flooding with Lugol's iodine solution (Appendix A-17) indicated the positive result. Gelatin hydrolysis was examined using JCM medium no. 377 agar containing 1% of gelatin but omitted casamino acid. The clear zones around colonies after flooding with 5-10 ml of 30% trichloroacetic acid (Appendix A-18) indicated the positive result. Tween 80 hydrolysis was examined using JCM medium no. 377 agar containing 1% of Tween 80. Clear zones area indicated the Tween 80 hydrolysis. Indole test was examined using tryptone broth (Appendix A-19), it was tested by adding 4 drops of Kovac's reagent (Appendix A-11), using *iso*-amyl alcohol as solvent. The ability to reduce nitrate to nitrite was determined using nitrate broth (Appendix A-10). After incubation the culture broth was added with solution A and solution B reagent (AppendixA-12). The pink to red color represented the presence of nitrite (positive). Acid from carbohydrate was performed in marine oxidation-fermentation medium (MOF) (Appendix A-5) as described by Leifson (1963) supplemented with 1% (w/v) carbon sources. The media were adjusted to pH 7.2 and 0.2% (w/v) of phenol red solution was added as an indicator solution. Growth was recorded daily for up to 7 days. The positive results were shown by color change of the indicator from red to yellow. The API ZYM was examined using API ZYM strip (bioMérieux).

3.2.3.2 Chemotaxonomic characteristics

Freeze-dried cells were used for chemotaxonomic analysis. Halophilic isolates were cultivated in JCM medium no. 377 at 37°C for 2 days. The culture broths were centrifuged at 10,000 rpm and discarded the supernatant. The cells were washed twice with sterile distilled water before freeze-drying.

- **Diaminopimelic (DAP) acid isomers analysis**

Diaminopimelic acid isomers were analyzed by cellulose thin-layer chromatography according to the method of Staneck 1974. Approximately 10 mg of dried cells were hydrolysed with 1 ml of 6 N HCl at 100°C for 18 and then the whole

cell hydrolysate were filtrated with a filter paper and evaporated to dryness and then dissolved with 0.3 ml of water. Three µl of each sample was applied on the base line of 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 spots were visualized by spraying with 0.2% ninhydrin solution and heated at $100^{\circ}C$ for 5 min. The dark-green spot of DAP isomers were appeared and disappeared in a few minutes. DL-diaminopimelic acid (DAP) which contains *meso*-DAP and LL-DAP isomers was used as the standard.

- **Cellular fatty acids analysis**

Cellular fatty acids analysis was performed by gas chromatography according to the method of Microbial Identification System (MIDI) Sherlock version 6.0 (Sasser 1990). Dry cell (40 mg) was suspended in 0.1 ml of reagent 1 (sodium hydroxide 15 g, methanol 50 ml and milli-Q water 50 ml) and vigorously mixed for 5-10 sec with a vortex mixer. The solution will be heated at $100^{\circ}C$ for 5 min, mixed and heated again at $100^{\circ}C$ for 25 min. After cooling, 2 ml of reagent 2 (6 N-HCl 65 ml, methanol 55 ml) was added to the test tube, mixed and heated at $80^{\circ}C$ for 10 min. The mixture was added with 1.25 ml of reagent 3 (n-hexane 50 ml, methyl-tert-butyl ether 50 ml) and mixed for 10 min. The upper layer was transferred to a new tube and added with 3 ml of reagent 4 (sodium hydroxide 1.2 g, milli-Q water 100 ml). The tube was mixed for 5 min and transferred 2/3 of the sample to a GC vial. The cellular fatty acids were analyzed by using gas chromatography.

- **Polar lipids analysis**

The polar lipids were extracted according to the method of Minnikin 1977. Dried cells (100-150 mg) were suspended in 3 ml of methanol-0.3% NaCl (100:10) and 3 ml of petroleum ether. The solutions were mixed for 15 min and the upper layer was removed. The lower layer was added with 1 ml of petroleum ether, mixed well and the upper layer was removed again. The lower layer was heated at $100^{\circ}C$

for 5 min. After cooling, 2.3 ml of chloroform-methanol-water (90:100:30) was added to the solution, mixed well for 15 min, centrifuged at 3000 rpm for 10 min and the supernatant was transferred to another tube. The lower layer was added with 2.3 ml of chloroform-methanol-water (50:100:40) twice and the supernatants was transferred to the tube in previous step. The combined supernatant 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 polar lipids will identified by using 2-dimension TLC technique, 10 µl of the samples will be applied to the corner of the silica-gel TLC plate (10 x 10 cm) and developed on the first solvent system chloroform-methanol-water (65:25:4, v/v) and the second dimension will be developed on the second solvent system chloroform-acetic acid-methanol-water (40:7.5:6:2, v/v). For comparison of chromatogram patterns, each TLC plate will be sprayed with specific reagents (Appendix B-2) including anisaldehyde reagent, Dittmer & Lester reagent, Dragendroff's reagent, ninhydrin reagent, and phosphomolybdic acid reagent. Anisaldehyde reagent was used for glycolipid detection. After spraying with the reagent and heating at 110°C for 10 min, glycolipids were presented as green yellow spots. Dittmer & Lester reagent was used for all phospholipid detection. After spraying with the reagent, all phospholipids were presented as blue spots. Dragendroff's reagent was used for choline-containing phospholipids (phosphatidyl choline, PC) detection. After spraying with the reagent, phosphatidyl choline was presented as a brown spot. Ninhydrin reagent was used for phosphatidylethanolamine (PE) and its derivative detection. After spraying with the reagent and heating at 110°C for 10 min, PE and its derivatives were presented as purple spots. Phosphomolybdic acid reagent was used for all polar lipid detection. After spraying with the reagent and heating at 130°C for 10 min, all polar lipids were presented as dark pots.

- **Analysis of quinone system**

The quinone components were extracted by the method of Collin 1977. Dried cells (100-300 mg) were extracted with 20ml of chloroform-methanol (2:2, v/v) and stirred overnight. The solvent was filtrated with a filter paper and the filtrate was evaporated and dissolved in a small amount of acetone. The acetone solution was applied to a silica-gel TLC plate (Merck kiesel-gel 60 F_{254}, 20 x 10 cm) and developed with benzene. The menaquinone spot were visualized by UV light at 254 nm, scraped off and extracted with acetone. The acetone extract was filtrated through 0.5 μm membrane and analyzed by HPLC using a cosmosil 5 C_{18} column, according to the method of Tamaoka 1983.

- **DNA base composition analysis**

The genomic DNA were extracted by the method described by Tamaoka 1994. One gram of wet cells were suspended in 2 ml of saline-EDTA with 5-10 mg of lysozyme and incubated at $37^{\circ}C$ for 30 min. The solution was added with 8 ml of Tris-NaCl (0.1M Tris and 0.1 M NaCl, pH9) and 0.05-0.1 ml of 10% SDS and then heated at $55-60^{\circ}C$ for 10 min. To separate the proteins, 5 ml of phenol-chloroform (1:1) was added to the tube, shaked for several minutes and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a small beaker and then added with 5-10 ml of 95% ethanol to precipitate the DNA. The DNA was spooled with a glass rod and dry in room temperature. After drying, the DNA was dissolved in 3 ml of 0.1 SSC (0.1 M NaCl and 0.015M Na-Citrate, pH7) and eliminated the RNA by adding 0.3 ml of RNase A solution, and incubated at $37^{\circ}C$ for 20 min. The DNA solution was added with 0.5 ml of 10 x SSC and extracted with phenol-chloroform (1:1) again. The DNA was precipitated from the supernatant, spooled with a glass rod, dry and re-dissolved in 0.1 SSC. The DNA solution was measured for the purity by using spectrophotometer at OD_{260}/OD_{280} (1.8 < OD_{260}/OD_{280}< 2.0).The DNA solution

(10 µg/ml) was heated at 100°C for 10 min. The denatured DNA was added with 10 µl of nuclease P1 solution and incubated at 50°C for 1 hour and then added 10 µl of alkaline phosphatase solution, incubated at 37°C for 1 hour. The DNA G+C content were determined by using HPLC according to the method of Tamaoka 1984.

3.2.3.3 Genotypic characteristics

- **16S rRNA gene sequencing and phylogenetic analysis**

The sequences of 16S rRNA gene were amplified by polymerase chain reaction (PCR) according to the method as described by Yamada 2000. The sequencing reaction of each sample was performed in a DNA thermal cycler (Gene Amp$^{®}$ PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96℃ followed by 25 cycles of 10 sec at 96℃ (DNA denaturation), 5 sec at 50℃ (primer annealing) and 4 min at 60℃ (DNA extension). The PCR products were checked by gel electrophoresis and compared the product size with 1 kb DNA marker. After gene amplification, the PCR products were sent to the Macrogen, Korea to analyze the nucleotide sequence of the PCR products. The following primers were used for sequencing: 27F (5' AGA GTT TGA TCA TGG CTC AG 3'), 518F (5' GTA TTA CCG CGG CTG CTG G 3'), 800R (5' TAC CAG GGT ATC TAA TCC 3'), 1492R (5' CGG TTA CCT TGT TAC GAC TT 3'). Sequence homology will be performed on the standard BLAST sequence similarity searching program from the web site http://eztaxon-e.ezbiocloud.net/ (Kim 2012). The sequence determined was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases employing CLUSTAL X version 1.83 (Thompson 1997). The alignment was manually edited, and positions with gaps and ambiguous bases were eliminated prior to reconstruction of a phylogenetic tree. The phylogenetic trees based on the neighbour-joining method (Saitou 1987), maximum-likelihood (Felsenstein 1985) and maximum-parsimony methods (Kluge 1969) were reconstructed in MEGA 6 software (Tamura et al. 2013).

The confidence values of branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein 1985) based on 1000 resamplings.

- **DNA-DNA hybridization**

DNA-DNA hybridization was determined according to Ezaki 1989. Genomic DNA was prepared by the method of Tamaoka (1994) as describe before. To fix the DNA on the 96-well plate, 0.2 ml of the DNA solution (0.1 mg/ml) was boiled for 10 minutes and immediately cooled in ice. The denature single-strand DNA solution was added with 1 ml of 2xPBS, 0.6 ml of distilled water and 0.2 ml of $MgCl_2$ and then mixed well. The DNA solution (0.1 ml) was dispensed in the microplate wells and incubated at $37^{\circ}C$ for 2 h for fixing the DNA. The solution in the well was removed and washed with 0.2 ml of PBS. The microplate was dried up at $60^{\circ}C$ for 2 h and preserved in the desiccators until used in the experiment.

The DNA probe was labeled with photobiotin. Briefly, 10 μl of DNA solution (1 mg/ml) was added with 15 μl of photobiotin solution (1mg/ml) and radiated with a sunlamp for 30 min. After the irradiation step, 0.2 ml of 0.1 M Tris-HCl buffer (pH 9.0) and 0.4 ml of 2- butanol was added to the DNA probe solution. The mixed solution was centrifuged at 12,000 rpm for 20 sec and the upper layer was discarded. The lower layer was added with 0.4 ml of 2-butanol, mixed well, and centrifuged at 12,000 rpm for 20 min. The lower layer was transferred to the new microtube and boiled for 15 min. After boiling, the solution was immediately cooled in an ice box and then sonicated for 3 min. The biotinylated DNA probe solution was dissolved in 10 ml of the hybridization solution and then 0.1 ml of the solution was dispended to each well of the microplate. The microplate was incubated at optimal temperature for 15 h, then the hybridization solution in the well was discarded and the well was washed with 0.2 ml of 0.2 x SSC, three times. After the washing step, the plate was added with 0.2 ml of solution 1, incubated at room temperature for 10 min and then removed the solution 1. The solution 2 (0.1 ml) was added to each microplate well,

incubated at 37°C for 30 min and discarded the solution 2. The microplate was analyzed with calorimetric method by adding 100 µl of peroxidase-streptavidin solution to each well and incubated at 37°C for 30 min and then the solution was discarded. The plate was washed twice with 300-400 µl of PBS. After washing, the plate was added with 100 µl of tetramethylbenzidine- H_2O_2 solution, incubated at 37°C for 5 min, the reaction was stopped by adding 100 µl of H_2SO_4 and measured the absorbance at 450 nm using the microplate reader (Microplate Reader Wallac 102, PerkinElmer^{TM}). The results were calculated for the percentage of DNA homology.

3.2.4 Optimization of lipase-producing selected halophilic bacteria

The modified JCM medium no. 377 (5% w/v NaCl) was used as the basal medium (Appendix A-3) for lipase production. One loopful of selected strain was cultivated in 50 ml of basal medium in 250 Erlenmeyer flask and incubated at 37°C under shaking condition (200 rpm) for 48 hour was used as an inoculum. The inoculums was inoculated in 50 ml of modified JCM medium no. 377 in 250 Erlenmeyer flask (initial cell grow at A_{600} ~ 0.2) and incubated at 37°C under shaking condition for 48 hour. The sample was assayed for lipase activity in cell-free supernatant and for growth by measuring an absorbance at 600 nm.

3.2.5.1 Effect of carbon source on lipase production

The selected halophilic bacteria, KN3-8-4 was inoculated in the basal medium containing different of carbon sources including coconut oil, olive oil, palm oil, sesame oil, soy bean oil, canola oil, sun flower oil, corn oil, camellia oil at 0.5% (v/v) and casamino acid at 0.5% (w/v). The culture was incubated at 37°C for 48 hour. After incubation, the growth and lipase activity was determined. The carbon source that was shown highest lipase activity was selected for next study.

3.2.5.2 Effect of nitrogen source on lipase production

Strain KN3-8-4 was inoculated in the basal medium containing 0.5% of palm oil as carbon source and different of nitrogen sources including yeast extract, peptone, tryptone of casein, beef extract, potassium nitrate, ammonium sulfate, urea and ammonium nitrate at 0.5% (w/v). The culture was incubated at 37°C for 48 hour. After incubation, the growth and lipase activity was determined. The nitrogen source that was shown highest lipase activity was selected for next study.

3.2.5.3 Effect of NaCl on lipase production

In order to investigate the influence of NaCl on growth and lipase production, Strain KN3-8-4 was grown in the suitable modified basal medium containing 0.5% (v/v) palm oil as carbon source, 0.5% (w/v) yeast extract as nitrogen source and various concentrations of NaCl (0, 1, 3, 5, 7, 9, 11, 13, 15%, w/v). The culture was incubated at 37°C for 48 hour. After incubation, the growth and lipase activity was determined. The concentration of NaCl that was shown highest lipase activity was selected for next study.

3.2.5.4 Effect of initial pH on lipase production

In order to investigate the influence of pH on growth and lipase production, Strain KN3-8-4 was grown in the suitable modified basal medium containing 0.5% (v/v) palm oil, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. The culture was incubated at varying initial pH (5, 6, 7, 7.5, 8, 8.5, 9 and 10). After incubation at 37°C for 48 hour, the growth and lipase activity was determined.

3.2.5.5 Effect of incubation temperature on lipase production

In order to investigate the influence of temperature on growth and lipase production, Strain KN3-8-4 was grown in the suitable modified basal medium (pH8.5) containing 0.5% (v/v) palm oil, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. The

culture was incubated at varying temperature (25, 30, 37, 40°C). After incubation for 48 hour, the growth and lipase activity was determined.

3.2.5.6 The kinetic of the growth and lipase production

The strain KN3-8-4 was inoculated on suitable modified basal medium with initial inoculum $A_{600} \sim 0.2$ in 250 Erlenmeyer flask and incubated at 40°C under shaking condition (200 rpm) for 60 hour. Samples were taken at 6 hour interval and assayed for lipase activity and monitored for growth by measuring an absorbance at 600 nm.

3.2.5 Purification of lipase from selected halophilic bacteria

3.2.5.1 Enzyme preparation

The strain KN3-8-4 was cultivated in suitable modified JCM medium no.377, which omitted 0.5% casamino acid but containing 0.5% (v/v) palm oil, pH 8.5 at 40°C under shaking condition (200 rpm). After 36 hour of cultivation, the cells from the cultures were removed by centrifugation (10,000 rpm, 20 min at 4°C). The supernatant was used for enzyme purification.

For enzyme preparation, the supernatant was precipitated with cold acetone according to the modified method of Peng 2010. Cold acetone (-20°C) was added to the supernatant to 40% (v/v) as a final concentration. After centrifugation, the enzyme remaining in the supernatant was precipitated by adding cold acetone to 60% (v/v) as a final concentration. The precipitate was collected and dissolved in 20 mM Tris-HCl buffer (pH8.0). The removal of salts were performed by using an Amicon® Ultra-15 centrifugal filter devices having Molecular weight cutoff (MWCO) (3,000 Da and spinned at 4°C, 5000 g for 30 min and then added the volume up to initial volume with 20 mM Tris-HCl buffer (pH8.0) and repeated again with same condition.

3.2.5.2 Enzyme purification

All of the following purification steps were performed at 4°C by using the ÄKTA explorer 100 purification systems (GE Healthcare). The sample was concentrated by Concentrator (Eppendorf) prior to size exclusion chromatography. First step, the concentrated supernatant was chromatographed by FPLC (Fast Protein Liquid Chromatography) with a Superose 12 10/300 GL gel filtration column (GE Healthcare), which was equilibrated with approximately two bed volumes of 20 mM Tris-HCl (pH8.0) containing 150 mM NaCl. The sample was loaded onto the column at flow rate 0.1 ml/min. The column was eluted with same buffer (20 mM Tris-HCl (pH8.0) containing 150 mM NaCl). Fractions of 1.0 ml were collected and the fraction was showed lipase activity from the Superose 12 10/300 GL column were pooled and purified in second step with HiTrap™ DEAE FF column (0.7 x 2.5 cm) (GE Healthcare). Each of fraction was measured an absorbance at 280 nm.

Second step, the pooled fractions with lipase activity from Superose 12 10/300 GL column was applied to a HiTrap™ DEAE FF column, which equilibrated with approximately five bed volumes of 20 mM Tris-HCl (pH8.0). The sample was loaded onto the column at flow rate 0.5 ml/min. The column was eluted with segments salt gradient (0-1M NaCl in 20 mM Tris-HCl (pH8.0) (Figure 3.1). Fractions (1.0 ml) were collected and subjected to A_{280} measurement. The lipase active fraction were pooled and used for further studies. The purity of the purified enzyme was analyzed by polyacrylamide gel electrophoresis.
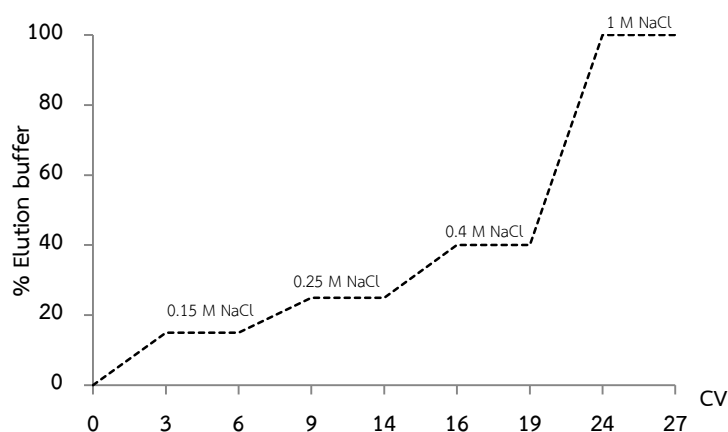
Figure 3.1 Elution profile of lipase on HiTrap^{TM} DEAE FF column. Elution was carried out with a segments gradient of 0-1 M NaCl in 20 mM Tris-HCl (pH8.0).

3.2.6 Protein determination

Protein concentration will be determined by Pierce^{TM} BCA protein assay kit (Thermo Scientific^{TM}, USA). A standard curve was drawn using bovine serum albumin.

3.2.7 Determination of lipase activity

Lipase activity was assayed by the colorimetric modified method of Lee 1999 by measuring the micromole of *p*-nitrophenol released from *p*-nitrophenyl butyrate (*p*-NPB). The substrate *p*-NPB was dissolved in ethanol and mixed with 900 μl of Universal buffer (Britton-Robinson buffer, pH 8.0) (Appendix C-1) to give a final concentration of 2 mM. After pre-incubation for 5 min, the reaction was initiated by adding 50 μl of enzyme solution to 50 μl of substrate solution, and incubation was carried out at 40℃ for 30 min. Following the addition of 100 μl of 20% Sodium dodecyl sulfate (SDS) to stop the reaction, the amount of *p*-nitrophenol (*p*-NP) released was measured at 405 nm against a blank. One unit (U) was defined as the amount of enzyme liberating 1 nanomole of *p*-NP per minute under the standard assay conditions.

3.2.8 Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE was performed by using 4% stacking gel and 12.5% running gel according to the modified method of Laemmli 1970. Samples were dissolved in a sample buffer containing 20% (v/v) glycerol, 2 mg coomassie blue, and 0.5 M Tris-HCl buffer (pH 6.8) and was not heated. The electrophoresis was carried out at a constant current of 20 mA per gel using an ATTO AE-6530 Dual mini-slab system in the cold condition. After separation, protein bands will be stained using PlusOne Silver staining kit (GE healthcare) (Appendix C-10).

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 2% BME; 0.002% Bromophenol blue) and boiled for 10 min. The samples were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 20 mA per gel using an ATTO AE-6530 Dual mini-slab system. After separation, protein bands will be stained using PlusOne Silver staining kit (GE healthcare) (Appendix C-10).

3.2.9 Enzymatic properties

Characterization of predominate enzyme from the strain KN3-8-4 including pH optimum, temperature optimum, NaCl concentration and substrate specificity were carried out.

3.2.9.1 Effect of pH on activity

The activity of purified lipase was assayed at 40°C for 30 min over the pH range of 5.0-10.0 using Universal buffer (Britton-Robinson buffer, Appendix C-1). The lipase activity was measured by using the method described above (3.2.7). Each of assays was performed in duplicate.

3.2.9.2 Effect of temperature on activity

The lipase activity was assayed at different temperatures (20, 25, 30, 37, 40, 45, 50, 55, 60 and 65°C) for 30 min in Universal buffer (pH 8.0) as the method described above (3.2.7). Each of assays was performed in duplicate.

3.2.9.3 Effect of NaCl concentration on activity

The optimum NaCl concentration of the purified enzyme was determined by varying the NaCl concentration from 0 to 15% (w/v) at 40°C, pH 8.0. The enzyme activity was determined by using the method described above (3.2.7). Each of assays was performed in duplicate.

3.2.9.4 Substrate specificity of purified lipase

To study of substrate specificity of the enzyme, different substrates 4-nitrophenyl butyrate (C:4), 4-nitrophenyl octanoate (C:8), 4-nitrophenyl decanoate (C:10), 4-nitrophenyl dodecanoate (C:12), 4-nitrophenyl myristate (C:14) and 4-nitrophenyl palmitate (C:16) was used and determined by using the method described above (3.2.7). The substrate concentration used was 2 mM. Each of assays was performed in duplicate.

3.2.9.5 Molecular mass

The enzyme separated on size exclusion chromatography (as described in 3.2.11.2) was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and purified lipase. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standard used included thyroglobulin (bovine) (M_r 670,000), γ-globulin (bovine) (M_r 158,000), ovalbumin (chicken) (M_r 44,000), myoglobin (horse) (M_r 17,000) and vitamin B_{12} (M_r 1,350).

Sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described above. Protein standard markers was used (Tricolor broad protein ladder, biotechrabbit, Germany) in range of 5.0-245.0 kDa.

3.2.10 Kinetic studies

The purified lipase was assayed with different final concentrations of p-nitrophenyl butyrate (50 µM - 20 mM). The V_{max}, K_m and K_{cat} values for the purified enzyme were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph (Lineweaver 1934).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Bacterial isolation

A total of 138 isolates were isolated from two fish sauce samples from Paknakhon fish sauce Factory, Nakhon Si Thammarat province (26 isolates), one fish sauce sample from Gold label brand, Chonburi province (14 isolates), one salting crab (*Poo-khem*) sample from Nakhon Si Thammarat province (21 isolates), two fish paste (*Keoy-pla*) samples from Mueang district, Nakhon Si Thammarat province (22 isolates), one fermented fish (*Kee-dee*) sample from Mueang district, Nakhon Si Thammarat province (14 isolates), one fermented fish entrails (*Tai-pla*) from Mueang district, Nakhon Si Thammarat province (15 isolates) and one Shrimp paste (*Ka-pi*) sample from Mueang district, Nakhon Si Thammarat province (26 isolates) by the standard dilution technique using JCM no. 377 medium (Table 4.1). A total of 138 isolates were isolated (Table 4.6). Seventy isolates of halophilic bacteria showed lipolytic activity as opaque halos of calcium salt on lipolytic agar (supplemented with 1% v/v tween 20, 40, 60, 80) when Tween was used (García-Lepe 1997) (Table 4.2). The seventy isolates were collected for determine lipase activity in liquid medium.

4.2 Screening of lipase activity

The isolates showed lipolytic activity when cultivated in the complex medium (CM) for 48 h at 37°C were shown in Table 4.2. The lipase activity was obtained from the culture medium ranged from 0.01±0.004 to 26.68±2.62 Unit/ml. The strain FN1-14 showed highest lipase activity and followed by FN1-1 (21.61±1.47 Unit/ml), NR1-1 (6.78±0.13 Unit/ml), NN2-2 (3.70±0.13 Unit/ml), NR3-3-3 (3.68±0.14 Unit/ml), NR3-3-1 (3.54±0.44 Unit/ml), FN1-8 (3.38±0.02 Unit/ml), CN2-9 (2.78±0.05 Unit/ml), FN1-13 (2.75±0.03 Unit/ml), NR1-3-1 (2.83±0.16 Unit/ml), NR1-3-4 (2.43±0.66 Unit/ml), FN6-6 (2.26±0.003 Unit/ml), FN2-3 (2.14±0.04 Unit/ml), KN3-8-4 (2.01±0.09

Unit/ml). The isolates showed lipase activity more than 1.00 Unit/ml were selected for identify in next study.

4.3 Identification of selected isolates

Thirty-six selected isolates were identified based on phenotypic characteristics and 16 rRNA sequence analyses of the representative strain in each of group. They were separated into 24 groups as described below and as shown in Table 4.3-4.5. The phylogenetic tree indicated their taxonomic position was shown in Figure 4.1-4.6.

4.3.1 Group I

Group I contained three isolates, FN1-8, NR1-1 and NR3-3-3. Cells are Gram-reaction-positive rods. The colonies were circular to slightly irregular and milky white in color after 2 days of incubation at 37°C on JCM no. 377 medium. They grew in 1–20% (w/v) NaCl, pH 6.0–9.5 and at 25–45 $^\circ$C. They produced acid from D-cellobiose, D-galactose, D-glucose, maltose, D-mannose, D-ribose, sucrose and D-xylose, but not from D-arabinose, D-fructose, lactose, D-mannitol, melibiose, raffinose, L-rhamnose and D-sorbitol. Hydrolysis of gelatin is positive, but nitrate reduction, Indole test, L-arginine and hydrolysis of Tween 80 and starch are negative (Table 4.3). Based on the 16S rRNA gene sequence, the representative isolate, FN1-8 in group I (1,373 bp) was identified as *Virgibacillus dokdonensis* (Figure 4.1) from its 99.63% sequence similarity to *V. dokdonensis* DSW-10^T (Yoon et al. 2005). This species is a halophilic bacteria where they often can be isolated from fermented foods such as *Pla-ra*, fish sauce, and shrimp paste (Tanasupawat 2014). Lipase and esterase are produced when assayed with the API zym system and reported as slightly halophilic bacteria (Yoon et al. 2005).

4.3.2 Group II

Group II contained one isolate, NR1-3-4. Cells are Gram-reaction-positive rods. The colonies were circular, cream in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew in 1-20% NaCl (w/v), pH 6.0-9.5 and at 25-40 $^{\circ}$C. This strain was showed the positive for hydrolysis of gelatin, but negative for hydrolysis of arginine, starch, Tween 80, nitrate reduction, Indole test and all of acid production (Table 4.3). Based on the 16S rRNA gene sequence, isolate NR1-3-4 in group II (1392 bp) was closely related to *Virgibacillus alimentarius* J18$^{\text{T}}$ (Figure 4.1) with 99.86% sequence similarity. Therefore, it was identified as *V. alimentarius* (Kim 2011).

4.3.3 Group III

Group III contained FN2-3 and FN6-6. Cells are Gram-reaction-positive rods. The colonies were circular and cream in color after 2 days of incubation at 37°C on JCM no. 377 medium. They grew in 0–20% (w/v) NaCl (w/v), pH 6.0–9.5 and pH 6.0– 9.0, and at 25–40 $^{\circ}$C and 25–45 $^{\circ}$C, respectively. They showed the positive for hydrolysis of gelatin, but negative for hydrolysis of arginine, starch, Tween 80, nitrate reduction, Indole test. They produced acid from D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-ribose, sucrose but not from D-arabinose, D-cellobiose, lactose, D-mannose, melibiose, raffinose, L-rhamnose and D-sorbitol, D-xylose (Table 4.3). Based on the 16S rRNA gene sequence, the representative isolate, FN6-6 in group III (1,406 bp) was identified as *Virgibacillus halodenitrificans* from its 99.86% sequence similarity to *V. halodenitrificans* DSM 10037$^{\text{T}}$ (Yoon 2004) (Figure 4.1). This species had been reported as moderately halophilic bacteria and produced lipase and esterase when assayed with the API zym system (Yoon 2004).

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

Source of Sample	Location	Isolate no.	No. of isolate
Fish sauce (I)	Poo Yim brand, Paknakhon fish sauce Factory, Nakhon Si Thammarat province	NN1-1, NN1-2, NN1-3, NN1-4, NN1-5, NN1-6, NN1-7, NN1-8, NN1-9, NN1-10, NN1-11, NN1-12, NN1-13, NN1-14, NN1-15	15
Fish sauce (II)	Silver label brand, Paknakhon fish sauce Factory, Nakhon Si Thammarat province	NN2-1, NN2-2, NN2-3, NN2-4, NN2-5, NN2-6, NN2-7, NN2-8, NN2-9, NN2-10, NN2-11	11
Fish sauce (III)	Gold label brand, Chonburi province	NR1-1, NR2-1, NR2-2, NR3-3-1, NR3-3-2, NR3-3-3, NR5-1, NR7-1, NR9-1, NR9-1, NR1-3-1, NR1-3-2, NR1-3-3, NR1-3-4	14
Salting crab (*Poo-khem*)	Mueang district, Nakhon Si Thammarat province	CN1-1, CN1-2, CN1-3, CN1-4, CN1-5, CN1-6, CN1-7, CN1-8, CN1-9, CN1-10, CN2-1, CN2-2, CN2-3, CN2-4, CN2-5, CN2-6, CN2-7, CN2-8, CN2-9, CN2-10, CN2-11	21
Fish paste (*Keoy-pla*) (I)	Mueang district, Nakhon Si Thammarat province	FN1-1, FN1-2, FN1-3, FN1-4, FN1-5, FN1-6, FN1-7, FN1-8, FN1-9, FN1-10, FN1-11, FN1-12, FN1-13, FN1-14	14
Fish paste (*Keoy-pla*) (II)	Cha-uat district, Nakhon Si Thammarat	FN2-1, FN2-2, FN2-3, FN2-4, FN2-5, FN2-6, FN2-7, FN2-8	8
Fermented fish (*Kee-dee*)	Mueang district, Nakhon Si Thammarat province	FN3-1, FN3-2, FN3-3, FN3-4, FN3-5, FN3-6, FN3-7, FN3-8, FN3-9, FN4-1, FN4-2, FN4-3, FN4-4, FN4-5	14
Fermented fish entrails (*Tai-pla*)	Mueang district, Nakhon Si Thammarat province	FN5-1, FN5-2, FN5-3, FN5-4, FN5-5, FN5-6, FN5-7, FN6-1, FN6-2, FN6-3, FN6-4, FN6-5, FN6-6, FN6-7, FN6-8	15
Shrimp paste (*Ka-pi*)	Mueang district, Nakhon Si Thammarat province	KN3-1-1, KN3-1-2, KN3-1-5, KN3-1-6, KN3-2-4, KN3-3-1, KN3-3-9, KN3-4-3, KN3-4-4, KN3-4-5, KN3-4-9, KN3-4-12, KN3-5-7, KN3-5-8, KN3-6-3, KN3-6-5, KN3-6-7, KN3-7-1, KN3-7-2, KN3-7-3, KN3-8-1, KN3-8-2, KN3-8-3, KN3-8-4, KN3-8-5, KN3-8-6	26
Total			138

Table 4.2 Lipolytic activity on lipolytic agar and CM medium.

Strain no.	Tween 20	Tween 40	Tween 60	Tween 80	Lipase activity (Unit/ml)
NN1-1	++	+++	+++	-	0.55±0.13
NN1-2	++	+++	+++	-	0.85±0.02
NN1-5	++	+++	+++	-	0.64±0.01
NN1-6	++	+++	+++	-	0.63±0.04
NN1-8	+++	+++	+++	-	0.98±0.02
NN1-10	+++	+++	+++	-	0.87±0.05
NN1-11	+++	+++	+++	-	0.64±0.002
NN1-12	+++	+++	+++	-	0.64±0.01
NN1-13	+++	+++	+++	-	0.62±0.02
NN2-2	+++	+++	+++	+	3.70±0.13
NN2-3	++	+++	+++	-	0.41±0.004
NN2-4	++	+++	+++	-	0.43±0.02
NN2-7	++	++	+++	+	0.39±0.03
NN2-8	-	++	++	-	0.60±0.08
NN2-9	+	++	++	-	0.67±0.01
NN2-10	-	-	+	-	1.63±0.01
CN1-1	+++	+++	+++	-	0.86±0.31
CN1-2	+++	+++	+++	-	1.31±0.17
CN1-3	-	+++	++	++	0.69±0.01
CN1-4	++	++	++	++	1.69±0.94
CN1-5	+++	+++	+++	-	0.65±0.01
CN1-8	+++	++	++	++	0.81±0.02
CN1-9	+++	+++	++	++	1.51±0.54
CN1-10	-	-	+++	-	1.28±0.12
CN2-5	+++	+++	+++	-	0.64±0.33
CN2-6	-	-	+++	+++	0.87±0.002
CN2-7	+	++	+++	++	1.12±0.31
CN2-9	+++	+++	++	-	2.78±0.05
FN1-1	+++	+++	+++	-	21.61±1.47
FN1-3	-	+++	+++	-	0.81±0.1
FN1-5	+++	+++	+++	-	1.03±0.01

+++, strong; ++, moderate; +, weak activity; -, no activity.

Table 4.2 Lipolytic activity on lipolytic agar and CM medium (Cont.).

Strain	Tween 20	Tween 40	Tween 60	Tween 80	Lipase activity (Unit/ml)
FN1-7	-	+++	+++	-	0.70±0.01
FN1-8	-	++	+	-	3.38±0.02
FN1-10	-	++	++	-	1.20±0.06
FN1-13	+++	+++	+++	-	2.75±0.03
FN1-14	+++	+++	+++	-	26.68±2.62
FN2-3	-	+++	+	-	2.14±0.04
FN3-4	-	+++	+++	+++	0.67±0.01
FN3-7	++	++	+++	++	1.43±0.002
FN3-9	++	++	+++	-	0.75±0.01
FN4-3	++	++	+++	-	0.54±0.01
FN4-4	+	++	++	-	0.49±0.01
FN5-1	-	++	+	-	0.33±0.03
FN6-1	-	++	++	-	1.88±0.04
FN6-6	-	++	++	-	2.26±0.003
FN6-7	-	++	-	-	1.71±0.04
FN6-8	-	++	-	-	1.60±0.01
NR1-3-4	++	++	++	-	2.43±0.66
NR1-1	+++	++	++	-	6.78±0.13
NR1-3-1	++	++	++	-	2.83±0.16
NR3-3-3	+++	++	++	-	3.68±0.14
NR1-3-2	++	++	++	-	1.12±0.06
NR3-3-1	++	++	++	-	3.54±0.44
KN3-1-1	+++	+++	+++	-	0.08±0.07
KN3-1-2	+	++	+++	-	1.03±0.01
KN3-1-5	++	++	++	-	0.04±0.002
KN3-1-6	+++	+++	+++	-	0.32±0.02
KN3-2-4	-	+++	+++	-	1.10±0.03

+++, strong; ++, moderate; +, weak activity; -, no activity.

Table 4.2 Lipolytic activity on lipolytic agar and CM medium (Cont.).

Strain	Tween 20	Tween 40	Tween 60	Tween 80	Lipase activity (Unit/ml)
KN3-3-1	-	-	+++	-	0.01±0.004
KN3-4-3	-	+++	++	-	1.50±0.03
KN3-4-4	++	+++	+++	-	1.57±0.06
KN3-4-5	-	-	+++	-	0.17±0.01
KN3-4-12	-	+++	+++	-	0.58±0.04
KN3-5-7	+	+++	+++	-	1.23±0.01
KN3-5-8	++	+++	+++	-	1.02±0.02
KN3-6-3	++	++	++	+	1.23±0.02
KN3-6-5	++	++	++	+	1.64±0.02
KN3-6-7	-	++	+++	-	0.42±0.08
KN3-7-1	+	+++	+++	-	1.11±0.02
KN3-8-4	-	+++	+++	+	2.01±0.09
Total					70

+++, strong; ++, moderate; +, weak activity; -, no activity.

4.3.4 Group IV

Group IV contained one isolate, NR1-3-1. Cells are Gram-reaction-positive rods. Colonies were circular, cream in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew in 1-20% NaCl (w/v), pH 5.0-9.0 and at 25-40 °C. This strain showed the positive for hydrolysis of gelatin, nitrate reduction but negative for hydrolysis of arginine, starch, Tween 80 and Indole test. This strain produced acid from D-fructose, D-glucose, D-ribose, sucrose and D-xylose, but not from D-arabinose, D-cellobiose, D-galactose, lactose, D- maltose, mannose, D-mannitol, , melibiose, raffinose, L-rhamnose and D-sorbitol (Table 4.3). Based on the 16S rRNA gene sequence, isolate NR1-3-1 (1525 bp) was closely related to *Lentibacillus juripiscarius* IS40-3[T] (Figure 4.1) with 98.86% sequence similarity. Therefore, it was identified as *L. juripiscarius* (Namwong et al. 2005).

4.3.5 Group V

Group V contained one isolate, FN1-10. Cells are Gram-reaction-positive rods. Colonies were circular and cream in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew in 0–20% NaCl (w/v), pH 6.0–9.5 and at 25–40 °C and showed the positive for hydrolysis of gelatin, Indole test and weak positive for hydrolysis of Tween 80 but negative for arginine, Nitrate reduction, hydrolysis of starch, Tween 80. Acid is produced from cellobiose, D-fructose, D-glucose, D-mannose, D-mannitol, D-sorbitol but not from D-arabinose, D-galactose, lactose, D-maltose, melibiose, raffinose, L-rhamnose D-ribose, sucrose and D-xylose (Table 4.3). Based on the 16S rRNA gene sequence (1,390 bp), it was identified as *Oceanobacillus iheyensis* (Figure 4.1) from its 99.78% sequence similarity to *O. iheyensis* HTE 831[T] (Lu 2001). It was reported as an extremely halotolerant and alkaliphilic bacteria (Lu 2001).

4.3.6 Group VI

Group VI contained one isolate, KN3-2-4. Cells are Gram-reaction-positive rods. Colonies were smooth, circular and light orange in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew at 25-45°C, 5-20% NaCl (w/v) and in pH 6.0-9.5 and showed the negative for Arginine, nitrate reduction, Indole test, hydrolysis of gelatin, starch and Tween 80. Acid is produced from D-fructose, D-glucose, D- maltose, but not from D-arabinose, D-cellobiose, D-galactose, lactose, mannose, D-mannitol, melibiose, raffinose, L-rhamnose D-ribose, D-sorbitol, sucrose and D-xylose (Table 4.3). Based on the 16S rRNA gene sequence (1,390 bp), it was identified as *Alkalibacillus almallahensis* (Figure 4.1) from its 99.93% sequence similarity to *Alkalibacillus almallahensis* S1LM8[T] (Perez-Davo 2014).

4.3.7 Group VII

Group VII contained one isolate, KN3-7-1. Cells are Gram-reaction-positive rods. Colonies were circular and orange in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew at 25-40°C, 5-20% NaCl (w/v) and in pH 6.0-9.5 and showed positive for hydrolysis of gelatin and negative for Arginine, Indole test, nitrate reduction, hydrolysis of starch, Tween 80. Acid is produced from D-fructose, D-galactose, D-glucose, maltose and sucrose but not from D-arabinose, cellobiose, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol and D-xylose (Table 4.3). Based on the 16S rRNA gene sequence (1,435 bp), it was identified as *Halobacillus trueperi* (Figure 4.1) from its 99.65% sequence similarity to *Halobacillus trueperi* DSM 10404[T]. It was reported as moderately halophilic bacteria (Spring 1996). The alkaline lipase from *Halobacillus trueperi* was purified and the molecular weight was 44 kDa (Sathishkumar 2015).

4.3.8 Group VIII

Group VIII contained one isolate, FN3-7. Cells are Gram-reaction-positive rods. It was circular and cream colonies and grew in 0–10% NaCl (w/v), pH 5.0–9.0 and at 25–45 $^\circ$C. This strain showed positive for Arginine, nitrate reduction, hydrolysis of gelatin, starch and Tween 80 and negative for Indole test. Acid is produced from cellobiose, D-fructose, D-glucose, D-mannose, D-mannitol, raffinose, D-ribose, sucrose, but not from D-arabinose, D-galactose, lactose, maltose, melibiose, L-rhamnose, D-sorbitol and D-xylose (Table 4.3). It was identified as *Bacillus amyloliquefaciens* subsp. *plantarum* (Figure 4.2) based on its 99.79% 16S rRNA gene sequence (1,436 bp) similarity to *B. amyloliquefaciens* subsp. *plantarum* FZB42[T] (Borriss 2011). It had reported ability to colonize plant rhizosphere, to suppress competing phytopathogenic fungi and bacteria and to stimulate plant growth (Niazi 2014).

4.3.9 Group IX

Group IX contained one isolate, NN2-10. Cells are Gram-reaction-positive rods. It was irregular and white colonies. It grew in pH 6.0-9.5, 3-15 % NaCl (w/v) and at 25-40°C. This strain showed positive for nitrate reduction, hydrolysis of gelatin and starch but negative for Arginine, Indole test and hydrolysis of Tween 80. Acid is produced from D-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-mannitol, sucrose and D-xylose but not from cellobiose, melibiose, raffinose, L-rhamnose, D-ribose and D-sorbitol (Table 4.4). Based on the 16S rRNA gene sequence (1,372 bp), it was identified as *Bacillus altitudinis* (Figure 4.2) from its 99.93% sequence similarity to *Bacillus altitudinis* 41KF2b^T (Shivaji 2006).

4.3.10 Group X

Group X contained one isolate, NN2-2. Cells are Gram-reaction-positive rods. It was irregular and cream-white colonies. It grew in pH 5.0-9.5, 0-10 % NaCl (w/v) and at 25-45°C and showed positive for hydrolysis of gelatin and negative for Arginine, Indole test, nitrate reduction, hydrolysis of starch, Tween 80. Acid is produced from D-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, D-mannitol, melibiose, D-ribose, sucrose and D-xylose but not from lactose, maltose, raffinose, L-rhamnose, and D-sorbitol (Table 4.4). Based on the 16S rRNA gene sequence (1,516 bp), it was identified as *Bacillus zhangzhouensis* (Figure 4.2) from its 99.73% sequence similarity to *Bacillus zhangzhouensis* DW5-4^T (Liu 2015b).

4.3.11 Group XI

Group XI contained one isolate, NR3-3-1. Cells are Gram-reaction-positive rods. Colonies was circular and cream in color. It grew in pH 5.0-9.0, 3-15 % NaCl (w/v) and at 25-40°C and showed negative for Arginine, Indole test, nitrate reduction, hydrolysis of gelatin, starch and Tween 80. Acid is produced from cellobiose, D-galactose, D-glucose, maltose, D-mannose, D-ribose, sucrose but not from D-

arabinose, D-fructose, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol and D-xylose (Table 4.4). Based on the 16S rRNA gene sequence (1,285 bp), it was identified as *Bacillus seohaeanensis* (Figure 4.2) from its 99.53% sequence similarity to *Bacillus seohaeanensis* BH724^T and this species was reported as halotolerant (Lee 2006a).

4.3.12 Group XII

Group XII contained FN1-1, FN1-14. Cells are Gram-reaction-positive rods. Colonies was circular and yellow in color. They grew in pH 5.0-9.0, 0-10 % NaCl (w/v) and at 25-40°C and showed positive for hydrolysis of Tween 80 and negative for Arginine, Indole test, nitrate reduction, hydrolysis of gelatin and starch. Acid is produced from D-galactose, D-glucose and D-ribose but not from D-arabinose cellobiose, D-fructose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose and D-xylose (Table 4.4). Based on the 16S rRNA gene sequence, the representative isolate, FN1-14 in group XII (1,312 bp), it was identified as *Corynebacterium falsenii* (Figure 4.3) from its 99.48% sequence similarity to *Corynebacterium falsenii* DSM 44353^T (Sjoden 1998). *Corynebacterium falsenii* sp. was first reported and characterized as such in 1998 (Sjoden 1998). It was recognized as a potential human pathogen and a clinically significant bacteremia occurring in an infant (Iroh Tam 2010).

Table 4.3 Phenotypic characteristics of isolates.

Characteristic	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII
Genera	VD	VA	VH	LJU	OI	AA	HT	BA
Cell shape	R	R	R	R	R	R	R	R
Pigmentation	MW	CR	CR	CR	CR	LO	OR	CR
Growth at pH 5.0	-	-	-	+	-	-	-	+
Growth at pH 9.5	+	+	-	-	+	+	+	-
Growth at 25 °C	+	+	+	+	+	+	+	+
Growth at 45 °C	+	-	+	-	-	+	-	+
0% NaCl (w/v)	-	-	+	-	+	-	-	+
10% NaCl (w/v)	+	+	+	+	+	+	+	+
15% NaCl (w/v)	+	+	+	+	+	+	+	-
20% NaCl (w/v)	-	+	+	+	+	+	+	-
Nitrate reduction	-	-	+	+	-	-	-	+
Arginine	-	-	-	-	-	-	-	+
Indole test	-	-	-	-	+	-	-	-
Hydrolysis of								
Tween 80	-	-	-	-	w	-	-	+
Gelatin	+	+	+	+	+	-	+	+
Starch	-	-	-	-	-	-	-	+
Acid from:								
D-Cellobiose	+	-	-	-	w	-	-	+
D-Fructose	-	-	+	+	+	+	+	+
D-Galactose	+	-	+	-	-	-	+	-
D-Glucose	+	-	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-
Maltose	+	-	w	-	-	+	+	-
D-Mannose	+	-	-	-	+	+	-	+
D-Mannitol	-	-	+	-	+	-	-	+
D-Raffinose	-	-	-	-	-	-	-	+
D-Ribose	+	-	+	+	-	-	-	+
Sucrose	+	-	+	+	-	-	+	+
D-Sorbitol	-	-	-	-	w	-	-	-
D-Xylose	+	-	-	+	-	-	-	-

VD, *Virgibacillus dokdonensis*; VA, *Virgibacillus alimentarius*; VH, *Virgibacillus halodenitrificans*; LJU, *Lentibacillus juripiscarius*; OI, *Oceanobacillus iheyensis*; AA, *Alkalibacillus almallahensis*; HT, *Halobacillus trueperi*; BA, *Bacillus amyloliquefaciens* subsp. *plantarum*; +, positive; - negative; w, weak positive; MW, milky white; CR, cream; LO, light orange; OR, orange; R, rod-shaped.
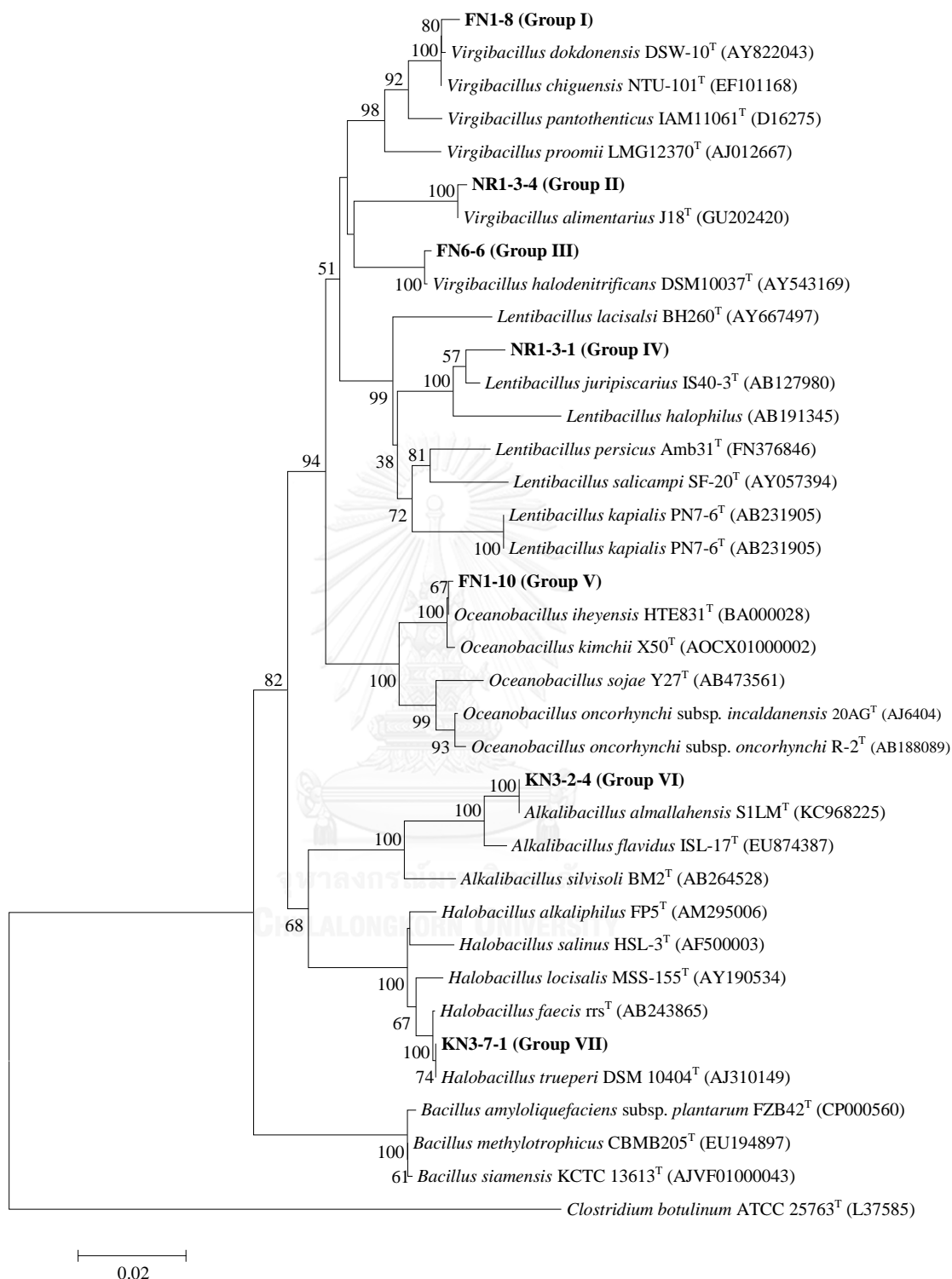
Figure 4.1 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Virgibacillus*, *Lentibacillus*, *Oceanobacillus*, *Alkalibacillus* and *Halobacillus* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.

4.3.13 Group XIII

Group XIII contained FN1-5, FN1-13. Cells are Gram-reaction-positive rods. Colonies was circular and cream in color. They grew in pH 6.0-9.0, 0-15 % NaCl (w/v) and at 25-40°C. and showed positive for Arginine and hydrolysis of Tween 80 and negative for Indole test, nitrate reduction, hydrolysis of gelatin and starch and negative for all of acid production (Table 4.4). Based on the 16S rRNA gene sequence, the representative isolate, FN1-13 in group XIII (1,373 bp), it was identified as *Corynebacterium variabile* (Figure 4.3) from its 99.85% sequence similarity to *Corynebacterium variabile* DSM 44702^{T} (Schroder 2011). This species is part of the complex microflora on the surface of smear-ripened cheeses and contributes to the development of flavor and textural properties during cheese ripening (Schroder 2011).

4.3.14 Group XIV

Group XIV contained KN3-6-3 and KN3-6-5. Cells are Gram-reaction-positive rods. Colonies was circular and light brown in color. They grew in pH 6.0-9.5, 0-20 % NaCl (w/v) and at 25-40°C and showed positive for nitrate reduction, hydrolysis of gelatin and starch and negative for Arginine, Indole test and hydrolysis of Tween 80. Acid is produced from D-glucose but not from D-arabinose cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose and D-xylose (Table 4.4). Based on the 16S rRNA gene sequence, the representative isolate, KN3-6-3 in group XIV (1,350 bp), it was identified as *Brevibacterium sediminis* (Figure 4.3) from its 99.70% sequence similarity to *Brevibacterium sediminis* FXJ8.269^{T} (Chen 2016). *Brevibacterium* sp. is the sole genus within the family *Brevibacteriaceae*, which belongs to the phylum *Actinobacteria*. Members of this genus are also reported to be degraders of organic pollutants such as phenol and 4-chlorophenol (Cui 2013).

Table 4.4 Phenotypic characteristics of isolates.

Characteristic	Group IX	Group X	Group XI	Group XII	Group XIII	Group XIV	Group XV
Genera	BAL	BZ	BS	CF	CV	BRS	PP
Cell shape	R	R	R	R	R	R	R
Pigmentation	W	CW	CR	Y	CR	LB	CW
Growth at pH 5.0	-	+	+	+	-	-	-
Growth at pH 9.5	+	+	-	-	-	+	+
Growth at 25 °C	+	+	+	+	+	+	+
Growth at 45 °C	-	+	-	-	-	-	+
0% NaCl (w/v)	-	+	-	+	+	+	+
10% NaCl (w/v)	+	w	+	+	+	+	+
15% NaCl (w/v)	+	-	+	-	+	+	-
20% NaCl (w/v)	-	-	-	-	-	+	-
Nitrate reduction	+	-	-	-	-	+	+
Arginine	-	-	-	-	+	-	-
Indole test	-	-	-	-	-	-	-
Hydrolysis of							
Tween 80	-	-	-	+	+	-	-
Gelatin	+	+	-	-	-	+	-
Starch	+	-	-	-	-	+	-
Acid from:							
D-Arabinose	+	+	-	-	-	-	-
D-Cellobiose	-	+	+	-	-	-	-
D-Fructose	+	+	-	-	-	-	-
D-Galactose	+	+	+	+	-	-	+
D-Glucose	+	+	+	+	-	+	+
Lactose	+	-	-	-	-	-	-
Maltose	+	-	+	-	-	-	+
D-Mannose	+	+	+	-	-	-	-
D-Mannitol	+	+	-	-	-	-	-
Melibiose	-	+	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Ribose	-	+	+	+	-	-	+
Sucrose	+	+	+	-	-	-	+
D-Sorbitol	-	-	-	-	-	-	-
D-Xylose	+	+	-	-	-	-	+

BAL, *B. altitudinis*; BZ, *B. zhangzhouensis*; BS, *B. seohaeanensis*; CF, *Corynebacterium falsenii*; CV, *Corynebacterium variabile*; BRS, *Brevibacterium sediminis*; PP, *Proteus penneri*;   CR, cream; W, white; CW, cream white; Y, yellow; LB, light brown; R, rod-shaped.
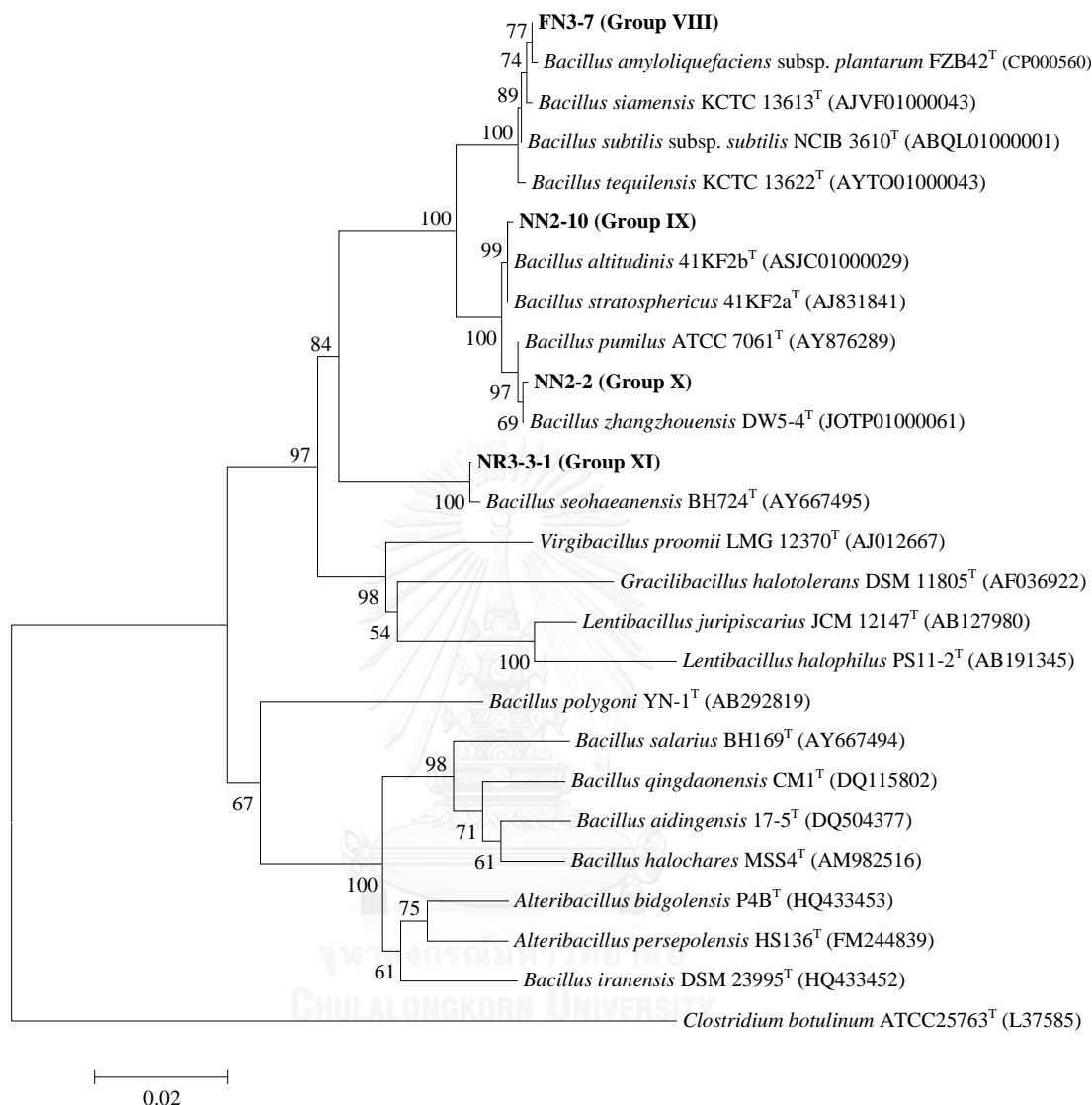
Figure 4.2 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Bacillus* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.
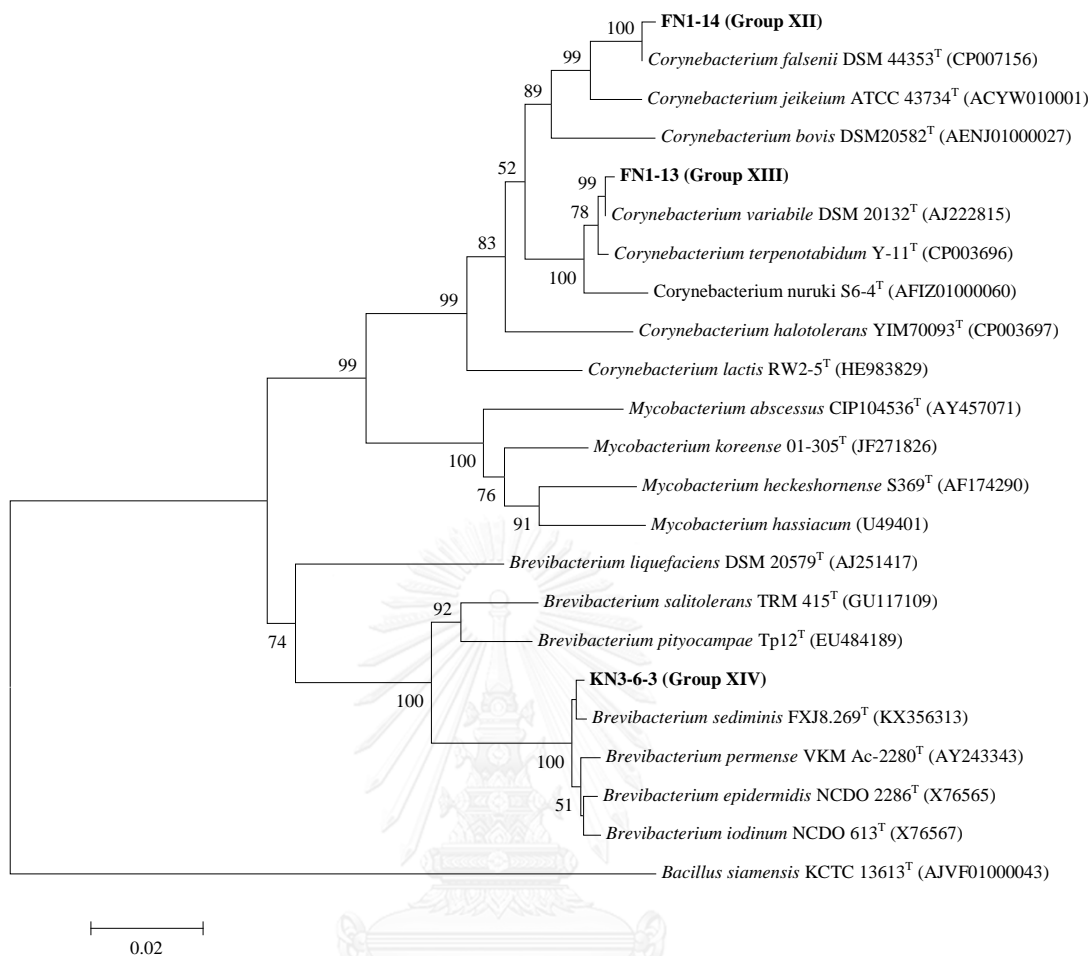
Figure 4.3 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Corynebacterium* and *Brevibacterium* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.

4.3.15 Group XV

Group XV contained one isolate, CN1-10. Cells are Gram-reaction-negative rods. Colonies was circular and cream white in color. It grew in 0-10% NaCl (w/v), pH 6.0-9.5 and at 25-45°C and showed positive for nitrate reduction and negative for Arginine, Indole test and hydrolysis of gelatin, starch and Tween 80. Acid is produced from D-galactose, D-glucose, maltose, D-ribose, sucrose and D-xylose but not from D-arabinose, cellobiose, D-fructose, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol (Table 4.4). Based on the 16S rRNA gene sequence (1,504 bp), it was identified as *Proteus penneri* (Figure 4.4) from its 99.71% sequence similarity to *Proteus penneri* NCTC 12737[T] (Hickman 1982). This species is an invasive pathogen capable of causing major infectious diseases. It usually infects urinary tract, blood, abdominal wound, groin, neck and ankle and has been isolated mostly from urine, wound and soft tissue exudates (Kishore 2012).

4.3.16 Group XVI

Group XVI contained FN6-1 and FN6-7. Cells are Gram-reaction-positive cocci. The colonies were circular and pale yellow in color after 2 days of incubation at 37°C on JCM no. 377 medium. They grew in 0–7% (w/v) NaCl, at 25–40 °C and pH 5.0–9.0 and 6.0-9.0, respectively. They showed positive for nitrate reduction but negative for Arginine, Indole test, hydrolysis of gelatin, starch and Tween 80. Acid is produced from D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-ribose and sucrose but not from D-arabinose, cellobiose, lactose, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol and D-xylose (Table 4.5). Based on the 16S rRNA gene sequence, the representative isolate, FN6-1 in group XVI (1,396 bp) was identified as *Staphylococcus saprophyticus* subsp. *bovis* (Figure 4.5) from its 100% sequence similarity to *Staphylococcus saprophyticus* subsp. *bovis* GTC843[T] (Hajek 1996).
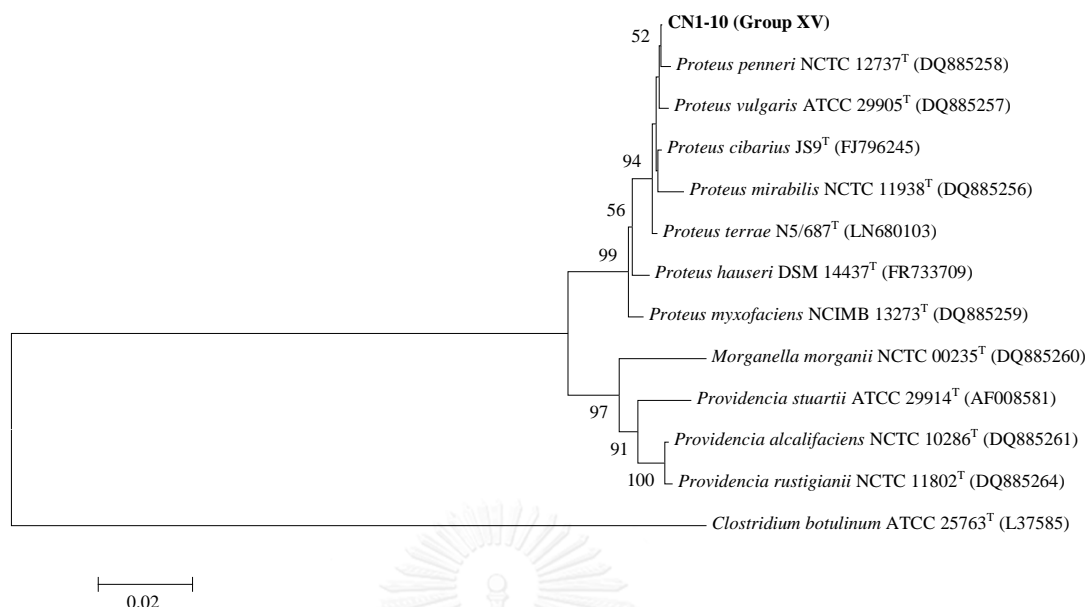
Figure 4.4 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Proteus* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.

4.3.17 Group XVII

Group XVII contained one isolate, FN6-8. Cells are Gram-reaction-positive and cocci-shaped. Colonies were circular and pale yellow in color after 2 days of incubation at 37°C on JCM no. 377 medium. It grew in 0–10% NaCl (w/v), pH 6–9 and at 25–40 °C and showed positive for hydrolysis of gelatin and negative for Arginine, Indole test, nitrate reduction, hydrolysis of starch, Tween 80. Acid is produced from D-arabinose, D-fructose, D-glucose, lactose, maltose, D-mannose, D-mannitol, sucrose and D-xylose, but not from cellobiose, D-galactose, melibiose, raffinose, L-rhamnose, D-ribose and D-sorbitol (Table 4.5). It was identified as *S. saprophyticus* subsp. *saprophyticus* (Figure 4.5) based on the 99.93% sequence similarity to *Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305[T] (Hajek et al. 1996) for the 16S rRNA gene sequence (1,429 bp). *Staphylococcus saprophyticus* is a

coagulase-negative facultative bacterium belongs to *Micrococcaceae* family. It is a unique uropathogen associated with uncomplicated urinary tract infections, especially cystitis in young women (Trivedi 2015).

4.3.18 Group XVIII

Group XVIII contained one isolate, KN3-1-2. Cells are Gram-reaction-positive and cocci-shaped. Colonies were circular and white in color after 2 days of incubation at 37°C on JCM no. 377 medium. . It grew in 0–7% NaCl (w/v), pH 6.0–9.0 and at 25–40 $^{\circ}$C and showed positive for nitrate reduction and negative for Arginine, Indole test, hydrolysis of gelatin, starch, Tween 80. Acid is produced from D-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, sucrose and D-xylose, but not from cellobiose, lactose, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose and D-sorbitol (Table 4.5). It was identified as *Staphylococcus nepalensis* (Figure 4.5) based on the 99.63% sequence similarity to *Staphylococcus nepalensis* CW1^{T} (Spergser 2003) for the 16S rRNA gene sequence (1,372 bp).

4.3.19 Group XIX

Group XIX contained one isolate, KN3-4-3. Cells are Gram-reaction-positive cocci-shaped. Colonies was circular and pink in color. It grew in 0-20% NaCl (w/v), pH 6.0-9.5 and at 25-40°C and showed positive for Arginine, nitrate reduction and hydrolysis of starch and negative for Indole test, hydrolysis of gelatin, and Tween 80. Acid is produced from D-fructose, D-glucose, maltose and D-ribose but not from D-arabinose, cellobiose, D-galactose, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose and D-xylose (Table 4.5). Based on the 16S rRNA gene sequence (1,357 bp), it was identified as *Salinicoccus salsiraiae* (Figure 4.5) from its 100% sequence similarity to *Salinicoccus salsiraiae* RH-1^{T} that it was reported as moderately halophilic bacteria (Franca 2006).

4.3.20 Group XX

Group XIX contained KN3-4-4, KN3-5-7 and KN3-5-8. Cells are Gram-reaction-positive cocci-shaped. Colonies was circular and orange in color. They grew in 3-20% NaCl (w/v), pH 6.0-9.0 and at 25-45°C and showed negative for Arginine, nitrate reduction, Indole test, hydrolysis of gelatin, starch and Tween 80. Acid is produced from D-fructose, D-glucose and D-ribose but not from D-arabinose, cellobiose, D-galactose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose and D-xylose (Table 4.5). Based on the 16S rRNA gene sequence, the representative isolate, KN3-5-8 in group XIX (1,366 bp), it was identified as *Salinicoccus siamensis* (Figure 4.5) from its 99.93% sequence similarity to *Salinicoccus siamensis* PN1-2[T] that it was reported as moderately halophilic bacteria and had been isolated from shrimp paste (Pakdeeto et al. 2007).

4.3.21 Group XXI

Group XXI contained CN1-2, CN1-4, CN2-7 and CN2-9. Cells are Gram-reaction-negative rod. Colonies was circular and cream in color. They grew in 0-10% NaCl (w/v), pH 6.0-9.5 and at 25-45°C and showed positive for Arginine, nitrate reduction, Indole test and hydrolysis of gelatin and negative for hydrolysis of starch and Tween 80. Acid is produced from cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, D-mannitol, D-ribose and sucrose, but not from D-arabinose, lactose, melibiose, raffinose, L-rhamnose, D-sorbitol, and D-xylose (Table 4.5). Based on the 16S rRNA gene sequence, the representative isolate, CN1-2 in group XXI (1,325 bp), it was identified as *Vibrio alginolyticus* (Figure 4.6) from its 99.40% sequence similarity to *Vibrio alginolyticus* NBRC 15630[T] (Liu 2015a). *Vibrio alginolyticus* as a human pathogen is a rare factor in human infections. These bacteria are a halophilic *Vibrio* sp. where they often can be isolated from the coastal flora of temperate seas and the rivers where they flow into the sea (Citil 2015).
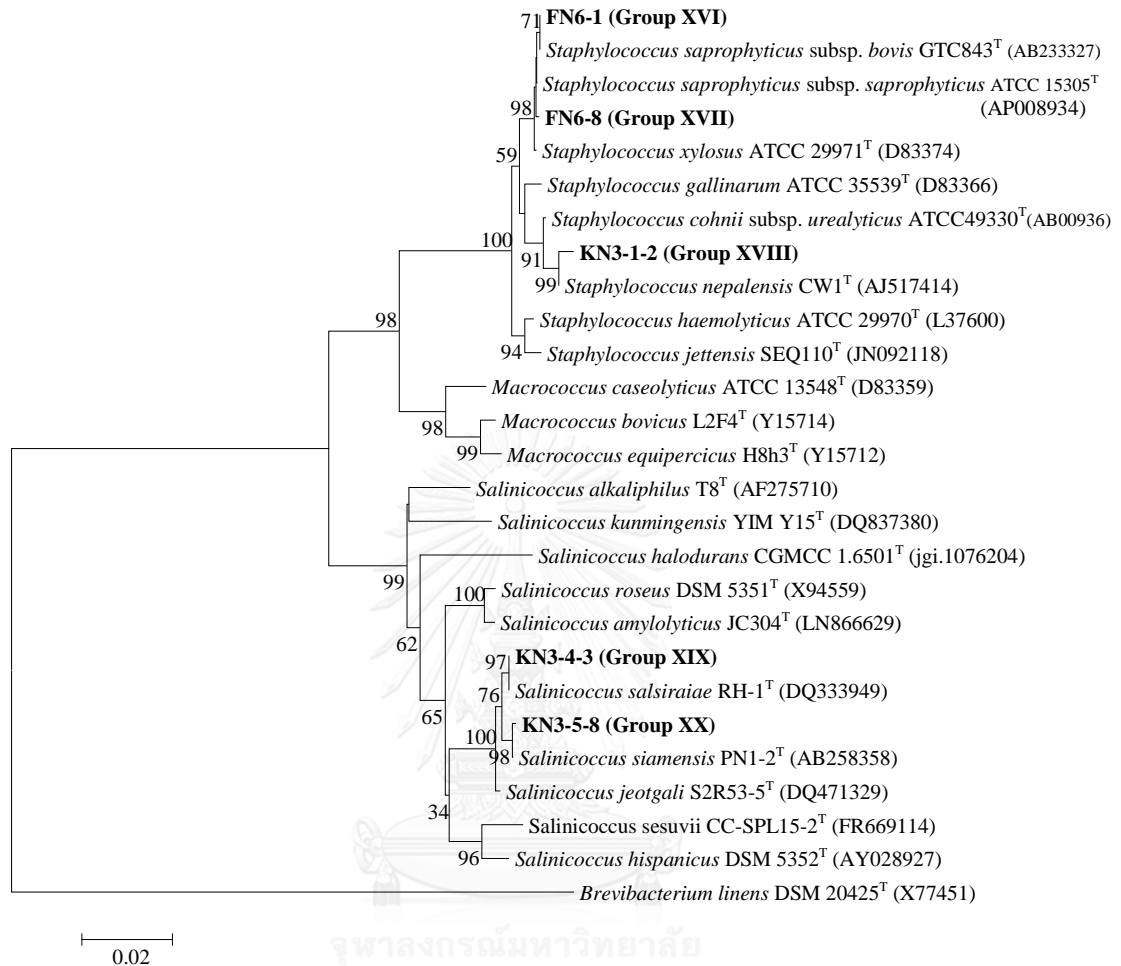
Figure 4.5 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Staphylococcus* and *Salinicoccus* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.

Table 4.5 Phenotypic characteristics of isolates.

Characteristic	Group XVI	Group XVII	Group XVIII	Group XIX	Group XX	Group XXI	Group XXII
Genera	SB	SS	SN	SAS	SASI	VA	SI
Cell shape	Co	Co	Co	Co	Co	R	R
Pigmentation	PY	PY	W	P	OR	CR	PB
Growth at pH 5.0	+	-	-	-	-	-	-
Growth at pH 9.5	-	-	-	+	-	+	+
Growth at 25 °C	+	+	+	+	+	+	+
Growth at 45 °C	-	-	-	-	+	w	-
0% NaCl (w/v)	+	+	+	+	-	+	+
10% NaCl (w/v)	-	+	-	+	+	+	+
15% NaCl (w/v)	-	-	-	+	+	-	-
20% NaCl (w/v)	-	-	-	+	+	-	-
Nitrate reduction	+	-	+	+	-	+	+
Arginine	-	-	-	+	-	+	+
Indole test	-	-	-	-	-	+	-
Hydrolysis of							
Tween 80	-	-	-	-	-	-	-
Gelatin	-	+	-	-	-	+	+
Starch	-	-	-	+	-	-	-
Acid from:							
D-Arabinose	-	+	+	-	-	-	-
D-Cellobiose	-	-	-	-	-	+	-
D-Fructose	+	+	+	+	+	+	-
D-Galactose	+	-	+	-	-	+	-
D-Glucose	+	+	+	+	+	+	-
Lactose	-	+	-	-	-	-	-
Maltose	+	+	+	+	-	+	-
D-Mannose	-	+	+	-	-	+	+
D-Mannitol	+	+	+	-	-	+	-
D-Ribose	+	-	-	+	+	+	+
Sucrose	+	+	+	-	-	+	-
D-Xylose	-	+	+	-	-	-	-

SB, *Staphylococcus saprophyticus* subs. *bovis*; SS, *Staphylococcus saprophyticus* subs. *saprophyticus*; SN, *Staphylococcus nepalensis*; SAS, *Salinicoccus salsiraiae*; SASI, *Salinicoccus siamensis*; VA, *Vibrio alginolyticus*; SI, *Shewanella indica*; PY, pale yellow; W, white; CW, cream white; P, pink; OR, orange; CR, cream; PB, pale brown; Co, cocci-shaped; R, rod-shaped.
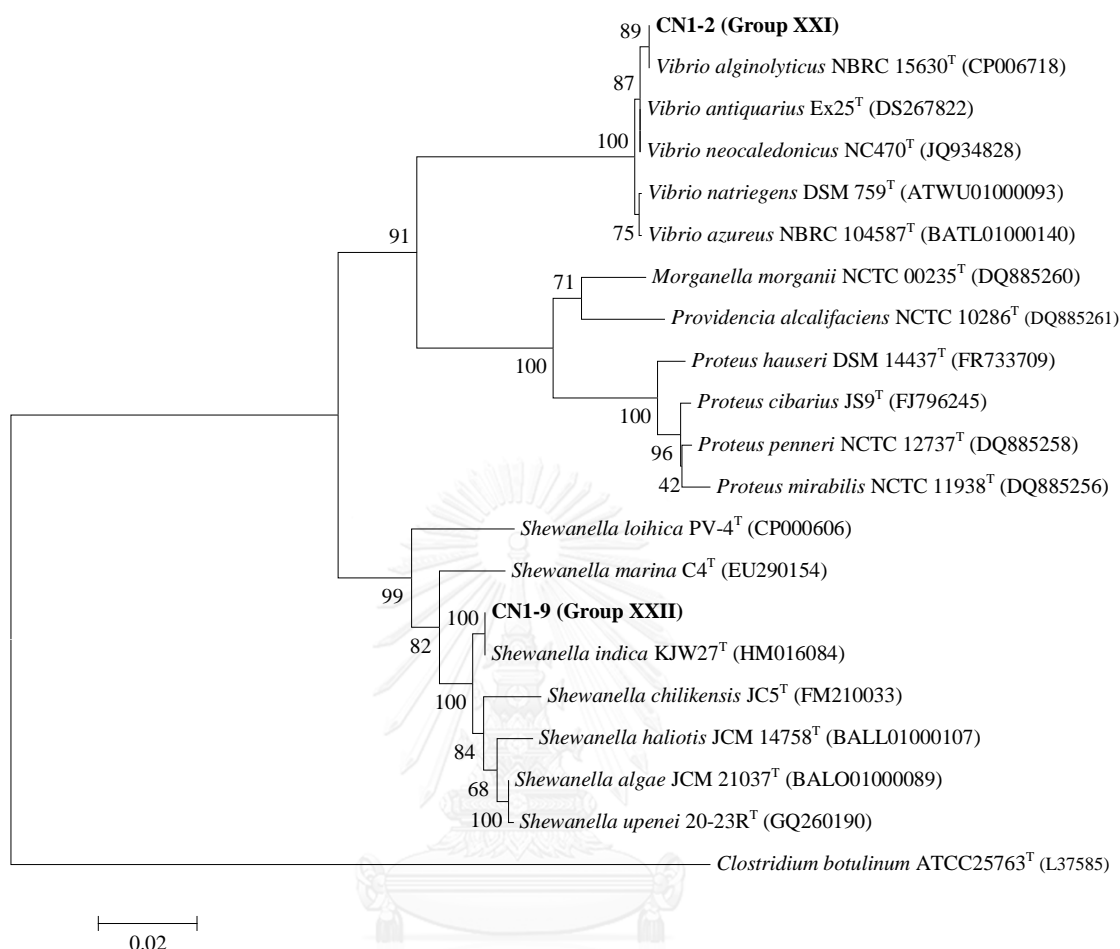
Figure 4.6 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among *Vibrio* and *Shewanella* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.02 substitutions per nucleotide position.

4.3.22 Group XXII

Group XXII contained one isolate, CN1-9. Cells are Gram-reaction-negative rod. Colonies was irregular and pale brown in color. It grew in 0-10% NaCl (w/v), pH 6.0-9.5 and at 25-40°C and showed positive for Arginine, nitrate reduction and hydrolysis of gelatin and negative for Indole test, hydrolysis of starch and Tween 80. Acid is produced from D-mannose and D-ribose, but not from D-arabinose,

cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose and D-xylose (Table 4.5). Based on the 16S rRNA gene sequence (1,365 bp), it was identified as *Shewanella indica* (Figure 4.6) from its 100% sequence similarity to *Shewanella indica* KJW27[T] (Verma 2011). *Shewanella* sp. have been isolated from a variety of sources, such as marine environments including seawater, sediment, Antarctic sea ice, fish and estuaries (Park 2009; Satomi 2007). It is rarely considered pathogenic in humans and implicated in fish spoilage (Vogel 2005).

4.3.23 Group XXIII

Group XXIII contained one isolate, NR1-3-2. Cells of strain NR1-3-2 were Gram-reaction-positive, endospore-forming rods and strictly aerobic. Ellipsoidal endospores were produced at central–subterminal positions in non-swollen sporangia (Figure 4.7). Colonies were circular to slightly irregular, cream in colour and 1–2 mm in diameter after growth on modified JCM medium no. 377 agar at 30 °C for 3 days. Cells were motile by peritrichous flagella. No growth was observed under anaerobic conditions. Catalase and oxidase activities were positive but L-arginine hydrolysis, nitrate reduction, and hydrolysis of starch, gelatin and Tween 80 were negative. The strain grew with 0–15 % (w/v) NaCl, at 21–48 °C and at pH 5.0–10.0 [optimally with 1–3 % (w/v) NaCl, at 37 °C and at pH 7.5]. It did not produce acid from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, methyl α-D-glucoside, glycerol, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose or D-xylose.
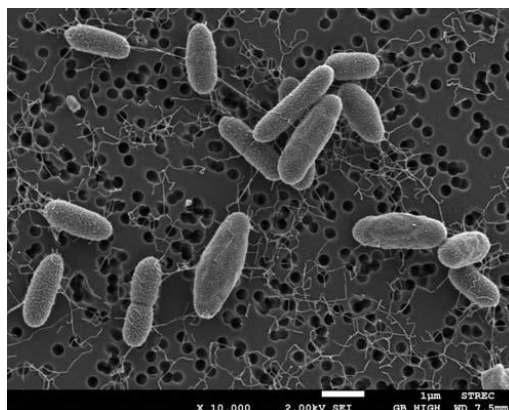
Figure 4.7 Scanning electron micrograph of cells of strain NR1-3-2[T] grown on modified JCM no. 377 agar for 3 days. Bar, 1 mm.

On the basis of 16S rRNA gene sequence analyses, strain NR1-3-2[T] (1453 nt) was closely related to *B. iranensis* DSM 23995[T] (97.4 % similarity), *Alteribacillus bidgolensis* P4B[T] (96.5 %), *B. salarius* BH169[T] (95.4 %) and *Alteribacillus persepolensis* HS136[T] (94.8 %) (Figure 4.9). The DNA G+C content of strain NR1-3-2[T] was 44.2 mol%, which was within the range reported for the genus *Bacillus* Slepecky 2006; however, it was higher than that of *B. iranensis* DSM 23995[T] (Table 4.6). It contained meso-diaminopimelic acid in the cell-wall peptidoglycan. The major menaquinone was MK-7. The major cellular fatty acids were anteiso-$C_{15:0}$ (53.1 %), iso-$C_{15:0}$ (15.8 %) and anteiso-$C_{17:0}$ (14.4 %) and the other fatty acids were iso-$C_{16:0}$ (5.0 %), iso-$C_{14:0}$ (1.2 %), iso-$C_{17:0}$ (4.1 %) and $C_{16:0}$ (2.5 %), a profile that matched *Bacillus* species (Kaneda 1968; Roohi 2014). The profile of fatty acids of strain NR1-3-2[T] matched that of *B. iranensis* DSM 23995[T] although the amounts of $C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:1}$ alcohol were different. The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid (Figure 4.8) while *B. iranensis* DSM 23995[T] displayed polar lipids consisting of diphosphatidylglycerol, phosphatidylglycerol, three phospholipids and two glycolipids (Bagheri et al. 2012). Strain NR1-3-2[T] and *B. iranensis* DSM 23995[T] exhibited reciprocally low DNA–DNA

relatedness (31.2–39.8 %) to each other. Levels of DNA–DNA relatedness between strain NR1-3-2[T] and its closest phylogenetic neighbors were well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne 1987).

Figure 4.8 Polar lipid profile of strain NR1-3-2[T] after two dimension TLC and detected with phosphomolybdic acid. DPG, diphosphatidylglycerol; PG, phosphatidglycerol and GL1, unknown glycolipid.

In addition, strain NR1-3-2[T] was differentiated from *B. iranensis* DSM 23995[T] based on growth in modified JCM medium no. 377 broth lacking NaCl and $MgSO_4.7H_2O$, nitrate reduction, acid production from D-mannitol, and acid phosphatase and esterase (C4) (Table 4.6). Differential properties between strain NR1-3-2[T] and *B. iranensis* DSM 23995[T], including NaCl, temperature and pH growth ranges, nitrate reduction, acid production from D-mannitol, acid phosphatase, esterase (C4) and DNA G+C content, are shown in Table 4.6.
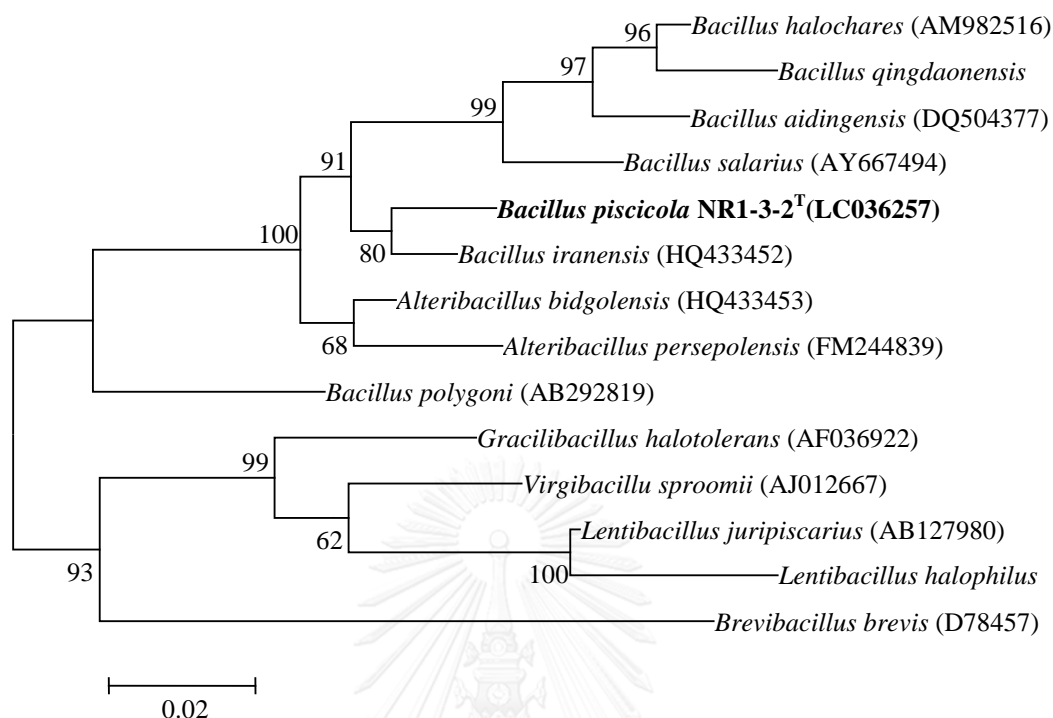
Figure 4.9 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain NR1-3-2[T] and closely related type strains of species of the genus *Bacillus*. Bootstrap values (>60 %) based on 1000 replications are given at branch nodes. *Brevibacillus brevis* JCM 2503[T] was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

As mentioned above and based on its phenotypic characteristics, phylogenetic analysis, DNA G+C content and DNA–DNA relatedness, strain NR1-3-2[T] is considered to represent a novel species of the genus Bacillus for which the name *Bacillus piscicola* sp. nov. is proposed (Daroonpunt 2016a).

Table 4.6 Characteristics that distinguished strain NR1-3-2^T from *B. iranensis* DSM 23995^T.

Characteristics	NR1-3-2^T	*B. iranensis* DSM 23995^T
NaCl concentration (%, w/v) range	0-15	1-15
Optimum	1-3	1-7
Temperature (°C) range	21-48	23-45
Optimum temp.	37	35
pH range	5-10	6-10
Optimum pH	7.5	7.0
Nitrate reduction	-	+
Acid from		
D-Mannitol	-	+
Acid phosphatase	-	+
Esterase (C4)	+	w
Quinones	MK-7	MK-7, MK6, MK-5
DNA G+C content (mol%)	44.2	42.3

All data are from this study. +, Positive; -, negative; w, weakly positive.

4.3.24 Group XXIV

Group XXIV contained one isolate, KN3-8-4. Strain KN3-8-4 was Gram-reaction-positive, rod-shaped (0.57–0.75x1.43–3.5 μm) and strictly aerobic; no growth was observed in anaerobic conditions. Terminal endospores were observed (Figure 4.10). Colonies were cream and circular to slightly irregular (1–2 mm in diameter) on modified JCM no. 377 agar after incubation at 30 °C for 3 days. Cells were motile by peritrichous flagella. Catalase and oxidase activities and hydrolysis of Tween 80 and starch were positive, but nitrate reduction and hydrolysis of gelatin and L-arginine

were negative. Strain KN3-8-4 grew in the presence of 0–15 % (w/v) NaCl, at 11–42 °C and at pH 4.5–10 (optimally in 1–5 % NaCl, at 37 °C, and at pH 7.5).

On the basis of 16S rRNA gene sequence analyses, strain KN3-8-4 (1446 nt) was closely related to *Virgibacillus olivae* JCM 30551[T] (97.85 % 16S rRNA gene sequence similarity), *Virgibacillus salexigens* JCM 30552[T] (97.82 %), *Virgibacillus salarius* JCM 12946[T] (97.78 %), *Virgibacillus marismortui* KCTC 3867[T] (97.71 %), *Virgibacillus xinjiangensis* KCTC 13128[T] (97.30 %), *Virgibacillus sediminis* KCTC 13193[T] (97.23 %), and *Virgibacillus litoralis* KCTC 13228[T] (97.06 %), respectively (Figure 4.11). The DNA G+C content of strain KN3-8-4[T] was 43.5 mol%, which was in the range of the genus *Virgibacillus* (Lee 2006b; An 2007; Quesada 2007) although it was higher than the closely related type strains (Table 4.8). Strain KN3-8-4[T] contained meso-diaminopimelic acid in the cell-wall peptidoglycan, and the major isoprenoid quinone was menaquinone with seven isoprene units (MK-7, 87.9 %); MK-6 (2.4 %) and MK-8 (9.7 %) were also detected. These chemotaxonomic properties are commonly found in other members of genus *Virgibacillus* (Arahal 1999, Arahal 2000; Heyrman 2003; Quesada et al. 2007; Hua 2008). The strain contained anteiso- $C_{15:0}$ (44.01 %), anteiso-$C_{17:0}$ (41.30 %), iso-$C_{15:0}$ (6.12 %), iso-$C_{16:0}$ (4.89 %), iso-$C_{17:0}$ (1.56 %) and iso-$C_{14:0}$ (1.04 %) in the cellular fatty acid profile (Table 4.7). The amount of iso-$C_{15:0}$ and anteiso-$C_{17:0}$ in strain KN3-8-4[T] was significant different from the closely related type strains. The polar lipids detected in strain KN3-8-4[T] were phosphatidylglycerol, diphosphatidylglycerol, two unknown phospholipids and one glycolipid (Figure 4.12).

The DNA–DNA relatedness of strain KN3-8-4[T] to *V. olivae* JCM 30551[T], *V. salexigens* JCM 30552[T], *V. salarius* JCM 12946[T], *V. marismortui* KCTC 3867[T], *V. xinjiangensis* KCTC 13128[T], *V. sediminis* KCTC 13193[T] and *V. litoralis* KCTC 13228[T] was 20.4, 19.3, 43.2, 22.2, 42.1, 39.0 and 40.7 %, respectively. Strain KN3-8-4[T] and *V. olivae* JCM 30551[T] exhibited reciprocally low DNA–DNA relatedness (20.4–28.8 %) to

each other. These DNA–DNA relatedness values of strain KN3-8-4[T] with the closest phylogenetic neighbours were well below the 70 % cut-off point recommended for the assignment of the strains to the same genomic species (Wayne et al. 1987).
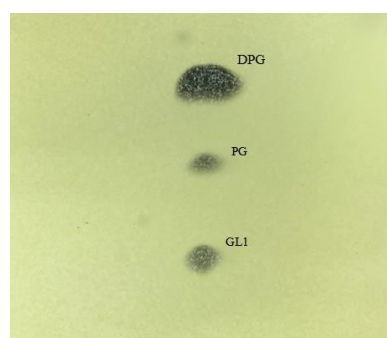
In addition, strain KN3-8-4[T] was differentiated from the phylogenetically closely related type strains by nitrate reduction ability, acid production from D-arabinose, D-mannitol, L-rhamnose and D-xylose, growth in modified JCM no. 377 broth omitting NaCl and $MgSO_4.7H_2O$, enzyme activity and DNA G+C content (Table 4.8). On the basis of the phenotypic characteristics, phylogenetic analysis using 16S rRNA gene sequences, DNA G+C content and DNA–DNA relatedness, strain KN3-8-4[T] represents a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus kapii* sp. nov. is proposed (Daroonpunt 2016b).
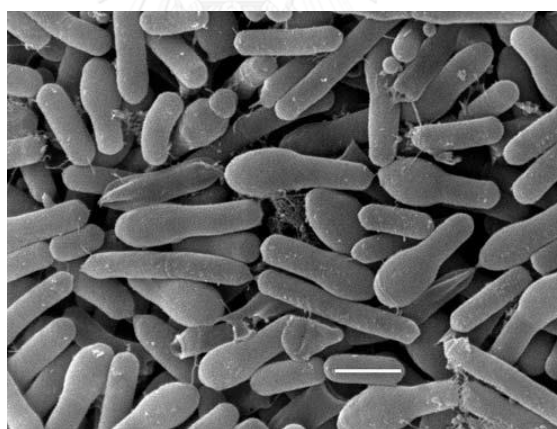
Figure 4.10 Scanning electron micrograph of strain KN3-8-4[T] grown on modified JCM no. 377 agar for 3 days. Bar, 1 μm.

Table 4.7 Cellular fatty acid compositions of strain KN3-8-4[T] and it closest phylogenetic neighbours Strains: 1, *Virgibacillus kapii* sp. nov. KN3-8-4[T]; 2, *V. marismortui* KCTC 3867[T]; 3, *V. salarius* JCM 12946[T]; 4, *V.olivae* JCM 30551[T]; 5, *V. salexigens* JCM 30552[T]. Data were taken from this study under the same conditions.

Fatty acid	Percentage of total[a]				
	1	2	3	4	5
Saturated straight-chain					
$C_{12:0}$	0.16		0.15	0.18	0.16
$C_{14:0}$			0.38	0.38	0.23
$C_{16:0}$	0.34	1.13	0.79	2.98	0.55
$C_{18:0}$				4.34	0.29
Saturated branched-chain					
anteiso-$C_{13:0}$	0.30		0.29	0.17	0.52
iso-$C_{13:0}$		0.82	0.87	0.21	0.34
iso-$C_{14:0}$	1.04	9.98	7.15	4.14	7.23
anteiso-$C_{15:0}$	44.01	34.15	41.13	41.32	48.70
iso-$C_{15:0}$	6.12	33.50	31.52	36.09	29.77
iso-$C_{16:0}$	4.89	5.25	4.23	1.87	2.12
anteiso-$C_{17:0}$	41.30	5.89	5.17	3.30	3.14
iso-$C_{17:0}$	1.56	2.12	1.36	0.96	0.50
Unsaturated straight-chain					
iso-$C_{15:1}$ ω9c			0.15		0.17
iso-$C_{17:1}$ ω10c		0.86	0.65	0.52	0.38
$C_{16:1}$ ω11c		0.48	0.61		0.24
$C_{16:1}$ ω7c alcohol	0.29	3.34	3.33	2.02	3.72
SF2[b]					0.15
SF4[c]		2.48	2.22	1.53	1.79

[a]Values are percentage of total cellular fatty acids. [b]Summed feature 2 contains iso-$C_{16:1}$I and/or $C_{14:0}$ 3OH. [c]Summed feature 4 contains iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.
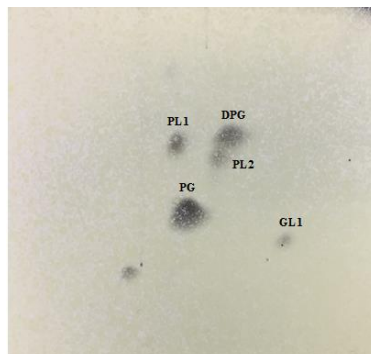
Figure 4.11 Polar lipid profile of strain KN3-8-4[T] after two dimension TLC and detected with phosphomolybdic acid. PG, phosphatidglycerol; DPG, diphosphatidylglycerol; PL1 and PL2, unknown phospholipids and GL1, unknown glycolipid.
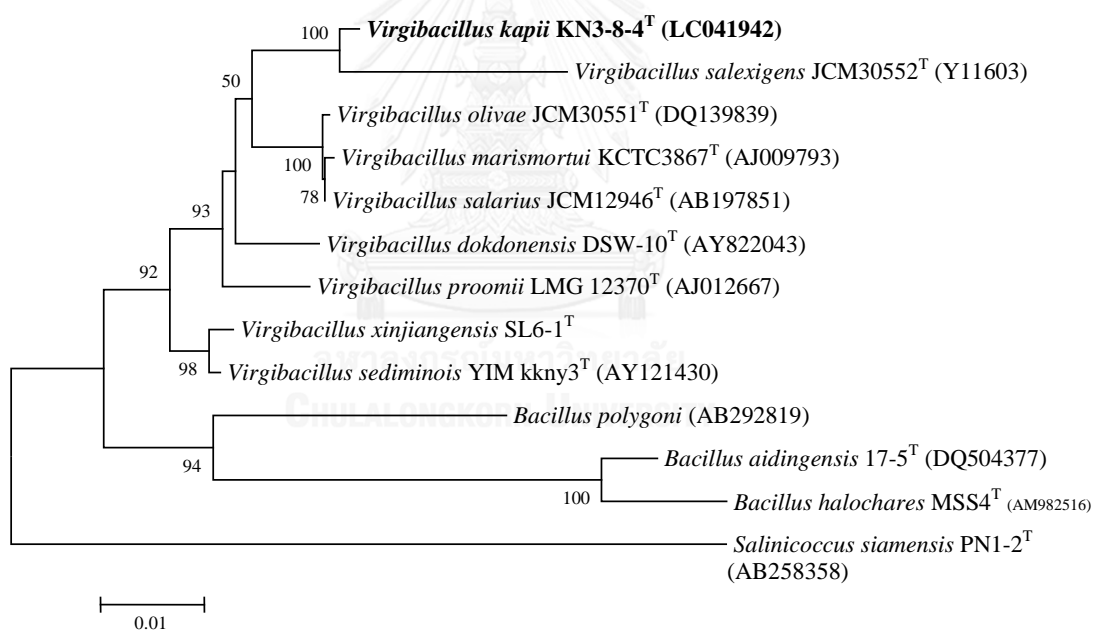
Figure 4.12 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain KN3-8-4[T] and closely related type strains of species of the genus *Virgibacillus*. Bootstrap values (>70 %) based on 1000 replications are given at branch nodes. *Salinicoccus siamensis* was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 4.8 Characteristics that distinguish strain KN3-8-4^T from its closest phylogenetic neighbours Strains: 1, *Virgibacillus kapii* sp. nov. KN3-8-4^T; 2, *V. marismortui* KCTC 3867^T; 3, *V. salarius* JCM 12946^T; 4, *V.olivae* JCM 30551^T; 5, *V. salexigens* JCM 30552^T.

Characteristic	1	2	3	4	5
Endospore position/shape*	T/E	T,ST/E	ST/E	ST/E	ST/E
NaCl concentration (%, w/v)					
Range	0-15	1-15	1-15	1-20	1-20
Optimum	1-5	1-10	1-7	3-5	3
Temperature (°C)					
Range	11-42	16.5-47	15-47	12-50	20-42
pH					
Range	4.5-10.0	5.0-10.0	5.0-9.5	5.0-9.0	5.0-10.0
Optimum	7.5	6.5	6.5	7.0	7.0
Nitrate reduction	-	+	-	+	-
Hydrolysis					
Tween 80	+	-	+	-	-
Gelatin	-	+	-	+	+
Starch	+	-	-	+	-
Acid production from:					
D-Arabinose	+	-	-	-	-
D-Fructose	+	+	+	+	-
D-Galactose	+	-	+	-	-
Inositol	-	-	+	+	-
Lactose	+	-	+	+	-
Maltose	+	+	+	+	-
D-Mannose	+	+	+	+	-
D-Mannitol	+	-	-	-	-
L-Rhamnose	+	-	-	-	-
D-Xylose	+	-	-	-	-
DNA G+C content (mol%)	43.58	38.09	38.12	38.21	38.24

*T/E, Terminal/Ellipsoidal; T,ST/E, Terminal, Subterminal/Ellipsoidal; ST/E, Subterminal/Ellipsoidal; +, positive; w, weakly positive; -, negative

4.4 Optimization of lipase-producing selected halophilic bacteria

Strain FN1-14 showed highest lipase activity. However it was identified as *Corynebacterium falsenii* which was recognized as a potential human pathogen and a clinically significant bacteremia occurring in an infant (Iroh Tam 2010). So, strain FN1-14 was not selected for further study. We selected novel species, *Virgibacillus sp.* KN3-8-4 for optimization, purification and characterization step.

Optimization of crude lipase production of the lipase-producing strain KN3-8-4 was carried out in the modified JCM medium no. 377 (as the basal medium). The influence of several factors (carbon source, nitrogen source, NaCl concentration, pH and temperature) on lipase production was studied.

4.4.1 Effect of carbon source on lipase production

Different carbon sources including coconut oil, olive oil, palm oil, sesame oil, soy bean oil, canola oil, sun flower oil, corn oil and camellia tea oil at 0.5% (v/v) and casamino acid at 0.5% (w/v) were tested which the major components of vegetable oils are triacylglycerols, which consist of glycerol molecules esterified with different of three long-chain fatty acids. Among these carbon sources tested, the maximal lipase production was achieved when 0.5% (v/v) palm oil (38.37 unit/ml), sesame oil (39.09 unit/ml), camellia tea oil (38.28 unit/ml), canola oil (38.20 unit/ml), sun flower oil (37.70 unit/ml) and coconut oil (37.49 unit/ml) was used in (Figure 4.13) and followed by olive oil, corn oil, soy bean oil and casamino acid (control). Palm oil was selected as best of carbon source because it was showed maximal lipase activity and cheapest price. The lipidic carbon source (such as oils, fatty acids or Tweens) was generally used to produce lipases (Gupta et al. 2004).
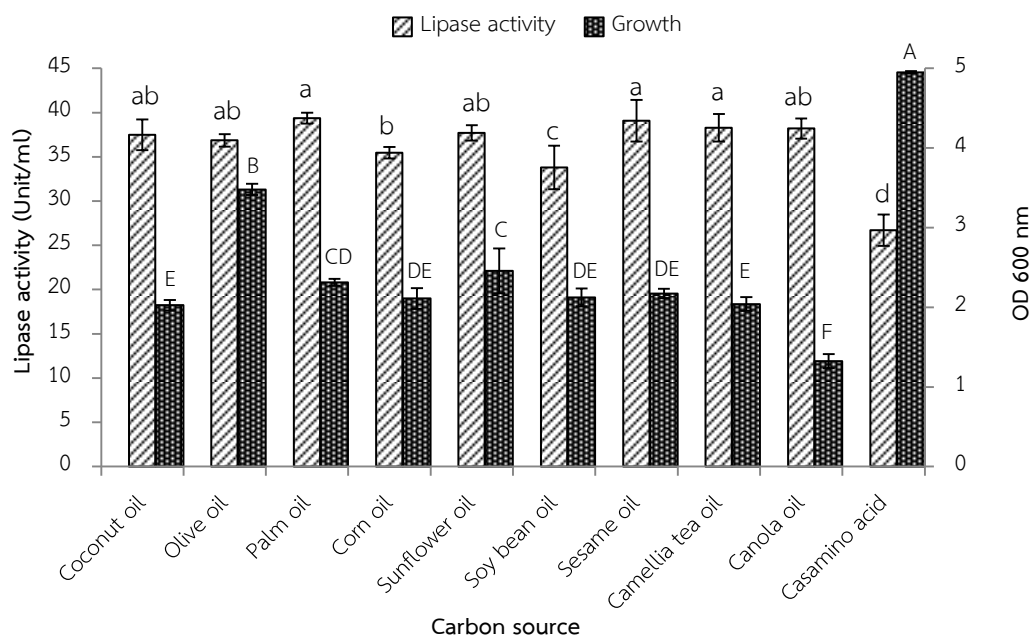
Figure 4.13 Effect of carbon source on production of lipase from strain KN3-8-4.

4.4.2 Effect of nitrogen source on lipase production

Different types of nitrogen sources (yeast extract, peptone, tryptone of casein, beef extract, potassium nitrate, ammonium sulfate, urea and ammonium nitrate at 0.5% (w/v) were tested to determine their effect on the production of lipase. The best lipase production was achieved with yeast extract (35.7±1.31 unit/ml) in the production broth and followed by tryptone of casein (16.0±0.59 unit/ml), beef extract (9.4±0.56 unit/ml), peptone (8.7±1.05 unit/ml), ammonium sulfate (3.5±0.23), urea (3.0±0.31 unit/ml), ammonium nitrate (2.9±0.38 unit/ml) and potassium nitrate (0.9±0.14 unit/ml) (Figure 4.14). The yeast extract was reported as best nitrogen source for lipase production in *Bacillus* sp. (Sidhu et al. 1998).
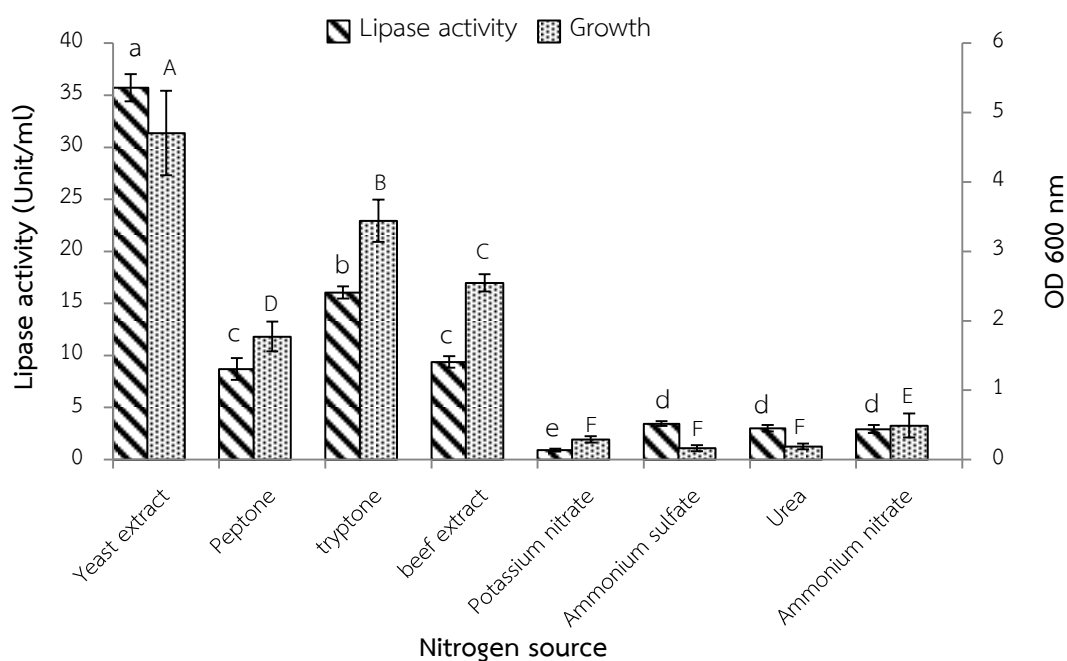
Figure 4.14 Effect of nitrogen source on production of lipase from strain KN3-8-4.

4.4.3 Effect of NaCl on lipase production

Strain KN3-8-4 was grown in the suitable modified basal medium containing 0.5% (v/v) palm oil as carbon source, 0.5% (w/v) yeast extract as nitrogen source and various concentrations of NaCl (0, 1, 3, 5, 7, 9, 11, 13, 15%, w/v), pH 7.5 and incubated at 37°C, shaking 200 rpm for 48 h. The optimal concentration of NaCl for lipase production was 1 and 3% w/v (60.7±0.74 and 58.4±2.92 unit/ml) shown in Figure 4.15. One percentage of NaCl concentration was selected for lipase production.

4.4.4 Effect of initial pH on lipase production

Strain KN3-8-4 was grown in the modified basal medium containing 0.5% (v/v) palm oil, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. The culture was incubated at varying initial pH (5, 6, 7, 7.5, 8, 8.5, 9 and 10) at 37°C, 48 h. The result showed that

the optimal initial pH for lipase production was in range of 5.0-8.5 (Figure 4.16). The pH of 8.5 (71.2±0.69 unit/ml) was selected for lipase production.
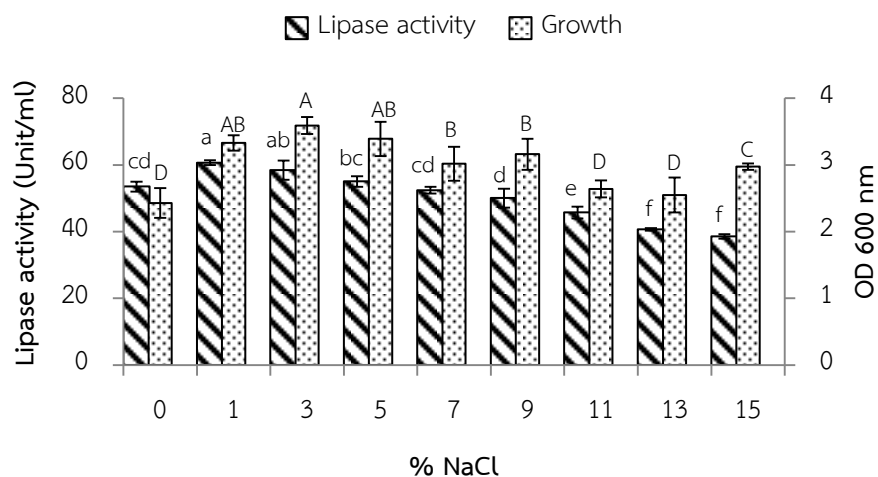
Figure 4.15 Effect of NaCl on production of lipase from strain KN3-8-4.
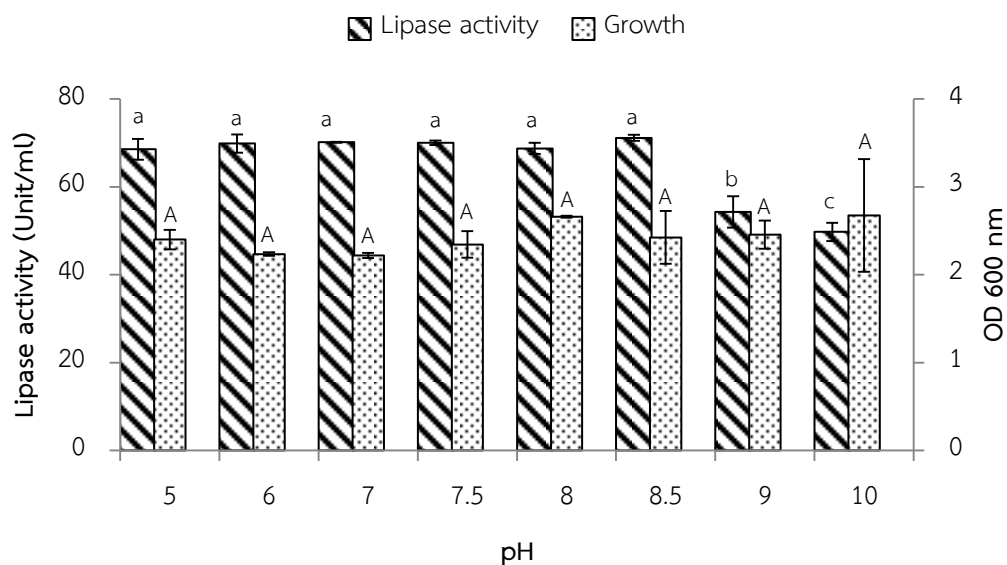
Figure 4.16 Effect of initial pH on production of lipase from strain KN3-8-4.

4.4.5 Effect of temperature on lipase production

Strain KN3-8-4 was grown in the suitable modified basal medium (pH 8.5) containing 0.5% (v/v) palm oil, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. The culture was incubated at varying temperature (25, 30, 37, 40°C) for 48 hour. The result was shown in Figure 4.17. The optimal temperature for lipase production was 40 °C (96.4±2.39 unit/ml).
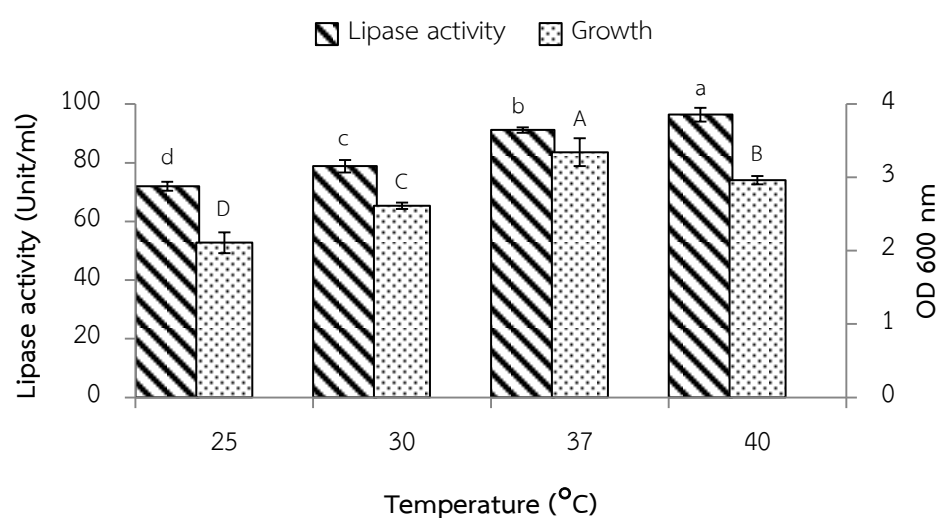
Figure 4.17 Effect of temperature on production of lipase from strain KN3-8-4.

4.4.6 The kinetic of the growth and lipase production

The kinetic of growth and lipase production were investigated. The lag phase of bacterial growth was 6 h and after 24 h the bacterial growth reached to the stationary phase. The maximum lipase production of KN3-8-4 in suitable basal medium was determined in the stationary phase. However, the lipase production was decreased during prolonged cultivation (Figure 4.18). At 36 hour of cultivation, it was selected for lipase production in purification step.
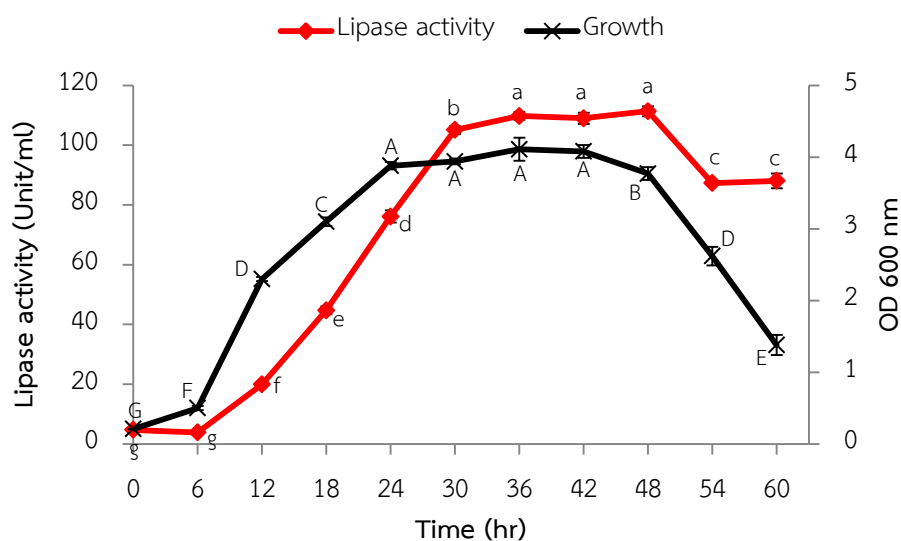
Figure 4.18 The kinetic of growth and lipase production of strain KN3-8-4.

4.5 Purification of lipase from selected halophilic bacteria

Purification step of KN3-8-4[T] lipase is summarized in Table 4.9. After the crude lipase was precipitated by acetone precipitation (40-60%), total activity (2,965.51 units) of approximately 23% remained, while 78% of total protein was removed. From this result, purity of 3.10 fold was achieved after precipitation. After removing salts by using Amicon[®] Ultra-15 centrifugal filter devices (MWCO 3,000 Da), approximately 18% of activity was retained and purification fold of 4.45 was obtained.

The sample was concentrated by concentrator and loaded onto the size exclusion chromatography using Superose 12 column, the column separated KN3-8-4 lipase from other protein by molecular size (Figure 4.19), leading to an increase in purity fold of 7.15 and specific activity of the active fraction increased to 166 units/mg protein. The fraction with highest activity were pooled (Fraction 13) and further purified by Anion exchange chromatography using Hitrap DEAE FF (1 ml) column. After loading onto Hitrap DEAE FF column, the column was eluted by 0-1 M NaCl segment gradients. Four protein peaks (A_{280}) was separated which an activity

peak was eluted at 0.15 M NaCl in the fraction 12 (Figure 4.20). Large amount of proteins was removed after elution with 0-1 M NaCl, leading to increase in purity fold of 18.7 with a yield of 1.2% in this step. This step effectively separated lipase from other protein contaminants.

Table 4.9 Summary of lipase purification from *Virgibacillus kapii* KN3-8-4.

Purification steps	Total activity (Units[*])	Total protein (mg[**])	Specific activity (Units/mg)	Purification fold	% Yield
Crude lipase	12,900	556	23.2	1.0	100
Acetone precipitation	2,965.5	40.7	72.9	3.1	23
Centrifugal filter (MWCO, 3 kDa)	2,334	22.6	103.2	4.5	18
Superose 12	361.8	2.2	166	7.2	2.8
Hitrap DEAE FF	155.9	0.4	433	18.7	1.2
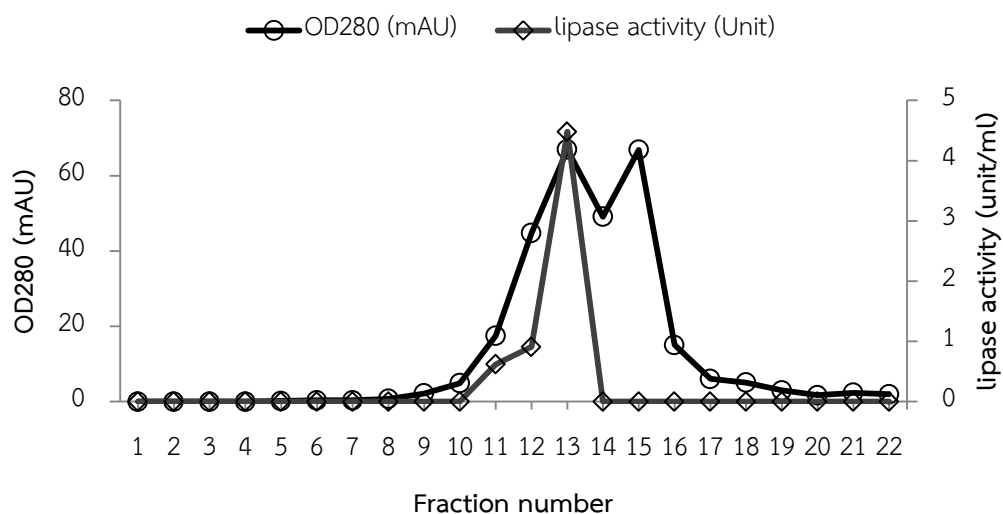
Figure 4.19 Elution profile of strain KN3-8-4 lipase on Superose 12 column. Elution was carried with 150 mM NaCl in 20 mM Tris-HCl, pH 8.0 at flow rate of 0.1 ml/min. Fraction of 1 ml were collected.
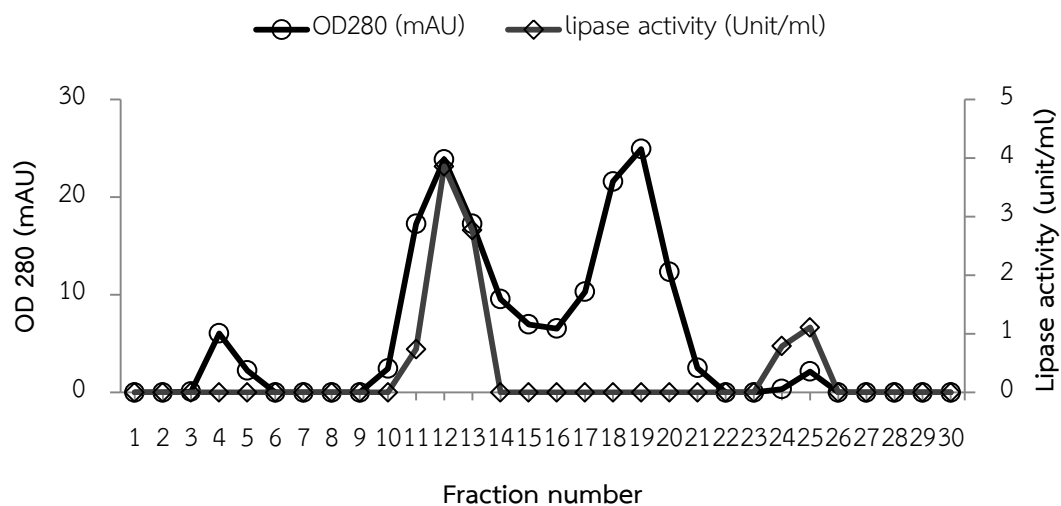
Figure 4.20 Elution profile of strain KN3-8-4 lipase on Hitrap DEAE FF column. Elution was carried with segment gradient of 0-1 M NaCl in 20 mM Tris-HCl, pH 8.0 at flow rate of 0.5 ml/min. Fraction of 1 ml were collected.

The purity of the purified lipase from KN3-8-4 was evaluated by Native-PAGE (Figure 4.21A). After subjected to Hitrap DEAE FF column (lane 4), the single protein band on native gel electrophoresis was obtained (Figure 4.21A).
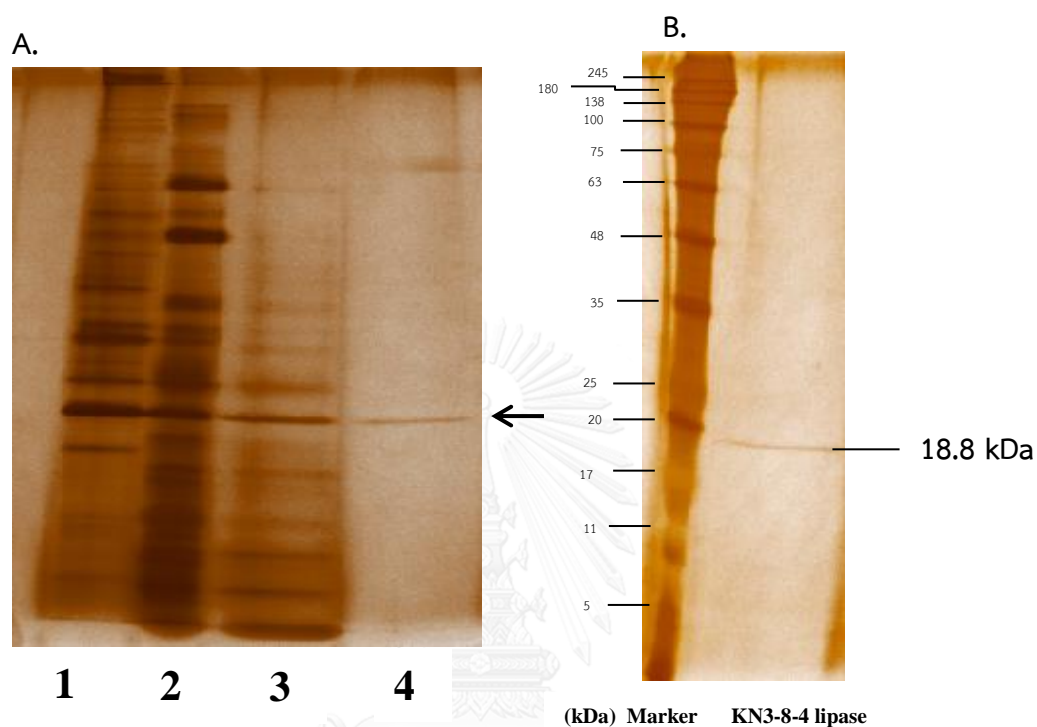
Figure 4.21 Protein pattern of the purified lipase from KN3-8-4 on A.) Native-PAGE. Lane 1, crude lipase; lane 2, Ultra-15 centrifugal filter (MWCO 3 kDa); lane 3, Superose 12 fraction; lane 4, Hitrap DEAE FF fraction (fraction no.12) and B.) SDS-PAGE. Approximately same amount (2 µg) of protein on each lane was applied to 12.5% polyacrylamide gel and electrophoresis was carried out for 90 min 20 mA per gel. The gel was stained with silver staining.

The molecular mass of the purified KN3-8-4 lipase was carried out by Superose 12 gel filtration chromatography, the molecular mass of the purified KN3-8-4 lipase was 19.5 kDa (Figure 4.22A). SDS-PAGE analysis showed that the purified enzyme migrated as a single band with the molecular weight of 19 kDa (Figure 4.21B and 4.22B). This result indicated that KN3-8-4 lipase was monomeric protein with

molecular weights of 19.5 kDa (gel filtration method) and 19.0 kDa (SDS-PAGE). The molecular weight of purified KN3-8-4 lipase was closely to lipase from *Bacillus licheniformis* (19 kDa) (Sharma and Kanwar, 2012); *Bacillus thermoleovorans* ID-1 (18 kDa) (Lee et al, 2001). Reported molecular weights of microbial lipases are variable, ranging from 11 kDa (Castro-Ochoa 2005) to 62 kDa (Kumar 2012).
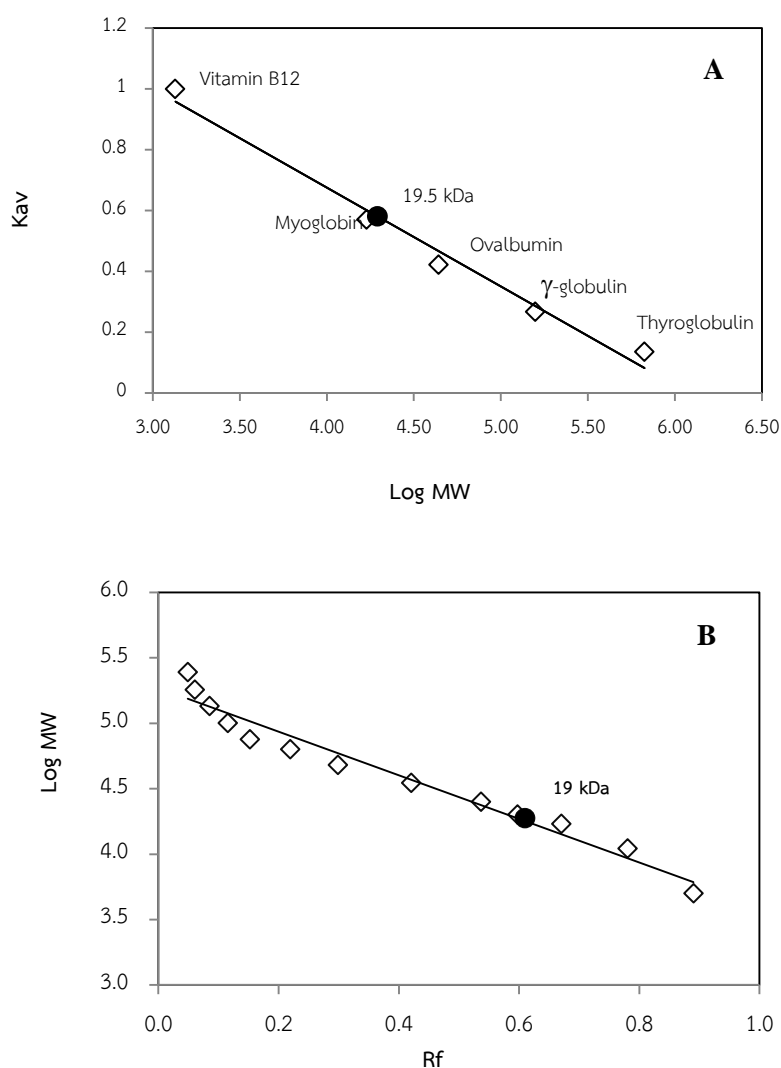
Figure 4.22 Calibration curve for the molecular weight determination on Superose 12 10/300 chromatography. (A) and SDS-PAGE of the purified lipase from KN3-8-4. ●, purified lipase from KN3-8-4.

4.6 Characteristics of the purified lipase from KN3-8-4

4.6.1 Optimal pH

An effect of optimal pH on the purified lipase from KN3-8-4 is shown in Figure 4.23. The activity was highest (4.64 Units) at pH 8.0. There was considerable loss of activity at pH ≤ 7.0 and at pH > 8.0. From these results the purified KN3-8-4 lipase might undergo denaturation at pH < 7.0 and at pH > 9.0. A number of reports showed lipase activity from some halophilic bacteria having optimal pH similar to the result from KN3-8-4[T] such as lipase from *Bacillus sphaericus* 205y (Sulong 2006), *Vibrio fischeri* (Ranjitha 2009), *B. stearothermophilus* AB-1 (Abada 2008), *Microbacterium* sp. (Tripathi 2014), *Pseudomonas stutzeri* (Parwata 2014); *Idiomarina* sp. W33 (Li et al. 2014).
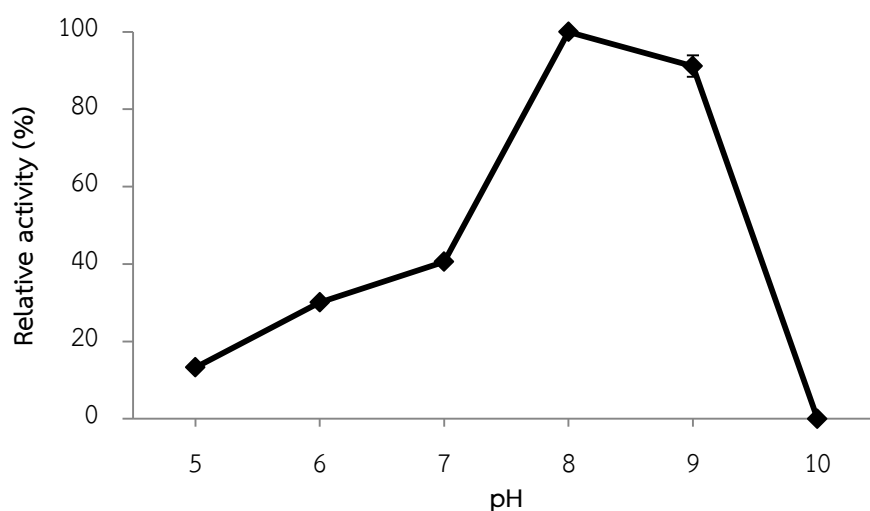
Figure 4.23 pH profile of the purified lipase from *V. kapii* KN3-8-4. Average±S.D. from a duplicate determination. The residual activity was analyzes using 4-nitrophenyl butyrate as a substrate for 30 min at 40°C in various pHs.

4.6.2 Optimal temperature

An effect of optimal temperature on purified lipase from KN3-8-4 is shown in Figure 4.24. The activity was highest at 40°C. An appreciable decrease in activity of enzyme was observed at temperature above 45°C, presumably as a result of thermal inactivation.
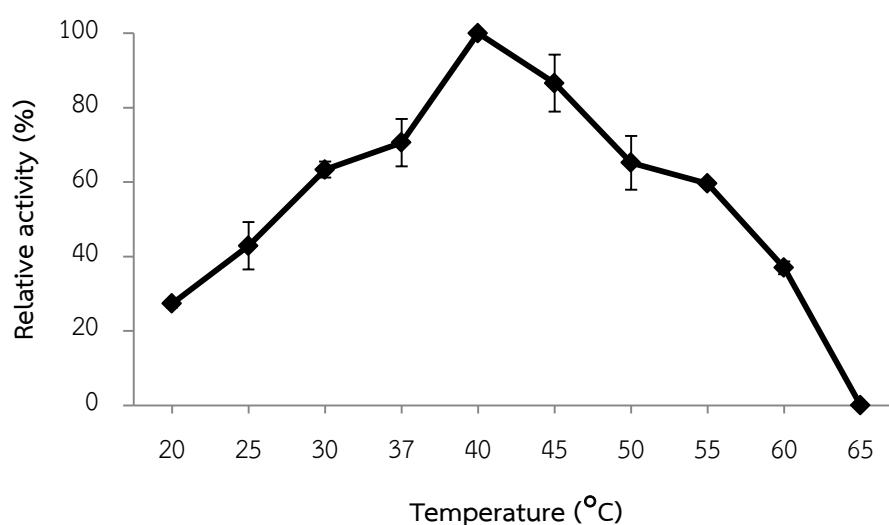
Figure 4.24 Temperature profile of the purified lipase from *V. kapii* KN3-8-4. Average±S.D. from a duplicate determination. The residual activity was analyzes using 4-nitrophenyl butyrate as a substrate for 30 min at pH 8.0 in various temperatures.

4.6.3 Optimal salt concentration

The lipase activity was determined in the presence of various NaCl concentrations (0-15%, w/v), the maximal activity was obtained in the presence 7% w/v NaCl (Figure 4.25). The residual activity that was higher than 50% of the maximal activity was detected in the presence of 11% w/v NaCl or in the absence of NaCl. The effect of NaCl concentration on activity was similar to another lipase halophilic bacteria such as lipase from *Idiomarina* sp. W33 (opt. 7% w/v) (Li et al. 2014);

Bacillus cereus AGP-03 (opt. 3-10% w/v, max. 4.5% w/v) (Ghati 2015); *Chromohalobacter* sp. LY7-8 (opt. 0-20% w/v, max. 12.5% w/v) (Li 2012); *Marinobacter lipolyticus* SM19 (opt. 0-3 M NaCl, Max. absence NaCl) (Pérez 2011).
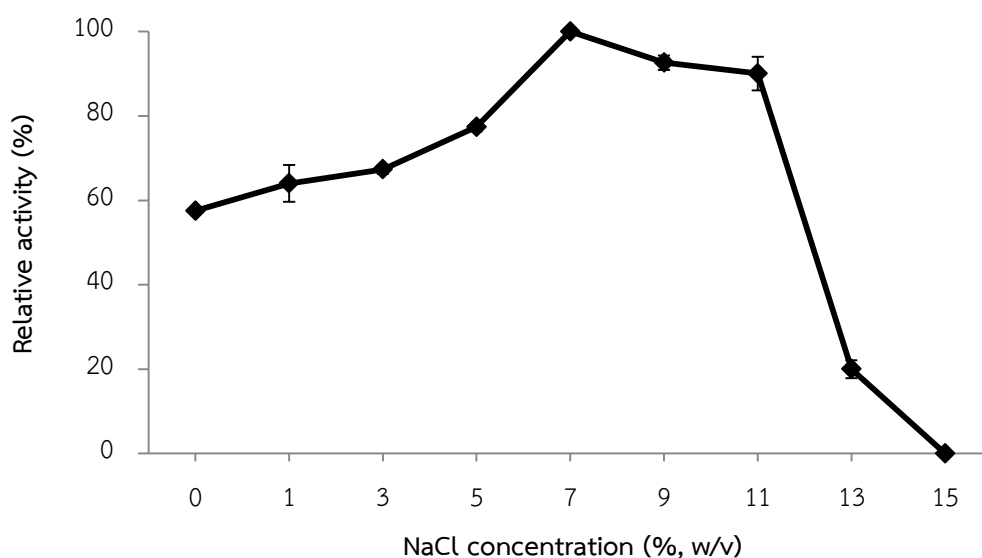
Figure 4.25 NaCl concentration profile of the purified lipase from *V. kapii* KN3-8-4. Average±S.D. from a duplicate determination. The residual activity was analyzes using 4-nitrophenyl butyrate as a substrate for 30 min at pH 8.0 and 40°C at the various NaCl concentration.

4.6.4 Substrate specificity

The purified KN3-8-4 lipase showed a variable specificity or hydrolytic activity towards various *p*-nitrophenyl esters in Figure 4.26. The low C-length (C:4) ester (*p*-NPB) was more efficiently hydrolyzed than other esters. These results indicated a preferential specificity of *Virgibacillus kapii* lipase towards short carbon chain length substrates as reported previously for a lipase from Thermophilic bacteria such as *Bacillus* sp. strain 398, *Bacillus* sp. H1, *B. thermoleovorans* ID-1, *Bacillus* sp. H-257 (Kim 1994; Handelsman 1994; Lee 1999; Imamura 2000).
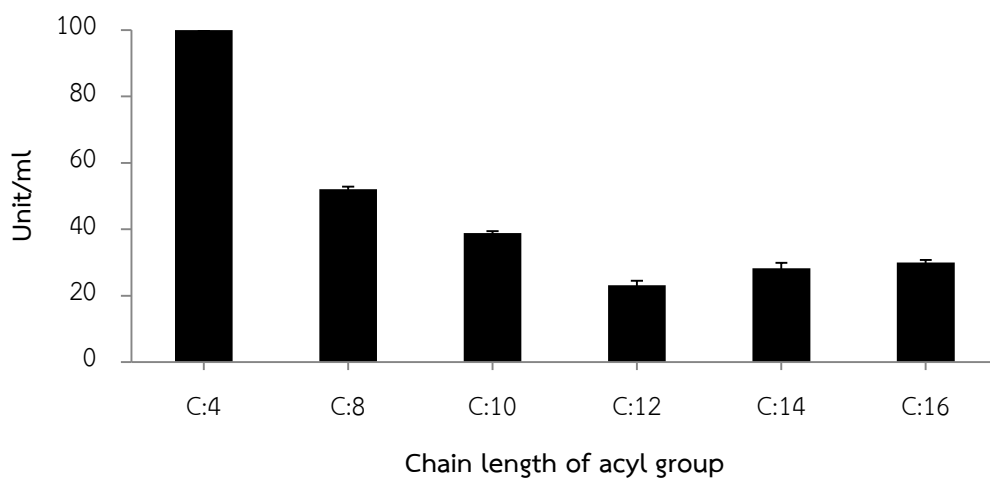
Figure 4.26 Substrate specificity of lipase from *V. kapii* KN3-8-4. Substrates used were 4-nitrophenyl butyrate (C:4), 4-nitrophenyl octanoate (C:8), 4-nitrophenyl decanoate (C:10), 4-nitrophenyl dodecanoate (C:12), 4-nitrophenyl myristate (C:14) and 4-nitrophenyl palmitate (C:16). Average±S.D. from a duplicate determination.

4.7 Kinetic studies

The analysis of these reactions was performed by keeping the concentration of enzyme constant and varying *p*-NPB. A set of these measurements was also performed at various fixed concentrations of *p*-NPB. These data can used to work out what the mechanism of the reaction is. The kinetic constants, K_m, V_{max} and k_{cat} were calculated by Lineweaver-Burk plots of enzyme activity vs. variable 4-NPB concentration (Figure 4.28). The Michaelis constants, K_m, V_{max} and k_{cat} values for 4-NPB were estimated to be 4.76 mM, 7.79 μmol/min and 15.58 (s^{-1}), respectively. The lower K_m value indicated the higher affinity of enzyme for substrate and higher V_{max} value indicated higher activity of enzyme. The purified lipase from *V. kapii* KN3-8-4 had higher Km value for *p*-NPB than lipase from *Bacillus pumilus* RK31 (1.83 mM) (Kumar 2012), lipase from Liver of Carp, *Cyprinus carpio L.* (0.17 mM) (Gorgun 2012), lipase from *Burkholderia anthina* NT15 (0.10 mM) (Jin 2012) and lipase from

Acinetobacter sp. AU07 (0.51 mM) (Gururaj 2016) (Table 4.10). This result suggests that purified lipase from *V. kapii* KN3-8-4 has lower affinity to *p*-NPB, compared to those of other source.
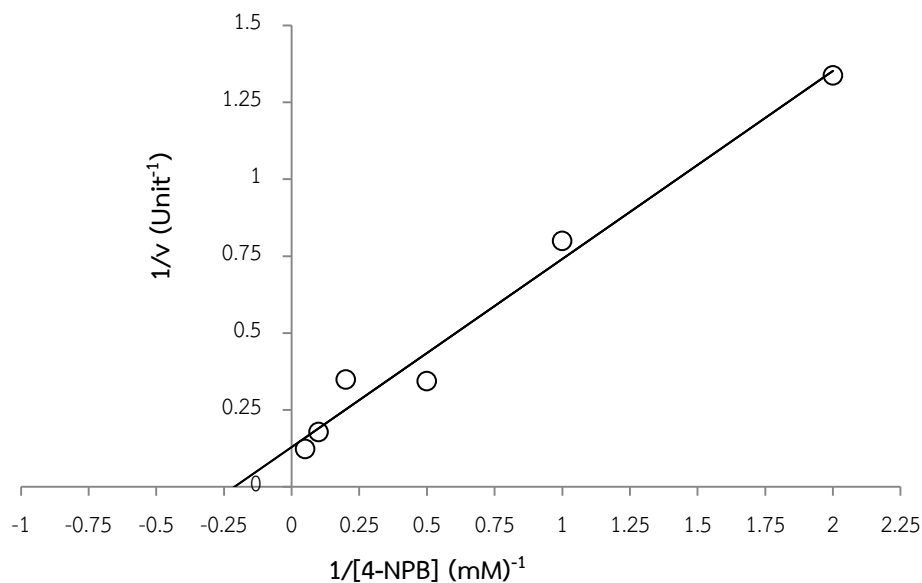
Figure 4.27 Kinetics of the reaction of the purified lipase from *V. kapii* KN3-8-4. Double reciprocal plots of enzyme activity against 4-NPB concentration. The assays were determined at pH 8.0 and 40 $^\circ$C in the presence of 1.2 M NaCl. The final enzyme concentration for assay was 0.01 mg/ml.

Table 4.10 Kinetic properties of lipase from various sources

Species	k_{cat}	K_m	Reference
Virgibacillus kapii KN3-8-4	15.58 s^{-1}	4.76 mM	
Bacillus pumilus RK31	ND	1.83 mM	Kumar 2012
Liver of Carp, *Cyprinus carpio L.*	ND	0.17 mM	Görgün 2012
Naranga aenescens	ND	28.4 mM	Zibaee 2012
Burkholderia anthina NT15	210 s^{-1}	0.10 mM	Jin 2012
Acinetobacter sp. AU07	ND	0.51 mM	Gururaj 2016

ND, not determined

CHAPTER 5
CONCLUSION

In the course of investigation of lipase-producing halophilic bacteria presented in fermented fish and shrimp paste, one hundred thirty eight strains were isolated and were screened. Thirty-six isolates were selected and they were divided into 24 groups based on their phenotypic characteristics and 16S rDNA sequence analysis. Thirty strains (Group I to XIV and XVI to XX) were Gram-positive rods belonged to genus *Virgibacillus* (7 isolates), *Bacillus* (5 isolates), *Alkalibacillus* (1 isolate), *Halobacillus* (1 isolate), *Oceanobacillus* (1 isolate), *Lentibacillus* (1 isolate), *Corynebacterium* (4 isolates), *Brevibacterium* (2 isolates) and Gram-positive cocci belonged to genus *Staphylococcus* (4 isolates) and *Salinicoccus* (4 isolates). Six of Gram-negative rods (Group XV, XXI and XXII) were *Vibrio* (4 isolates), *Proteus* (1 isolates) and *Shewanella* (1 isolate). They were identified as *Virgibacillus dokdonensis* (3 isolates) isolated from *Tai-pla* collected from Nakhon Si Thammarat province and fish sauce collected from Chonburi province (Gold label brand), *Virgibacillus alimentarius* (1 isolate) isolated from fish sauce collected from Chonburi province (Gold label brand), *Virgibacillus halodenitrificans* (2 isolates) isolated from *Tai-pla* and *Keoy-pla* (II) collected from Nakhon Si Thammarat province, *Lentibacillus juripiscarius* (1 isolate) isolated from fish sauce collected from Chonburi province (Gold label brand), *Oceanobacillus iheyensis* (1 isolate) isolated from *Keoy-pla* (I) collected from Nakhon Si Thammarat province, *Alkalibacillus almallahensis* (1 isolate) and *Halobacillus trueperi* (1 isolate) isolated from shrimp paste collected from Nakhon Si Thammarat province, *Bacillus amyloliquefaciens* subsp. *plantarum* (1 isolate) isolated from *Kee-dee* collected from Nakhon Si Thammarat province, *Bacillus altitudinis* (1 isolate) and *Bacillus zhangzhouensis* (1 isolate) isolated from fish sauce collected from a factory in Nakhon Si Thammarat province (Silver label

brand), *Bacillus seohaeanensis* (1 isolate)) isolated from fish sauce collected from Chonburi province (Gold label brand)**,** *C. falsenii* (2 isolates) and *C. variabile* (2 isolates) isolated from *Keoy-pla* (I) collected from Nakhon Si Thammarat province, *Brevibacterium sediminis* (2 isolates) isolated from shrimp paste collected from Nakhon Si Thammarat province, *Proteus penneri* (1 isolate) isolated from *Poo-khem* collected from Nakhon Si Thammarat province, *Staphylococcus saprophyticus* subsp. *bovis* (2 isolates) and *Staphylococcus saprophyticus* subsp. *saprophyticus* (1 isolate) isolated from *Tai-pla* collected from Nakhon Si Thammarat province, *S. nepalensis* (1 isolate), *Salinicoccus salsiraiae* (1 isolate) and *Salinicoccus siamensis* (3 isolates) isolated from shrimp paste collected from Nakhon Si Thammarat province, *Vibrio alginolyticus* (4 isolates) and *Shewanella indica* (1 isolate) isolated from *Poo-khem* collected from Nakhon Si Thammarat province. *Corynebacterium falsenii* FN1-14 showed highest lipase activity (26.68±2.62 unit/ml) and followed by *C. falsenii* FN1-1 (21.61±1.47 unit/ml), *V. dokdonensis* NR1-1 (6.78±0.13 unit/ml), *B. zhangzhouensis* NN2-2 (3.70±0.13 unit/ml) and *V. dokdonensis* NR3-3-3 (3.68±0.14 unit/ml).

The novel species in Group XXIII, *Bacillus* sp. NR1-3-2 isolated from fish sauce and Group XXIV, *Virgibacillus* sp. KN3-8-4 isolated from shrimp paste (*ka-pi*) were characterized based on polyphasic taxonomy. Strain NR1-3-2 contained anteiso-$C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{17:0}$ as major cellular fatty acids and had diphosphatidyl glycerol (DPG), phosphatidyl glycerol (PG) and one glycolipid as polar lipids. DNA G+C content was 44.2 mol%. The 16S rDNA sequence analyses indicated that strain NR1-3-2 highest similarity with *B. iranensis* (97.4%). The DNA -DNA relatedness between strain NR1-3-2 and *B. iranensis* DSM 23995[T] was 39.8%, therefore it was proposed as *Bacillus piscicola* sp. nov. Strain KN3-8-4 contained anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$ as major cellular fatty acids and had PG, DPG, two unknown phospholipids and one glycolipid as polar lipids. The DNA G+C content was 43.58 mol%.The 16S rDNA sequence analyses indicated that strain KN3-8-4 closely related to *V. olivae* (97.85%).

The DNA -DNA relatedness between strain KN3-8-4^T and *V. olivae* JCM 30551^T was 28.8%, therefore it was proposed as *Virgibacillus kapii* sp. nov.

Among 70 isolates, strain KN3-8-4 in Group XXIV produced extracellular lipase was selected for lipase purification due to its novel species. The maximum lipase production of strain KN3-8-4 was at stationary phase and could be achieved when cultivated in a JCM no. 377 medium (pH 8.5) that casamino acids was replaced with 0.5% palm oil (w/v), incubated in 1% NaCl (w/v), at 40°C for 36 h. The KN3-8-4 lipase was purified by cold acetone precipitation, gel filtration (on Superose 12 10/300 column) and anion exchange chromatography (on Hitrap DEAE FF column) with 18.66-fold purification. The purified lipase from KN3-8-4 was monomeric protein with the molecular mass of about 19.5 kDa by gel filtration and 19 kDa by SDS-PAGE. The enzyme had a maximal activity in the presence of 7% w/v NaCl, pH 8.0 at 40°C. The enzyme exhibited a variable specificity activity towards various *p*-nitrophenyl esters especially *p*-Nitrophenyl butyrate (C4).

The present lipase had optimal activity in wide range of NaCl concentration, substrate specificity and an alkaline environment.

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APPENDIX A
Culture media and reagents for identification

All media were dispensed and sterilized in autoclave for 15 min at 15 pounds pressure and 121°C except for acid production from carbon sources test which was sterilized at 110°C, 10 min.

1. JCM medium no. 377

Casamino acids	5	g
Yeast extract	5	g
Sodium glutamate	1	g
Trisodium citrate	3	g
$MgSO_4.7H_2O$	20	g
KCl	2	g
NaCl	100	g
$FeCl_2.4H_2O$	0.362	g
$MnCl_2.4H_2O$	0.0362	g
Agar	20	g
Distilled water	1	L

Adjust pH to 7.2 with 1 N NaOH

2. Modified JCM medium no. 377 broth

Casamino acids	5	g
Yeast extract	5	g
Sodium glutamate	1	g
Trisodium citrate	3	g
$MgSO_4.7H_2O$	20	g
KCl	2	g
NaCl	50	g

FeCl$_2$.4H$_2$O	0.362	g
MnCl$_2$.4H$_2$O	0.0362	g
Distilled water	1	L

Adjust pH to 7.2 with 1 N NaOH

3. Basal medium for lipase production

Yeast extract	5	g
Sodium glutamate	1	g
Trisodium citrate	3	g
MgSO$_4$.7H$_2$O	20	g
KCl	2	g
NaCl	50	g
FeCl$_2$.4H$_2$O	0.362	g
MnCl$_2$.4H$_2$O	0.0362	g
Distilled water	1	L

Adjust pH to 8.0 with 1 N NaOH

4. Complex medium (CM)

Casein peptone	7.5	g
Yeast extract	10	g
Sodium citrate	3	g
MgSO$_4$.7H$_2$O	20	g
KCl	2	g
FeSO$_4$.7H$_2$O	0.01	g
NaCl	50	g
Distilled water	1	L

Adjusted pH to 7.0

5. Marine oxidation-fermentation medium (MOF)

Casitone (Difco)	1	g
Yeast extract	0.1	g
Ammonium sulfate	0.5	g
Tris	0.5	g
NaCl	50	g
Phenol red 0.001%		
Distilled water	1	L
Adjusted pH to 7.5		

6. L-arginine agar medium

Peptone	1	g
K$_2$HPO$_4$	0.3	g
NaCl	50	g
L-(+) arginine hydrochloride	1	g
Phenol red, 0.1% aq. solution	1	ml
Agar	3	g
Distilled water	1	L
Adjust pH to 7.2		

7. Starch agar

JCM medium no. 377 agar	100	ml
Starch	1%	(w/v)
Dissolve and adjust pH to 7.2		

8. Gelatin agar

JCM medium no. 377 agar (omitted casamino acid)	100 ml	
Gelatin	1%	(w/v)
Dissolve and adjust pH to 7.2		

9. Tween 80 agar medium

        JCM medium no. 377 agar (omitted casamino acid)   100 ml

Tween 80	1%	(w/v)

        Dissolve and adjust pH to 7.2

10. Nitrate broth

Beef extract	10	g
Peptone	10	g
KNO_3	1	g
NaCl	5	g
Distilled water	1	L

        Adjust pH to 7.2

11. Kovac's reagent

p-dimethylaminobenzaldehyde	5	g
Amyl alcohol	75	g
Conc. HCl	25	ml

Distilled the aldehyde in the alcohol by gently warming in a water bath (about 50-55°C). Cool and acid with care. Protect from light and store at 4°C.

12. Nitrate test reagent

Solution A: 0.33% sulphanilic acid in N-acetic acid dissolve by gentle heating

Solution B: 0.6% dimethyl-**α**-napthylamine in N-acetic acid dissolve by gentle heating

13. PCR reaction mixture

Stock		1 Volume (100 µL)
Forward Primer: 20F	10 pmol/µl	4
Reverse Primer: 1492R	10 pmol/µl	4
10 x Taq buffer (NH_4SO_4-$MgCl_2$)10 x		10
dNTP	2.0 mM	2

	MgCl$_2$	25 mM	8
	Taq DNA polymerase	5 Unit/µl	0.5
	Milli-Q water	-	66.5
	Template	Undilute	5

14. 1X Tris-acetate (TAE) buffer

50X Tris-acetate (TAE) buffer	20	ml
Distilled water	980	ml

15. 0.8% Agarose gel

Agarose	0.8	g
Distilled water	100	ml

Melt the mixture with the microwave.

16. Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	ml

17. Lugol's iodine

KI	40	g
Distilled water	100	ml

18. 30% Trichloroacetic acid

Trichloroacetic acid (TCA)	15	g
Distilled water	100	ml

19. Tryptone broth

Tryptone	5%	(w/v)
NaCl	5%	(w/v)

Adjust pH to 7.2

APPENDIX B

Reagents for chemotaxonomic characteristic

1. 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 salmon sperm DNA (10 mg/mL)		0.1		ml
Distilled water	2.9			ml

   Hybridization solution (10 ml)

Prehybridization solution	100	ml
Dextran sulfate	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

3,3',5,5' Tetramenthylbenzidine (TMB)		
(10 mg/ml in DMFO)	100	ml
0.3% H_2O_2	100	ml

0.1 M citric acid + 0.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMFO 5 ml 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		

100 x Denhardt solution

Bovine serum albumin	2	g
Polyvinylpyrrolidone	2	g
Ficoll400	2	ml

Dissolve in 100 ml Nanopure water and store at 4°C until used

Salmon sperm DNA (10 mg/mL)

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.

20X Saline sodium citrate (SSC)

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

1X Saline sodium citrate (SSC)

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

        <u>Reagent A</u>

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

        <u>Reagent B</u>

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 (50ml) plus solution B (50 ml) plus 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 sodium chloride)

Sodium chloride saturated in Milli-Q water

4.   RNase A solution

RNase A	20	ng
0.15 M NaCl, pH 5.0	10	ml

Dissolve RNase A in 0.15 M NaCl, pH 5.0 and heat at 95 °C for 5-10 min. Keep RNase A solution at -20 °C

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°C for 5 min. Keep RNase T solution at -20°C

APPENDIX C

Buffers and reagents for lipase determination and purification

1. Universal buffer (Britton-Robinson buffer, pH2-12)

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

2. Polyacrylamide gel electrophoresis (PAGE) reagents

    Monomer solution

Acrylamide	30%
(w/v)	
Bisacylamide	0.8%
(w/v)	

    Made up to 100 ml with distilled water

3. 4x Resolving gel buffer

Tris(Hydroxymethyl)aminomethane	18.15	g
Milli-Q water	90	ml

    Dissolve and adjust the pH to 8.8 by using 0.1 N HCl. Made up to 100 ml with Milli-Q water

    Note: The solution can be store up to 3 months at 4°C in the dark.

4. 4x Stacking gel buffer

Tris(Hydroxymethyl)aminomethane	6	g
Deionized water	90	ml

    Dissolve and adjust the pH to 6.8 by using 0.1 N HCl. Made up to 100 ml with Milli-Q water

    Note: The solution can be store up to 3 months at 4°C in the dark.

5. 10x Tank buffer for SDS-PAGE

Tris(Hydroxymethyl)aminomethane	30.28	g
Glycine	144.13	g
Sodium dodecyl sulfate	10	g
Distilled water	900	ml

Dissolve and made up to 1 L with distilled water

6. 10% Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate	10	g
Milli-Q water	90	ml

Dissolve and made up to 1 L with milli-Q water

7. 2x Sample buffer for SDS-PAGE

4x Stacking gel buffer	2.5	ml
Glycerol	2	ml
10% (w/v) SDS	4	ml
Bromophenol blue (2 mg/ml)	1	ml
β-mercaptoethanol	0.2	ml

Dissolve and made up to 10 ml with milli-Q water

Note: The reagent should be filtered before use. 2x Sample buffer for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

8. 12.5% Running gel for SDS-PAGE

Milli-Q water	4.1314	ml
4x Running gel buffer	3.25	ml
Monomer solution	5.4171	ml
10% (w/v) SDS	130	µl
10% (w/v) Ammonium persulfate	65	µl

TEMED	6.5	µl

<u>Note</u>: 12.5% Running gel for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

9. 4% Stacking gel for SDS-PAGE

Milli-Q water	3.053	ml
4x Stacking gel buffer	1.25	ml
Monomer solution	677	µl
10% (w/v) SDS	50	µl
10% (w/v) Ammonium persulfate	25	µl
TEMED	5	µl

<u>Note</u>: 4% Stacking gel for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

10. Silver staining kit reagent (GE Healthcare)

<u>Fixing solution TCA</u>

Trichloroacetic acid	50	g

Add Milli-Q water to 250 ml

<u>Sensitizing solution</u>

Ethanol	75	ml
Sodium thiosulphate (5% w/v)	10	ml
Sodium acetate	17	g

Add Milli-Q water to 250 ml

Note: Before use, Add 1.25 ml glutardialdehyde (25% w/v)

<u>Silver solution</u>

Silver nitrate solution (2.5% w/v)	25	ml

Add Milli-Q water to 250 ml

<u>Developing solution</u>

Sodium carbonate	6.25	g

Add Milli-Q water to 250 ml and vigorously stir to dissolve the sodium carbonate

Note: Before use, Add 0.2 ml formaldehyde (37% w/v)

Stop solution

EDTA-Na$_2$.2H$_2$O	3.65	g

Add Milli-Q water to 250 ml

Washing solution

Milli-Q water

Preserving solution

Glycerol (87% w/w)	25	ml

Add Milli-Q water to 250 ml

Staining procedure

1. Fixation: 30 min

   Soak the gel in fixing solution for 30 minutes.

2. Washing: 3 x 5 min

   Wash the gel 3 times in distilled water for 5 minutes each time.

3. Sensitizing: 30 min

   Remove the solution. Add sensitizing solution and leave shaking for at least 30 minutes.

4. Washing: 3 x 10 min

   Remove the solution. Add distilled water and wash the gel 3 times for 10 minutes each time.

5. Silver reaction: 20 min

   Add silver solution and leave shaking for 20 minutes.

6. Washing: 2 x 1 min

   Remove the silver solution. Rinse twice in distilled water for one minutes each time.

7. Developing: 2 to 5 min

   Add developing solution and leave shaking for 2 to 10 minutes. Transfer the gel to stopping solution when the bands have reached desired intensity.

8. Stopping: 10 min

   Leave gels shaking in stopping solution for 10 minutes.

9. Washing: 3 x 5 min

   Remove the stop solution. Add distilled water and wash the gel 3 times for 5 minutes each time.

10. Preserving: 20 min

    Add preserving solution and leave shaking for 20 minutes.

11. Drying: Overnight

    Put the gel on a glass plate and wrap it in Cellophane sheet. Leave the gel to dry overnight at room temperature. Do not put the gel in a heating cabinet (the silver stain bleaches at elevated temperatures).

APPENDIX D

16S rDNA sequence of strains

1. Strain FN1-8 (1373 bp)

AAGCAGATCTCCTTCGGGAGTGACGCTTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTGTAAGACTGGG
ATAACTCCGGGAAACCGGGGCTAATACCGGATGAAACAAAGCGTCGCATGACGCAATGTTAAAAGGCGGCATATGCTGTCACTTAC
AGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCCATTCGAATAGGTTGGCACCTT
GACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTTAACCGTGGAGGGCCATTGGAAACTGGAGGA
CTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGAC
TCTCTGGTCTGTAACTGACGCTGAGGTGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCT
GAAACTCAAAAGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTCTGACACCCCTAGAGATAGGGCATTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTAGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACA
AAGGGAGCGAAGCCCCGAGGCCAAGCAAAATCCCATAAAACACATTCTCAGTTCGGATTGCAGGCTGCAACTCGCACTGCATGAAA
CCGGAATCGCTAGTAATCACGGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTAC

2. Strain NR1-3-4 (1392 bp)

CCCCTTCGGGGGGTGACGTTTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATTGGGATAACTCG
CGGAAACGTGAGCTAATACCGAATGACACTTTTCATCGCCTGATGGGAAGTTAAAAGGCGGCATTTGCTGTCACTTACAGATGGG
CCCGCGGCGCATTAGCTAGTTGGTGAGATAAAAGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTATTGTTCGAATAGGACAGTACCTTGACGGT
ACTTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGT
AAAGCGCGCGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCCACGGCTTAACCGTGGAGGGTCATTGGAAACTGGAGGACTTGAG
TACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GTCTGTAACTGACGCTGAGGTGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC
TAGGTGTTAGGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAAAGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGCTATCGGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGGTGCATGGTTGTCGTCAGCTCGTGTCCT
GAAAATGTTGGGGTTAAGTCCCGTAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGA
ACAAAGGGCAGCGAAGCGGCAACGTGTTAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG
AAGCAGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGA
GAGTTGGCAACACCCGAAGTCGGTGAGGTAAC

3. Strain FN6-6 (1406 bp)

CGAGCGCGGGAAGCAAGCTGATCCTCTTCGGAGGTGACGCTTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCT
GCCTGTAAGACTGGGATAACCCCGGGAAACCGGGGCTAATACCGGATAATACTTTTCATCACCTGATGGGAAGTTGAAAGGTGGC
TTTTAGCTACCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGATGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTC
GAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAG
CGTTGTCCGGAATTATTGGGAGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT
CATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA
CCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCTCTGCAATCGGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGATGGAACAAAGGGAAGCAAAACCGCGAGGTCAAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGG
CTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGCCTTGTACACAC
CGCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAA

4. Strain NR1-3-1 (1525 bp)

AGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCAGGCAAACACCCTTTCGGGGTGTGGCGCCTGTG
GAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTCGCGGAAACGTGAGCTAATACCGGA
TAATGTCTTTCCTCGCATGAGGGAGGGCTGAAAGACGGCCTTTGTGCTGTCACTTACAAATGGGCCCGCGGCGCATTAGTTAGTT
GGTGAGGTAAGAGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTC
GGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGCGTGGTTCGAATAGGGCCATGCCTTGACGGTACCTGACCAGAAAGCCCCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTT
CTTAAGTCTGATGTGAAATCTTGCGGCTTAACCGCAAGTGGTCATTGGAAACTGGGAGGCTTGAGTACAGAAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
CGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGAGTGCTAGGTGTTAGGGGGTTTCC
GCCCCTTTGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAGCGGCAGA
GATGCCGTGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
TAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCGAAGCCGTGAG
GTGTAGCAAATCCCACAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGTATGAAGCCGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGAAGTCGGT
GAGGTAACCTTTTTGGAGCCAGCCGCCGAAGGTGGGGCCAATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGG

5. Strain FN1-10 (1390 bp)

TCTTCGGAGGTGATGATAATGGAATGAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTAAGACTGGGATAACTCGTG
GAAACGCGAGCTAATACCCTGATAACACTTTTCATCTCCTGATGAGAAGTTGAAAGGCGGCTTTTGCTGTCACTTACAGATGGGCC
TGCGGCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACTATAGTAACTGATAGTACCTTGACGGTACC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAA
GCGCTCGCAGGCGGTTCTTTAAGTCTGATGTGAAATCTTACGGCTCAACCGTAAACGTGCATTGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAG
GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCA
AAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC
CTCTGAACACTCTAGAGATAGAGTTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGG
GAAGCGAACCCGCGAGGTCAAGCAAATCCCACAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGA
ATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGT
AACACCCGAAGTCGGTGAGGTAACCGTAA

6. Strain KN3-2-4 (1390 bp)

GAGCAGAAGCCCTTCGGGGTGGACGCTCGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTGTGAGACGGG
GATAACTCCGGGAAACCGGGGCTAATACCGGATAACGCATCGAACCGCATGGTTCGATGATCAAAGATGGCTTCTAGCTATCACT
CACAGATGGGCCCGCGGCGCATTAGTTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCATCCGCAATGGACGCAAGTCTGACG
GTGCAACGCCGCGTGAGCGATGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTGAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGC
ACCTTGACGGTACCTCACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGGCCACAGCTCAACTGTGGAGGGCCATTGGAAACTG
GGGAACTTGAGGACAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAA
GGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGAGTGCTAGGTGTTAGGGGGTCCAACCCCTTAGTGCTGCAGTTAACGCAATAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACC
AGGTCTTGACATCTTCGGACAGCCCAAGAGATTGGGTCTTCCCTTCGGGGACCGAATGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTGAGTTGGGCACTCTAGGATG
ACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GATGGTACAAAGGGAAGCCAAACCGCGAGGTCGAGCTAATCCCATAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCT
GCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCAGGCCTTGTACACACCGCCCGTCACAC
CACGAGAGTTGGCAACACCCGAAGTCGGTGGGG

7. Strain KN3-7-1 (1435 bp)

ACATGCAAGTCGAGCGCGGGAAGCGAGTGGCTCCCTTCGGGGTGAAGCTCGTGGAACGAGCGGCGGACGGGTGAGTAACACGTG
GGCAACCTGCCTGTAAGATCGGAATAACCCCGGGAAACCGGGGCTAATGCCGGGTAATACTTTCTTTCGCATGAAGGAAAGTTGA
AAGATGGCTTCTAGCTATCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACA
AGTACCGTGCGAATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATG
TGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGCTTCCACCCCTTAGTGCTGAAGTTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTGGACATCCCTAGAGATAGGGCTTTCCCTTCGGGGACCAAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTAATCTTAGTTGCCAGC
ATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTGTAGCAAATCCCATAAAACCATTCTCAGTTC
GGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCG

8. Strain FN3-7 (1436 bp)

GGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAA
AAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC
GGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGC
ATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCC

9. Strain NN2-10 (1372 bp)

AGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
AGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCACCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCTGAGTGATG
AAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAG
AGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC
GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGG
GTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGACATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACC
CTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCG
CAAGGTTTAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATC
GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTC
GGTGAGGTAA

10. NN2-2 (1516 bp)

TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGC
ATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCTCAC
CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTT
GTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAG
CCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGG
GACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCGAATCCCATAAAT
CTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGGCCGCGGGTG
AATACGTTCCCGGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAATTCGGTGAGGTAACCCTTTA
TGGAGCCAGCCCGCCGAAGGTGGGGGCAGATGATTTGGGGGTGAAAGTCGTAAACAAAGGTAGCCCTTATCGG

11. Strain NR3-3-1 (1285 bp)

GGGATAACTCGCGGGAAACCGGGGCTAATACCGGATAACATCTTTCTTTGCATAAAGAGAGATTGAAAGATGGCTTTTTGCTATCA
CTTATAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGTTCGAATAGGGCGG
TACCTTGACGGTACCTAACCAGGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC
TGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTTGGAGGAACACCAGTGGCG
AAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAAGTGTTTAGGGGGGTTTCCGCCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAATTTGACGGGGGCCCGCACAAGCGGTGGAACCATGGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGCTACCTCTAGAGATAGAGGGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGG
GCACTCTAAGATGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACGGTACAAAGGGCAGCGAGACCGCGAGGTTTAGCCAATCCCATAAAACCGTTCTCAGTTCGGATTGTAGG
CTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTC

12. Strain FN1-14 (1312 bp)

TTGCTTGCAGGGGGTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCTTGTACTTCGGGATAAGCCTGGGAAACTG
GGTCTAATACCGGATAGGACCATGCTTTAGTGTGTGTGGTGGAAAGTTTTTTCGGTACAAGATGAGCCCGCGGCCTATCAGCTTGT
TGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTACGGCCACATTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTTGAGGGATGACGGCC
TTCGGGTTGTAAACCTCTTTCGCTAGGGAAGAAGCCACTTTGTGGTGACGGTACCTGGATAAGAAGCACCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTCCTCTG
TGAAATCCCGGGGGCTTAACTCCGGGCGTGCAGGCGATACGGGCATAACTGGAGTGCTGTAGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCAT
GGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCTAGGTGTGGGGATCTTTCTACGATTTCCGTG
CCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATATGCAGGATCGCTGCAGAGATGTAGTTT
CCCTTGTGGTCTGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTCTTGTGTTGCCAGCACGTTATGGTGGGGACTCGCGAGAGACTGCCGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGAGGGTCGCGATACCGTGAGGTGGAGCTAAT
CCCTGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAGTCGCTAGTAATCACAGATCAGCAACG
CTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGGAAAGTTGGCTAACACCCCGAAGGCCAGTGGCCC
CAAA

13. Strain FN1-13 (1373 bp)

TCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGAAGGCCCTGCTTGCAGGGTACTCGAGTGGCGAACGG
GTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACCATCGTTT
AGTGTCGGTGGTGGAAAGTTTTTCGGTGCAGGATGAGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGC
GTCGACGGGTAGCCGGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGAAGGCCTTCGGGTTGTAAACTCCTTTC
AACCATGACGAAGCATTATGTGACGGTAGTGGTAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTCGTCTGTGAAATTCCGGGGCTTAAC
TCCGGGCGTGCAGGCGATACGGGCATAACTTGAGTGCTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGA
TATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCATGGGTAGCGAACAG
GATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCTAGGTGTGGGGGTCTTCCACGACTTCTGTGCCGTAGCTAAC
GCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAG
CATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATATGCCGGATCGGCGCAGAGATGCGTTTTCCCTT
GTGGTCGGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGTCTTGTGTTGCCAGCACGTTATGGTGGGGACTCGCGAGAGACTGCCGGGGTTAACTCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGTGGGTTGCTACACCGTGAGGTGGTGCT
AATCCCTTAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCCAATCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAG
CAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTT

14. Strain KN3-6-3 (1350 bp)

TTGCTGTTGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGATTTCGGGATAAGCCTGGGAAACTG
GGTCTAATACCGGATACGACCAACCCTCGCATGAGGGTTGGTGGAAAGTTTTTCGATCGGGGATGGGCTCGCGGCCTATCAG
CTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCCAATGGGGGAAACCCTGATGCAGCGACGCAGCGTGCGGG
ATGACGGCCTTCGGGTTGTAAACCGCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGTACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGTTGGTC
ACGTCTGCTGTGGAAACGCAACGCTTAACGTTGCGCGTGCAGTGGGTACGGGCTGACTAGAGTGCAGTAGGGGAGTCTGGAA
TTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGACTCTGGGCTGTAACTGACACTG
AGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGAC
ATTCCACGTTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACT
GGACCGTTCTGGAAACAGTTCTTCTCTTTGGAGCTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGAACTCATAGGAGACTGCCGGGGT
CAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTTATGTCTTGGGCTTCACGCATGCTACAATGGCTGGTAC
AGAGAGAGGCGAACCCGTGAGGGTGAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATCGTAGTCTGCAATTCGACTACGTG
AAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTC
ACGAAAGTCGGTAACACCCGAAGCCGGTGTCCCAACCCC

15. Strain CN1-10 (1504 bp)

GCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGC
GGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATGACGTCTACGG
ACCAAAGCAGGGGCTCTTCGGACCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGG
CGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGG
GAGGAAGGTGATAAAGTTAATACCTTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGA
GCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGTCTTGAACCGTGGCTTCTGGAGCTAACGCGTT
AAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGCTGA
GACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTG
CCAGCGCGTGATGGCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC
TTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTCTGT
CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTT
ACCACTTTGTGATTCATGACTGGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCT

16. Strain FN6-1 (1396 bp)

GAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGA
TAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAG
ATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCACGTCTTGA
CGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACT
TGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTT
TCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTG
AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCT
TGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAA
TACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGA
AGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAG
AGTTTGTAACACCCGAAGCCGGTGGAGTAACCA

17. Strain FN6-8 (1429 bp)

GCCTAATACATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGG
TTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGC
GAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAG
TAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTC
ATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACAC
CAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
AGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGG
GCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACAC
ACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTC
TGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCG

18. Strain KN3-1-2 (1372 bp)

GAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAACC
GGGGCTAATGCCGGATAATATTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGT
ATTAGCTAGTTGGTGGGGTAATGGTTCCAGGCAACGATACGTAGCCGACCTGAGAGGGATGGCCCCTGGAACTGAGACACGGTCC
AGATCCTACGGGAGGCAGCAGTAGGGATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTC
GGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATT
CCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTG
CGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCC
CCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATA
GAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTAACTTATTGCCAGCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCA
TGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCA
GCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTCTGTAACACCCGAAGCCGGTGGAGT
AACCATTT

19. Strain KN3-4-3 (1357 bp)

TTGCTCCGGGTTCTGCGAGTGGCGGACGGGTGAGTAACACGTAGGCAACCTACCCATCAGACTGGGATAACCGCGGGAAACCGTG
GCTAATACCGGATGATCCTGATCCCCGCAGGGGGATGAGTTGAAAGGCGGTCTTTGACTGCCACTGATGGATGGGCCTGCGGCGC
ATTAGCTAGTTGGTGGGGTAAGGGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGA
AGAAGGGTTTCGGCTCGTAAAACTCTGTTGTCAGGGAAGAACGCCGGTGGGAGTAACTGTCCATCGGGTGACGGTACCTGACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCG
TAGGCGGTTCGTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGCGAACTTGAGTGCAGAAGAG
GAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACT
GACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAG
GGGGTTTCCGCCCCTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTCTGAC
CGCCATGGAGACATGGCTTCCCTTCGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAGGTTACAAAGGGCAGCTA
CGCCGCGAGGCCAAGCGAATCCCATAAAACTGTTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCCATGAAGCTGGAATCGCTA
GTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGGAAGTCGGTAACAC

20. Strain KN3-5-8 (1366 bp)

TGCTCTGGGGTCTGCGAGTGGCGGACGGGTGAGTAACACGTAGGCAACCTACCCATCAGACTGGGATAACCGCGGGAAACCGTGG
CTAATACCGGATAACCCTGATCCCCGCAGGGGGATGAGTTGAAAGGCGGTCTTTGACTGCCGCTGATGGATGGGCCTGCGGCGCA
TTAGCTAGTTGGTGGGGTAAGGGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGAA
GAAGGGTTTCGGCTCGTAAAACTCTGTTGTCAGGGAAGAACGCCGGTGGGAGTAACTGTCCATCGGGTGACGGTACCTGACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGT
AGGCGGTTCGTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGCGAACTTGAGTGCAGAAGAGG
AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTG
ACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGG
GGGTTTCCGCCCCTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT
GACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTCTGACC
GCCATGGAGACATGGCTTCCCTTTTGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCCAGCATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAGGTTACAAAGGGCAGCTAC
GCCGCGAGGCCAAGCGAATCCCATAAAACTGTTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCCATGAAGCTGGAATCGCTAG
TAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGGAAGTCGGTAACACCTG
AAGCCGG

21. Strain CN1-2 (1325 bp)

CGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGAT
GCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCT
CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT
TCAGTCGTGAGGAAGGCGTTGTCGTTAATAGCGGCATCGTTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA
GCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAA
AGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTG
AAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCT
AACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGG
AACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC
CTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCA
TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGCGGCCAACTTGCGAAAGTGAGCGAATCCCAAAAA
GTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGATTGGG

22. Strain CN1-9 (1365 bp)

GCTTGCTTTTGAAGATGACGAGCGGCGGACGGGTGAGTAATGCCTGGGAATTTGCCCATTTGTGGGGGATAACAGTTGGAAACGA
CTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCTGATGGATAAGCCCAGGTGGGATTAGCTA
GTAGGTGAGGTAATGGCTCACCTAGGCAACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGGTGTAAGTTAATACCTTACATCTGTGACGTTACTCGCAGAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTT
TGTTAAGCGAGATGTGAAAGCCCCGGGCTCAACCTGGGAACCGCATTTCGAACTGGCAAACTAGAGTCTTGTAGAGGGGGGTAGA
ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTCGGAGTTTGGTGTCTTGA
ACACTGGGCTCTCAAGCTAACGCATTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAG
ATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCCTATCCTTACTTGCCAGCGGGTAATGCCGGGAACTTTAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTG
GGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCGAAGCCGCGA
GGTGGAGCTAATCTCATAAAGCCGGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT
GGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCTACCAGAAGTAG
ATAGC

23. Strain NR1-3-2 (1453 bp)

TCAGGACGAACCCTGGCGGCGTGCCTAATACATGGCAAGTTCGGCGCGGGAAGCAGGCAGATCCCTTCGGGGTGATGCCTGTGGA
ACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTTAGAGACGGGGATAACTCCGGGAAACCGGGGCTAATACCCGAT
AATCGGCAAGATCACATGATCATGTCGTAAAAGTGGGGACTTGTTCCTCACACTCTGAGATGGGCCCGCGGCGCATTAGCTAGTT
GGGGAGGTAACGGCTTCCCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCATCCGCAATGGGCGAAAGCCTGACGGTGCAACGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAGCTCTGTTATCCGAGAAGAACAAGTACCGGTCGAATAGGCCGGTACCTTGACGGTACCGGATCAGAAAGCCCCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCGCGCAGGCGGTTT
CCTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGATGGTCATTGGAAACTGGGGAACTTGAGTACAGGAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGTAACTGACGCTGAGG
CGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGAC
GCCCTTAGTGCCGAAGCAAACGCATTAAGCACTCCGCCTGGGGACTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGCTACTTCCAGAG
ATGGAAGGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAGAGGGAAGCGAAGCCGTGA
GGTGTACGAATCTCGAAAAGCCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGCTTGCAACACCCGAAGTCGGT
GAGGTAACCC

24. Strain KN3-8-4 (1446 bp)

CAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCAGGCAGAACTCCTTCGGGAGTGATGCCTGTGG
AACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACCCCGGGAAACCGGGGCTAATACCGGA
TAACACTTTTTGGTACATGCCAAGAAGTTGAAAGGCGGCCTTTTTGGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTT
GGTAGGGTAACGGCCTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCCCGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCT
TTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGATGGCCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGT
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGAGTGCTAGGTGTTAGGGGGTTTCCGC
CCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACACCCCTAGAGA
TAGGGCATTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTGAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGCAGCGAAACCGCAAGGT
CAAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTCAACTCCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGT
AA

APPENDIX E

Chemical and physical properties of shrimp paste

Methodology

- Chemical, physical properties and microbial cells determination of shrimp paste (*Ka-pi*)

Two and eleven shrimp paste (*Ka-pi*) samples were collected from Songkhla and Nakhon Si Thammarat provinces, respectively. These samples were directly obtained from the market and supermarket which packed in plastic bag at room temperature, and they were maintained at 4°C until analysed.

Chemical analyses were immediately performed after the samples were brought to the laboratory. Fermentation period, sampling location and other information are provided in Appendix E-1). Ten samples of *Kapi* (indicated as SP) were collected from the markets and three samples (indicated as KS) were collected from the supermarkets.

- Proximate analyses

Shrimp paste samples (5 g) were homogenized thoroughly with 25 ml of distilled water (w/v) prior to determine salt concentration and pH. Salt concentration was measured by handy salt concentration meter (APAL-ES2, AS ONE) and pH was measured by pH meter (C-62, AS ONE). Water content was determined by using moisture analyzer (AND MX-50 using manual) while water activity (A_w) of each sample was measured at 25 °C using a water activity analyzer (Aw SPRINT TH-500, Novasina AG, Switzerland). Nitrogen content (N-content) analysis was performed by using an automated nitrogen and carbon analyzer (Sumigraph NC-220F, Sumika Chemical Analysis Service, Ltd.). Protein was calculated by 6.25 x N-content.

- LC/MS measurement of free amino acids

In brief, free amino acids were extracted from shrimp paste. Samples were diluted 1:100 with pure water and centrifuged for 15 min at 15,000 rpm and 4 °C. The supernatant was passed through a 0.20 μm filter (Kurabo, Osaka, Japan); the filtrate was injected directly into an Accurate-Mass Q-TOF LC/MS with an Agilent 6530 (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1260 Infinity HPLC (Santa Clara, CA, USA). To identify the Amino Acid Standard (Agilent Technologies, Logistics Center - USA). The accurate mass data was detected at high resolution and high mass accuracy (± 2 ppm), which was essential for accurate chemical formula assignment. Total ion spectra were collected over a mass range of m/z 60–400 in positive mode at an acquisition rate of 5.0 spectra/s. The drying gas temperatures and flow rate were 300 °C and 6.0 L/min, respectively. The sheath gas temperature and flow rate were 350 °C and 12.0 L/min, respectively. The nebulizer gas pressure, skimmer voltage, octopole RF, and fragmentor voltage were 50 psi, 60 V, 750 V, and 110 V, respectively. The capillary voltage was 3.5 kV. Continuous internal calibration was performed during analysis to achieve the desired mass accuracy of recorded ions with the ions of m/z of 112.9855. Chromatographic separation of the analytes was done using an Intrada Amino Acid (50 x 3 mm i.d., particle size 3.0 μm) (Imtakt Corpration, Kyoto) using 100 mM ammonium formate (A) and acetonitrile/formic acid 0.1% (v/v) (B) as eluent. A gradient was delivered at 600 μl/min starting from 86% phase B; it was maintained for 3 min at 86% phase B and then raised to 100% in 10 min; and then the column was equilibrated back to the initial condition in 4 min. Thus, the total analysis time was 14 min, including column washing and re-equilibration. All data, acquired in the positive ion mode, were collected and processed using MassHunter Workstation Software Quantitative Analysis ver. B.07.00 for TOF software (Agilent Technologies).

- Measurement of color

Color of shrimp paste samples were measured by spectrophotometer CM-5 (Konika Minolta) and reported in the CIE system. L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness).

- Histamine analysis

Histamine concentration of shrimp paste samples was determined by colorimetric enzymatic assay (Kikoman Biochemifa Company, Japan, 2014). The colored tetrazolium salt was created in the presence of 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) and measured at 470 nm.

- Bacterial cell count

A 1 g of sample stored in a refrigerator was homogenized with 9 ml normal saline containing 8.5 g kg^{-1} NaCl. Ten-fold dilutions of homogenates (100µl) were spread on standard agar. medium (composed of 5.0 g peptone, 2.0 g yeast extract, 1.0 g glucose and 15.0 g agar in 1 L distilled water) for general bacteria and de Man, Rogosa, and Sharpe (MRS) agar medium for lactic acid bacteria (LAB), and incubated at 30 °C for 2-7 days. Viable counts (colony-forming unit; cfu) were reported.

Results and discussions

- Chemical, physical properties and microbial cells determination of shrimp paste (*Ka-pi*)

Ten and three samples of shrimp paste were collected from the local market and the supermarket in Thailand, respectively. Sample number, SP1, SP2, SP3, SP4, SP6, SP7, SP8, SP9, KS1, KS2 and KS3 were collected from Nakhon Si Thammarat province while SP5 and SP10 were collected from Songkhla province (Appendix E-2).

The water content and water activity (A_w) of the shrimp paste samples collected from the local market were 33.95-52.19 % and 0.64-0.72 while the samples

from supermarket were 44.54-48.95 % and 0.66-0.69, respectively (Appendix E-2). A_w can be related to the consistency of shrimp paste which varied from soft and pasty to dry and hard. A_w of the final shrimp paste product depend on period which the sample was dried under the sun (Pongsetkul 2014). The shrimp paste can be classified as an intermediate moisture food, with an A_w of about 0.7 (Fennema 1996). The low A_w of shrimp paste products could be associated with increase the shelf-life and preserve the product from microbial spoilage at ambient temperature (Goulas 2005; Prapasuwannakul 2015). In addition, the low A_w would prevent rancidity of the product and limited the growth of food pathogens (Hajep 2012). Salt concentration of thirteen shrimp paste samples ranged from 7.00 to 10.85 %. The salt content would enhance both the shelf-life and the flavor of shrimp paste products. All shrimp paste samples had a neutral to slightly alkaline pH ranging from 7.02-8.31. The slightly alkaline might have been caused by the formation of volatile base compounds such as ammonia or other degradation products, the degradation products generated during fermentation. The shrimp paste collected from the local market contained 2.87-6.79 % of N content and 23.9-42.4 % protein while the local market contained 4.47-6.85 % of N-content and 27.9-42.8 % protein.

Generally, the nitrogen content has been used to indicate the degree of protein hydrolysis which can be the indicator for the level of the cleavage of peptides (Angeles Navarrete del Toro 2002). The highest nitrogen contents were 6.85, 6.79 and 6.61 % in KS1, SP1 and SP3 samples, respectively. This was higher than the nitrogen contents found in fish sauce which ranged from 0.3 to 3.0 % (w/v) (Park 2001; Tungkawachara 2003; Xu 2008). The protein content ranged from 17.9-42.8 %. The sample KS1 showed the highest protein content (42.8 %) followed by 42.4 and 41.3 %, in SP1 and SP3, respectively. So, the protein content could be indicated the good source of proteins.

The amino acid contents of the shrimp paste collected from the local market and the supermarket were variable (Appendix E-3). The predominant amino acids of thirteen shrimp paste products were glutamic acid (70.1-593.9 µg/g), lysine (112.7-546.3 µg/g) and leucine (29.5-544.9 µg/g) (Appendix E-3), compared to the content of glutamic acid in shrimp paste and fish sauce which ranged from 3.1-7.0 % (w/w) and 0.5-1.5 % (w/v), respectively (Kim 2014; Park et al 2001) while to the Cambodian traditional fermented fish products which contained 4.9 g kg^{-1} glutamic acid as reported by Chuon 2014. SP4 was showed highest glutamic acid followed by KS1 and SP2 that agreed with our study. The high amounts of glutamic acid were indicated the rich of umami taste. In 1990, Kim and Rhee reported that arginine, aspartate, isoleucine, lysine, proline, serine, threonine and valine were related to taste and flavor (Kim 1990). In addition, γ-aminobutyric acid (GABA) was detected in all samples (0.7-2.6 µg/g). The GABA containing in food has reported to play role as health benefits in many kinds of fermented products (Dhakal 2012).

The different shrimp paste products had different colors, L^* (lightness), a^* (redness) and b^* (yellowness) ranged from 29.6-39.48, 6.01-9.15 and 8.33-17.91, respectively (Appendix E-4). These samples were purple-brown to dark brown in color. The difference in color might be caused by the difference pigment contents in raw materials, process as well as ingredients added. The carbonyl groups of aldehydes and ketone, the oxidation products, could react with amino groups of free amino acids or peptides generated during hydrolysis, leading to yellow or brown color development (Yarnpakdee 2014). Brown development of shrimp paste products may be occurred by enzymatic and non-enzymatic reactions such as active polyphenoloxidase or Maillard reaction. Moreover, the oxidation of free astaxanthin resulted in the pale discoloration of products (Chaijan 2012).

The histamine concentration of the shrimp paste collected from the local market and the supermarket ranged from < 20-46.70 and 25.11-53.85 ppm,

respectively (Appendix E-5). The histamine detected was below the limit of 200 ppm according to Codex Alimentarius Vallejos 2012 that would not exhibited the toxicity. Low histamine content here supported the procedure used for shrimp paste production as the shrimps were immediately mixed with salt at the time that the raw materials reached the shore because histamine associated with incorrect handling and the storage of raw materials (Pilapil 2015). The level of histamine of all samples were differed which can be attributed to differences in quality of the raw materials, endogenous microflora, improper handling and environment during the production and the free amino acids (histidine) presented as a precursors (Singh 2012).

Total bacterial cell count of the samples varied from 1.3×10^{3}-2.9×10^{5} cfu g^{-1} (Appendix E-5) while the number of lactic acid bacteria (LAB) in thirteen shrimp paste products was not detected. Based on A_w, bacteria distributed as the rest cell or spore. Therefore, these shrimp pastes are stable for long period though the maximum bacterial number was 10^{5} order.

Table **E-1** Thai traditional shrimp paste (*Ka-pi*) products analysed in this study.

Sample source	Sample no.	Fermentation period	Sampling location
Market	SP1	<2 months	Amphoe Meuang, Nakhon Sri Thammarat province
	SP2	<2 months	Amphoe Khanom, Nakhon Sri Thammarat province
	SP3	<1 month	Tambon Banglaung, Amphoe Meuang, Nakhon Sri Thammarat province
	SP4	1 month	Amphoe Tha sala, Nakhon Sri Thammarat province
	SP5	<2 months	Amphoe Satingpra, Songkhla province
	SP6	<2 months	Amphoe Pak Phanang, Nakhon Sri Thammarat province
	SP7	<3 months	Amphoe Hua Sai, Nakhon Sri Thammarat province
	SP8	3 months	Amphoe Sichon, Nakorn Sri Thammarat province
	SP9	2 months	Tambon Pak Nakhon, Amphoe Meuang, Nakorn Sri Thammarat province
	SP10	Several months	Songkhla province
Supermarket	KS1	Several months	M-Peaw Brand, Kapi Chumchon Bangyai, Tambon Bangchak, Amphoe Meuang, Nakorn Sri Thammarat province
	KS2	3 months	Tonmai and Konmek shop, Amphoe Ronpiboon, Nakorn Sri Thammarat province
	KS3	Several months	Kapi Namprikkungtadum brand, Tambon Pakpoon, Amphoe Meuang, Nakorn Sri Thammarat province

Table E-2 Proximate composition of thirteen Thai shrimp paste (*Ka-pi*) products.

Sample no.	Water content (%) [*]	Water activity (A_w) [*]	Salt Concentration (%) [*]	pH [*]	N-content (%) [*]	Protein (%) [*]
SP1	46.32	0.65	7.15	7.67	6.79	42.4
SP2	44.64	0.66	7.00	6.96	5.55	34.7
SP3	36.63	0.65	8.90	8.31	6.61	41.3
SP4	33.95	0.64	8.65	8.11	6.23	38.9
SP5	42.52	0.68	8.90	7.81	4.75	29.7
SP6	45.49	0.69	8.90	7.04	2.87	17.9
SP7	45.74	0.68	9.05	6.94	3.19	19.9
SP8	52.19	0.72	7.54	7.02	4.76	29.8
SP9	48.53	0.70	8.60	7.05	3.83	23.9
SP10	46.72	0.68	9.50	7.84	6.15	38.4
KS1	44.54	0.66	10.85	7.72	6.85	42.8
KS2	48.95	0.69	10.15	7.73	4.47	27.9
KS3	47.69	0.68	8.25	7.35	4.68	29.2

*Standard variation value < 0.01

Table E-3 Amino acid contents (μg/g) of thirteen Thai shrimp paste (*Ka-pi*) products.

Sample no.	Phe	Leu	Ile	Met	Pro	Val	Ala	Glu
SP1	112.1	296.1	197.9	97.3	99.0	161.4	173.9	83.2
SP2	118.7	252.3	158.0	66.1	74.6	142.9	147.2	524.8
SP 3	131.6	324.6	183.0	95.6	71.8	160.5	162.8	399.1
SP 4	164.7	454.8	264.5	124.2	93.8	212.3	229.2	593.9
SP 5	56.8	135.4	82.7	38.7	64.6	90.2	118.7	298.3
SP 6	79.8	166.4	84.4	44.7	48.5	93.4	101.5	70.1
SP 7	121.2	301.5	200.7	86.2	68.3	149.7	126.9	310.7
SP 8	179.9	383.6	237.2	113.6	33.8	204.8	174.5	427.5
SP 9	123.1	246.5	140.5	76.4	66.3	143.2	120.5	253.9
SP 10	255.8	544.9	364.3	162.9	135.4	269.7	245.0	260.8
KS1	153.0	277.9	139.1	77.2	107.3	205.5	182.3	591.3
KS2	114.6	297.1	168.1	70.4	70.2	128.3	140.5	450.9
KS3	13.4	29.5	18.5	8.2	28.3	29.5	90.1	151.9

Sample no.	Gly	Gln	Asp	His	Lys	Arg	GABA
SP1	137.6	16.2	212.4	27.5	311.9	41.5	1.9
SP2	98.4	14.6	129.2	15.8	196.6	30.8	1.9
SP 3	187.9	5.2	151.6	23.7	301.4	22.0	2.1
SP 4	220.3	9.4	221.7	32.1	458.0	30.5	2.6
SP 5	94.9	5.8	139.8	11.3	165.6	24.9	1.5
SP 6	80.6	14.4	96.5	12.1	143.1	50.2	0.7
SP 7	89.3	2.2	174.4	11.3	196.7	10.0	1.1
SP 8	133.0	11.3	209.8	27.8	315.0	28.9	2.0
SP 9	97.1	6.2	155.1	12.9	195.7	18.2	1.0
SP 10	206.6	46.0	281.7	37.6	546.3	224.4	2.6
KS1	136.1	18.3	274.7	41.3	376.7	56.1	2.6
KS2	109.0	29.0	169.5	31.5	229.6	138.2	1.4
KS3	93.4	7.4	124.2	11.1	112.7	33.8	1.3

Phe, Phenylalanine; Leu, Leucine; Ile, Isoleucine; Met, Methionine; Pro, Proline; Val, Valine; Ala, Alanine; Glu, Glutamic acid; Gly, Glycine; Gln, Glutamine ; Asp, Aspartic acid; His, Histidine; Lys, Lysine; Arg, Arginine; GABA, gamma-Aminobutyric acid.

Table E-4 CIELAB colorimetric values of thirteen Thai shrimp paste (*Ka-pi*) products.

Sample no.	L^*	a^*	b^*
SP1	39.22	6.78	14.62
SP2	35.51	8.5	12.67
SP3	34.37	6.01	9.23
SP4	32.6	6.34	8.33
SP5	35.87	6.74	12.5
SP6	29.6	8.28	10.8
SP7	30.61	7.03	9.04
SP8	32.93	9.51	9.58
SP9	29.78	9.2	11.07
SP10	39.48	7.91	10.4
KS1	36.76	6.4	12.39
KS2	32.89	8.66	10.36
KS3	28.56	8.29	17.91

L^*, lightness; a^*, redness-greenness; b^*, yellowness-blueness

Standard variation value < 0.01

Table E-5 Bacterial numbers and histamine concentration of thirteen Thai shrimp paste (*Ka-pi*) products.

Sample no.	Bacterial number (cfu/g)	Histamine concentration (ppm)
SP1	2.9×10^4	46.70
SP2	1.7×10^4	30.73
SP3	1.3×10^5	29.19
SP4	2.1×10^5	31.06
SP5	3.4×10^4	32.60
SP6	8.2×10^4	ND **
SP7	4.3×10^4	26.65
SP8	6.1×10^4	ND **
SP9	6.8×10^4	24.45
SP10	1.3×10^5	29.19
KS1	2.9×10^5	30.29
KS2	7.0×10^4	53.85
KS3	1.1×10^5	25.11

**ND, <20 ppm

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PUBLICATIONS:

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