อนุกรมวิธาน และ เอนไซม์โปรติเอสของแบคทีเรียชอบเค็ม
ที่แยกได้จากน้ำปลา

นางสาว ศิริลักษณ์ นามวงษ์

TAXONOMY AND PROTEASE OF HALOPHILIC BACTERIA
ISOLATED FROM FISH SAUCE

Miss Sirilak Namwong

Thesis Title   TAXONOMY AND PROTEASE OF HALOPHILIC
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ศิริลักษณ์ นามวงษ์: อนุกรมวิธาน และ เอนไซม์โปรติเอสของแบคทีเรียชอบเค็มที่แยกได้จาก น้ำปลา (TAXONOMY AND PROTEASE OF HALOPHILIC BACTERIA ISOLATED FROM FISH SAUCE) อาจารย์ที่ปรึกษา: รศ. ดร. สมบูรณ์ ธนาศุภวัฒน์ อาจารย์ที่ปรึกษาร่วม: PROF. KOHEI ODA, PH. D., และ MR. TAKASHI ITOH, PH. D. 162 หน้า. ISBN: 974-17-5502-3

ในการศึกษาเพื่อหาสายพันธุ์แบคทีเรียชอบเค็มที่สามารถผลิตเอนไซม์โปรติเอสจากน้ำปลาที่ผลิตในจังหวัดสมุทรปราการ สมุทรสงคราม ระยองและ สิงห์บุรี และจากบูดูในจังหวัดปัตตานี พบว่าสามารถแยกเชื้อได้จำนวน 40 ไอโซเลต จากผลการศึกษา ลักษณะทางฟีโนไทป์ และทางอนุกรมวิธานเคมีรวมทั้งการวิเคราะห์ลำดับเบสในช่วง 16S rDNA สามารถพิสูจน์เอกลักษณ์ของเชื้อ แบคทีเรียชอบเค็มปานกลางจำนวน 14 สายพันธุ์ ได้เป็น *Lentibacillus salicampi* 3 สายพันธุ์, *L. juripiscarius* sp. nov. 5 สายพันธุ์, *Chromohalobacter salexigens* 4 สายพันธุ์, *Halobacillus* sp. 1 สายพันธุ์, *Filobacillus* sp. 1 สายพันธุ์ แบคทีเรีย *Lentibacillus* และ *Filobacillus* มีกรด *meso*-diaminopimelic ในผนังเซลล์ และมี phosphoglycerol และ diphosphoglycerol แบคทีเรีย *Lentibacillus*, *Filobacillus* และ *Halobacillus* มีปริมาณ G+C ของ DNA อยู่ในช่วง 42.5-43.5, 36.5 และ 40.3 mol% ตามลำดับ พบมีกรดไขมันส่วน ใหญ่แบบ anteiso-$C_{15:0}$ และ iso-$C_{15:0}$ ใน *Lentibacillus* และ *Filobacillus* สำหรับแบคทีเรียชอบเค็มปานกลางสายพันธุ์ *Chromohalobacter salexigens* มีกรดไขมันส่วนใหญ่แบบ cyclo $C_{19:0}$ ω8c และ $C_{16:0}$ มี phosphoglycerol, diphosphoglycerol และ phosphatidylamine และมีปริมาณ G+C ของ DNA อยู่ในช่วง 61.7-63.5 mol% พบแบคทีเรียชอบเค็มสูงจำนวน 26 สายพันธุ์ พิสูจน์ เอกลักษณ์ได้เป็น *Halobacterium salinarum* 10 สายพันธุ์, *Halococcus saccharolyticus* 1 สายพันธุ์ และ *Halococcus thailandensis* sp. nov. 15 สายพันธุ์ เชื้อแบคทีเรีย *Halobacterium* มี $C_{20}C_{25}$ เป็นอนุพันธ์ของ phosphatidylglycerol และ phosphatidylglycerol methylphosphate มี MK-8(H_2) และมีปริมาณ G+C ของ DNA อยู่ในช่วง 61.3-63.5 mol% เชื้อ *Halococcus* มี $C_{20}C_{25}$ และ $C_{20}C_{20}$ เป็น อนุพันธ์ของ phosphatidylglycerol และ phosphatidylglycerol methylphosphate มี MK-8(H_2) และมีปริมาณ G+C ของ DNA อยู่ ในช่วง 60.2-63.2 mol% ในการศึกษาครั้งนี้พบแบคทีเรียชอบเค็มสปีชีส์ใหม่ จำนวน 2 สปีชีส์ ได้แก่ *Lentibacillus juripiscarius* sp. nov. และ *Halococcus thailandensis* sp. nov โดย *L. juripiscarius* 5 สายพันธุ์ มีความแตกต่างจาก *L. salicampi* ทั้งทางด้านลักษณะ ทางฟีโนไทป์ มีความเหมือนของลำดับเบสในช่วง 16S rDNA น้อยกว่า 95% และ DNA-DNA ในระดับต่ำ (12 to 32%) ส่วน *H. thailandensis* 5 สายพันธุ์ มีความแตกต่างจาก *H. dombrowskii*, *Halococcus morrhuae* และ *Halococcus* สายพันธุ์อื่นๆ ทั้งทางด้าน ลักษณะทางฟีโนไทป์ ความเหมือนของลำดับเบสในช่วง 16S rDNA น้อยกว่า 98.6% และ DNA-DNA ในระดับต่ำกว่า56.3%

แบคทีเรียชอบเค็มสูง 9 สายพันธุ์ และแบคทีเรียชอบเค็มปานกลาง 7 สายพันธุ์ ซึ่งคิดเป็น 39% ของแบคทีเรียชอบเค็ม ทั้งหมด สามารถสร้างเอนไซม์โปรติเอสเพื่อย่อยนม (skim milk) โดยสายพันธุ์ SR5-3 ถูกคัดเลือกเพื่อเป็นตัวแทนสำหรับการศึกษาการ แยกเอนไซม์โปรติเอสให้บริสุทธิ์ และ ศึกษาคุณสมบัติของเอนไซม์ดังกล่าว เนื่องจากสายพันธุ์ SR5-3 สามารถสร้างเอนไซม์โปรติเอส ได้มากสุดที่ 10% NaCl จากผลของลำดับเบสในช่วง 16S rDNA พบว่า SR5-3 เป็นแบคทีเรียสปีชีส์ใหม่ในสกุล *Halobacillus*, *Halobacillus* sp. SR5-3 สามารถผลิตเอนไซม์โปรติเอสเพิ่มขึ้น 100 เท่า เมื่อเติม aspartic acid, glutamic acid และ glycine ในอาหาร JCM No 377 เอนไซม์โปรติเอสที่ผลิตได้จากแบคทีเรียดังกล่าวเมื่อนำไปทำให้บริสุทธิ์และศึกษาลักษณะ พบว่ามีน้ำหนักโมเลกุล 43 kDa มีสถาวะที่เหมาะสมของกิจกรรมของเอนไซม์ที่ 50 °C, pH 9-10 และ 20% NaCl เมื่อใช้สารตั้งต้นสังเคราะห์ Suc-Ala-Ala-Phe-Pro-MCA เพื่อหากิจกรรมของเอนไซม์ พบว่ากิจกรรมของเอนไซม์เพิ่มขึ้น 2.5 เท่าเมื่อเติม 25-30% NaCl แต่กิจกรรมของเอนไซม์ ถูกยับยั้ง โดย phenylmethane sulfonyl fluoride (PMSF), chymostatin, และ α-microbial alkaline protease inhibitor (MAPI) การศึกษา fluorescence resonance energy transfer substrate (FRETS) combinatorial libraries แสดงให้ทราบว่าเอนไซม์สามารถย่อยโปรตีนได้ดี สุดเมื่อ ตำแหน่ง P_1 เป็น leucine, glutamine และ alanine และ P_2 ของโปรตีนเป็น isoleucine

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ   ลายมือชื่อนิสิต.....................................

ปีการศึกษา 2548   ลายมือชื่ออาจารย์ที่ปรึกษา.....................................

SIRILAK NAMWONG: TAXONOMY AND PROTEASE OF HALOPHILIC BACTERIA ISOLATED FROM FISH SAUCE. THESIS ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, PH. D., THESIS CO-ADVISOR: PROF. KOHEI ODA, PH. D., AND MR. TAKASHI ITOH, PH. D. 162 pp. ISBN: 974-17-5502-3

In order to isolate and screen for protease-producing halophiles, 40 isolates were isolated from fish sauce samples collected in Samutprakarn, Samutsongkram, Rayong and Singburi and from bu-du in Pattani provinces. On the basis of their phenotypic and chemotaxonomic characteristics, DNA-DNA similarity and the phylogenetic analysis using 16S rDNA sequences. 14 isolates of moderate halophiles belonged to *Lentibacillus salicampi* (3 isolates), *L. juripiscarius* sp. nov (5 isolates), *Chromohalobacter salexigens* (4 isolates), *Halobacillus* sp. (1 isolate), and *Filobacillus* sp. (1 isolate). *Lentibacillus* and *Filobacillus* isolates contained *meso*-diaminopimelic acid in cell wall-peptidoglycan, MK-7 as predominant menaquinone, and phosphoglycerol and diphosphoglycerol as major polar lipids. The DNA G+C contents of *Lentibacillus*, *Filobacillus*, and *Halobacillus* isolates were 42.2 to 43.5, 36.5, and 40.3 mol%, respectively. The major cellular fatty acids of *Lentibacillus* and *Filobacillus* isolates were anteiso-$C_{15:0}$ and iso-$C_{15:0}$, respectively. *Chromohalobacter salexigens* isolates contained *meso*-diaminopimelic in cell wall-peptidoglycan, cyclo $C_{19:0}$ ω8c and $C_{16:0}$ as the major cellular fatty acids, and phosphatidylamine as a polar lipid. The DNA G+C contents were 61.7 to 63.5 mol%. Twenty-six isolates of extreme halophiles were belonged to *Halobacterium salinarum* (10 isolates), *Halococcus saccharolyticus* (1 isolate) and *Halococcus thailandensis* sp. nov. (15 isolates). *Halobacterium* isolates comprised $C_{20}C_{25}$ derivatives of phosphatidylglycerol and phosphatidylglycerol methylphosphate and MK-8 as the predominanat menaquinone. The DNA G+C contents were 63.4 to 64.7 mol%. *Halococcus* isolates contained $C_{20}C_{25}$ and $C_{20}C_{20}$ derivatives of phosphatidylglycerol and phosphatidylglycerol methylphosphate, and MK-8(H_2). The DNA G+C contents were 60.2 to 63.2 mol%. Two novel species of halophilic bacteria are proposed. In this study, five isolates of *L. juripiscarius* sp.nov. were differentiated from *L. salicampi* by several biochemical properties, low 16S rDNA sequence similarity less than 95% and low DNA-DNA similarity (12 to 32%). Fifteen isolates of *H. thailandensis* sp.nov. were related to *H. dombrowskii* and *H. morrhuae* with 99.1 and 98.6 % 16s rDNA sequence similarity, respectively. They were distinguished from *H. dombrowskii*, *Halococcus morrhuae* and the other *Halococcus* species by several phenotypic characteristics and DNA-DNA similarity ($\leq$56.3%).

Nine isolates of extreme halophiles and seven isolates of moderate halophiles corresponding to 39% of the total isolates showed caseinolytic forming colonies on the plate. SR5-3 showed the highest protease activity in the presence of 10% NaCl. This isolate was selected for purification and characterization of protease. Based on the 16S rDNA sequence similarity, SR5-3 is assumed as a new species of *Halobacillus*, *Halobacillus* sp. SR5-3. Production of protease of the SR5-3 was enhanced to 100-fold by the addition of aspartic acid, glutamic acid, and glycine into the JCM No 377 medium. The protease from this bacterium was purified and characterized. Molecular weight of the purified enzyme was estimated as 43 kDa. The enzyme showed the highest activity at 50°C and pH 9~10, and highly stable in the presence of 20-35% NaCl by using Suc-Ala-Ala-Phe-Pro-MCA as substrate. The protease activity was increased about 2-fold and 2.5-fold by the addition of 5% and 20-35% NaCl, respectively using Suc-Ala-Ala-Pho-Pro-MCA as a substrate. The activity was strongly inhibited by phenylmethane sulfonyl fluoride (PMSF), chymostatin, and α-microbial alkaline protease inhibitor (MAPI). Substrate specificity of the enzyme was determined by using fluorescence resonance energy transfer substrate (FRETS) combinatorial libraries. One of the unique features was that the enzyme absolutely preferred Ile at the P2 position of the substrates and it was found to prefer three amino acids, Leu, Gln and Ala at the P1 position.

Field of study Pharmaceutical Chemistry and Natural Products

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Advisor's signature....*Somboon Tanasupawat*....

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LIST OF ABBREVATIONS AND SYMBOLS

α	=	Alpha
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
ATP	=	Adenosine triphosphate
˚C	=	Degree celsius
$C_{20}C_{20}$	=	2,3-di-*O*-phytanyl-*sn*-glycerol
$C_{20}C_{25}$	=	2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol
$CaCl_2$	=	Cacium chloride
cm	=	Centimeter
DAN	=	Diazoacetyl-DL-norleucine methylester
DAP	=	Diaminopimelic acid
D-A_2pr	=	D-2,3-diamino propionic acid
DCL	=	3,4-dichloroisocoumarin
DDBJ	=	DNA Data Bank of Japan
DFP	=	Diisopropylfluorophosphate
DMF	=	Dimethyl formamide
DNase	=	Deoxyribonuclease
Dnp	=	2, 4-dinitrophenyl
DSMZ	=	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
E 64	=	L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane
EDTA	=	Disodiumethylenediaminetetraacetate
EMBL	=	European Molecular Biology Laboratory
EPNP	=	1, 2-epoxy-3-(*p*-nitrophenoxy) propane
FRETS	=	Fluorescence resonance energy transfer substrate
g	=	Gram
GalNAc	=	N-acetylgalactosamine
Gal	=	Galactose
Glc	=	Glucose
GlcN	=	*N*-acetylglucosamine
GlcNAc	=	*N*-acetylglucosamine

Gly	=	Glycine
GulNUA	=	N-acetylgulosaminuronic acid
GenBank	=	National Institute of Health genetic sequence database
h	=	Hour
H_3BO_3	=	Boric acid
HCl	=	Hydrochloric acid
HPTLC	=	High performance thin layer chromatograhphy
H_2O	=	Water
H_2O_2	=	hydrogen peroxide
H_2SO_4	=	Sulfuric acid
JCM	=	Japan Collection of Microorganisms
KB	=	A human epidermoid carcinoma cell line of the nasopharynx
kDa	=	kilo Dalton
KH_2PO_4	=	Potassium hydrogenphosphate
KNO_3	=	Potassium nitrate
KOH	=	Potassium hydroxide
L	=	Liter
Man	=	Mannose
MAPI	=	α-microbial alkaline proteinase inhibitor
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
meso-DAP	=	*meso*-Diaminopimelic acid
Methyl-PE	=	methyl-Phosphatidylethanolamine
M	=	molar
Min	=	Minute
μg	=	Microgram
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
μL	=	Microliter
mL	=	Milliliter
μm	=	Micrometer
mm	=	Millimeter

NaCl	=	Sodium chloride
NaHCO$_3$	=	Sodium hydrogencanate
NaOH	=	Sodium hydroxide
Na$^+$	=	Sodium ion
NAG	=	N-acetly glucose amine
NAM	=	N-acetyl muramic acid
nm	=	Nanometer
O$_2$	=	Oxygen
OH-PE	=	Hydroxyl-phosphatidylethanolamine
%	=	Percent
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PC	=	Phosphatidylcholine
PCR	=	Polymerase chain reaction
PCMB	=	*p*-chloromercuribenzoate
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PGP-Me	=	Phosphatidylglyceol methylphosphate
PGS	=	Phosphatidylglyceol sulphate
PMSF	=	Phenylmethylsulfonyl fluoride
rDNA	=	Ribosomal deoxynucleic acid
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
sec	=	Second
SEM	=	Scanning electron microscope
Si Gel	=	Silica gel
SDS	=	Sodium dodesylsulfate
sp.	=	Species
SSC	=	Standard sodium citrate
S-TGD-1	=	Sulfated-triglycerol diether (Gal is removed from S-TeGD)
S-TeGD	=	(1-*O*-[β-D-galactose-(3'-SO$_3$H)-(1'→6')-α-D-mannose-(3'→1')- α-D-Galactofuranose)- (1'→2')- α-D- Glucose]-2,3-di-*O*-phytyl- *sn*-glycerol

TAE	=	Tris-acetate
TBE	=	Tris-borate
TCA	=	Trichloroacetic acid
TGD-1	=	Triglycosyl diether (desulfated product of S-TDG-1)
TLCK	=	Tosyl-L-lysine chloromethyl ketone
TLC	=	Thin layer chromatography
TCA	=	Trichloroacetic acid
UA	=	Uronic acid
UV	=	Ultraviolet
v/v	=	volume / volume
v/w	=	volume / weigh
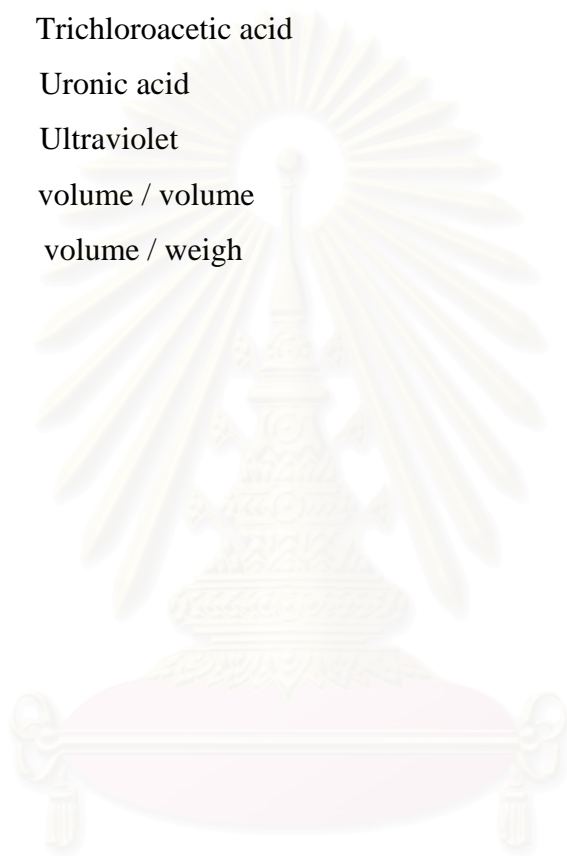
CHAPTER I

INTRODUCTION

Fermented foods play an important role in the Thai diet and in the life of Thai people. Fermented fish products such as nam-pla (fish sauce) have been consumed daily by Thai people since ancient times (Phithakpol, 1995). Among Southeast Asian countries, Thailand is the largest producer, where 390 fish sauce factories are located. The annual production of fish sauce is approximately 459,700 kiloliters (for domestic consumption is approximately 6,600 million baht and exports are around 1,056 million baht) (Krung Thai Bank, 2005). Fish sauce is a clear brown liquid hydrolysate of salted fish obtained after about 1 year of salting. The pH, NaCl and lactic acid content of fish sauce are pH 5.3–6.7, 22.5–29.9 g/L, and 0.06–0.48 g/L, respectively (Lopetcharat *et al.*, 2001). This clear brown, salty liquor is an important sources of peptides, amino acids, minerals and vitamins. Most proteins in fish are hydrolyzed into small peptides and amino acids by the action of both halophilic bacteria and fish proteases during fermentation process (Park *et al.*, 2001).

Based on the chemical properties, fish sauce developed halophilic bacteria such as *Halobacterium salinarum, Halococcus* species*., Tetragenococcus halophilus, T. muriaticus* (Chaiyanan *et al.*, 1999; Lopetcharat *et al.*, 2001; Thongthai *et al.*, 1992; Thongsanit *et al.*, 2002). The halophilic bacteria are salt-loving organisms that were classified according to their salt requirement, slight halophiles grow optimally at 2-5% (w/v) NaCl such as *Jeotgalicoccus* strains(Yoon *et al.*, 2005); moderate halophiles grow optimally at 5-20% (w/v) NaCl such as *Lentibacillus*, *Filobacillus*, *Halobacillus* and *Tetragenococcus* strains (Schlesner *et al.*, 2001; Spring *et al.*, 1996; Thongsanit *et al.*, 2002; Yoon *et al.*, 2002); and extreme halophiles grow optimally above 20-30% (w/v) NaCl such as the strains of *Halobacterium* and *Halococcus* (Gruber *et al.*, 2004; Stan-Lotter *et al.*, 2002).

In addition, halophiles produced proteases showing optimal activities at the different values of salt concentrations and temperature. Several extracellular proteases from extreme halophiles have been studied and characterized e.g., *Halobacterium halobium* S9, *Natrialba magadii* ATCC 43099, *Halobacterium* sp. TuA4, *Halobacterium halobium*, *Natronococcus occultus* NCMB 2192, *Halobacterium halobium* ATCC 43214 and *Halobacterium mediterranei* 1538 and *Haloferax mediterranei* R4 (Capiralla *et al*., 2002; Izotova *et al*., 1983; Gimenez *et al*., 2000; Kamekura *et al*., 1993; Kamekura *et al*., 1996; Ryu *et al*., 1994; Schmitt *et al*., 1990; Stepanov *et al*., 1992; Studdert *et al*., 1997;). These proteases required 3-4 M NaCl to obtain the best activity as well as for the best growth. On the other hand, a few extracellular proteases from the moderate halophiles have been characterized in details. For example, proteases from *Pseudomonas* sp. A-14, *Pseudoalteromonas* sp. CP76 and *Salinivibrio costicola* 18 AG have been purified and characterized showing optimal activity at low salt concentration, but their activity also retained at high salt (Lama, *et al*. 2005; Qua et. al., 1981; Sanchez-Porro et. al., 2003. Recently, *Bacillus* sp., *Halobacterium salinarum* and *Halobacillus thailandensis* isolated from fish sauce were reported as the protease producing-bacteria. Among them, the moderately halophilic bacterium *Halobacillus thailandensis*, produced a serine protease was characterized partially (Chaiyanan *et al*., 1999). The advantage of halophilic enzymes showed optimal activities at high salt concentrations are possibly application in industrial processes i.e., laundry, dishwashing detergents, treatment of agricultural waste and waste from food processing industries. In recent years, the isolation of halophilic bacteria and the characterization of protease enzyme produced by these microorganisms from fish sauce, have acquired enormous interest. Hence, the taxonomic studies and the characterization of protease of halophilic bacteria from fish sauce in Thailand were subjected for this investigation.

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The main objectives of this presence study were as followed:

      1. To isolate and screen the protease-producing halophilic bacteria from fish sauce.

      2. To identify and characterize the protease-producing halophilic bacteria from fish-sauce based on the phenotypic and chemotaxonomic characteristics including DNA-DNA relatedness and 16S rDNA sequencing.

      3. To purify and characterize the protease from SR5-3 strain

CHAPTER II

LITERATURE REVIEW

1. FISH SAUCE (NAM-PLA)

Fermented fishery products have been consumed since ancient times. Roman fermented fish sauce (*garum*) was originally made from the viscera and blood of mackerel. In the present of high salinity, mackerel blood coagulates rapidly and is broken down slowly by halotolerant enzymes from viscera. After a 9-month fermentation period, *garum* was obtained from the clear brown liquid drained from the fermentation tank. Garos, a fish sauce produced in Greece, was made from the liver of Scomber colias. Aimeteon was another fish sauce made during the ancient Greek period (Lopetcharate *et al*., 2001).

The present time, fish sauce produce from fish and heavily salt and is a clear brown liquid with an unique characteristic, salty flavor and fishy smell. It is commonly used as seasoning for cooking and dipping seafood and oriental food. Fish sauce is currently very popular in Southeast Asia and with Asian people in Western countries and its name is depending on the country. In Malaysia, fish sauce is called *budu*; in the Philippines, *patis*; in Indonesia, *ketjap-ikan*; in Burma, *ngapi*; in Cambodia and Vietnam, *nouc-mam* (or *nouc-nam*); in Thailand, *nampla*; in Japan, *ishiru* or *shottsur*; in India and Pakistan, *colombo-cure*; in China, *yeesu*; and in Korea, *aekjeot* (Park *et al*., 2001)

In Thailand, fish sauce is classified by the Thai Public Health Ministry (2532) into three types based on the production process: pure fish sauce, hydrolyzed fish sauce, and diluted fish sauce. Pure fish sauce is derived from fresh fish or fish residue obtained from fish fermented with salt or brine. Hydrolyzed fish sauce can be obtained from the hydrolysates of fish or other kinds of animals, which are often treated with hydrochloric acid (HCl) or other hydrolyzing processes that are approved by the Thai Public Health Ministry. Diluted fish sauce is obtained from pure fish sauce or hydrolyzed fish sauce, but is diluted using approved additives or flavoring agents.

1.1 FISH SAUCE PRODUCTION

Fish sauce production starts with cleaning fresh saltwater fish, *Stolephorus* spp. (Anchovy), *Sardinella* spp. (Sardine), *Rastrelliger* spp. (Mackeral), *Ristrelliger* spp., (Herring) and *Clupea* spp. or freshwater fish with cold water to remove impurities and to reduce the quantity of microorganisms in the raw materials. The saltwater fish can be found in bountiful supply in the coastal waters of the Gulf of Thailand and the South China Sea. Generally, cleaned fish will be mixed with salt in a 2:1 or 3:1 ratio (fish:salt) (w/w), depending on the area of production. Then, salt-mixed fish is transferred to a fermentation tank where a bamboo mat is laid on the bottom of the tank. Another layer of bamboo mat is placed on top of the fish and loaded with heavy weight to keep the fish flesh in the brine that is extracted from the fish during fermentation. Brine will reach the top of the fish flesh within the first week of fermentation. The tank are covered with bamboo and left in a sunny location for at least a year. From time to time, the tank expose to the air with directly hot sunshine, which helps digest the fish protein to a small molecules (Figure 1). (Saisithi, 1994).

After enough months have passed, the liquid showing a clear reddish brown color and a fragrant aroma, is removed from the jars, preferably through a spigot on the bottom of the jars, so that it passes through the layers of fish remains. Any sediments are strained out with a clean cloth. The filtered fish sauce is filled into other clean jars and allowed to air out in the sun for a couple of weeks to dissipate the strong fish odors. It is then ready for bottling. The finished product is 100-percent, top-grade, genuine fish sauce. Second and third grade fish sauces are made by adding salt water to cover the fish remains for 2-3 months, then filtering before bottling. Finally, the fish remains are boiled with salt water to produce the lowest grade fish sauce. Because flavor is substantially reduced with each fermentation, so top-grade fish sauce is frequently added to the lower grades to improve their flavor. In fact, many manufacturers do not market top-grade, 100-percent fish is mixed with second and third grade sauces in order to produce larger quantities to sell that can still qualify as genuine fish sauce.

Fresh water fish or marine fish

↓

Mixing with salt (2:1 or 3:1) (fish:salt, w/v)

↓

Fermentation (12-18 month)

↓

Filtering

Extract/unripened fish sauce   Fish residue

↓

Ripening under the sun   Saturated brine → Fermentation 1-4

months

↓

Top-grade fish sauce   Filtering

Second-grade fish sauce   Fish residue

↓

Added saturated brine

↓

Boiling and filtering

↓

Caramel or additives

↓

Diluted fish sauce

Figure 1. Process of traditional making fish sauce.

1.2 CHEMICAL COMPOSITIONS OF FISH SAUCE

There are five major factors influencing fish sauce quality: fish species, type of salt, the ratio of fish and salt, minor ingredients, and fermentation conditions. Fish species al**so** affects the type of proteins that serve as nutrients for microorganisms and substrates for enzymes which hydrolyze proteins into the small peptides, free amino acids, ammonia, and trimethylamine (TMA) contribute to the specific aroma and flavor in fish sauce. The release of free amino acids from proteins and large polypeptides cause the pH value (5.3-6.7) drops during process. Fish sauce contains essential amino acid more than 13 types i.e., Aspartic acid, glutamic acid, threonine, alanine and glycine, especially Lysine (Table 1). In addition, every fish has a slightly different fatty acid profile that is hydrolyzed to low molecular weight volatile fatty acids, especially ethanoic and n-butanoic acids caused to the cheesy aroma in fish sauce. The fish to salt ratio is another factor affecting fish sauce quality. Because of at different salt concentrations, bacterial and enzymatic activity are changed, resulting in different flavors and digestive products. The average NaCl content in fish sauce was 26±3.7%.

Another component of fish sauce a mineral (potassium (K), phosphorus (P), sulfur (S), sodium (Na), magnesium (Mg), calcium (Ca), iron (Fe) and vitamin (thiamin, riboflavin, niacin, and vitaminsB_6 andB_{12}), vitamin B_{12}, which is essential for healthy diet (Lopetcharat $et\ al.$, 2001; Park $et\ al.$, 2001). Often fish sauces are known to contain high levels of histamine, biogenic amine. This compounds is generally vasoactive and can cause changes in blood pressure, severe headache, hypertension, renal intoxication, intracerebral hemorrhage and eventually death. The Food and Drug Administration (FDA) established an advisory level of 500 ppm to be hazardous to human health (FDA, 1998). However, high histamine concentration may not cause severe illness since a comsumers consume fish sauce less than 23.5 g/day/person (Yongsawatdigul $et\ al.$, 2004)

Table 1. Chemical compositions of Fish sauce.

Characteristics	Concentration (g/L)
pH	5.3-6.7
NaCl	225-299
Total amino acids	27-70
Amino acids	
Aspartate	5.8
Threonine	3.84
Serine	2.33
Glutamate	14.89
Proline	1.35
Glycine	2.65
Alanine	5.74
Cysteine	0.17
Valine	4.78
Methionine	2.22
Isoleucine	3.34
Leucine	4.39
Tyrosine	0.91
Phenylalanine	3.23
Lysine	7.67
Histidine	2.75
Arginine	0.03
Total organic acids	2.1-2.3
Acetic acid	0-20
Lactic acid	0.6-4.9
Succinic acid	0.2-1.9
Reducing sugar	trace
Alcohol	tract
Moisture (%)	6.37
Total nitrogen	1.63
Nitrogen recovery (%)	64.3

2.   HALOPHILIC BACTERIA

2.1   PHYSIOLOGY

For millions of years, the hypersaline habitats develope a specialized halophilic bacteria. They are classified according to their salt requirement to be three groups, halotolerant, moderately halophilic bacteria and halophilic archaea (Holt *et al*., 1994; Ventosa *et al*., 1998; Vreeland and Hochstein, 1993). The halophilic bacteria can balance the osmotic pressure of the environment and resist the denaturing effects of salts and the considerable water stress by developing two mechanisms of osmoregulatory solute accumulation (i) Accumulation of inorganic ions, (ii) Accumulation of organic solutes (Martin *et al*., 1999; Roebler *et al*., 2001). For halotolerant and moderately halphilic, potassium ions (K^+) is accumulated an early response to an increase in external NaCl. However, the increased K^+ is often transient and is superseded by the accumulation of organic solutes i.e., polyols (glycerol, arabitol and mannitol), low molecular-weight nonionic carbohydrates (sucrose, trehalose, and glucose), free amino acids and their derivatives (proline, glutamate, glycine, g-aminobutyrate, taurine, and β-alanine), unique organic zwitterions (tetrahydropyrimidines such as ectoine, methylamines (Figure 2). In contrast, *Halobacteriaceae*, halophilic archaea have high intracellular concentrations of K^+ for all life time i.e., *Halobacterium salinarum* contained 4 M and 4.57 of the external Na^+ and intracellular of K^+, respectively and also accumulate the organic osmolytes as the major classes as for eubacteria and eukaryotes to balance such contiditions.
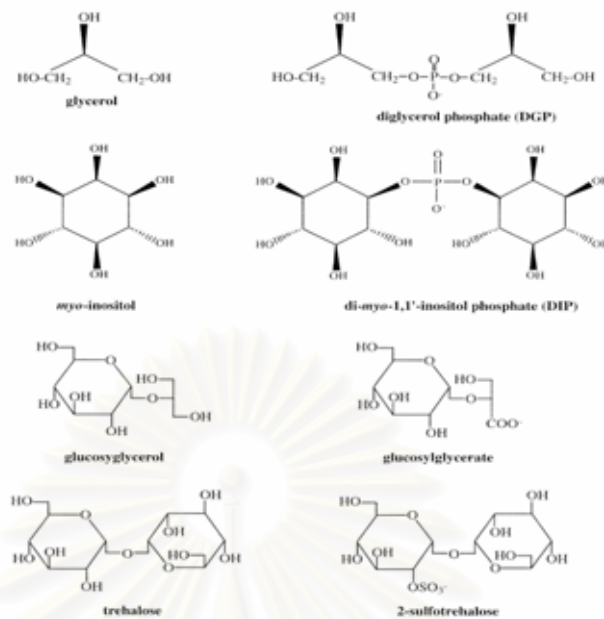
Figure 2. The compatible solutes and the counterparts of halophilic bacteria and plants.

2.2 TAXONOMY

Archaea is the microorganism that grow optimally in extreme conditions. Based on their physiology, the Archaea can be organized into six types: methanogens (prokaryotes that produce methane); extreme halophiles (prokaryotes that live at very high concentrations of salt (NaCl); extreme (hyper) thermophiles (prokaryotes that live at very high temperatures); extreme acidophiles and basidophiles (prokaryotes that live at very low pH or high pH). They consist of ethers of glycerol and hydrocarbon chains and their cell walls are absence of peptidoglycan. In contrast, bacteria grow optimally in the presence of 5-15% NaCl, but their growth is partially inhibited in the presence of more than 15% NaCl as called moderate halophile. Polar lipids of bacteria (moderate halophiles and halotolerant) are esters of glycerol and fatty acids and contain chemically peptidoglycans.

2.2.1 Halophilic archaea or extreme halophiles

They live in natural environments where the salt concentration is very high (as high as 5 M or 25 % NaCl) and grow optimally at 3.4–5.1 M (20–30%) NaCl. These prokaryotes require salt for growth and will not grow at low salt concentrations. Their cell walls, ribosomes, and enzymes are stabilized by Na^+. The halophilic

archaea is strictly aerobic even high salt conditions that are less availability of O_2 for respiration. They adapt to the high-salt environment by the development of purple membrane, actually patches of light-harvesting pigment in the plasma membrane. The pigment is bacteriorhodopsin contains 25% lipids and 75% protein. It reacts with light resulting a proton gradient on the membrane just as in the case of the respiratory chain allowing the synthesis of ATP. Accordingly, the extreme halophiles can produce efficiently ATP by nomal way (respiration) and adaptation one (bacteriorhodopsin). Currently, members of the aerobic, extremely halophilic archaea are classified in 20 genera; *Halobacterium, Haloarcula, Halobaclucum, Halobiforma, Halococcus, Haloferax, Halogeometricum, Halomicrobium, Haloquada, Halorhabdus, Halorubrum, Halosimplex, Haloterrigena, Hatrialba, Natrinema, Natronobacterium, Natronocuccus, Natronolimnobius, Natronomonas and Natronorubrum* .

The *Halobacteriaceae* have archaeal-type lipids based on branched 2,3-di-*O*-phytanyl-*sn*-glycerol ($C_{20}C_{20}$) and sometimes also 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol ($C_{25}C_{20}$) chains, bound to glycerol by ether bonds. The lipids are expected to form 'zip' type bilayer membranes as exemplified in Figure 3. All known species contain the diether derivatives of phosphatidylglycerol (PG) and methyl ester of phosphatidylglycerol methylphosphate (PGP-Me) (Fig.4a and b). The glycolipids and sulfolipids have been found in different members of the *Halobacteriaceae* (Table 2).
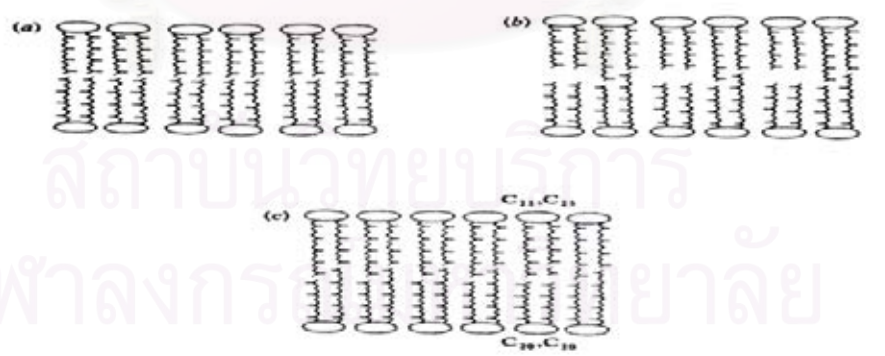
Figure 3. Halophile membrane structure. (a) Bilayer membrane structure formed by $C_{20}C_{20}$ diether lipids proposed for *Halobacterium*. (b) Zip type I membrane formed by $C_{20}C_{20}$ and $C_{20}C_{25}$ diethers proposed for certain haloalkaliphiles. (c) Zip type 2 membrane proposed for haloalkaliphiles which $C_{20}C_{20}$ and $C_{20}C_{25}$ diethers.
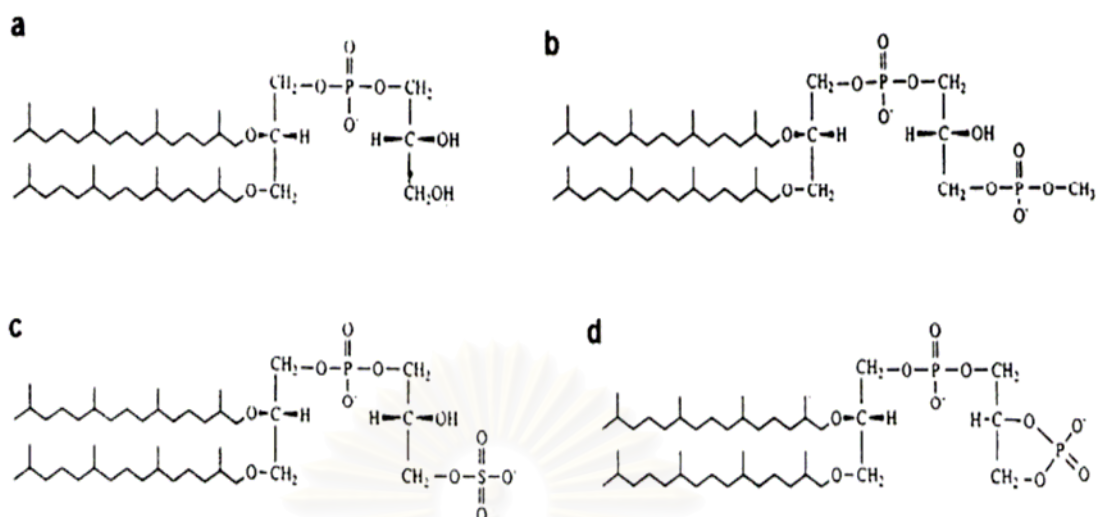
Figure 4. Structure of the phytyl diether derivatives of PG (a), Me-PGP (b), phosphatidylglycerosulfate (PGS) (c), and the cyclic PGP of extreme halophiles.

Table 2. Major glycolipids of different members of the *Halobacteriaceae*.

Genus and species	Major glycolipids present; minor components in parentheses
Halobacterium	S-TGD-1, S-TeGD (TGD-1, TeGD)
Haloarcula (except *Haloarcula mukohataei*)	TGD-2 (DGD-2)
Haloferax	S-DGD-1
Halococcus	S-DGD-1, S-TGD[a]
Halobaculum	S-DGD-1
Halorubrum	S-DGD-1, S-DGD-3 or S-DGD-5
Halogeometricum	Unidentified glycolipids
Natrialba asiatica	S_2-DGD-1
Natrinema	Unidentified glycolipids
Haloterrigena	Unidentified glycolipids
Natronobacterium	None, Small amounts of DGD-4 have been detected in an Indian isolate
Natronomonas	None
Natronococcus	None

2.2.2.1 Characterization of *Halobacterium*

The genus *Halobacterium*, *H. salinarum* was proposed by Elazari-Volcani (1957). *Halobacterium* species does not have peptidoglycan. Their rod shape is maintained by glycoprotein that contain negatively charged acidic amino acids in their cell walls which counteract the positive charges of the high Na^+ environment. If the salt concentration drops much below 4 M, cells will change form rod through irregular transition forms to spheres, and the spheres undergo lysis. The first step is due to disintegration of the glycoprotein envelope.

The member of this genus are proteolytic capabilities even heavily salt, hence, Thongthai (1992) isolated the halophilic bacteria from fish sauce. It was identified as *H. salinarum* by high DNA-DNA similarity (>70%) with *H. salinarum* (Wayne *et al*., 1987). It produced protease, so Thongthai (1992) suggested that it may show a significant role in the fish sauce fermentation and flavor and aroma producing process.

In 2004, the new species of the genus *Halobacterium* was proposed. *H. noricense* DSM 15987^T isolated from a bore core from a salt mine in Austria. (Gruber *et al.,* 2004). Gram-negative, motile and aerobic extremely halophilic archaea occurred growth in media containing 3.0-5.2 M NaCl and grew best at 3.5-4.5 M NaCl. Polar lipids consist $C_{20}C_{20}$ derivatives of PG, Me-PGP, S-TeGD and TGD and Menaquinone, MK-8. Cells are rod shaped measuring 1.2-2.0x1.0-6.0 µm, growing in liquid media as single cell. These features are relating to the genus *Halobacterium*. It is susceptible to anisomycin and novobiocin and resistant to ampicillin, chloramphenicol, gentamycin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin. The different characteristics from the known species of the genus *Halobacterium* are shown Table 3. Low DNA-DNA similarity among the strain and the referent strains (*H. salinarum* and *Halobacterium* sp. NRC-1) supported that it is the new species.

Table 3. Differential characteristics of *Halobacterium* species (Gruber *et al*., 2004; Thongthai *et al*., 1992).

Characteristics	*H. salinarum* DSM 3754^T	*Halobacterium* sp. NRC-1	*H. noricense* DSM 15987^T
pH range for growth	5.5-8.0	5.2-8.0	5.2-7.0
Temperature range for growth (°C)	22-50	22-50	28-50
Anaerobic growth in the presence of			
Arginine	+	+	+
DMSO	+	+	+
Nitrate	+	+	+
Enzyme assays (API-ZYM)			
Alkaline phosphatase	+	+	-
Esterase (C4)	-	-	+
Cysteine arylamidase	-	-	+
Valine arylamidase	-	-	+
Acid phosphatase	+	+	-
Hydrolysis of			
Gelatin	-	-	-
Starch	-	-	-
G+C content (mol%)	57-60	57-60	54.5

+, positive; -, negative

2.2.1.2 Characterization of *Halococcus*

The genus *Halococcus* have been created by Scoop (1935), comprised the red and extremely halophilic bacteria such as *Micrococcus morrhuae*. Kocur and Hodgkiss (1973) transferred *M. morrhuae* to the genus *Halococcus* as *H. morrhuae*, the type species of genus. The main lipids were both $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of PG and PGP-Me. Menaquinone MK-8(H_2) was the main component. Cells are not lysis, when suspended in distilled water since *Halococcus* species are absence of glycoprotein in cell wall. On the other hand, they have a thick heteropolysaccharide cell wall comprising uronic acid, glucosamine, N-

acetylgalactosamine, galactose, glucosamine, glycine, mannose, N-acetylglucosamine and N-acetylgulosaminuronic acid (Figure 5), that does not require high salt concentrations to maintain sphere shape.
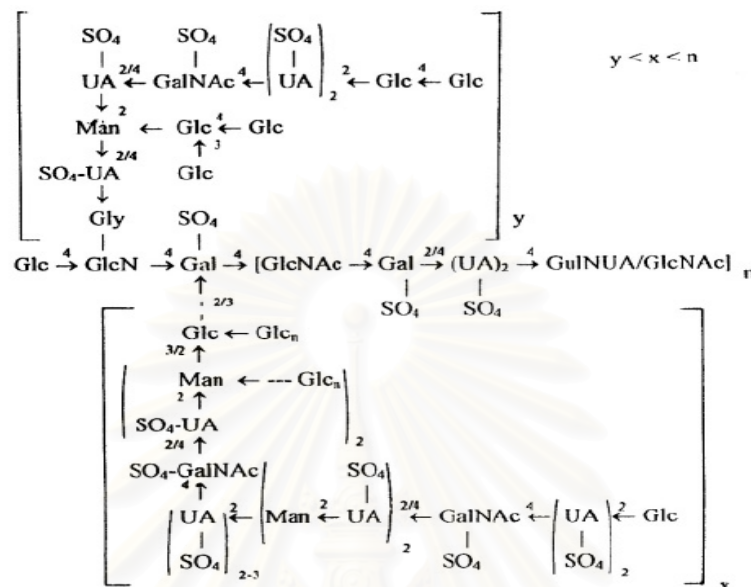
Figure 5. A model of the cell wall of *Halococcus morrhuae*. UA, uronic acid;

   GlcN, glucosamine; GalNAc, N-acetylgalactosamine; Gal, galactose;

   Glc, glucose; GlcNAc, N-acetylglucosamine; Gly, glycine

    GulNUA, N-acetylglucosaminuronic acid; Man, mannose.

In 1989, the non spore-forming, motile and strictly aerobic halophilic archaea isolated from marine salterns. It produced oxidase and catalase. The strain was cocciod microorganism of about 0.8-1.5 µm in diameter, occurring in pairs, tetrads, sarcina pockets of irregular. It was the second member of the genus *Halococcus* and classified as *H. saccharolyticus* (Montero *et al.*, 1989). Danner *et al.* (1994) isolated halophilic bacteria from Austrian salt mine, was proposed as *H. salifodinae*. Recently, Stan-Lotter *et al.* (2002) taxonomically studied the extreme halophiles isolated from Austrian salt mine. It required at least 2.5 M NaCl for growth and 3.5-4.5 M NaCl for best growth. They were susceptible to anisomycin and novobiocin and resistant to ampicillin, chloramphenicol, gentamycin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin. Its properties differed from the known species of the genus *Halococcus* (Table 4), warrants that it is the new species of the genus *Halococcus, H. domdrowskii*.

Table 4. Differential characteristics of *Halococcus* species (Danner *et al*., 1994; Montero *et al*., 1989; Stan-Lotter *et al*., 2002).

Characteristics	*H. morrhuae*	*H. saccharolyticus*	*H. salifodinae*	*H. dombrowskii*
pH range	5.5-8.0	6.0-8.0	6.8-9.5	5.2-8.0
Temperature range (°C)	28-50	28-42	28-50	28-50
Cysteine arylamidase	-	ND	ND	+
Acid production from				
D-glucose	-	+	ND	-
Lactose	-	-	ND	-
Utilization of				
L-arabinose	-	+	+	-
D-glucose	+	+	+	-
D-xylose	-	-	+	+
L-arginine	+	+	ND	-
L-glutamic acid	+	+	ND	-
Hydrolysis of				
Gelatin	+	+	-	+
Tween 80	+	-	+	ND

+, positive; -, negative; ND, not determined

2.2.2 Moderate halophiles

Kushner (1987) defined microorganism that grow in media containing approximately 3 to 15% NaCl are moderately halophilic bacteria. All moderately halophilic bacteria require salt for growth and are able to tolerate high salt concentrations. The example of moderately halophilic bacteria from different genera. Archaeal bacteria; *Methanohalophilus, Halomethanococus*, Gram-negative; *Deleya, Arhodononas, Chromohalobacter, Flavobacterium, Haloanaerobium, Halobacteroides, Haloincola, Halomonas, Pseudomonas, Sporohalobacter, Vibrio, Volcaniella*, Gram-positive; *Halobacillus, Bacillus, Marinococcus, Micrococcus,*

Salinicoccus, *Lentibacillus*, *Filobacillus*, *Virgibacillus*, and *Sporosarcina*, and facultative anaerobe; *Flavobacterium salegens* and *Arhodomonas aquaeolei*. All the members of moderately halophilic bacteria and halotolerant, contain chemically peptidoglycans comprising peptides (short amino acids chains) and glycans, *N*-acetyl(glycolyl)muramic acid (NAM or M) and N-acetylglucosamine (NAG or G). Some of the amino acids are only found in cell walls, but not in other cellular proteins i.e., D- amino acids, e.g D-alanine and diaminopimelic acid, DAP. The family *Bacillaceae* contain meso-diaminopimelic acid as the diagnostic cell wall peptidoglycan. The family *Halomonaceae* is the presence of the hydrophobic amino acid leucine, which is expected to add to the overall hydrophobicity of the cell wall.

Polar lipids of halophilic bacteria are glycerol bound to fatty acids with ester links. The lipid side chains are fatty acids i.e., Saturated fatty acid ($C_{13:0}$, $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$) and Unsaturated fatty acids (iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$). The major polar lipids present in most species are phosphoglycerol (PG) and diphosphoglycerol (DPG). Additional types of lipids may occur such as are phosphatidylcholine (PC) and phosphatidylethonalamine (PE), diphosphatidylglycerol (cardiolipin, CL) and glycolipids. Generally the content of negatively charged phospholipids (PC, CL) increases at the expense of neutral phospholipids (PE) as salinity increases (Vreeland *et al*., 1992).

2.2.2.1 Characterization of *Chromohalobacter*

Some halophilic strains that had been assigned in the genus *Halomonas* were reclassified as those of another genus *Chromohalobacter* by Ventosa *et al*. (1989). The *Chromohalobacter* genus, the sub-group γ-Proteobacterial, *Halomonadaceae, C. canadensis*, *C. israelensis*, *C. marismortui* and *C. salexigens* have been recognized in the Dead sea, Maditerranean saltern and solar salt land, respectively. (Arahal *et al*., 2001a; Arahal *et al*., 2001b; Ventosa *et al*., 1989). They were motile and rod-shaped moderately halophilic bacterium. The rod shape was measured about 0.6-1.2X1.5-4.2 µm and occur singly, in pairs and in short chains. It was aerobic, non-spore forming and Gram-negative. Cells were straight or sometimes slightly curved. They were tolerate at salt concentrations up to 30%(w/v). The broader ranges of temperature and pH observed for growth were 5-45 °C and pH 5.0-10. Strains reduced nitrate, but H_2S was not produced. Urease and phenylalanine deaminase were negative, but not nitrate reduction, catalase and oxidase. Casein,

DNA, aesculin, gelatin, starch and Tween 80 were not hydrolyzed. The cell wall peptidoglycan contain diaminopimelic acid. The predominant menaquinone is MK-7. The differentiated characteristics of *Chromohalobacte*r species show in Table 5.

Table 5. Characteristics of species belonging to the genus *Chromohalobacter* (Arahal *et al*., 2001a; Arahal *et al*., 2001b; Ventosa *et al*., 1989).

Characteristics	*C. marismortui*	*C. salexigens*	*C. canadensis*	*C. israelensis*
Pigmentation	Yellow	Cream	White	Cream
NaCl range (%)	1-30	0.9-25	3-25	3.5-20
Acid production from				
D-galactose	+	+	ND	ND
Maltose	+	+	-	+
Sucrose	+	+	-	+
D-glucose	+	+	ND	ND
D-trehalose	+	-	-	-
D-Xylose	+	+	ND	ND
Lysine decarboxylase	-	-	+	+
G+C contents (mol%)	62.1-64.9	64.2-66	62	65

+, positive; -, negative; ND, not determined

2.2.2.2 Characterization of *Halobacillus*

In 1992, the genus *Halobacillus* was first described by Spring *et al*. (1992) who isolated two strictly aerobic, Gram-positive, spore-forming, short rod-shaped moderately halophilic bacteria from the Great salt Lake in Utah. The cell wall contained peptidoglycan of the Orn-D-Asp type. The major fatty acids are anteiso $C_{15:0}$ and iso $C_{16:0}$. The predominant menaquinone is MK-7. Two strains were named as *H. trueperi* and *H. litoralis*, respectively. Spring *et al*. (1992) transferred *Sporosarcina halophila* to the genus *Halobacillus*, *H. halophilus*. In Thailand, the moderately halophilic bacteria isolated from fish sauce were identified as the new species of the genus *Halobacillus*, *Halobacillus thailandensis* (Chaiyanan *et al*., 1999). It was motile by means of flagella and chemoorganotroph. Catalase and oxidase are produced. Urease and Voges-Proskauer are negative. Nitrate was not reduced to nitrite. Endospore resisted heating at 75°C for at least 10 min. *H.*

thailandensis was the protease-producing bacteria, so its protease may be significant role in fish sauce fermentation similar to *Halobacterium salinarum*. Subsequencely, *Halobacillus aidingensis, Halobacillus dabanensis, Halobacillus karajensis, Halobacillus locisalis, Halobacillus salinus,* and *Halobacillus yeomjeoni* have been recognized as novel species based on the differentiated properties shown in Table 6 (Amoozegar *et al*., 2003; Yoon *et al*., 2004).

Table 6. Phenotypic characteristics of species belonging to the genus *Halobacillus* (Amoozegar *et al*., 2003; Spring *et al*., 1996; Yoon *et al*., 2004).

Characteristics	*H. halophilus*	*H. litoralis*	*H. trueperi*	*H. karagensis*	*H. locisalis*	*H. salinus*
Morphology	Cocci	Rods	Rods	Rods	Rods	Rods
Pigmentation	Orange	Orange	White	White	Orange	Orange
NaCl range (%)	2-20	0.5-25	0.5-30	1-24	1-23	1-23
Maximum temp (C°)	37	43	44	49	42	45
Acid production from						
D-fructose	-	+	+	+	+	+
D-galactose	-	-	+	-	-	w
Maltose	-	+	+	+	-	+
Sucrose	-	+	+	-	+	+
D-glucose	-	+	+	+	+	+
D-mannitol	-	+	-	+	-	+
D-trehalose	-	+	-	ND	+	+
D-Xylose	-	+	+	-	-	-
Hydrolysis						
Aesculin	-	-	-	+	+	+
Casein	+	-	-	+	-	+
Gelatin	+	+	+	+	-	+
Starch	+	-	-	+	+	-
G+C contents (mol%)	40.1-40.9	42	43	41.3	44	45

+, positive; -, negative

2.2.2.3 Characterization of *Lentibacillus*

Halophilic bacteria, SF-20 isolated from a salt field in Korea related to the recently described genera *Virgibacillus, Gracilibacillus* and *Halobacillus* based on the phylogenetic tree analysis (Yoon *et al.*, 2002). However, chemotaxonomic properties, cell wall peptidoglycan of the genus *Halobacillus* was Orn-D-Asp type that differed from SF-20 containing meso-diaminopimelic acid. The differences between SF-20 and the genera *Virgibacillus* was the cellular fatty acid profiles. Strain SF-20 had a minor component of iso-$C_{16:0}$, but it was the major component of the genera *Virgibacillus*. DNA-DNA similarity warrants that strain SF-20 distinguish from the members of related genera. They grew optimally in the presence of 4–8% NaCl. Catalase and oxidase were positive but not Urease. The predominant menaquinone was MK-7. The major fatty acids were anteiso-$C_{15:0}$. The cellular phospholipids were PG and PGP. In 2005, three new species of genus *Lentibacillus* have been proposed, *L. salaries* and *L. lacisalsi* (Jeon *et al.*, 2005; Lim *et al.*, 2005). The differentiated characteristics of the genus *Lentibacillus* show Table 7.

Table 7. Salient features of genus *Lentibacillus* (Jeon *et al.*, 2005; Lim *et al.*, 2005; Yoon *et al.*, 2002).

Characteristics	*L. salaries* KCTC 3911[T]	*L. salicampi* JCM 11462[T]	*L.lacisalsi* KCTC 3915[T]
Maximum growth (^{o}C)	50	40	40
NaCl range (%)	1-20	3-25	5-25
Hydrolysis of:			
Aesculin	+	-	-
Casein	-	+	-
Tween 80	-	+	-
Acid production from:			
D-Glucose	+	+	-
D-Fructose	+	-	+
Maltose	+	w	-
D-Mannose	+	w	-
D-Ribose	+	-	+
Salicin	-	w	-
D-Trehalose	w	-	-
D-Xylose	+	-	w
G+C content (mol%)	43	42	44

+, positive; -, negative

2.2.2.4 Characterization of *Filobacillus*

The genus *Filobacillus* have been created by Schlesner *et al.* (2001). A spore-forming, motile, strictly aerobic and rod-shaped halophilic bacterium was isolated from the beach of Palaeochori Bay near to a shallow water hydrothermal vent area, Milos, Greece (Schlesner *et al.*, 2001). Spores were spherical and located terminally in sporangium swollen. Oxidase, Voges-Proskauer and KOH test were negative, but not catalase. It did not grow in media without the addition of NaCl. Acid was not produced from D-glucose. Nitrate was not reduced to nitrite. The G+C content. of 35 mol%. A tree constructed by the neighbour-joining method revealed that it was nearest with *Alkalibacillus haloalkaliphilus*. The cell wall peptidoglycan of strain was type L-Orn-D-Glu, but meso-diaminopimelic acid was observed in *A. haloalkaliphilus*. In addition, the unknown strain differed from *A. haloalkaliphilus* in hydrolyzing DNA, but not casein and gelatin and by its negative oxidase (Table 8). It was classified as *F. milosensis*.

Table 8. Phenotypic characteristics species belonging to the genus *Filobacillus* and *Alkalibacillus haloalkaliphilus* (Schlesner et al., 2001)

Characteristics	*F. milosensis*	*A. haloalkaliphilus*
Morphology	Rods	Rods
Pigmentation	Yellow	Cream
Spore shape	Spherical	Spherical
Spore position	Terminal	Terminal
NaCl range (%)	2-23	ND
Maximum temp (C°)	42	45
Oxidase	+	+
Catalase	+	+
Acid production from		
D-xylose	-	-
Casein	-	-
Gelatin	-	+
G+C contents (mol%)	36.7	37-38

+, positive; -, negative; ND, not determined

2.2.2.5 Halotolerant

They were tolerant high salt concentration but do not require them for growth. Flannery (1996) defined The slight halophile as those that grow optimally in the media containing <2% salt but may grow in a NaCl concentration of 10% of more, such as *Jeotgalicoccus, Pseudomonas, Moraxella, Flavobacterium, Acinetobacter* and *Vibrio*, they isolated from sea fish and shell.

In contrast, nonhalophiles grow optimally at less than 0.2 M NaCl. Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors.

3. PROTEASE

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Protease catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins (Figure. 6).
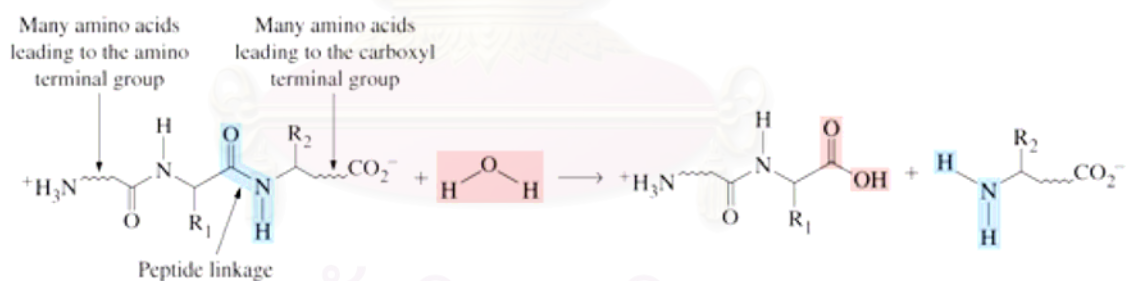
Figure 6. Catalytic reaction of protease

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories: 1) Limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein such as conversion of prohormones to hormones. 2) Unlimited proteolysis, in which proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. This

modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists in the compartmentation of proteases such as in lysosomes. Proteins transferred into this compartment undergo a rapid degradation.

3.1 CLASSIFICATION OF PROTEASE

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of two major criteria (Barett, 1994).

3.1.1 Type of reaction catalyzed

Proteases are grossly subdivided into two major groups (Table 9), i.e., exopeptidases and endopeptidases, depending on the location of the enzymatic action, either exopeptidase or endopeptidase. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate.

3.1.2 Chemical nature of the catalytic site

Based on the functional group present at the active site, proteases are further classified into four prominent groups. There are serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Guzzo *et al*., 1990)

Serine proteases: Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. This class comprises two distinct families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the subtilisin family which include the bacterial enzymes such as subtilisin.

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the

active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Aspartic proteases. Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin, and enzymes from pararetroviruses.

The aspartic proteases are inhibited by pepstatin (Fitzgerald *et al*., 1990). They are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methylester (DAN) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp. Cysteine/thiol proteases. Cysteine proteases occur in both prokaryotes and eukaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine.
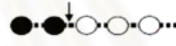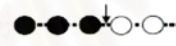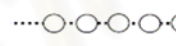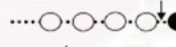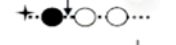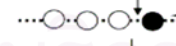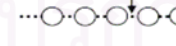
Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents. Metalloproteases. Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity. The metallo proteases may be one of the older classes of proteases and are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Because of they require a metal ion for their activity, so inhibited by metal chelating

agents such as EDTA but not by sulfhydryl agents or DFP. For example carboxypeptidase, thermolysin, collagenase.

3.2 EVOLUTIONARY RELATIONSHIP WITH REFERENCE TO STRUCTURE

Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawling *et al*., 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.

Table 9 Classification of protease

Protease	Mode of action[a]	EC no.
Exopeptidases		
Aminopeptidases		3.4.11
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidase		3.4.16-3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase		3.4.15
Dipeptidase		3.4.13
Omega peptidases		3.4.19
		3.4.19
Endopeptidases		3.4.21-3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown Catalytic mechanism		3.4.99

[a] Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.

3.3 MICROBIAL PROTEASE

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996) (Fig. 7). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

3.3.1 Bacteria

Most commercial proteases, mainly neutral and alkaline protease, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant protease inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

3.3.2 Fungi

Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal

proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process.

3.3.3 Viruses

Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic, and cysteine peptidases are found in various viruses (Rawling and Barrett, 1993). All of the virus-encoded peptidases are endopeptidases; there are no metallopeptidases. Retroviral aspartyl proteases that are required for viral assembly and replication are homodimers and are expressed as a part of the polyprotein precursor. The mature protease is released by autolysis of the precursor. An extensive literature is available on the expression, purification, and enzymatic analysis of retroviral aspartic protease and its. Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS.

Thus, although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. In contrast, non-genetic manipulation, halophilic microorganisms produce stable proteases capable of functioning under conditions that lead to precipitation or denaturation of most proteins. Halophilic proteins compete effectively with salts for hydration, a property that may result in resistance to other low-water-activity environments, such as in the presence of organic solvents. For that reason, it would be of great importance to have available enzymes for biotechnology applications.
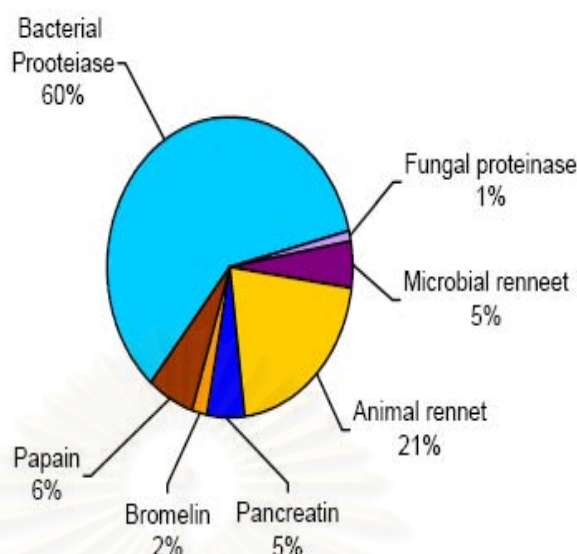
Figure 7. Piechart of the industrial protease market. Market shares for each protease
are given as percent of a total estimated sales turnover of US$242 m.
Microbial proteases are depicted in pie slices with shading.

4. PROTEASE-PRODUCING HALOPHILIC BACTERIA

Halophiles produce a large variety of stable and unique biomolecules that may
be useful for practical applications. Halophilic microorganisms produce stable
enzymes including many hydrolytic enzymes such as proteases capable of functioning
under conditions that lead to precipitation or denaturation of most proteins. Halophilic
proteins compete effectively with salts for hydration, a property that may result in
resistance to other low-water-activity environments, such as in the presence of organic
solvents. Therefore, halophilic microorganisms have attracted much attention in
recent years, most studies have been performed in halobacteria. However, moderately
halophilic bacteria represent an excellent model of adaptation to frequent changes in
extracellular osmolality and constiute an interesting group of microorganisms from a
biotechnological point of view. Thus, many of them accumulate intracellular organic
osmolytes named "compatible solutes" which can be used as stabilizers of enzymes
and whole cells (Ventosa *et. al*., 1998; Nieto *et. al*., 2000) and they produce halophilic
exoenzymes that could of commercial interest and could be used in biodegradation
processes. They have the advantage that most species are able to grow in a wide range
of salinities, in contrast to the more strict requirements of salt presented by

halobacterium. For example *Gracilibacillus* and *Tetragenococcus* strains can growth in absence NaCl and cell reduce size in low concentration of salts(Thongsanit *et al*., 2002)

4.1 PROTEASE-PRODUCING EXTREMELY HALOPHILIC BACTERIA

everal extracellular proteases from extremely halophiles have been studied and characterized e.g., an endopeptidase from *Halobacterium halobium* S9, a chymotrypsin-like serine protease from *Natrialba magadii* ATCC 43099, a serine protease from *Halobacterium* sp. TuA4, *Halobacterium halobium*, *Natronococcus occultus* NCMB 2192, *Halobacterium halobium* ATCC 43214 and *Halobacterium mediterranei* 1538 and a subtilisin-like protease (halolysin R4) from *Haloferax mediterranei* R4. A gene of halolysin R4 has been cloned and expressed in *Haloferax volcanii* WFD11 (Capiralla *et al*., 2002; Izotova *et al*., 1983; Gimenez *et al*., 2000; Kamekura *et al*., 1993; Kamekura *et al*., 1996; Ryu *et al*., 1994; Schmitt *et al*., 1990; Stepanov *et al*., 1992; Studdert *et al*., 1997). The enzymes from extraordinally halophilic bacteria represent a fascinating example of adaptations summarized in Table 9

The extracellular protease of the extreme halophile *H. halobium* had Mw of 66,000 and is highly dependent upon salt concentrations near saturation for catalytic activity and stability (Ryu, *et al*., 1995). The enzyme activity is dramatically affected by the salt concentration, with a loss of 2.5 fold in the absence of NaCl. The enzyme has a broad acyl donor substrate specificity and can effectively use amino acid esters of Phe, Tyr, Trp, Ser, Gly, and Ala.

An unidentified halophilic archaebacterium strain 172 P1 produced three extracellular proteases in media containing 15-27% salts. The 44 kDa of purifed enzyme was a serine protease and salt-enhanced activity. In the presence of 25% NaCl, high activity was obtained at 75-80 °C revealed that its activity was stabilized by salt (Kamekura and Seno, 1990)

Gimenez *et al*. (2000) isolated and characterized a serine protease secreted by *Natrialba magadii*. The Mw estimated 45 kDa and had chymotrypsin-like activity. The extracellular protease was dependent on high salt concentrations for activity and stability in range of basic pH. The activities were activated by thiol-containing reducing agents such as dithiotreitol (DTT) and 2-mercaptoethanol.

Studdert *et al.* (1997) purified a serine protease from *Natronococcus occultus* and characterized at the biochemical level. Approximately 130 kDa of purified enzyme was chymotrypsin-like activity that stable and active in a broad pH range (5.5-12), dependent on high salt concentrations for activity and stability (1-2 M NaCl or KCl). The amino acid sequences of three tryptic peptides obtained from *Natronococcus occultus* protease did not show significant similarity to other known proteolytic enzymes. Therefore, the extracellular protease may be a novel enzyme.

Izotova *et al.* (1980) isolated an extracellular serine protease from the *Halobacterium halobium*. The enzyme activity was completely and irreversibly lost in salt concentration less than 2 M. Polypeptide chain with a molecular weight of 41,000. enriched in Asx and Glx content. The protease shows a preference for leucine in the carboxylic side of the scissile bond of the substrate.

In 2002, *Halobacterium halobium* S9 produced an extracellular endopeptidase that has very high salt requirements in the range of 4 M NaCl. N-terminal amino acid sequence analysis shows LVPNDAR as the probable residues. The endopeptidase exhibited significant stability under wide variations of pH and temperature. Substrate specificity studies using peptides reveal high specificity for peptides containing Pro in the penultimate position and preference for hydrophobic amino acids in the carboxy terminal side of the peptides. The action of endopeptidase on the complex peptide mixture of a tryptic digest of b-casein resulted in the degradation of hydrophobic peptides. Thus, these characteristics of the purified peptidase make it suitable for an array of industrial applications with special impetus in debittering of meso, cheese and protein hydrolysates in food processing industry (Capiralla *et al.*, 2002).

4.2 PROTEASE-PRODUCING MODERATELY HALOPHILIC BACTERIA

Protease from moderately halophilic bacteria have not been extensively studied. Some of their works on protease from moderately halophilic bacteria are as follows : Duong Van Qua *et al.* (1981), Sanchez-Porro *et al.* (2003), and Hiraga *et al.*, (2005) shown Table 15. Previously study, only studies extracellular protease produced by unidentified moderately halophilic bacterium, designated *Psseudomonas* sp. strain A-14, was purified. The molecular weight of this enzyme was estimated to be 12 kDa, The optimum pH for activity was 8.0, and the enzyme presented maximal at 18% NaCl concentration (Qua *et. al.*, 1981).

In 2003, the protease CP1 produced by the moderately halophilic bacterium, *Pseudoalteromonas* sp. CP76 has been purified and characterized in detail by Sanchez-Porro *et. al.* (2003). The enzyme is a homodimer with a subunit size 38 kDa. The enzyme is moderately thermophile , presenting optimum activity at 55 ℃, at pH 8.5. An interesting feature of this protease is salt tolerance over a wide range of NaCl concentration (0-20% NaCl). These characteristic make the protease CP1 interesting for its application in biotechnological process. This study constitutes the first report on the purification and in-depth characterization of proteolytic enzyme from a moderately halophilic microorganism. In a recent study, Lama, *et al*. (2005) describe the identification and characterization of a proteolytic enzyme from a haloalkaliphilic microorganism belonging to *Salinivibrio costicola* subsp. *costicola*. The optimal protease yield was concomitant with optimal growth conditions at pH 9.0, 30 ℃, 12% NaCl concentration and agitation. Approximately 38 kDa of purified enzyme was active even without NaCl and over a wide range of NaCl concentrations (up to 15%). Its protease is a moderately thermo active enzyme showing optimal activity at 60 ℃ in the presence of calcium and NaCl. This characteristic makes the protease of strain 18AG an interesting candidate for application in biotechnological processes

In addition, the protease-producing bacteria isolated from fish sauce were studied and characterized its properties (Hiraga *et al*., 2005). An isolated moderately halophilic bacterium, RF2-5 was identified and named as *Filobacillus*. The molecular weight of the purified enzyme was estimated to be 49 kDa. The enzyme showed the highest activity at 60 ℃ and pH 10-11 under 10%NaCl and was highly stable in the presence of about 25% NaCl. The activity was strongly inhibited by PMSF, Chymostatin, and α-microbial alkaline protease inhibitor (MAPI). The N-terminal 15 amino acid sequence of the purified enzyme showed about 67% identity of the serine protease from *Bacillus subtilis* 168 and *Bacillus subtilis* (Natto). The protease from *Filobacillus* sp RF2-5. might be useful for the degradation of fish protein during fermentation at high salt concentrations and might be useful in reduction of the fermentation period.

4.3 INDUSTRIAL APPLICATION

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in

leather treatment and in several bioremediation processes. The worldwide requirement for enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used.

The halophilic enzymes may become the enzymes of choice in biotechnological potential for novel applications. For example, an extracellular protease from *Halobacterium halobium* has been exploited for efficient peptide synthesis in water/N0-N0-dimethylformamide (Kim and Dordick, 1997). The protease from *Halobacterium halobium* (ATCC 43214) is significantly more selective for the amino acid amide, preferring Gly in the P~ site. A series of glycine-containing oligopeptides have been prepared in yields up to 76% without degradation due to secondary. From practice, it is an excellent candidate as a catalyst for peptide synthesis, particularly for glycine-containing peptides. On the other hand, they rapidly denature and dissociate at NaCl/KCl concentrations below 1 M and can be irreversible. This feature, rather than stability at high salt concentrations, has been proposed as the defining feature of halophilicity. Accordingly, the effect of reducing water activity, enzymes from the halophilic archaea are thought to be important biocatalysts in aqueous/organic and nonaqueous media such as DMF. Based on the aw of saturated NaCl is 0.75, corresponding to a DMF concentration of 60 vol%. Although enzyme activity can be observed in such media, it is generally low, and the high specific activity of halophilic enzymes at such an aw is an important property (Burg, 2003)

Table 9. Properties of protease from extreme halophiles

Strains	Mw (kDa)	Optimal condition			Inhibitor
		NaCl	pH	Temp (°C)	
Extremely halophile					
Natrialba magadii	45	1.5 M	8-10	60	DFP,PMSF, chymostatin
Halophilic archaebacterium 172P1	42-46	-	10.7	75	-
Halobacterium mediterranei 1538	41	4.5M	8-8.5	55	-
Halobacterium halobium ATCC 43214	66	4M	10	-	DMF
Natronocuccus occultus NCMIB 2129	45	1-2M	-	-	DFP,PMSF, chymotrypsin
Halobacterium sp. strain TuTA	60	-	-	-	PMSF, DFP, leupeptin
Moderately halophiles					
Pseudoalteromonas sp. CP76	38	-	8.5	55	PMSF, EDTA
Salinivibrio costicola subsp. *costicola*.	38	2%	8.0	60	PMSF, DTT
Filobacillus sp. RF2-5	49	10%	10-11	60	PMSF, α-MAPI, chymostatin

CHAPTER III

EXPERIMENTAL

1.   **Sample collection and isolation of Halophilic bacteria**

      The halophilic bacteria were isolated from the fish sauce samples (nam-pla) which were collected from factories, except for bu-du that was collected from a market. The sample, aliquots of 100 μL were spread onto JCM No.168 and JCM No.169 agar containing 20 and 25% NaCl, respectively (Appendix A) and were incubated at 37 °C for 1-2 weeks. A single colony of the extremely and moderately halophilic bacteria were transferred to a JCM medium No. 169 and JCM medium No. 377 containing 10% NaCl (Appendix A), respectively and incubated at 37 $^{\circ}$C for 3-14 days.   The stock cultures were kept in cold room at 4 $^{\circ}$C at the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.   **Identification methods**

      Morphological, cultural, physiological, and biochemical characteristics of the isolates were determined using the methods described by Barrow and Feltham (1993), Oren (1997), Leifson (1963), Namwong (2005) and Stan-Lotter (2002) along with several supplementary tests.

      2.1 Morphological and cultural characteristics

      The cells of the isolates cultivated on the JCM No. 169 and 377 after 3 days or 14 days incubation at 37°C were examined for Gram staining as method of Hucker and Conn, (1923) for moderately halophilic bacteria. Gram staining of the extremely halophilic isolates was performed by using air-dried slides which were fixed and simultaneously desalted in 2% acetic acid for 5 min, dried before staining by standard procedures (Oren *et al*., 1997). Flagella of moderate halophiles were stained by the solution containing 0.4 g of basic fuchsin, 0.2 g of acid fuchsin, 0.2 g of tannic acid, 0.5 g of aluminum sulfate, 2 mL of 95% ethanol, 0.5 ml of glycerol and 7.5 mL of Tris-buffer (Forbes, 1981).   Flagella arrangement in motile extremely halophilic bacteria was observed after cells were stained with 0.5% uranyl acetate in 25% NaCl

for 30 sec (Oren *et al*., 1997). Cells of moderately halophilic bacteria were negatively stained with 1% (w/v) phosphotungstic acid (Yoon *et al*., 2002). Scaning electron microscope (SEM) used for morphological examinations was prepared according to the method of Bozzola and Russell (1999).

2.2 Physiological and biochemical characteristics

Growth at different temperatures, pHs, and NaCl concentrations were observed in the broth of JCM No. 169 or 377 at 37°C , 45°C and 50°C;   in the same medium adjusted pH to 5, 6, 8 and 9; and on the agar plate containing 0, 5, 10, 15, 20, 25 and 30% NaCl incubated for 1-2 weeks.

Oxidase test was determined by dropping small amount of 1% tetramethyl-p-phenylenediamine on sterile filter paper disc, the colonial appearance of dark-purple colour revealed the positive result.   Catalase test was performed by flooding the colonies with 3% hydrogen peroxide (H_2O_2) then became the gas bubbles denoted a positive reaction. Hydrolysis of L-arginine and aesculin were determined by inoculating the bacteria into L-arginine agar medium and aesculin broth, respectively (Appendix A-(5-6) and incubated for 1-2 weeks. A positive reaction for hydrolysis L-arginine was shown by a colour change of the indicator to red. For hydrolysis of casein, gelatin, starch and tyrosine were determined by addition of 1% skim milk, gelatin and starch and 5% tyrosine, respectively in JCM No. 169 or 377 agar plates and incubated for 1-2 weeks as recommended by Barrow and Feltham (1993). The Casamino acids in JCM medium no. 377 were omitted from the medium used to test for the hydrolysis of gelatin and casein. Clear colourless zones after the plates were flooded with Lugol's iodine or 10% trichloro acetic acid indicated the hydrolysis of starch and gelatin, respectively. Clear zone indicate areas of the hydrolysis of casein and tyrosine. Deoxyribonuclease (DNase) activity, indole test, nitrate reduction were observed by inoculating the cultures on DNase test agar (Difco) supplemented with 10 and 20% NaCl, tryptone broth and nitrate broth (Appendix A-(10-12) for 7 days or 14 days. After incubation, the DNase plates were flooded with 1 N HCl. The clear zone around the growth appeared indicated the positive result. The indole broth was inoculated and then 4 drops of Kovacs' reagent was added (Appendix B-42). If the nitrite is present a pink colour would develope within 5 minutes.

The acid production from carbon sources were performed in marine oxidation-fermentation medium or MOF (Appendix A-4) as described by Leifson (1963) for moderate halophiles. In case of extreme halophiles, modified Leifson agar medium were used for determination of acid production by omitting casitone and Tris-HCl and decreasing yeast extract to 0.01%. Both media were supplemented with the different carbon sources at final concentration 0.5% (w/v). The following 22 different carbon sources were used including L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, *myo*-Inositol, maltose, D-mannitol, mannose, melibiose, melizitose, raffinose, rhamnose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose, D-xylose. Both media were adjusted pH to pH 7.2 and phenol red 0.2% solution (w/v) was added as an indicator solution. The 2 ml of agar was dispensed into a test tube. Growth was recorded daily for up to 14 days. The positive results were shown by colour change of the indicator from red to yellow.

The utilization of nutritions of extreme halophiles were performed in modified Leifson medium by omitting Tris-HCl and supplementing 1% of the respective carbohydrates (L-arabinose, D-galactose, D-glucose, D-fructose, raffinose, D-xylose) or 0.01% of the respective amino acids (L-arginine, L-glutamic acid, L-serine). The media were adjusted pH to pH 7.2. The 2 mL liquid was dispensed into a test tube. The broth was inoculated with two drops of bacterial cell suspensed in saline and incubated at 37ºC for 14 days. Growth was measured at 660 nm .

All extremely halophilic bacteria were tested the susceptibility of antibiotics by spreading cell suspensions onto agar plates and then applying paper discs (6 mm in diameter) on which the antibiotics were dispensed in 30 µg amounts (rifampicin, chloramphenicol, tetracycline, ampicillin, gentamicin, kanamycin, nalidixic acid, streptomycin), with the exception of novobiocin and bacitracin, which was used in 5 and 10 µg, respectively. Zones of inhibition were measured following 2 weeks incubation at 37 °C; sensitivity was deemed strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc (Stan-Lotter *et al*., 2002).

2.3 Chemotaxonomic characteristics

2.3.1 Diaminopimelic acid analysis

Dried cells (10 mg) were hydrolyzed with 6N HCl at 100 ˚C for 18 h. The hydrolyzed solution was filtered and evaporated. The 400 µL of distilled water was added into dried sample. The solution was loaded onto cellulose HPTLC plate

no.5787 and developed with MeOH:H_2O:6N HCl:Pyridine (80:26:4:10). Finally, the cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol for detection (Komagata and Suzuki, 1987).

2.3.2 Amino acid composition of peptidoglycan

2 g of wet cells were suspended in 6 ml of phosphate buffer (pH 7.2) and sonicated them for 45 min. The unbroken cells were removed by centrifugation (4000 rpm for 10 min) and 1ml of 25% SDS was added to the supernatant and then heated at 100°C for 40 min. The solution mixtures were centrifuged at 10000 rpm for 1h and collected the precipitates. The precipitates were washed two times with phosphate buffer pH 7.2. The broken cells were collected by centrifugation at 4000 rpm for 10 min and collected the supernatant and then centrifuged at 10000 rpm for 1h . The broken cells were suspended in 2 ml of phosphate buffer pH 7.6 and 100 µL of Pronase E solution and incubated at 37°C for 2h. After centrifuge at 4000 rpm for 10 min, the precipitate was washed with phosphate buffer pH 7.6 two times and then resuspended in 2 ml of 5% TCA solution. The mixture was reacted at 100 °C for 20 min and then centrifuged at 4000 rpm for 10 min. The precipitate was again washed with distilled water for three times and dried in vacuum. The cell wall (1 mg) was hydrolyzed with 6N HCl at 100 °C for 18 h and then filtered and dried in the rotary evaporator. Finally, 100 µL of distilled water was added into the sample. For normal amino acid analysis: mix 10 µL of cell wall solution was mixed with 240 µL of 0.02 N HCL and detected with amino acid analyzer (Komagata and Suzuki, 1987).

2.3.3 Cellular fatty acids

Dried cells (40 mg) were put into screw-cap tube and added with 1 mL of reagent 1 (Appendix B). This suspension was shaken well before heating at 100 °C for 30 min and then cooling to room temperature in water. The reagent 2 (Appendix B) was added into the suspension and mixed for 5 to 10 sec with vortex mixer. The suspension was heated at 80 °C for 10 min and cooled to room temperature in water. Then, reagent 3 was added (Appendix B) and the suspension was mixed for 10 min and was transferred the upper layer to another tube. The reagent 4 (Appendix B) was added into the suspension and mixed for 5 min, if it became to emulsion form, the reagent 5 was added (Appendix B) into the suspension. The upper layer was transferred to the vial for GC to detect cellular fatty acids (Minnikin *et al*., 1984).

2.3.4 Polar lipids

Three ml of MeOH:0.3%NaCl aq. (100:10) and 3 mL of petroleum ether were added to the dried cells (150-300g). The solution was mixed for 15 min. The lower layer was added with 1 mL of petroleum ether and then the solution was mixed for 2-5 min. The lower layer was heated at 100 °C for 5 min and cooled immediately at 37 °C for 5 min.   The suspension was added with Chloroform:MeOH:water (90:100:30) and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with Chloroform:MeOH:water (50:100:40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 ml of chloroform and water. The final lower layer was dried with N_2 gas (<37 °C). The polar lipid fraction was dissolved with 60 µL of chloroform:MeOH (2:1) and applied to two-dimensional silica HPTLC no. 1.05633 and was developed with the following solvent systems. The first solvent system : chloroform : MeOH : Water (65:25:4). The second solvent system : chloroform : acetic acid : MeOH : Water (40:7.5:6:2). HPTLC was sprayed with iodine until polar lipid appeared. Subsequently, the first plate was sprayed with Ninhydrin reagent (Appendix B) and then heated at 110 °C for 10 min. Dittmer and Lester reagent (Appendix B) was sprayed onto the plate and then blue spots were detected on the plates containing all phospholipids. The second plate was sprayed with Anisaldehyde reagent (Appendix B) and then heated at 110 °C for 10 min after spraying. Green-yellow spots and blue spots were detected on plates containing glycolipids and other lipids, respectively. The third plate was sprayed with Dragendroff's reagent (Appendix B) and then orange spots were detected on plate containing phosphatidyl chroline (Sasser *et al*., 1990; Kämpfer and Kroppenstedt, 1996).

2.3.5 Quinones (Komagata and Suzuki, 1987)

Dried cells (100-500 mg) were extracted with chloroform:MeOH (2:1) overnight. The suspension was then filtered and dried under rotary evaporator. The dried sample was dissolved with a small amount of acetone and applied onto a silica gel TLC (Merck no.1.05744). The applied TLC was then developed by 100% benzene and the band of menaquinone was detected by using a UV lamp (254 nm). The menaquinone band was scraped and dissolved with HPLC acetone. The suspension was filtered and dried up with N_2 gas. The menaquinone sample was analyzed by HPLC.

2.3.6 DNA base composition

Chromosomal DNA was isolated from cells grown in JCM No. 169 and No. 377 broth for 3-14 days according to the method of Saito and Miura 1963 and Tamaoka (1994). Cells were harvested and suspended in 10 ml of saline-EDTA buffer pH 8.0 (Appendix B). The cell suspension was inoculated with 20 mg of lysozyme at 37 °C for 30 min followed by the incubation period of 10 min at 50 °C with 1.0 mL of 10% SDS. The phenol extraction was then carried out by adding an equal volume of phenol:chloroform (1:1) (Appendix B) to the sample for removal of protein and other debris. The upper layer of the mixture was collected after centrifugation at 10,000 rpm for 20 min. Chromosomal DNA was precipitated with two volumes of ice cold absolute ethanol. DNA was dissolved with 0.1x SSC (Appendix B) and treated with RNase A, RNase T_1 and protease K solution at 37 °C for 1 h for removal of RNA and protein, respectively. Chromosomal DNA was stored in 0.1x SSC at 4 °C.

The 10 µL of heated DNA (1 mg/mL) was hydrolyzed with 10 µL nuclease P_1 at 50 °C for 1 h and followed by the incubation period of 1 h at 37 °C with 10 µL of alkaline phosphatase. The hydrolyzed DNA was determined using the HPLC method of Tamaoka and Komagata (1984). An equimolar mixture of nucleotides (Yamasa Shoyu, Choshi, Japan) was used as the quantitative standard for analysis of DNA base composition.

2.4 DNA-DNA hybridization

DNA labeling probe with photobiotin was started by mixing 10 µl of purified DNA solution (1 mg/mL) and 15 µL of photobiotin solution (1 mg/mL) in an Eppendorf tube and then the mixture was irradiated with sunlamp for 30 min on ice. After irradiation, the excess photobiotins were removed by the addition of 100 µl of 0.1 M Tris-HCl buffer pH 9.0, and 100 µl of n-butanol. The upper layer was removed. A 100 µL of n-butanol was added and mixed well and removed the upper layer. The biotinylated DNA solution was boiled for 15 min and immediately cooled in ice. The solution was sonicated for 3 min and dissolved with hybridization solution (Appendix B).

DNA-DNA hybridization solution was performed by Ezaki, Hashimoto, and Yabuuchi (1989). 100 µL of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno™ Plate: MaxiSorp™ surface) and fixed by

incubation at 37 ˚C for 2 h. After incubation, the DNA solution was removed. 200 µL of a prehybridization solution (Appendix B) was added to microdilution wells. The microdilution plate was incubated at hybridization temperature, 41.7˚C (Group I), 42.1˚C (Group II), 50.1˚C (Group III), 41.9˚C (Group IV), 39.2˚C (Group V), 50.5˚C (Group VI), 50.2˚C (Group VII) and 48.9˚C (Group VIII) for 1-2 h. The prehybridization solution was removed from the wells and replaced with 100 µL of a hybridization mixture containing biotinylated DNA. The microdilution plate was incubated at hybridization temperature of each Group for 15-18 h.

After hybridization, the microdilution wells were washed three times with 200 µl of 0.2xSSC buffer. Then 200 µL of solution I (Appendix B) was added to microdilution wells and incubated at 30 ˚C for 10 min. Solution I was removed from the wells and replaced with 100 µL of solution II (Appendix B). The microdilution plate was incubated at 37 ˚C for 30 min. After incubation, the microdilution plate was washed for three times with 200 µL of PBS. 100 µL of solution III (Appendix B) was added, and the plate was incubated at 37 ˚C for 10 min. The enzyme reaction was stopped with 100 µL of 2M H_2SO_4 (Appendix B) (Verlander, 1992). The absorbance was measured at 450 nm with Microplate Reader (Microplate Manager[R] 4.0 Bio-Rad Laboratories, Inc) and calculated for the value of percentage DNA homology (Appendix B) (Tanasupawat *et al*., 2000).

2.5 16S rDNA analysis

2.5.1 16S rDNA amplification by PCR

The PCR was performed in a total volume of 50 µl containing 1 µL of DNA sample, 0.25 µL of *Taq* DNA polymerase, 5 µL of 10xpolymerase buffer, 4 µl of dNTP mixture, 2.5 µL of 10 µM forward and reverse primers (Appendix C) and 34.75 µL of Milliq water. A DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 3 min at 95 ˚C followed by 30 cycles of 30 sec at 95 ˚C (denaturing of DNA), 15 sec at 55 ˚C (primer annealimg), and 1 min at 72 ˚C (polymerization) and a final extension for 5 min at 72 ˚C. The PCR amplified products were analyzed by running 5 µL of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer (Appendix C). Agarose gel was stained in an ethidium bromide solution (0.5 mg/mL) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rDNA band.

2.5.2 16S r DNA sequencing

The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for 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 ˚C followed by 25 cycles of 10 sec at 96 ˚C (denaturing of DNA), 5 sec at 50 ˚C (primer annealimg), and 4 min at 60 ˚C (polymerization). Sequencing for each sample was carried out in both forward and reverse directions (Appendix C).

3.5.3 16S rDNA sequence analysis and phylogenetic tree construction

Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequences at the GenBank/EMBL/DDBJ databases. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods (Kluge and Farris, 1969) in the MEGA program version 2.1 (Kumar *et al.*; 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.   Primary screening of isolates for protease-producing bacteria

Protease producing ability of the cultures was screened qualitatively in casein agar (Appendix A-7) and incubated at 37 ºC for 7-10 days. Colonies with clear zones were selected for the further study.

A loopful of strains that produced clear zone on casein medium (Appendix A-7) was inoculated in 500-mL Erlenmeyer flask containing 100 mL of JCM No. 169 and 377. After 3 days of incubation at 37°C in the rotary shaker operated at 200 rpm, the cell-free supernatant recovered by centrifugation at the speed of 10,000 rpm at 4°C

for 20 min was used for the extracellular protease activity (caseinolytic activity) assayes described by Oda and Murao (1974) and Hiraga *et al*. (2000). Sample (0.5 mL) was incubated with 1.5 mL of 1.33%(w/v) Hammarsten casein in 50 mM Tris-HCl, pH 7.5 buffer containing 10% NaCl for SR5-3 strain and 25%NaCl for HSD2-5 and HIB20-2 at 37º C for 60 min. The enzymatic reaction was stopped by adding 2 mL of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 2,500 rpm for 10 min to remove the precipitate. After that, 0.5 mL of the supernatant was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of the Folin-Ciocalteu reagent. The absorbance of the color developed at 37º C for 20 min was measured at 660 nm. A correlation between an absorbance at 660 nm and tyrosine concentration was constructed for an estimation of tyrosine produced.

One unit of protease was defined as the amount of the enzyme yielding the equivalent of 1 μmol of tyrosine per minute under the defined assay conditions. A blank was run in the same manner except that the enzyme was added after the addition of TCA solution. Total protein in the supernatant was measured by the method as described by Lowry *et al*. (1951). The details of the analytical method is described clearly in Appendix C.

4. Protease production of HDS2-5, HIB20-2 and SR5-3

Production of protease were tested by adding a variety of nitrogen sources and carbon sources such as ami ami, casamino acid, polypeptone, yeast extract, soybean powder, lactose, starch, and sucrose in JCM No. 169 and 377. After glutamic acid fermentation, the byproduct containing 4% of glutamic acid was referred to ami ami. A 150 μL of the inoculum was inoculated in 3 mL of the modified medium in 10X 150 mm test tubes and incubated with shaking (200 rpm) at 30ºC for 2 days. Samples were obtained periodically and their protease activities were assayed using casein as substrate in the presence of 10% NaCl.

5. Purification and characterization of SR5-3 protease

5.1 Characterization of crude SR5-3 protease

5.1.1 Effect of protease inhibitors on crude protease activity

The effects of various protease inhibitors on protease activity were examined. After preincubation of the crude enzyme with inhibitors (PMSF, EDTA,

EGTA, TLCK, TPCK, E-64, leupeptin, chymostatin and MAPI) at 37 °C for 60 min. The residual activity was assayed using casein as substrate. Protease activity (caseinolytic activity) assay was determined by the method described by Oda and Murao (1974) and Hiraga *et al*. (2000).

5.1.2 Effect of NaCl concentration on crude protease activity

Protease activity of crude enzyme was measured at various concentrations of NaCl (0 to 30% (w/v)) using casein or MCA-peptides as substrate at pH 7.5.

Assay of protease activity by using synthetic substrate (Suc-Ala-Ala-Pro-Phe-MCA) were determined as described by Capiralla *et al*. (2002). The protease activity was assayed in the presence of optimal NaCl concentration of 20% NaCl. Fifty μL of diluted enzyme and 440 μL of 25 mM Tris-HCl buffer containing 20% NaCl, pH 7.5 were preincubated at 37 °C for 30 min. After preincubation, the reaction was started by adding 10 μl of 98 μM of MCA-peptide substrate and incubated at 37 °C for 20 min. The enzyme reaction was terminated by the addition of 2 ml of 15% (v/v) acetic acid. The fluorescence intensity was measured at λex = 360 nm and λem = 460 nm. One relative fluorescence unit of enzyme activity was defined as the enzyme quantity that liberates 1 μmole of AMC per ml of the reaction mixture per minute. Protein concentration was estimated by BCA protein assay (Smith *et al*., 1985) with crystalline bovine serum albumin (Sigma Chemical) as a standard.

5.2 Purification of SR5-3 protease

The selected isolate, SR5-3, was cultivated in modified JCM No. 377 medium, which contained 1% (w/v) yeast extract, 0.32% (w/v) aspartic acid, 0.32% (w/v) glycine and 2% (v/v) ami ami per liter, pH 7.2 at 30 °C. Culture broth after 48 h cultivation was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was used for the following enzyme. Solid ammonium sulfate was added into 2700 ml of the supernatant to make an 80% saturated final concentration (561.1 g/l) and kept overnight at 4 °C. The precipitate was collected by centrifugation (8000 rpm for 20 min at 4 °C). Two hundred ml of 50 mM Tris-HCl buffer, pH 8.0 (buffer A) was added to dissolve the precipitate for loading onto Bacitracin-Sepharose column.

Bacitracin-Sepharose column was prepared as follows: 60 mg/ml of bacitracin in 0.1 M NaHCO$_3$ containing 0.5 M NaCl, pH 8.5 was mixed with the NHS-activated Sepharose 4 Fast Flow matrix with 1:1 (v/v). After coupling, the column was blocked

with glycine solution.   The Bacitracin-Sepharose 4 Fast Flow was packed in the column (2 x 20 cm) with a bed volume of 50 ml.   The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.0 containing 4 M NaCl and 1 mM $CaCl_2$ (buffer B). The sample diluted with buffer B was loaded onto the column, and then the column was washed with buffer B.   The proteases were eluted by stepwise method with 150 ml of buffer B containing 15% (v/v) and then buffer B containing 15%(v/v) ethanol were changed to 150 ml of buffer B containing 20% (v/v) ethanol.   Three ml of fractions were collected at a flow rate of 1 ml/min. Active fractions were pooled and concentrated by ultrafiltration (pore size = 3000 kDa). The purity of the purified enzyme was analyzed by 12.5% SDS-PAGE and 16% native-PAGE (Laemmli, 1970). Purified enzyme were directly loaded onto Native polyacrylamide gel.   Native gel were run at 120 volt for 12 h at 4 °C. The purified enzyme was also analyzed by SDS-PAGE after heating the samples at 100 °C for 1 min. Following native and SDS-PAGE, the proteins were stained with Commassie blue (0.1%). Protease activity was detected by zymogram as previously reported (Sanchez-Porro *et al*., 2003).   In the case of zymogram, casein were added in SDS-PAGE solution.   After preparation the gels, purified enzyme were directly loaded onto the gels. Following the SDS-PAGE, the gels were rinsed in 2.5% Triton X-100 in order to remove SDS for 30 min and they were incubated in 25 mM Tris-HCl, pH 7.5.   After incubation for 1 h at 37 °C, the gels were stained with amido black.

5.3 Analysis of molecular mass and the amino-terminal sequence

A 12.5% polyacrylamide gel was used in the determination of the purity of the purified protease. Protein bands were stained with Coomassie brilliant blue R-250. The molecular mass of the purified protease was determined by comparison of its electrophoretic mobility with that of marker proteins. The amino-terminal amino acid sequence of the purified protease was identified by the method of Matsudaira (1987) using an Applied Biosystems Model 476A protein sequencing system.

5.4 Characteristics of the purified SR5-3 protease

Optimum pH: Protease activity of the purified enzyme was measured at various pH values under standard assay condition using MCA-peptide as a substrate at 37 °C for 20 min.

Optimum temperature: Protease activity of the purified enzyme was assayed at different temperatures (30 to 70 °C) at pH 7.5 for 20 min.

pH stability: the pH of the protease solution was adjusted in the range of pH 3.0 to 10.0 using 25 mM citrate/NaOH, KH_2PO_4/NaOH, and H_3BO_3/ NaOH buffers containing 20% (w/v) NaCl. The enzyme was incubated at 37 °C for 3 h, and the residual protease activity was assayed at pH 7.5 for 20 min.

Thermal stability: the purified protease was incubated at various temperatures at pH 7.5 for 2h, and the remaining activity was measured at pH 7.5 for 20 min at 37 °C.

Effect of NaCl on activity: protease activity of the purified enzyme was measured at various concentrations of NaCl (0 to 30% (w/v)) using casein (final conc. = 1.0% (w/v)) or Suc-Ala-Ala-Pro-Phe-MCA (final conc. = 2 μM) as a substrate at pH 7.5.

Effect of NaCl on stability: the purified enzyme was incubated at various concentrations of NaCl at 37 °C and pH 7.5 for 24 h. In the case of Suc-Ala-Ala-Pro-Phe-MCA as a substrate, the purified enzyme was incubated at various concentrations of NaCl at 37 °C and pH 7.5 for 96 h. After the incubation, the NaCl concentration was adjusted to 10% (w/v) for casein as substrate and 20% for MCA-peptide substrate, respectively, and the remaining activity was assayed.

Effects of protease inhibitors on activity: the effects of various protease inhibitors on protease activity were examined. After preincubation of the purified enzyme with inhibitors (PMSF, EDTA, EGTA, TLCK, TPCK, E-64, leupeptin, chymostatin and MAPI) at 37 °C for 30 min, the residual activity was assayed using MCA-peptide substrate.

5.5 Analysis of substrate specificity using FRETS-25Xaa-libraries

FRETS (fluorescence resonance energy transfer substrate) combinatorial libraries (Tanskul *et al*. 2003) were used for the analysis of substrate specificity. FRETS-25Xaa contains a highly fluorescent 2-(*N*-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal D-2,3-diamino propionic acid (D-A_2pr) residue, which is efficiently quenched by a 2, 4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys as shown below.

$$P_3 \quad P_2 \quad P_1$$

D-A_2pr(Nma)-Gly-Zaa-Yaa-Xaa-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg

Xaa represents the fixed position of the 19 natural amino acids excluding Cys. A mixture of 5 amino acid residues (P, Y, K, I and D) was incorporated at the Yaa position along with a mixture of 5 amino acid residues (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combinations of each Xaa series, resulting in a combinatorial library with a total of 475 peptide substrates in 19 separate pools.

The preference at the P_1 position was determined at 37 °C for 5 min in 50 mM H_3BO_3/NaOH buffer, pH 7.5 (Tanskul *et al*., 2003). The fluorescence intensity at λex 360 nm and λem 465 nm were measured. Then the P_1 position was fixed with the favored amino acid residue and subjected for determination of preference at the P_2 and the P_3 position by LC-MS analysis (Tanskul *et al*., 2003).

CHAPTER IV

RESULTS AND DISCUSSIONS

1. **Bacterial isolates and sources of isolation**

    A total of 40 halophilic isolates were isolated from fish sauce samples collected from the factories in Samutprakarn, Samutsongkram, Rayong, and Singbury; and from bu-du samples collected in Patany (Table 10). The pH of the fish sauce samples ranges from 5.5-6.0.

2. **Identification of isolates**

    On the basis of phenotypic characteristics (Table 11), forty halophilic isolates could be classified into eight groups. The rods were found in the isolates of Group I to V. Their colonies were cream-white for Group I and II, cream-yellow for Group III and Group IV isolates while white pigmented was in Group V. All could grow in the wide range of NaCl (0.5 to 25%) and were refered as the moderately halophilic bacteria (Kushner, 1978). The red-pigmented isolates of Group VI, VII and VIII grew optimally in the presence of the high salt concentration (20-30%) but did not grow in the medium contained NaCl less than 15% and were refered as the extremely halophilic bacteria or halophilic archaea (Grant and Larsen, 1991).

    Group VI isolates were rod-shaped and red colonies that were translucence and lysed in distilled water. The remaining red pigmented isolates were cocci and appeared as single cells or diplococci but their cells did not lyze within 1-2 h when suspended in distilled water (Group VII-VIII). Group I, II, V isolates had spherical/oval, oval and spherical endospores (Figure 8D and 17B) respectively, observed at terminal positions in the swollen sporangia. Group IV had oval endospores at subterminal positions (Figure 14B). Non spore forming bacteria were observed in Group III and VI- VIII. Group I, IV and V isolates were motile but the remaining groups were non motile. The cultural characteristics of all isolates were summarized in Table 12.

Table 10. Factory, fermentation times, isolate no. and number of isolate.

Factory	Fermentation times (days)	Isolate no.	Number of isolate
1. Sindhu Samuth Fish sauce Factory (Squid Brand) LTD., Samutprakarn	0	I0-1	1
	10	HIS10-2, HIS10-4, HIS10-5	3
	20	HIB20-2	1
	30-35	HIS30-1 KS35-3	2
	40	IS40-2, IS40-3	2
	50	HIS50-1(1)	1
	60	HIB60-1	1
	87	KS87-3, KS87-5	2
	270	PS9-2	1
	333	KS11-1 HKS333-2, KS333-3B	3
	360	PB12	1

Table 10 (continued)

Factory	Fermentation times (days)	Isolate no.	Number of isolate
2. Thai fish sauce (Squid Brand) Co., LTD., Samutsongkram	30	HDB1-1, HDB1-4, HDB1-11, HDB1-31	4
	60	HDS2-5	1
	150	HDB5-2	1
	180	HDS6-1A, HDB6-2, HDB6-6	3
	210	HDS7-4	1
	240	HDB8-5, HDB8-2	2
	300	HDB10-5, HDB10-5R, HDS10-5	3
	780	DS26-2	1
3. Rayong Fish Sauce Industry Co., Ltd., Rayong	60	RF2-5	1
	180	HRF6	1
4. Rungarun Fish Sauce Industry Co., Ltd., Singbury	150	SR5-3	1
5. Budu from Patany		BN2-2	1
Total			40

**Table 11**. Differential phenotypic characteristics of isolates.

Group	Isolate no.	Pigment/ shape	Spore	NaCl (%)		Hydrolysis of		
				10	25	Skim milk	Starch	Tyrosine
I (3 isolates)	BN2-2 DB8-4 KS333-2B	Cream/ rods	Spherical or Oval/ terminal	+	+	-	-	-
II (5 isolates)	IS40-3 IS40-2 IS10-5 PS9-2 IO-1	Cream/ rods	Oval/ terminal	+	+	+	-	+
III (4 isolates)	DS26-2 KS11-1 KS87-5 PB12	Cream/ rods	-	+	+	-	+	-
IV (1 isolate)	SR5-3	Cream yellow/ rods	Oval/ subterminal	+	+	+++	-	ND
V (1 isolate)	RF2-5	White/ rods	Spherical / terminal	+	+	++	-	ND
VI (10 isolates)	HIS1-4 HIS10-5 HIB20-2 HIS50-1(2) HIB60-1 HDB1-1 HDB1-4 HDB1-31 HDS2-5 HDB1-5R	Red and translucence/ rods	-	-	+	++	ND	ND

Table 11 (Continued)

Group	Isolate no.	Pigment/ shape	Spore	NaCl (%)		Hydrolysis of		
				10	25	Skim milk	Starch	Tyrosine
VI I (1 isolate)	HRF6	Red/ Cocci	-	-	+	+	ND	ND
VIII (15 isolates)	HIS10-2	Red/ Cocci	-	-	+	+	ND	ND
	HKS35-3							
	HKS333-2							
	HDB1-4							
	HDS4-1							
	HDB5-2							
	HDS6-1A							
	HDB6-2							
	HDS6-6							
	HDS7-4							
	HDB8-2							
	HDB8-5							
	HDB10-5							
	HDS10-5							
	HKS87-3							

2.1 Group I and II isolates

Group I contained three isolates (BN2-2, DB8-4 and KS333-3B). Group II comprised of five isolates, including IS40-3, IS40-2, IS10-5, IO-1 and PS9-2. They were spore-forming rods and white-cream colonies as shown in Figure 8. The cells of the isolates were rod-shaped, measuring approximately 0.4–0.5x 2–6 mm for Group I and 0.4–0.5 x 1.5–6 mm for Group II. The Group I isolates and *L. salicampi* JCM 11462[T] occurred singly, in pairs or in short chains, whereas the Group II isolates tended to form crooked chains comprising several cells. Group II isolates hydrolyzed tyrosine, but not for Group I isolates. Other morphological, cultural, physiological and biochemical properties were shown in Table 12.

(A)   (B)

(C)   (D)
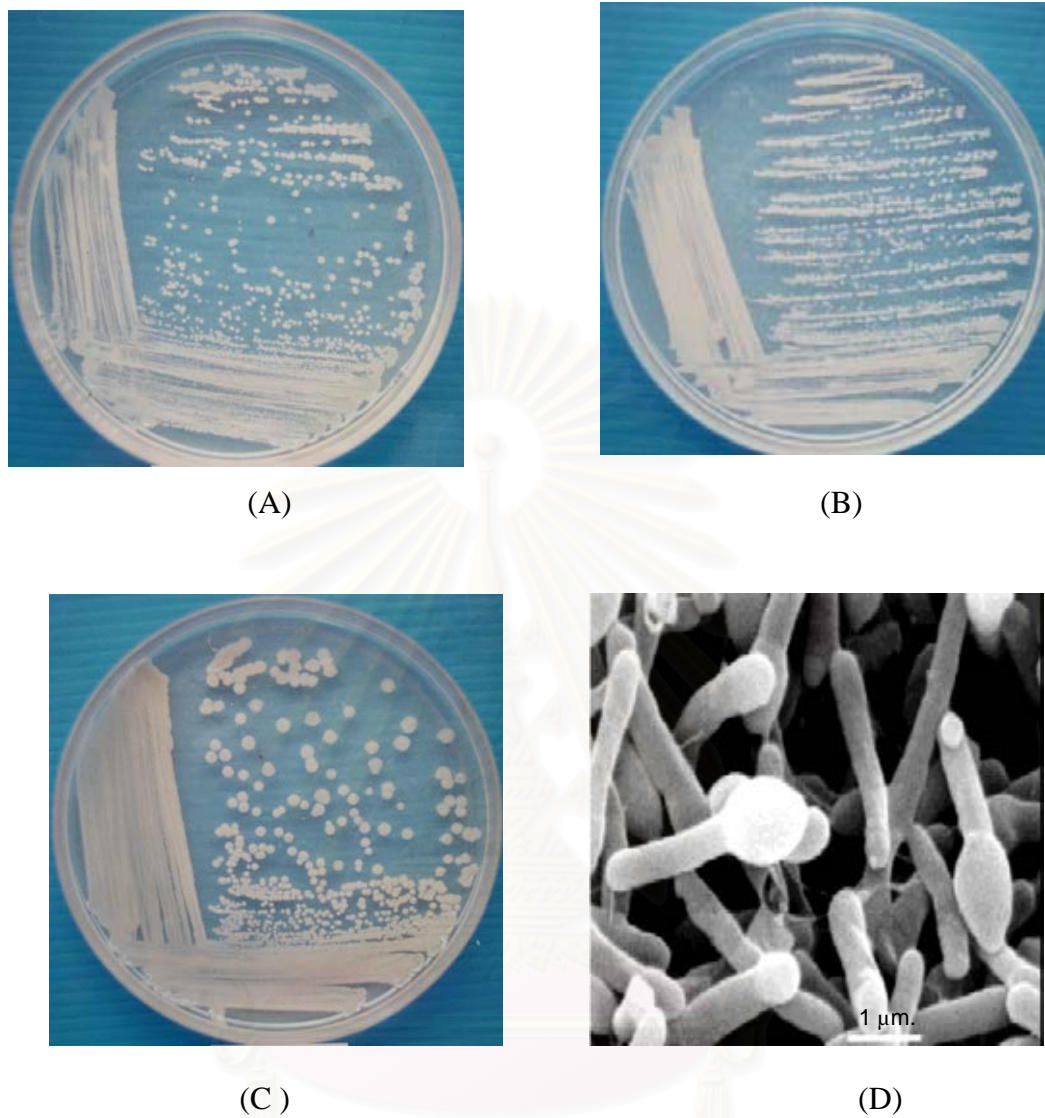
Figure 8. The colonial appearance of Group I (KS333-2B), *Lentibacillus*
               salicampi JCM 11462[T] and Group II (IS40-3) grown on JCM No. 377 at
               37 ºC for 5 days. A, KS333-2B; B, *L. salicampi* JCM 11462[T] and C,
               IS40-3   and   D, scanning   electron   micrograph   of   IS40-3.

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Table 12. Differential characteristics of Group I, II and *Lentibacillus salicampi* JCM 11462[T].

Characteristics	Group I (3 isolates)	Group II (5 isolates)	*L. salicampi* JCM 11462[T]
Spore shape	Spherical or oval	Spherical or oval	Spherical or oval
Spore position	Terminal	Terminal	Terminal
NaCl range (%)	3-25	3-30	3-25
Maximum temperature for growth (°C)	40	45	40
Urease	+	+	+
Nitrate reduction	+	+	+
H$_2$S formation	-	-	-
methyl red reaction	-	-	-
Acid production from			
Cellobiose	+	-	+
D-Fructose	+	-	+
D-Glucose	+	+	+
Glycerol	+	+	+
D- Mannose	+	-	+
D-Ribose	+	+	+
Salicin	+	-	+
Sucrose	-	+	-
D-Xylose	+	+	+
Hydrolysis			
Arginine	-	-	-
Casein	+	+	+
Gelatin	+	+	+
Tyrosine	-	+	-
Phenylalanine	-	-	-

+, positive; - negative

Group I and Group II isolates including *L. salicampi* JCM 11462[T] contained *meso*-diaminopimelic acid (*meso*-DAP) as a diagnostic diamino acid as well as glutamic acid and alanine in the cell wall composed of peptidoglycan. The predominant isoprenoid quinone found was menaquinone-7 (Table 34). The polar lipids analysis revealed the presence of phosphoglycerol (PG), diphosphatidylglycerol (DPG) and an unidentified glycolipid (Figure 9 and Table 32). The cellular fatty acid profile tested for all isolates contained following components (%): anteiso-$C_{15:0}$ (38-54%), iso-$C_{16:O}$ (13-22%), anteiso-$C_{17:0}$ (13-18%), iso-$C_{15:0}$ (3-18%) and iso-$C_{14:0}$ (5-13%) as shown in Table 35. The G+C content of DNAs of Group I and Group II were 42.2 to 42.5 and 43.2 to 43.5 mol% G+C, respectively, whereas that of *L. salicampi* JCM 11462[T] was 42.4. The chemotaxonomic profiles of Group I and II isolates were agreed with that of the genus *Lentibacillus* (Yoon *et al*., 2002).

Almost complete 16S rDNA sequence of representative of Group II, IS40-3 determined in this study contained 1524 nucleotide positions (Figure 36). Eliminating ambiguous and unalignable bases, 1222 positions were compared in the phylogenetic revealed that the representative isolate of Group II, IS40-3 was included in a cluster containing aerobic, spore-forming halophilic bacteria represented by the genera *Halobacillus*, *Gracilibacillus*, *Lentibacillus*, and *Virgibacillus*, as shown in Figure. 10. The close relationship between isolate IS40-3 and *L. salicampi* JCM 11462[T] was supported by high bootstrap values (≥96%), and high 16S rDNA sequence similarity (95.2 %) according to Stackebrandt and Goebel (1994) and Stackebrandt *et al*. (2002). Isolate IS40-3 showed levels of 16S rDNA similarities (%) of 93.0-95.3, 93.3-94.1, 93.5-94.1, 92.2 to members of the genera *Virgibacillus*, *Gracilibacillus*, *Halobacillus* and *Bacillus halophilus*, respectively (Table 14). As shown in Table 13, *L. salicampi* JCM 11462[T] showed high DNA-DNA relatedness to the Group I isolates (96-102 %). Also, high DNA-DNA relatedness values were detected between the Group II isolates (85-118 %), however, those values between the Group II isolates and the Group I isolates (or *L. salicampi* JCM 11462[T]) were low (<32 %). Furthermore, the high DNA-DNA relatedness with *L. salicampi* JCM 11462[T] (>70%) and several differentiated properties (Table 12) warranted the Group I isolates being included in *L. salicampi* JCM 11462[T] (Wyne *et al*., 1987). The DNA-DNA hybridization study supported that the Group II isolates were separated from *L. salicampi* JCM 11462[T]. Therefore, Group II was unrelated to *L. salicampi* JCM 11462[T], and it should be

classified as a new species of the genus *Lentibacillus* and a name *Lentibacillus juripiscarius* sp. nov. is proposed for this group.
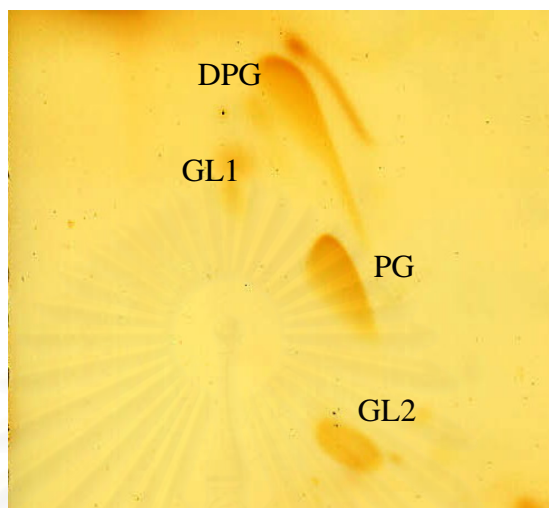
Figure 9. Thin-layer chromatogram of the total polar lipids of IS40-3
(Group II). DPG, diphosphoglycerol; PG, phosphoglycerol;
GL, glycolipids.

Table 13. DNA G+C content and DNA-DNA Similarity among Group I, Group II
and *L. salicampi* JCM 11462[T].

Isolate no.	G+C content	% Similarity with labelled isolates		
	(mol%)	JCM 11462[T]	IS40-3[T]	PS9-2
Group I				
1. BN2-2	42.5	96	1	12
2. DB8-4	42.3	71	1	19
3. KS333-3B	42.5	102	14	8
Group II				
1. IS40-3	43.4	12	100	113
2. IS40-2	43.5	32	98	111
3. IS10-5	43.3	27	96	118
4. IO-1	43.2	13	82	85
5. PS9-2	43.3	14	79	100
L. salicampi JCM 11462[T]	42.4	100	13	11
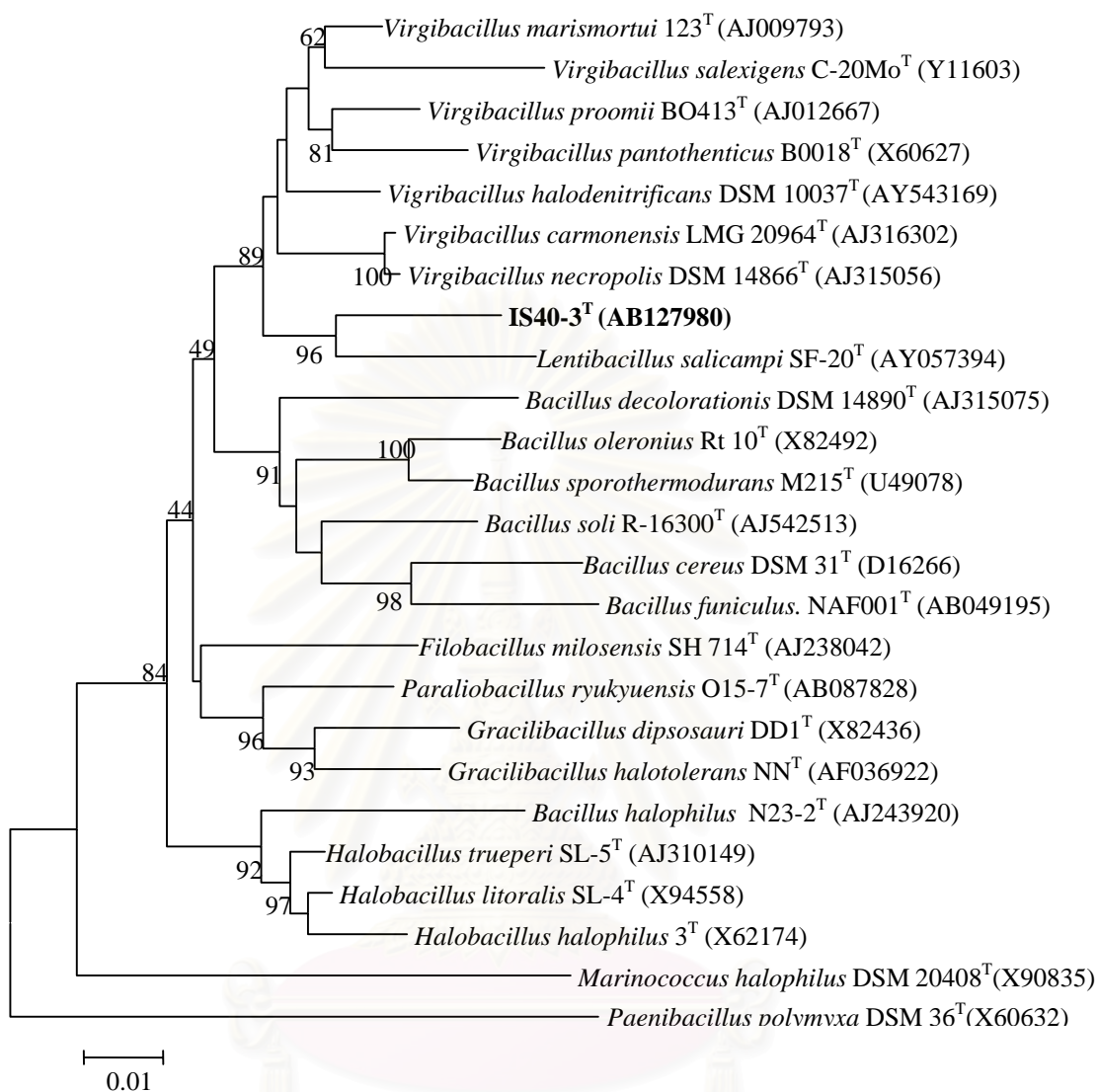
Figure. 10. Neighbour-joining-tree showing the phylogenetic position of

IS40-3 and related taxa based on 16S rRNA sequences. Bar, 0.01

substitutions per nucleotide position. Bootstrap values expressed as

percentages of 1000 replication.

Table 14. Percentage similarities of IS40-3 (Group II) and related taxa.

Accession no.	% Similarity																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. X94558	100																								
2. X62174	98.4	100																							
3. AJ310149	99.2	97.9	100																						
4. AJ243920	95.6	95.1	95.9	100																					
5. X90835	90.7	90.4	91.1	90.4	100																				
6. AJ238042	94.7	93.8	94.9	92.8	90.8	100																			
7. X82436	94.5	93.4	94.9	92.4	88.9	94.5	100																		
8. AF036922	94.5	93.6	95.1	92.8	89.2	94.4	96.5	100																	
9. AB087828	95.3	94.5	95.3	93.3	89.5	94.7	96.2	95.7	100																
10. D16266	92.4	91.6	92.4	89.6	88.4	92.4	91.6	91.6	92.4	100															
11. AB049195	92.7	92.1	92.8	90.4	89	92.5	91.9	91.6	92.4	95.4	100														
12. AJ542513	93.9	93.1	94	91.4	89.2	93.4	92.7	92.5	93.6	94.4	95.1	100													
13. AJ315075	94.1	93	94.3	92.4	89.6	93.6	92	92.7	92.6	93.1	93.2	95.2	100												
14. X82492	94	92.8	93.7	90.9	88.7	93.2	93.8	93.6	94.1	94.4	93.4	94.8	94.3	100											
15. U49078	94.5	93.3	94.1	91.1	89.1	94.2	93.2	92.8	93.5	94.9	94.4	95.7	94.7	98.1	100										
16. AB127980 (IS40-3)	94.2	93.4	94.1	92.3	90.4	92.9	93.6	94.3	94.5	92	92.1	93.2	92.7	94.4	93.9	100									
17. AY057394	93.4	92.5	93.3	91.2	89.1	92.8	93.1	93.2	93.7	91.6	90.4	92.1	91.9	92.5	92.5	95.4	100								
18. AJ009793	95.9	94.5	95.9	92.9	90.4	94.6	94.1	94.3	95.1	93.8	93.2	95.3	94.6	94.2	94.9	95.3	95.1	100							
19. Y11603	93.6	92.5	93.6	90.8	88.3	93.5	92.6	92.8	93.2	92.2	90.9	93.2	92.2	92.4	93.2	93.1	93.3	96.5	100						
20. AJ012667	95.1	94.3	95.1	92.3	90.1	95	94.5	94.5	95.1	93.1	92.4	94.8	93.9	94	94.7	95.4	94.8	97.7	95.7	100					
21. AY543169	95.4	94.4	95.4	92.7	90.4	94.6	94.4	94.3	94.9	94.1	93.3	95.2	94.8	94.8	94.9	95.3	94.9	97.9	95.7	96.6	100				
22. AJ316302	95.2	94.3	95.5	92.7	90	94.4	94	94.5	95.1	93.6	92.9	94.4	93.9	94.2	94.8	95.6	95	97.2	95.5	96.8	97.3	100			
23. AJ315056	95.3	94.2	95.5	92.9	90	94.3	94.1	94.5	94.9	93.2	92.7	94.2	93.6	93.9	94.6	95.6	95.3	96.8	95.2	96.8	97	99.5	100		
24. X60627	94.8	94	94.6	91.7	89.9	94.3	93.6	93.8	94.8	93.2	92.8	94.5	93.6	93.4	94.2	94.7	94.7	97.2	94.9	97.2	96.5	95.7	95.7	100	
25. X60632	88.4	88.2	88.1	86.8	86.7	88.2	86.8	88.3	88.8	87.6	88.1	88.3	88.3	88.2	87.8	88.4	87.8	88.2	86.7	88.6	87.8	89.1	88.8	88	100

2.1.1 Characteristics of *Lentibacillus salicampi* isolates (Group I)

The three spore-forming, aerobic, gram-positive, moderately halophilic rods and *L. salicampi* JCM 11462^T occurred singly, in pair or short chains. They were motile and facultatively anaerobic, spore-forming, gram-positive rods. They grew in a range from 3 to 25% NaCl and optimally in the presence of 10% NaCl. Group I isolates and *L. salicampi* JCM 11462^T grew in the temperature range between 10 and 40 °C. They produced acid form Cellobiose, D-fructose, D-glucose, glycerol, D-mannose, D-ribose, Salicin and D-xylose, but not sucrose. They were positive for hydrolysis of casein, gelatin, Tween 80 and negative for tyrosine, arginine, hypoxanthine, phenylalanine and starch. The minimum pH for growth was 5. Group I isolates contained *meso*-DAP as a diagnostic diamino acid as well as glutamic acid and alanine in the cell wall peptidoglycan. The predominant isoprenoid quinone found was menaquinone-7 (MK-7). The polar lipids analysis revealed the presence of phosphoglycerol (PG), diphosphatidylglycerol (DPG) and an unidentified glycolipid. The cellular fatty acid profile tested for all isolates contained following components (%): anteiso-$C_{15:0}$ (38-54%), iso-$C_{16:O}$ (13-22%), anteiso-$C_{17:0}$ (13-18%), iso-$C_{15:0}$ (3-18%) and iso-$C_{14:0}$ (5-13%). The G+C content of DNAs of Group I was 42.2 to 42.5 mol%G+C.

2.1.2 Characteristics of *Lentibacillus juripiscarius* sp. nov. isolates (Group II)

Group II isolates were spore-forming, aerobic, gram-positive and rods. They formed crooked chains of several cells. They were non-motile and no flagella attached to the cells. Growth occurred in the media containing 3 to 30% NaCl. All isolates grew best at 10% NaCl. They were positive for oxidase, catalase, urease, nitrate reduction and hydrolysis of casein, gelatin, tyrosine and Tween 80. Methyl red, Voges-Proskauer, H_2S formation, hydrolyzed arginine, hypoxanthine, starch, tributyrin and xanthine were negative. They produced acid form, D-glucose, glycerol, D-ribose, sucrose and D-xylose, but not Cellobiose, D-fructose, D-mannose and salicin. The maximum temperature for growth was 45 ^{o}C. The minimum pH for growth was 5. Group II isolates contained *meso*-DAP as a diagnostic diamino acid as well as glutamic acid and alanine in the cell wall peptidoglycan. The predominant isoprenoid quinone found was menaquinone-7 (MK-7). The polar lipids analysis revealed the presence of phosphoglycerol (PG), diphosphatidylglycerol (DPG) and an

unidentified glycolipid. The cellular fatty acid profile tested for all isolates contained following components (%): anteiso-$C_{15:0}$ (38-54%), iso-$C_{16:O}$ (13-22%), anteiso-$C_{17:0}$ (13-18%), iso-$C_{15:0}$ (3-18%) and iso-$C_{14:0}$ (5-13%). The G+C contents of DNAs of Group II were 43.2 to 43.5 mol%G+C.

2.2 Group III isolates

Group III constained four isolates. They were DS26-2, KS87-5, PB12 and KS11-1. They were non spore forming, rods and cream in colour as shown in Figures 11. Group III isolates and *Chromohalobacter salexigens* KTCC 12941^T grew over a large range of salinity between 0.9 and 25% (w/v), with optimal growth at 10%. Optimal NaCl concentration was 10% NaCl and maximum temperature for growth was 45°C. Hydrolysis of starch was positive but gelatin, casein, Tween 80 and tyrosine were negative. The phenotypic characteristics and other properties of Group III isolates and *C. salexigens* KTCC 12941^T were summarized in Table 15.

Table 15. Phenotypic characteristics of Group III and *Chromohalobacter salexigens* KTCC 12941^T.

Characteristics	DS26-2	KS87-5	KS11-1	PB12	KTCC 12941^T
Cell shape	Rods	Rods	Rods	Rods	Rods
Temperature range (^{o}C)	10-45	10-45	10-45	10-45	10-45
NaCl range (%)	3-25	3-25	3-25	3-25	0.9-25
pH range	6-9	6-9	6-9	6-9	5-10
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Acid production from					
L-Arabinose	+	+	+	+	+
D-Cellobiose	-	w	-	w	w
D-Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Glycerol	-	-	-	-	-

Table 15 (continued).

Characteristics	DS26-2	KS87-5	KS11-1	PB12	12941[T]
Acid production from					
Inulin	-	-	-	-	-
myo-Inositol	-	-	-	-	-
Lactose	-	-	-	-	-
Maltose	-	-	-	-	-
Mannitol	-	-	-	-	-
Mannose	-	-	-	-	w
Melibiose	-	-	-	-	-
Melizitose	-	-	-	-	-
Raffinose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Ribose	+	+	+	+	+
Salicin	-	-	-	-	-
Sucrose	-	-	-	-	-
Sorbitol	-	-	-	-	-
Trehalose	-	-	-	-	-
Xylose	+	+	+	+	+
Urease	+	+	+	+	-
Hydrolysis of					
Tyrosine	-	-	-	-	-
Gelatin	-	-	-	-	-
Casein	-	-	-	-	-
Starch	+	+	++	+	-
Dnase	-	-	-	-	-
Indole	-	-	-	-	-

+, positive; - negative

(A)
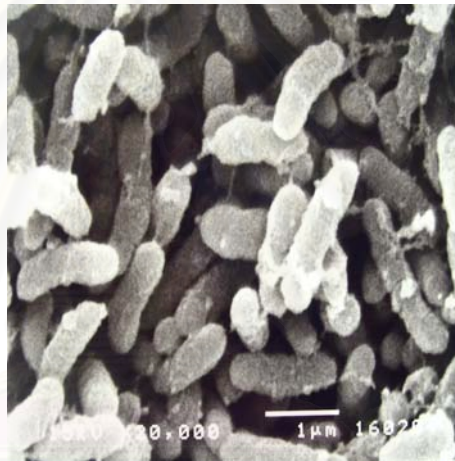
(B)

Figure 11. The colonial appearance (A) and scanning electron micrograph (B) of
DS26-2 grown on JCM No. 377 at 37 ℃ for 5 days.

The Group III isolates contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant respiratory menaquinones found in the representative isolates, DS26-2 and KS87-5 had ubiquinone with nine isoprene units (Q-9) as shown in Table 34. The major fatty acids were Cyclo C19:0 ω8c and C16:0 as shown Table 35. The cellular polar lipids found in at least four isolates were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidyl ethanolamine (PE) and unidentified glycolipids (Figure 12 and Table 32). The DNA G-C content of isolates, determined by HPLC, were 63.1 – 63.5 mol%. The chemotaxonomic profiles of Group III isolates were agreed with that of the genus *Chromohalobacter* (Aral *et al*., 2001a; Aral *et al*., 2001b).

Almost complete 16S rDNA sequence of representative of Group III, DS26-2 determined in this study contained 1458 nucleotide positions (Figure 37). Eliminating ambiguous and unalignable bases 1240 positions were compared in the phylogenetic showed that DS26-2 falls within the radiation of the cluster comprising the members of the family *Halomonadaceae* and the genus *Chromohalobacter* (Figure. 13). From the result of 16S rDNA sequence analysis, isolates DS26-2 was closely related to *C. salexigens* KTCC 12941^T. Isolate DS26-2 exhibited sequence similarity values of 99.3% and 99.5% with *C. salexigens* KTCC 12941^T and C. *israelensis* ATCC 43985^T, respectively (Table 17). A DNA–DNA hybridization experiment revealed that the Group III isolates were closely related among themselves (100-102.9% DNA similarity), suggesting that they belong to the same species, but they exhibited relatively high levels of hybridization with *C. salexigens* KTCC 12941^T (97.4-106.1%) (Table 16). In addition, their phenotypic characteristics were similar with *C. salexigens* KTCC 12941^T (Table 15). Based on high DNA-DNA similarity and phenotypic properties indicated strongly that the Group III isolates were *C. salexigens* KTCC 12941^T.
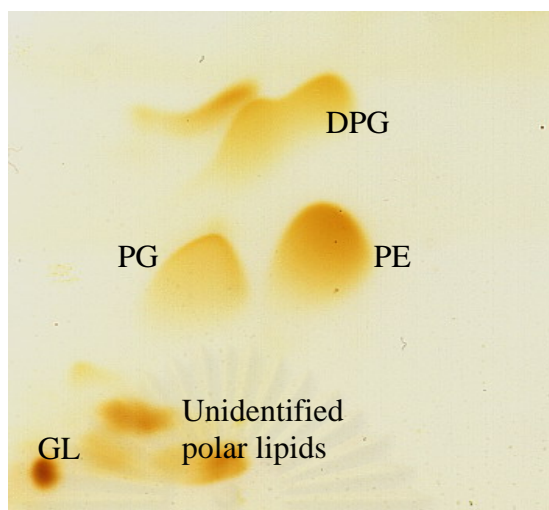
Figure 12. Thin-layer chromatogram of the total polar lipids of DS26-2
(Group III).

Table 16. DNA G+C content and DNA-DNA Similarity among Group III and
Chromohalobacter salexigens KTCC 12941[T].

Isolate no.	G+C content	% Similarity with labeled isolates	
Group III	(mol%)	KTCC 12941[T]	DS26-2
1. DS26-2	63.2	97.4	100.0
2. KS11-1	63.4	106.1	102.9
3. KS87-5	63.5	102.3	101.1
4. PB12	63.1	105.6	100.6
5. *C. salexigens* KTCC 12941[T]	65.0	100.0	85.6
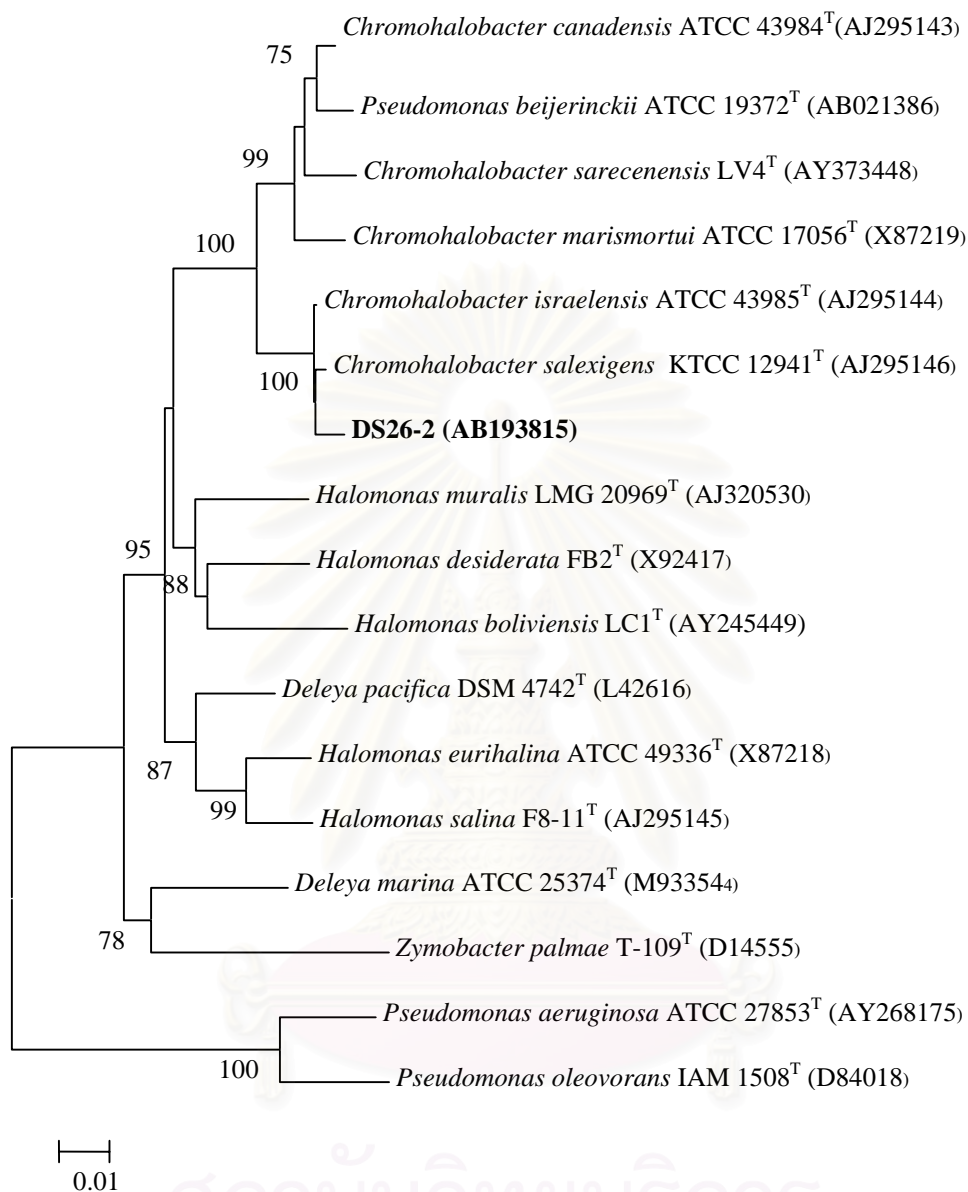
Figure 13. Neighbour-joining-tree showing the phylogenetic position of DS26-2 and related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

Table 17. Percentage similarities of DS26-2 (Group III) and related taxa.

Accession no.	% Similarity																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. X87218	100																
2. AJ295145	97.4	100															
3. L42616	96.1	96.4	100														
4. AJ295144	94.3	93.9	94.4	100													
5. AJ295146	94.3	93.8	94.4	99.8	100												
6. AB193815 (DS26-2)	94	93.5	94	99.3	99.2	100											
7. AY373448	94.6	94.2	93.8	97.1	96.9	96.7	100										
8. AJ295143	94.7	94.4	94.4	97.2	97	96.6	98.5	100									
9. AB021386	94.1	93.8	93.9	97.1	96.9	96.4	97.9	98.9	100								
10. X87219	94.1	93.6	94.4	96.9	96.8	96.5	97.3	98.5	98.1	100							
11. X92417	94.6	94.4	95.2	94.4	94.2	94.1	93.7	94	93.7	94.6	100						
12. AY245449	93.8	94.3	94.4	93.5	93.4	93.1	93	93.7	93.7	93.4	95.4	100					
13. AJ320530	93.8	93.4	95.4	95.1	94.9	94.6	93.9	94.1	93.8	94.5	95.7	94.7	100				
14. M93354	93.3	93.7	94.7	93.4	93.1	92.7	92.3	93.1	92.7	92.3	93.2	93.4	93.4	100			
15. D14555	90.9	91.3	92.6	91.7	91.4	91.1	90.5	90.8	90.8	90.9	92	90.3	91.6	93	100		
16. AY268175	87.7	88.1	89.2	88.2	88.2	87.8	87.2	87.2	87	87.4	87.9	87	88.2	87.6	87.2	100	
17. D84018	87.1	87.1	88.9	88	87.8	87.3	86.9	87.2	86.9	87.2	87.7	87.2	88.2	87.9	87.2	96.1	100

2.2.1 Characteristics of *Chromohalobacter salexigens* isolates (Group III)

Group III isolates grew over a large range of salinity between 3 and 25% (w/v), with optimal growth at 10%. At the optimal NaCl concentration, the highest temperature for growth was 45 °C. The growth was possible between pH 5.0 and pH 9.0. Catalase, oxidase, urease, and nitrate reduction were positive. Hydrolysis of arginine and starch were positive, but not gelatin, casein, Tween 80 and tyrosine. They produced acid from arabinose, glucose, galactose, fructose, ribose and xylose, but not amygdalin, cellobiose, glycerol, inulin, myo-inositol, lactose, maltose, mannitol, mannose, melibiose, melizitose, raffinose, rhamnose, salicin, sucrose, sorbitol and trehalose. The Group III isolates contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant respiratory menaquinones found in DS26-2 and KS87-5 was ubiquinone with nine isoprene units (Q-9). The major fatty acids were Cyclo C19:0 ω8c and C16:0. The major cellular polar lipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidyl ethanolamine (PE) and unidentified glycolipids. The DNA G-C content of isolates were 63.1 to 63.5 mol%.

2.3 Group IV isolates

One isolate (Group IV), SR5-3 was spore-forming short rods and cream-yellow colonies as shown in Figure 14. Major polar lipids were PG, DPG and unidentified glycolipids (Figure 15 and Table 32). The DNA G+C content was 40.3 mol%. The phylogenetic analysis revealed that the halophilic bacteria group IV (SR5-3) belonged to the genus *Halobacillus* and closest with *Halobacillus trueperi* DSM 10404[T] (Figure. 16). The 16S rDNA sequence similarity of the SR5-3 isolate showed 97.1% to *H. trueperi* DSM 10404[T], 97.0% to *H. karajensis* DSM 14948[T], and 96.8% to *H. litoralis* SL-4[T], respectively (Table 19). Isolate SR5-3 showed several differentiated physiological and biochemical properties to *H. trueperi* DSM 10404[T], *H. karajensis* DSM 14948[T] and *H. halophilus* ATCC 35676[T] were summarized in Table 18. Colonies of SR5-3 were yellow-cream in colour but another species of genus *Halobacillus* were white (*H. karajensis* DSM 14948[T]) and orange in colour (*H. trueperi* DSM 10404[T], and *H. halophilus* ATCC 35676[T]). Isolate SR5-3 produced acid produced from L-arabinose, D-galactose, sucrose, trehalose and D-xylose and

hydrolyzed tyrosine. In contrast, *H. trueperi* DSM 10404[T], *H. karajensis* DSM 14948[T] and *H. halophilus* ATCC 35676[T] did not hydrolyzed tyrosine and produced acid from those sugars. The DNA G-C content of isolate, determined by HPLC, were 40.3 mol%. Based on these data suggested that SR5-3 isolate was the new species of the genus *Halobacillus*.
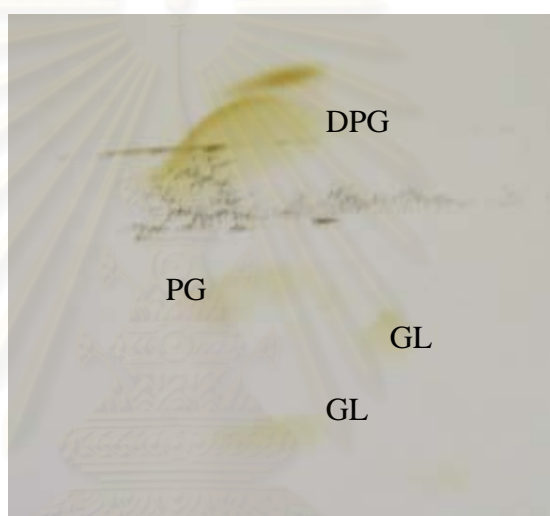
Figure 15. Thin-layer chromatogram of the total polar lipids of SR5-3
(Group I V).
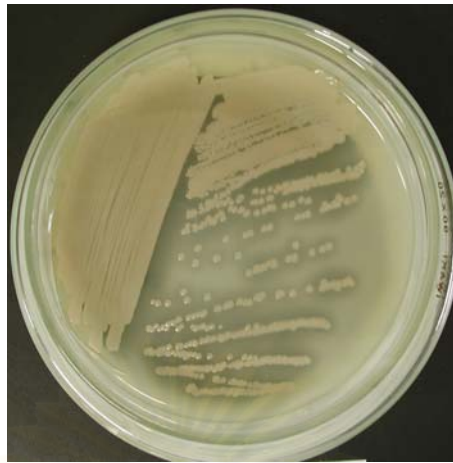
(A)
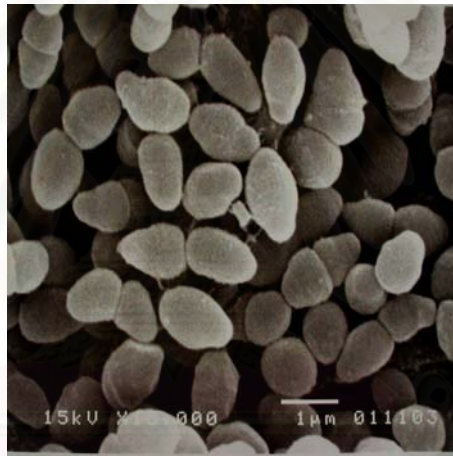
(B)

Figure 14. The colonial appearance (A) and scanning electron micrograph (B) of
SR5-3 grown on JCM No. 377 supplemented 1% skim milk at 37 ºC for
5 days.

Table 18. Differential characteristics between Group IV and *Halobacillus* species (Amoozegar *et al*., 2003; Spring *et al*., 1996).

Characteristics	Group IV	*H. karajensis* DSM 14948[T]	*H. halophilus* ATCC 35676[T]	*H. trueperi* DSM 10404[T]
Cell shape	Rods	Rods	Cocci	Rods
Spore shape	spherical	spherical	spherical	spherical
	ellipsoidal	ellipsoidal		ellipsoidal
Spore position	Subterminal	Central or	Central or	Central or
		Subterminal	Subterminal	Subterminal
Pigmentation	Cream Yellow	White	Orange	Orange
NaCl range (%)	2-30	1-24	2-20	0.5-30
Acid production				
L-Arabinose	+	-	ND	ND
D-Fructose	+	+	-	+
Galactose	+	-	-	+
Glucose	+	+	-	+
Maltose	+	+	-	+
Mannitol	+	+	+	-
Mannose	+	+	ND	ND
D-Raffinose	w	+	ND	ND
Sucrose	+	-	-	+
Trehalose	+	-		+
D-Xylose	+	-	-	-
Hydrolysis of				
Casein	+	+	+	-
Gelatin	+	+	+	+
Starch	-	+	+	-
Tyrosine	+	ND	-	-

+, positive; - negative
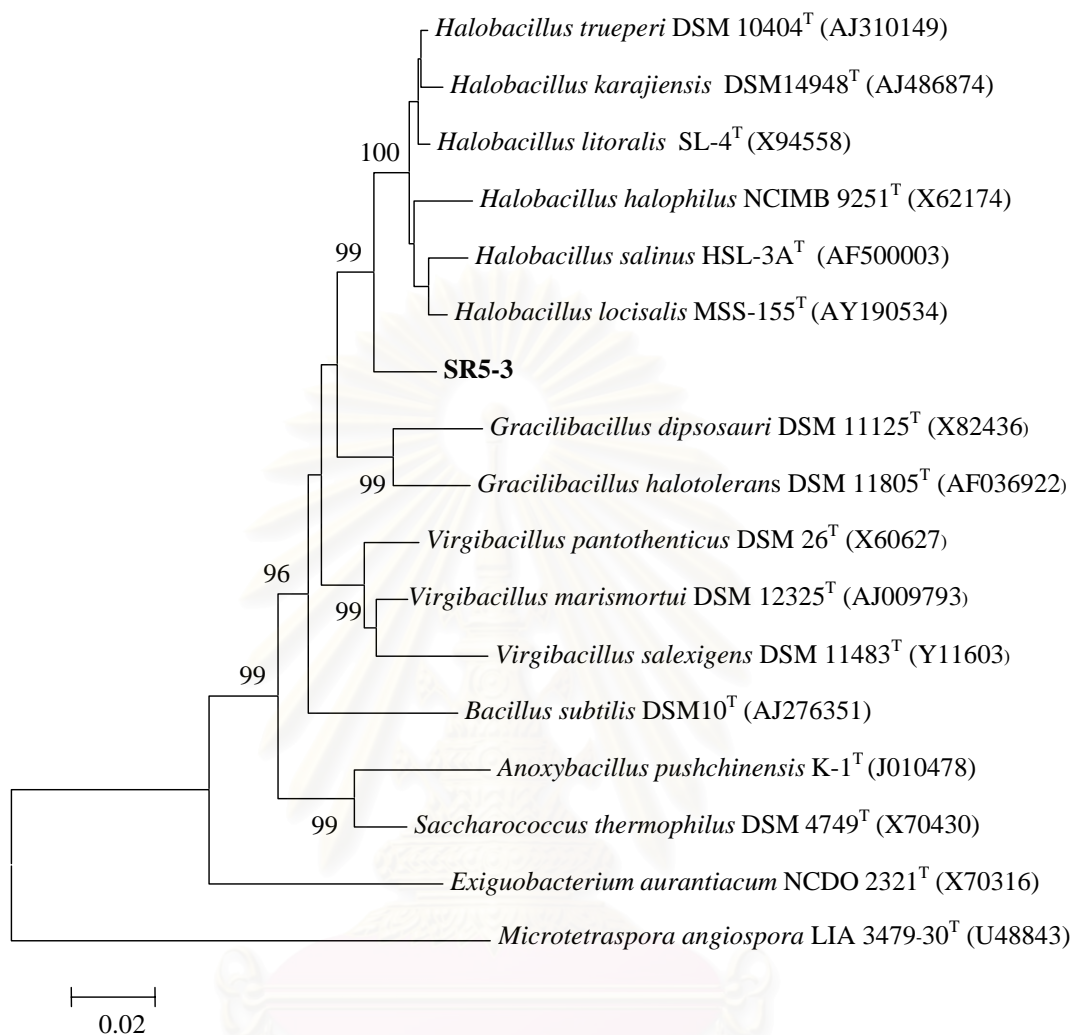
Figure. 16. Neighbour-joining-tree showing the phylogenetic position of SR5-3 and related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

Table 19. Percentage similarities of SR5-3 (Group IV) and related taxa.

Accession no.	% Similarity														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. AJ310149	100														
2. AJ486874	99.3	100													
3. AY190534	98.8	98.3	100												
4. X62174	98	97.6	97.6	100											
5. SR5-3	97.1	96.9	96.5	96.4	100										
6. X82436	94.3	94.1	94.2	93.3	94.3	100									
7. AF036922	94.8	94.4	94.3	94	94.3	96	100								
8. AJ009793	95.3	94.9	95.1	94.6	95.5	93.8	94.2	100							
9. Y11603	93.4	93.2	92.9	92.9	93.4	92.5	92.9	96.6	100						
10. X60627	94.8	94.6	94.8	94.8	94.9	93.7	94.3	97.6	95.5	100					
11. AJ276351	93.4	93	93.6	92.3	93.1	93.2	92.7	94.2	92.6	93.8	100				
12. AJ010478	91.2	90.9	91.1	90.8	91.8	90.2	90.1	91.6	90	91.9	90.8	100			
13. X70430	93.4	93.1	93.5	92.9	93.9	92.3	92.2	93.6	92.1	93.9	92.7	95.4	100		
14. X70316	89	88.9	89.4	89.1	89.5	88.5	89.2	89.9	88.3	90.2	89.6	89.2	89.6	100	
15. U48843	81.2	80.9	80.9	80.6	81.9	80.3	81	81	80.7	80.4	80.3	80.3	81.7	80.6	100

2.3.1 Characteristics of *Halobacillus* sp. SR5-3 (Group IV)

Group IV isolate was short-rods and cream- yellow-pigmented halophilic bacteria occurred singly, in pair or short chains. It produced spherical and ellipsoidal spores at subterminal position in the cells. The isolate showed positive reactions to hydrolysis of casein, gelatin, tyrosine, and DNA. Acid was produced from L-arabinose, D-fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, trehalose, and xylose, but not D-cellobiose, glycerol, inulin, myo-inositol, melibiose, rhamnose, ribose, salicin, sorbitol. Indole formation, nitrate reduction, MR-VP, urease, utilization of starch, and arginine were negative. The SR5-3 isolate grew up to 30% NaCl and its optimal growth was at 10% NaCl. Major polar lipids were PG and DPG. The G+C contents were 40.3 mol%.

2.4 Group V isolate

One isolate, RF2-5 was spore-forming rods and white-cream-colonies (Figure 17). It contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant respiratory menaquinone was MK-7 (Table 34). The major fatty acids were anteiso $C_{15:0}$ and iso $C_{15:0}$ (Table 35). The cellular polar lipids found in at least four isolates were phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Figure 18 and Table 32). The DNA G-C content of isolate, determined by HPLC, was 36.5mol%. The chemotaxonomic profiles of Group V isolate was agreed with that of the genus *Filobacillus* (Schlesner *et al*., 2001).

The phylogenetic tree showed that RF2-5 and *F. milosensis* JCM 12288 [T] were included in a monophyletic cluster together with *Alkalibacillus haloalkaliphilus* DSM 5271[T] with high bootstrap values (95%) as shown in Figure 19. Furthermore, the comparison of 16s rDNA sequence with known aerobic gram positive, spore forming halophilic bacteria revealed that isolate RF2-5 was closest to *F. milosensis* JCM 12288 [T] with 97.0% similarity (of 1491 positions shown Figure 39) as shown Table 21. Whereas, Group V isolate had several unrelated characteristics from *F. milosensis* JCM 12288[T] was shown in Table 21. Group V isolate showed positive reactions to hydrolysis of casein and gelatin but negative for *F. milosensis* JCM 12288[T]. According to the DNA-DNA hybridization experiments, labeled DNA from *F. milosensis* JCM 12288 [T] showed 18.75% DNA-DNA similarity to RF2-5, and reciprocally, that of RF2-5 showed 31.25% DNA-DNA similarity to *F. milosensis*

JCM 12288 [T] (Table 22) including several distinguished characteristics (Table 20) suggested that Group V isolate was a new species.

(A)
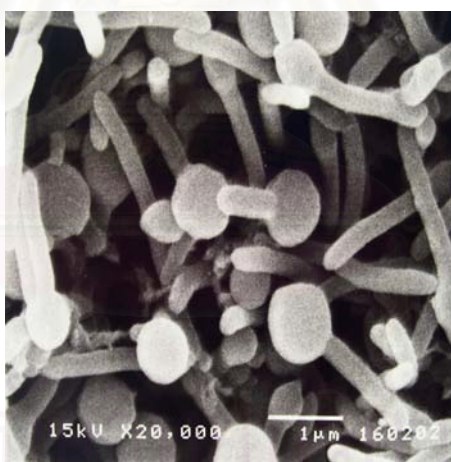
(B)

Figure 17. The colonial appearance and scanning electron micrograph of RF2-5 grown on JCM No. 377 bat 37 ℃ for 5 days.
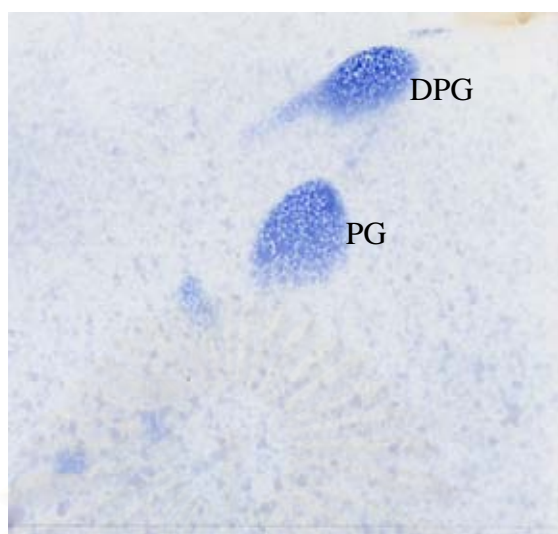
Figure 18. Thin-layer chromatogram of the total polar lipids of RF2-5
(Group V).

Table 20. DNA G+C content, and DNA-DNA Similarity of RF2-5 and
Filobacillus milosensis DSM 13259[T].

Isolate no.	G+C content	% Similarity with labelled isolates	
Group V	(mol%)	JCM 12288[T]	RF2-5
1. RF2-5	36.5	31.25	100.0
2. *F. milosensis* JCM 12288[T]	35.7	100	18.7
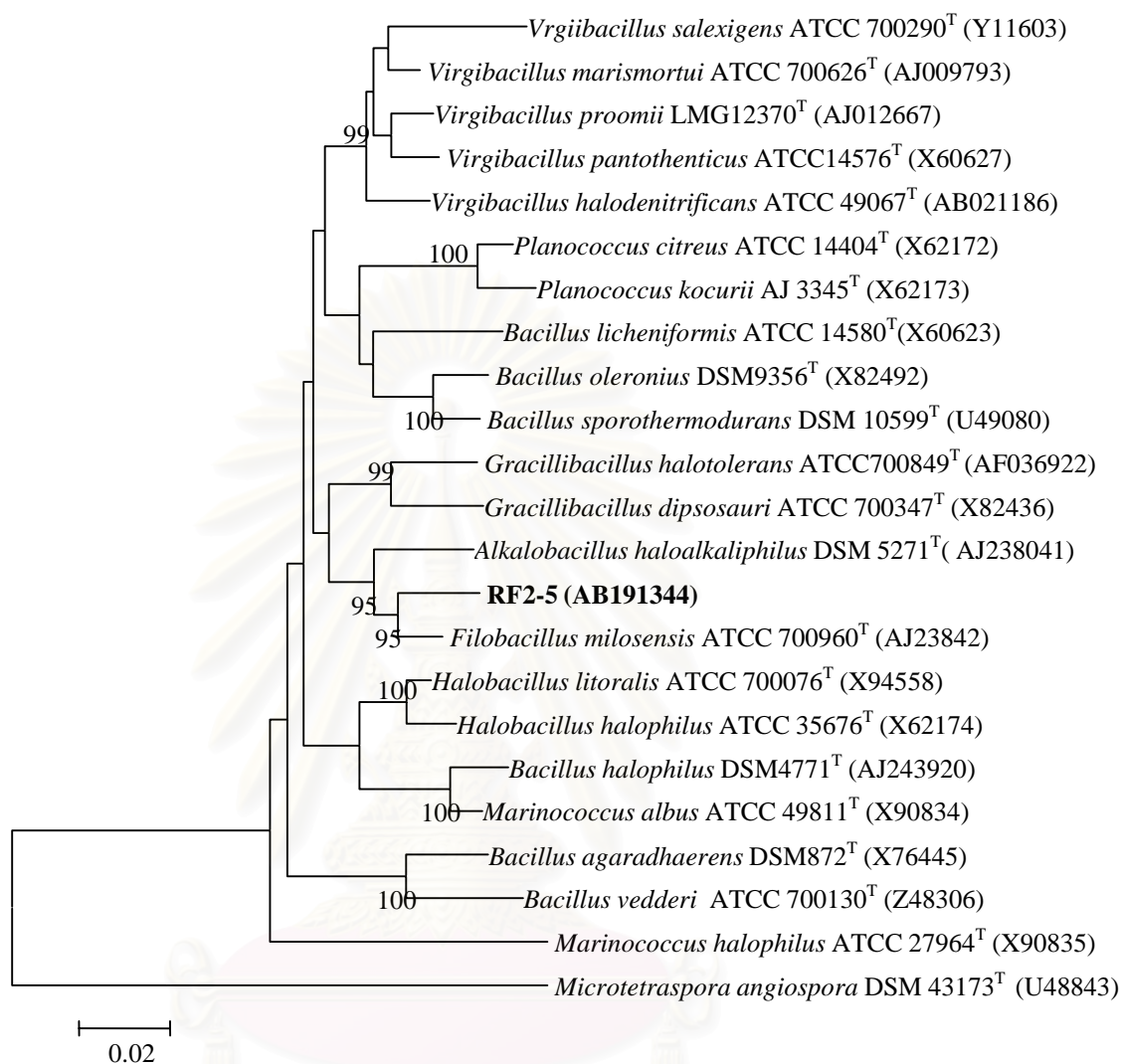
Figure 19. Neighbour-joining-tree showing the phylogenetic position of RF2-5 and
related taxa based on 16S rDNA sequences. Bar, 0.02 substitutions per
nucleotide position. Bootstrap values expressed as percentages of 1000
replications.

Table 21. Percentage similarities of RF2-5 (Group V) and related taxa.

Accession no.	% Similarity																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. Y11603	100																							
2. AJ009793	96.4	100																						
3. AJ012667	95.4	97.5	100																					
4. AB021186	95.4	97.8	96.7	100																				
5. X60627	95	97.5	97.7	96.8	100																			
6. X60623	92.2	94.3	94.7	94.3	94.7	100																		
7. X62172	91.6	94	94.3	93.8	93.9	93.8	100																	
8. X62173	91.2	93.4	94	93.2	93.2	92.9	97.7	100																
9. X82492	92.5	94.3	94.3	94.3	93.8	94.9	93.8	92.8	100															
10. U49080	92.6	94.5	94.3	94.8	93.9	95.4	93.9	92.8	97.9	100														
11. AB191344 (RF2-5)	91.9	93.7	93.3	93.7	93.2	93	92	91	93.8	93.2	100													
12. AB194046	91.9	93.8	93.6	93.7	93.5	93.1	92.1	91.1	94	93.4	99.3	100												
13. AJ238042	93.2	94.6	94.6	94.4	93.9	93.2	92.4	92.1	93.2	94.3	97.3	97.1	100											
14. AJ238041	92.2	94.1	93.9	94.3	93.6	93.3	92.1	91.2	93.3	94	95.6	95.9	96.4	100										
15. AF036922	92.7	94.4	94.6	94.5	94	92.1	91.7	91.4	93.2	92.6	93.9	94.1	94.3	92.9	100									
16. X82436	92.2	94.2	94.3	94.4	93.5	92.6	91.5	90.4	93.8	93.6	94.6	94.8	94.6	93.7	96.3	100								
17. X94558	93.2	95.9	94.9	95.3	95	93.3	93.2	92.1	93.5	94.1	93.2	93.6	94.4	93.7	94.3	94.3	100							
18. X62174	92.5	95.2	94.9	94.6	95.2	92.6	92.6	91.9	92.8	93.1	92.8	93.3	94	93.4	94	93.4	98.5	100						
19. AJ243920	91.2	93.7	92.9	93.1	92.6	91.1	90.8	90.4	91.1	91.1	92.2	92.5	93.1	93.2	93	92.7	95.4	95	100					
20. X90834	91.7	94.3	93.6	94	93.6	92.1	92.1	91.5	91.5	92.1	92.4	92.6	93.7	93.4	93.2	92.8	95.9	95.9	98.1	100				
21. X76445	91.4	93.2	93	93.1	92.9	91.9	91.5	91	91.2	91.8	91.5	91.3	92.3	91.7	92.1	91.6	92.8	92.5	92.1	92.1	100			
22. Z48306	90.7	92.4	92.5	92.1	91.9	92.1	91.2	90.8	90.8	91.4	91	91.1	92.1	91.6	90.9	90.5	91.5	91	90.5	91	95.9	100		
23. X90835	89	91.4	90.7	90.8	90.3	89.3	89.5	88.9	89.3	89.4	90.1	90.2	91.1	91.5	89.9	89.6	91.3	91.1	91.4	92.1	90.6	90	100	
24. U48843	80.8	81.8	82	81.6	81	80.6	81.7	81.5	81.1	81	81.1	81	81.3	81.6	81.7	81.1	81.7	81.5	81.5	81.6	80.6	80.5	80	100

Table 22. Differential characteristics of RF2-5, *Filobacillus milosensis* DSM 13259[T], and *Alkalibacillus haloalkliphilus* NCIMB 9251[T] (Schlesner *et al*., 2001).

Characteristics	RF2-5	*F. milosensis* DSM 13259[T]	*A. haloalkaliphilus* NCIMB 9251[T]
Cell shape	Rods	Rods	Rods
Spore shape	Spherical	Spherical	Spherical
Spore position	Terminal	Terminal	Terminal
Pigmentation	White	White	Cream white
NaCl range (%)	0-25	2-23	0-25
Acid production			
D-Fructose	+	-	ND
D-Galactose	+	-	
D-Glucose	+	-	-
Maltose	-	-	-
Mannitol	-	-	-
Sucrose	+	-	ND
Trehalose	+	-	-
D-Xylose	+	-	-
Nitrate reduction	-	-	-
Urease	+	ND	-
Hydrolysis of			
Casein	+	-	+
Gelatin	+	-	+
Starch	-	-	w

+, positive; -, negative; ND, not determined

2.4.1 Characteristics of *Filobacillus* sp. RF2-5 (Group V)

RF2-5 was rods occurred singly, in pair or short chains. It produced white-pigmented colonies and spherical spores at terminal position in the cells. It showed positive reactions to catalase, oxidase, urease and hydrolysis of casein and gelatin. Nitrate reduction and hydrolyzed starch was negative. The maximum temperature for growth was 45°C. Well growth was observed in 0 to 25% (w/v) NaCl. Acid was produced from D-glucose, D-galactose, D-fructose, sucrose, trehalose, and D-xylose, but not maltose, mannitol and mannose. This isolate contained diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant respiratory menaquinone was MK-7. The major fatty acids were anteiso $C_{15:0}$ and iso $C_{15:0}$. The cellular polar lipids found in at least four isolates were phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). The DNA G-C content was 36.5mol%.

2.5 Group VI isolates

Group VI comprised eleven isolates included HDB1-1, HDB1-11, HDB1-31, HDS2-5, HDB10-5B, HIB20-2, HIB60-1, HIS10-4, HIS30-1, and HIS50-2(1). They grew optimally in the presence of 20–30% (w/v) NaCl. Growth did not occur in the presence of less than 15% NaCl. They grew optimally at 37 °C and no growth was observed at 50 or at 10°C. Some isolates hydrolyzed casein, and gelatin.

The representative isolates of Group VI, HDS2-5, HDB1-1 and HIB20-2 contained MK-8 as the predominant respiratory menaquinone (Table 34). Two-dimensional TLC revealed that the major polar lipids present phytanyl-sesterterpenyl moieties (2,3-di-O-phytanyl-sn-glycerol; $C_{20}C_{25}$) of Phosphatidylglycerol sulfate (PGS), phosphatidylglycerol methylphosphate (PGP-Me) and phosphoglycerol (PG). Glycolipids were present glycolipids (S-TGD-1, and S-TeGD) (Figure 20 and Table 33). The G-C contents of the genomic DNAs from the representative isolates of Group VI, HDB1-1, HDB1-31, HDB2-5 and HIB20-2 were 64.2, 63.4, 63.7 and 64.7 mol%, respectively. The data corresponds to the *Halobacterium* genus (Thongthai *et al*., 1992).
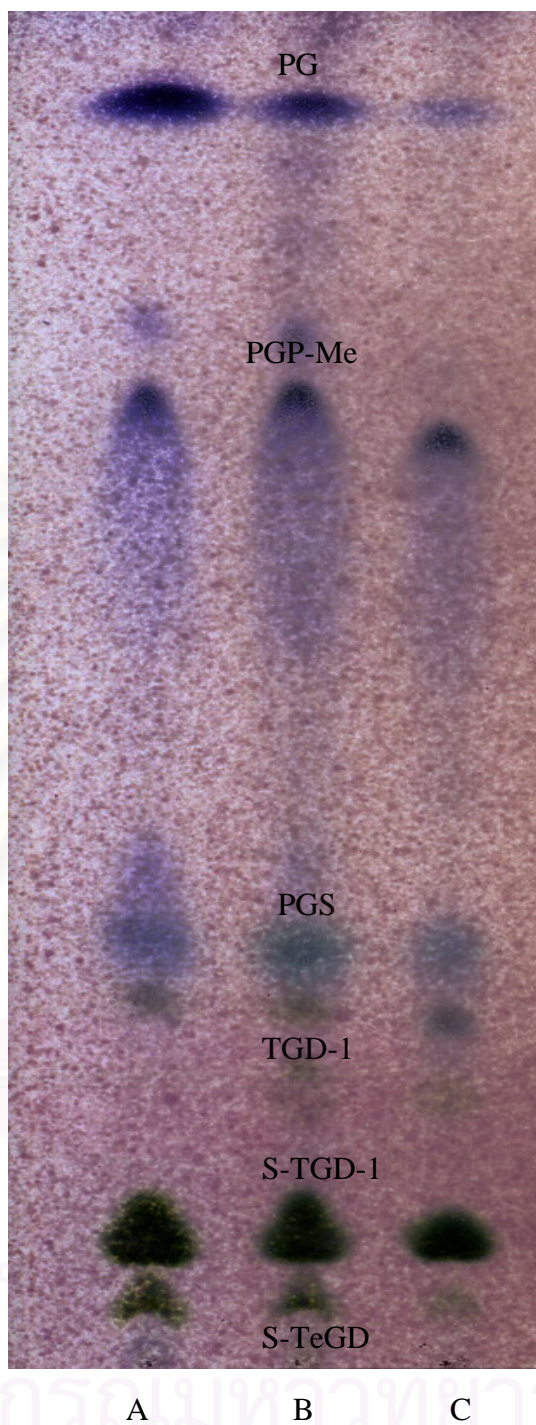
Figure 20. Thin-layer chromatogram of the total polar lipids of
Halobacterium salinarum JCM 8978 [T] (A) and
HDS2-5 in Group VI (B) and HIB20-2 in Group VII (C).

A tree constructed by the neighbour-joining method, depicting the phylogenetic relationships of the representative isolates for Group VI, HDS2-5 (1382 nucleotides shown Figure 40), is shown in Figure 21 and demonstrates that it represents the *Halobacterium* genus. HDS2-5 was closest to the *H. salinarum* JCM 8978[T] with 99.9% similarity (Table 23). The DNA-DNA similarity of Group VI isolates to *H. salinarum* JCM 8978[T] and *Halococcus morrhuae* JCM 8876[T] were shown in Table 24. DNA-DNA hybridization indicated that the isolates had low DNA –DNA similarities to *H. morrhuae* JCM 8876[T] (1.6-16.7%) as shown in Table 23. On the other hand, all isolates showed high levels of DNA-DNA similarities with *H. salinarum* JCM 8978[T] (87.3-101.1). The DNA-DNA similarities with *H. salinarum* JCM 8978[T] was higher than 80% in all cases including phenotypic (Table 25) and chemotaxonomic characteristics warranted that Group VI isolates belonged to the known species of *Halobacterium* and they were identified as *H. salinarum*.

Table 23. DNA G+C content, and DNA-DNA similarity among Group VI,
H. salinarum JCM 8978[T] and *H. morrhuae* JCM 8876[T].

Isolate no.	G+C content	% Similarity with labelled isolates	
Group VI	(mol%)	JCM 8978[T]	JCM 8876[T]
1. HDB1-1	64.2	101.1	15.9
2. HDB1-11	63.3	98.5	1.6
3. HDB1-31	63.4	92.3	4.7
4. HDS2-5	63.7	89.5	3
5. HDB10-5R	ND	98.8	2.7
6. HIB20-2	64.7	94.5	13.9
7. HIB60-1	ND	92.9	8.9
8. HIS10-4	ND	87.3	2.5
9. HIS30-1	63.7	94.6	4.8
10. HIS50-2(1)	63.8	97.3	16.7
JCM 8978[T]	57 or 60	100	8.8

ND, not determined.
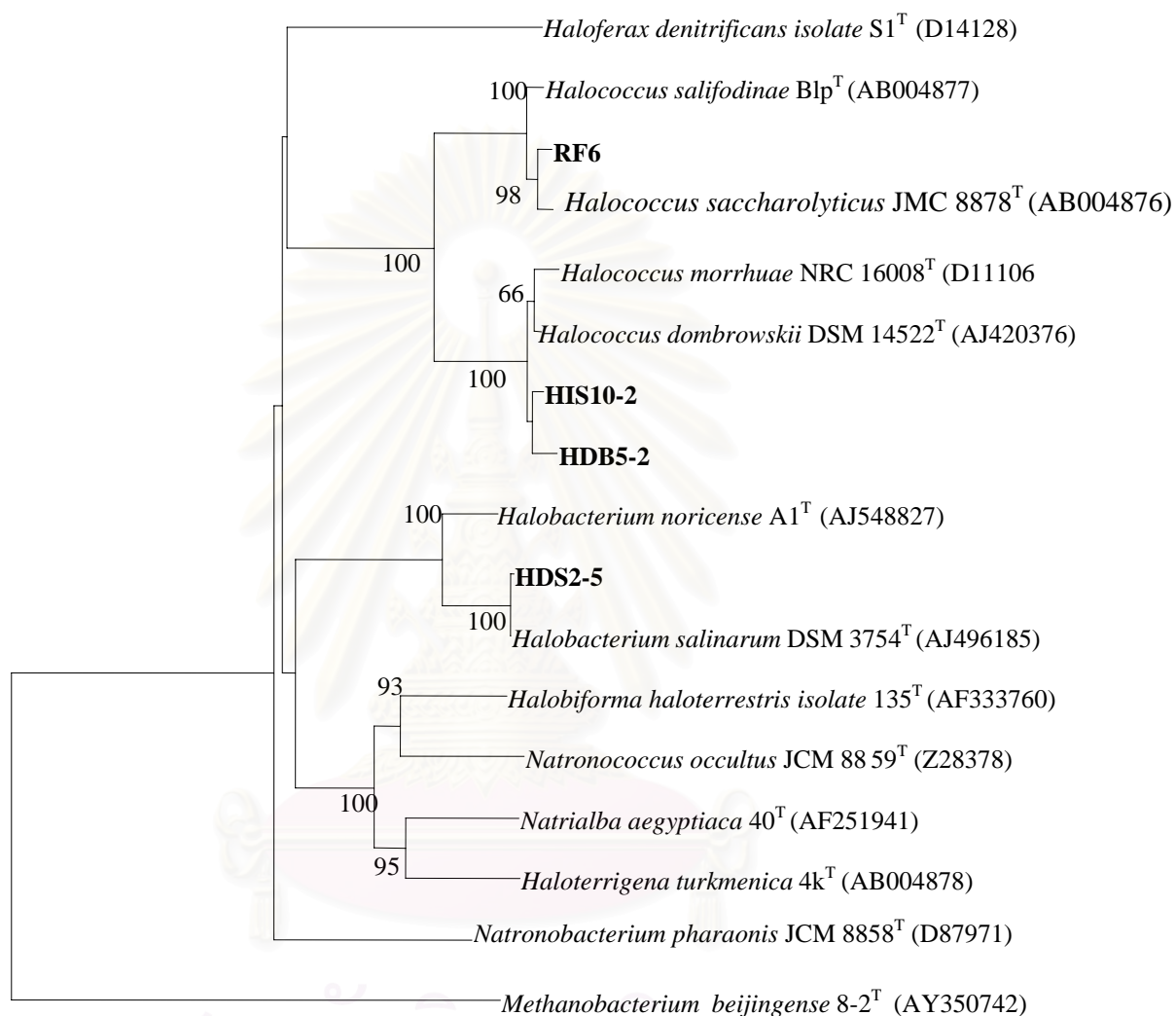
Figure. 21. Neighbour-joining-tree showing the phylogenetic position of HDS2-5 (Group VI), HRF6 (Group VII), HDB5-2 (Group VIII), HIS10-2 and related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

Table 24. Percentage similarities of HDS2-5 (Group VI), HRF6 (Group VII) and HDB5-2 (Group VIII) and related taxa.

Accession no.	% Similarity																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. AB004878	100																
2. AF251941	94.46	100															
3. Z28378	92.21	93.26	100														
4. AF333760	93.17	93.02	94.36	100													
5. AJ496185	87.04	88.29	88.98	89.45	100												
6. HDS2-5	86.95	88.2	88.9	89.36	99.92	100											
7. AJ548827	88.71	89.21	89.98	90.27	97.06	96.98	100										
8. HDB5-2	87.63	86.96	86.58	87.95	86.78	86.69	86.53	100									
9. HIS10-2	88.25	87.23	87.3	88.48	87.06	86.97	87.07	99.16	100								
10. AJ420376	87.99	87.32	86.58	88.39	87.24	87.15	87.16	99.16	99.08	100							
11. D11106	87.63	86.96	86.02	87.85	86.79	86.7	86.8	98.7	98.62	99.24	100						
12. AB004876	88.12	86.95	87.1	87.52	87.49	87.4	87.04	94.29	94.21	94.62	94.2	100					
13. HRF6	87.75	87.15	87.18	87.8	87.58	87.49	87.22	94.37	94.29	94.7	94.12	99.31	100				
14. AB004877	88.03	87.42	87.29	88.17	88.12	88.04	87.5	94.29	94.38	94.78	94.21	98.93	99	100			
15. D14128	88.9	87.82	86.84	87.01	86.76	86.67	87.49	85.83	86.2	86.56	86.01	87.19	86.9	87.2	100		
16. D87971	87.49	87.44	88.23	88.31	89.15	89.07	90.05	87.67	88.29	88.3	87.66	87.51	87.68	87.96	88.34	100	
17. AY350742	70.46	71.25	70.12	70.87	71.31	71.21	72.11	69.89	70.35	70.7	70.27	67.71	67.66	68.45	69.85	72.34	100

2.5.1 Characteristics of *Halobacterium salinarum* isolates (Group VI)

Group VI isolates grew optimally in the presence of 20–30% (w/v) NaCl. Growth did not occur in the presence of less than 15% NaCl. Cells of Group A lysed in distilled water. They grew optimally at 37 °C and no growth was observed at 50 or at 10°C. Optimal growth occurred at pH 6.0 to 7.0, and the pH range for growth was broad (pH 5.0 to 10.0). At least 100 mM $MgCl_2$ were required for growth and grew optimally at 200-300 mM $MgCl_2$. Best growth was obtained at $MgCl_2$ concentrations between 3 to 5%.The isolates showed catalase and oxidase activities. Casein, and gelatin were hydrolyzed, but did not hydrolyze arginine and Tween 80. Growth on single carbon sources was never observed. Acid was not produced from arabinose, cellobiose, glucose, lactose, mannitol, melibiose, raffinose, sucrose and trehalose. The isolates were susceptible to Bacitracin (10 mg), rifampicin (30 mg) and novobiocin (5 mg), but resistant to chloramphenicol, tetracycline, ampicillin, gentamicin, kanamycin, nalidixic acid and streptomycin. The DNA G-C contents of HDB1-1, HDB1-31, HDB2-5 and HIB20-2 were 64.2, 63.4, 63.7 and 64.7 mol%, respectively. The representative isolates of Group VI, HDS2-5, HDB1-1 and HIB20-2 contained MK-8 as the predominant respiratory menaquinone. Two-dimensional TLC revealed that the major polar lipids present phytanyl-sesterterpenyl moieties (2-O-sesterterpanyl-3-O-phytanyl-sn-glycero; $C_{20}C_{25}$) of sulfate phosphatidylglycerol (PGS), phosphatidylglycerol (PG) and phosphatidylglycerol methylphosphate (PGP-Me). Glycolipids were present S-TGD-1, S-TeGD and TGD. The data corresponds to *Halobacterium salinarum* (Thongthai *et al*., 1992).

Table 25. Differential characteristics of Group VI, *H. salinarum* JCM 8978[T], Group VII, and *H. saccharolyticus* JCM 8878[T].

Characteristics	Group VI	*H. salinarum* JCM 8978[T]	Group VII	*H. saccharolyticus* JCM 8878[T]
NaCl range (%)	20-30	20-30	20-30	20-30
$MgCl_2$ range (%)	0.5-5	0.5-5	0.5-5	0.5-5
pH	5-10	5-10	5-10	5-10
Catalase	+	+	+	+
Oxidase	+	+	+	+
Acid from				
Arabionose	-	-	-	-
Cellobiose	-	-	-	-
Glucose	-	-	-	-
Lactose	-	-	-	-
Mannitol	-	-	-	-
Melibiose	-	-	-	-
Raffinose	-	-	-	-
Sucrose	-	-	-	-
Trehalose	-	-	-	-
Utilization of				
Arabinose	-	-	+	+
Glucose	-	-	+	+
Fructose	-	-	+	+
Galactose	-	-	+	+
Raffinose	-	-	-	-
Xylose	-	-	-	-
Arginine	-	-	+	+
Glutamic acid	-	-	+	+
Serine	-	-	+	+

+, positive; -, negative

2.6 Group VII isolate

One isolate, HRF6 grew optimally in the presence of 20–30% (w/v) NaCl. Growth did not occur in the presence of less than 15% NaCl. It grew optimally at 37 °C and no growth was observed at 50 or at 10°C. Casein and gelatin were hydrolyzed, but not from Tween 80.

Thin-layer chromatography of lipid extracts of Group VII isolate revealed that this organism contained two polar lipids, the glycerol diether analogs (2-O-sesterterpanyl-3-O-phytanyl-sn-glycerol; $C_{20}C_{25}$ and 2,3-di-O-phytanyl-sn-glycerol; $C_{20}C_{20}$) of phosphatidylglycerol (PG) and phosphatidylglycerol methylphosphate (PGP-Me) and unidentified glycolipids (Figure 22 and Table 33). The major menaquinone was MK-8(H_2) (Table 34). The G-C contents of the genomic DNAs from the representative isolates of Group B, HRF6 was 63.2 mol%. These characteristics seemed to agree with those of the genus *Halococcus* (Stan-Lotter *et. al.*, 2002).

The phylogenetic tree, which was constructed by comparing the representative isolate of Group VII, HRF6, 16S rRNA sequence comprised 1376 nucleotides (Figure 41) with the 16S rRNA sequences of other halophilic archaea, showed that the nearest phylogenetic relative of HRF6 corresponds to *H. saccharolyticus* JCM 8878[T] (Figure. 21). The HRF6 16S rRNA gene exhibited 99.3% sequence similarity to *H. saccharolyticus* JCM 8878[T] (Table 26). The DNA-DNA similarity was assessed between *H. saccharolyticus* JCM 8878[T], *H. morrhuae* JCM 8876[T] and HRF6 shown in Table 27. HRF6 exhibited low levels of DNA-DNA similarity of 8.3 % with *H. morrhuae* JCM 8876[T], but high levels of DNA-DNA similarity of 88.5% with *H. saccharolyticus* JCM 8878[T]. Based on the data, HRF6 of Group VII was identified as *H. saccharolyticus*.
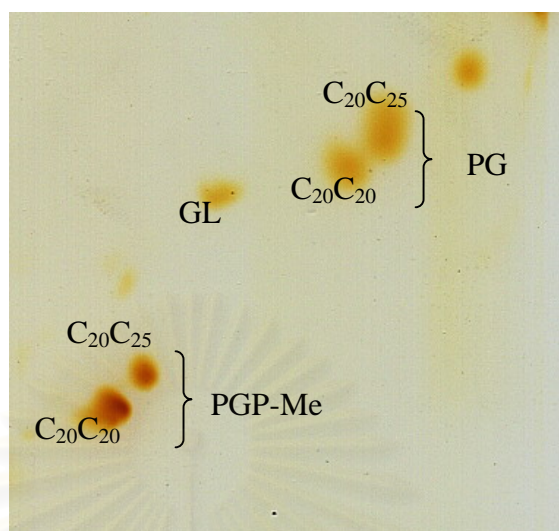
Figure 22. Thin-layer chromatogram of the total polar lipids of HRF6
(Group VII).

Table 26. DNA G+C content, and DNA-DNA Similarity of HRF6 (Group VII) and
H. sacchalolyticus JCM 8878[T].

Isolate no.	G+C content	% Similarity with labeled isolates	
Group VII	(mol%)	HRF6	JCM 8878[T]
1. HRF6	63.2	100	79.9
H. saccharolyticus JCM 8878[T]	59.5	88.5	100
H. morrhuae JCM 8876[T]	57.8	8.3	6.5

**2.6.1 Characteristics of *Halococcus saccharolyticus* HRF6
(Group VII).**

HRF6 (Group V) grew optimally in the presence of 20–30% (w/v) NaCl.
Growth did not occur in the presence of less than 15% NaCl. It grew optimally at 37
°C and no growth was observed at 50 or at 10°C. Optimal growth occurred at pH 6.0

to 7.0, and the pH range for growth was broad (pH 5.0 to 10.0). At least 100 mM $MgCl_2$ were required for growth. The isolates showed catalase and oxidase activities. Casein, and gelatin were hydrolyzed, but did not hydrolyze arginine and Tween 80. Growth on single carbon sources was never observed. Acid was not produced from arabinose, cellobiose, glucose, lactose, mannitol, melibiose, raffinose, sucrose and trehalose. The isolates were susceptible to Bacitracin, rifampicin and novobiocin but resistant to chloramphenicol, tetracycline, ampicillin, gentamicin, kanamycin, nalidixic acid and streptomycin. Thin-layer chromatography of lipid extracts of Group VII isolate revealed that this organism contained two polar lipids, the glycerol diether analogs (2-O-sesterterpanyl-3-O-phytanyl-sn-glycerol; $C_{20}C_{25}$ and 2,3-di-O-phytanyl-sn-glycerol; $C_{20}C_{20}$) of phosphatidylglycerol (PG) and phosphatidylglycerol methylphosphate (PGP-Me). The major menaquinone was MK-8(H_2). The G-C contents of the genomic DNAs from the representative isolates of Group B, HRF6 was 63.2 mol%.

2.7 Group VIII isolates

Group VIII contained fifteen isolates (HDB5-2, HIS10-2, HDS4-1, HKS35-3, HDB8-2, HDS7-4, HDS10-5, HDB10-5, HDS6-1A, HDS6-2, HKS333-2, HDB1-4, HDS6-6, HDB8-5 and HKS87-3). Group VIII isolates were Gram-positive and nonmotile. They were coccus, red-orange in colour and non spore-forming shown in Figures 23. Maximum temperature for growth was 45°C. The growth of *Halococcus morrhuae* JCM 8876[T] and *H. dombroskii* JCM 12289[T] were completely inhibited in this temperature. The highest growth rate was observed in the presence of 1-5% of $MgCl_2$. In case of *H. morrhuae* and *H. dombrowskii* showed the maximum growth occurred in the presence of 2-5% of $MgCl_2$. The tested isolates used glutamic acid and fructose as the sole energy source and produced acid from cellobiose, glucose, lactose, mannitol, raffinose and trehalose, but not for *H. morrhuae* JCM 8876 [T] and *H. dombroskii* JCM 12289[T]. The different phenotypic characteristics of the isolates with *H. morrhuae* JCM 8878[T] and *H. dombroskii* JCM 12289[T] were summerized in Table 2.
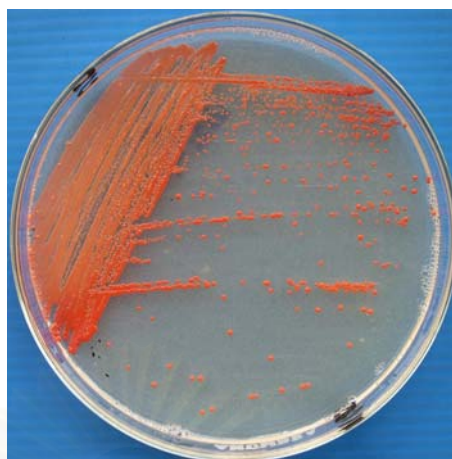
(A)

(B)

Figure 25. The colonial appearance (A) and scanning electron micrograph (B) of HDB5-2 grown on JCM No. 169 at 37 ºC for 14 days.

Table 27. Differential characteristics among Group VIII, *H. morrhuae* JCM
8876[T] and *H. dombrowskii* JCM 12289[T].

Characteristics	DB5-2[T] group (15 isolates)	*H. morrhuae* JCM 8876[T]	*H. dombrowskii* JCM 12289[T]
NaCl range (%)	20-30	20-30	20-30
pH range	5-10	5-10	5-10
MgCl$_2$ range (%)	0-5	0-5	0-5
Nitrate reduction	-	-	+
Acid from			
Arabinose	+	+	+
Cellobiose	+	-	-
Glucose	+	-	-
Lactose	+	-	-
Mannitol	+	-	-
Melibiose	-	-	-
Raffinose	+	-	-
Sucrose	-	-	-
Trehalose	+	-	-
Utilization			
Arabinose	-	-	+
Fructose	+	-	+
Glucose	V	-	+
Galactose	-	-	+
Raffinose	-	-	-
Xylose	V	-	+
Glutamic acid	+	-	-
Methionine	-	-	-
Serine	-	-	-
Hydrolyzed			
Casein	-	-	ND
Gelatin	-	-	+
Tween	-	-	ND

+, positive; -, negative; v, variable; ND, not determined

The ether lipids of the representative isolates for Group VIII, HDB5-2, HDS4-1 and HKS333-2 comprised only diether lipids, tetraether lipids being absent. The diether lipids present were the diphytanyl derivative $C_{20}:C20$ and the phytanyl sesterpanyl derivative $C_{20}:C_{25}$. Phosphatidylglycerol (PG), phosphatidylglycerol methylphosphate (PGP-Me) and unidentified glycolipids (Figure 24 and Table 33) were present in greatest abundance, similar to *H. morrhuae* JCM 8878[T] and *H. dombrowskii* JCM 12289[T]. The major menaquinone was MK-8(H_2) (Table 34). DNA G+C content ranged from 60.2 to 61.3. These characteristics seem to agree with those of the genus *Halococcus*.
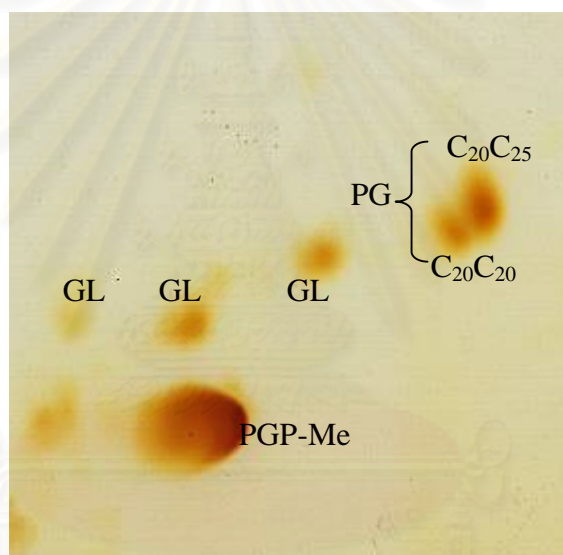
Figure 24. Thin-layer chromatogram of the total polar lipids of
the representative isolate of HDB5-2 (Group VIII).

The almost complete sequences of the 16S rDNA from the representative isolates of Group VIII, HDB5-2 and HIS10-2, with respective lengths of 1384 and 1416 bp, respectively (Figure 42 and 43), were determined and compared to the sequences of members of the genus *Halococcus* and other closely related halobacteria. A total of 1325 unambiguous nucleotides were used for computing evolutionary distance. The phylogenetic tree placement of the two isolates (Figure 21) indicated that two isolates were members of the genus *Halococcus* and constituted a subgroup with the two described halophilic species (*H. morrhuae* JCM 8876^T and *H. dombrowskii* JCM 12289^T). The similarity values of the sequence between HDB5-2 with *H. morrhuae* JCM 8876^T and *H. dombrowskii* JCM 12289^T were 98.7 and 99.1%, respectively (Table 25). The DNA-DNA hybridization study indicated that HDB5-2 and HIS10-2 showed the high levels of DNA-DNA similarities to the remaining isolates with more than 70 % (Wayne *et al.,* 1987), but only low levels to *H. morrhuae* JCM 8876^T (30-49%) and *H. dombrowskii* JCM 12289^T (21.6-47.9%), as shown in Table 28. Physiological, biochemical characteristics and DNA-DNA similarity, that would differentiate the isolates from the known *Halococcus* species shown Table 29. Thus, these 15 isolates should represent a new species and the name *Halococcus thailandensis* sp. nov. is proposed.

Table 28. DNA G+C content, and DNA-DNA Similarity among Group VIII, *H. dombrowskii* JCM 12289[T] and *H. morrhuae* JCM 8878[T].

Isolate no. Group VIII	G+C content (mol%)	% Similarity with labeled isolates		
		8876[T]	12289[T]	DB5-2[T]
1. HDB5-2	60.2	36.1	41.0	100.0
2. HIS10-2	60.7	30	21.6	91.3
3. HDS4-1	60.0	42.3	42.3	98.4
4. HKS35-3	61.2	49.4	45.6	78.9
5. HDB8-2	61.8	38.6	36.7	81.1
6. HDS7-4	ND	43.5	47.9	97.1
7. HDS10-5	ND	43.5	46.6	97.5
8. HDB10-5	ND	45.7	41.3	86.6
9. HDS6-1A	61.6	41.5	40.3	84.5
10. HDS6-2	61.3	39.8	24.3	83.8
11. HKS333-2	61.7	43.0	35.6	85.9
12. HDB1-4	61.7	49.0	31.6	90.2
13. HDS6-6	ND	44.6	34.1	88.7
14. DB8-5	61.5	46.3	44.4	84.2
15. HKS87-3	ND	49.9	32.7	91.5
H. morrhuae JCM 8876[T]	57.8	ND	34.1	25.7
H. dombrowskii JCM 12289[T]	61.3	ND	100.0	42.3

ND, not determined.

2.7.1 Characteristics of *Halococcus thailandensis* sp. nov. isolates (Group VIII)

Group VIII isolates were red in color and cocci. They produced catalase and oxidase. Growth was observed at NaCl concentrations between 20 and 30% (w/v), with optimal growth at 25 to 30% (w/v). Growth occured at 45°C but not at 10 and 50°C. Growth was possible between pH 6.0 to 10.0 but optimal at pH 6.0 to 8.0. The isolates grew in the absence of $MgCl_2$ but optimal at 200-1000 mM MgCl2. Anaerobic growth did not occur on JCM No.169 and on JCM no.169 with 1% KNO3. The isolates did not hydrolyze arginine, casein, gelatin, Tween 80, and starch. The tested isolates did not utilize arabinose, galactose, raffinose and serine as the sole energy source, but not glutamic acid and fructose. The isolates produced acid from arabinose, cellobiose, glucose, lactose, mannitol, raffinose and trehalose, but not melibiose and sucrose. The isolates were susceptible to Bacitracin, rifampicin and novobiocin, but resistant to chloramphenicol, tetracycline, ampicillin, gentamicin, kanamycin, nalidixic acid and streptomycin. The ether lipids of the the representative isolates for Group VIII,   HDB5-2, HDS4-1 and HKS333-2 comprised only diether lipids, tetraether lipids being absent. The diether lipids present were the diphytanyl derivative $C_{20}:C20$ and the phytanyl sesterterpanyl derivative $C_{20}:C_{25}$. phosphatidylglycerol (PG) and phosphatidylglycerol methylphosphate (PGP-Me) and unidentified glycolipids were present in greatest abundance and minor components. The major menaquinone was MK-8(H_2). DNA G+C content ranged from 60.2 to 61.3 mol%.

In this study, 40 isolates were isolated and taxonomically studies from fish sauce. Seventeen isolates of protease-producing halophilic bacteria in *Lentibacillus juripiscarius*, *Halobacterium salinarum*, *Halobacillus* species and *Filobacillus* species were found at the initial stage of fermentation (30-60 days). In the middle stage until complete process of fermentation (150 days until 360 days), 4 isolates of protease-producing halophilic bacteria in *Lentibacillus salicampi* and *Halococcus saccharolyticus* and 19 isolates of non protease-producing halophilic bacteria in *Chromohalobacter salexigens* and *Halococcus thailandensis* isolates were observed. Therefore, a variety of halophilic bacteria occured to grow in fish sauce fermentation. In the past time, a few reports studied and identified the microorganisms from fish sauce as *Halobacillus thailandensis*, *Tetragenococcus halophilus*, *T. muriaticus*, the moderately halophiles and *Halobacterium salinarum*, an extremely halophilic bacteria (Chaiyanan *et al*., 1999; Thongthai *et al*., 1992; Thongsanit *et al*., 2002). The important roles of bacteria in fish sauce were protein degradation and flavor-aroma development. Consequently, when fish sauce was produced under aseptic conditions or hydrolysis of fish protein by acid, typical fish sauce aroma was not developed (Lopetcharat *et al*., 2001). The non protease-producing halophilic bacteria might involve in flavor and aroma development. Most of protease from extreme halophiles most were inactive in the absence of salt (Capiralla *et al*., 2002; Izotova *et al*., 1983). On the other hand, protease from moderate halophiles could fully function in low salt environment, but their activity decreased to about 40% in high salt environment (Sanchez-Porro *et. al*., 2003). More details of protease from fish sauce isolate will be provided later.

3.   Screening of protease-producing halophilic bacteria

The halophilic isolates were screened using JCM No. 169 and JCM. No.377 agar plates supplemented with 1% skim milk and were incubated at 37°C for 3-14 days. The results of caseinolytic halo-forming colonies on the plate were showed in Figures 25 and 26. Nine extreme halophiles exhibited caseinolytic activity on the plate containing 25% NaCl as in Table 29. On the other hand, the eight moderate halophiles hydrolyzed skim milk and gelatin on plate containing 10% NaCl as in Table 29. HDS2-5 and SR5-3 showed the highest activity on the plate containing 25% and 10% NaCl, respectively.

Coolbear *et al*. (1991) reported that there is not necessarily a good correlation between the clear zone around colonies on milk-agar plates and the levels of protease activity produced. To assess protease production more quantitatively, the casein assay method was used for determination of the protease activities from the supernatant fluid of each isolate. Therefore, the protease producing-extremly halophilic bacteria were cultivated in broth of JCM. No. 169 for 14 days and the moderately halophiles were in JCM. No. 377 for 2 days at 37°C, respectively. The supernatant was determined for the protease activities. The extreme halophilic bacteria, HDS2-5 (0.15) and HIB20-2 (0.10 U/ml) were the highest protease-producing bacteria in the presence of 25% (w/v) NaCl.   In case of the moderate halophilic bacteria (SR5-3) showed the highest protease production (0.29 U/ml) by addition of 10% NaCl in the reaction mixtures. The isolate HDS2-5, HIB20-2 and SR5-3 were selected for further study.

Table 29. Casein and gelatin hydrolysis of halophilic bacteria.

No.	Isolate no.	Protease activity (clear zone)	
		Casein (25% NaCl)	Gelatin (25% NaCl)
1	HDS2-5	+++	+++
2	HDB1-1	+	+
3	HDB1-31	+	+
4	HIB20-2	++	+
5	HDB10-5R	++	+
6	HKS87-2	+	+
7	HDS6-1A	+	+
8	HRF6	++	+
9	HIS40-3B	+	+
		Casein (10 % NaCl)	Gelatin (10% NaCl)
1	SR5-3	+++	+++
2	IS40-3	+	+
3	IO-1	+	+
4	PS9-2	+	+
5	RF2-5	++	++
6	RF5-1	+	+
7	RF5-2	+	+
8	RF12-1S	+	+

+++, strong; ++, moderate; +, weak reaction
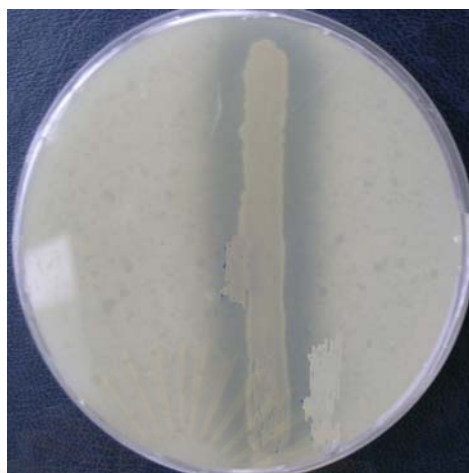
IS40-3
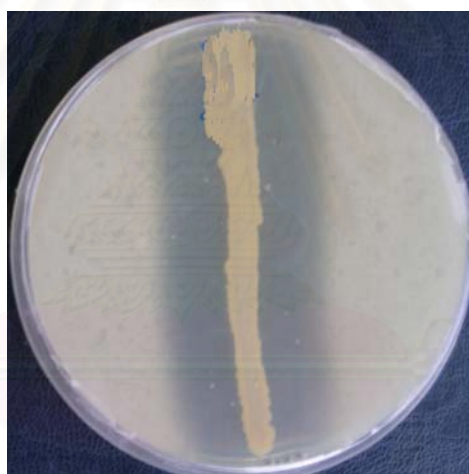
SR5-3

Figure 25. Colonial appearance of IS40-3 and SR5-3 on JCM No. 377
with 1% skim milk at 37°C for 3 days.

HDS2-5

HIB20-2

HRF6

Figure 26. Colonial appearance of HDS2-5, HIB20-2 and HRF6 on JCM.

No 169 with 1% skim milk at 37°C for 14 days.

4.   Protease production of HDS2-5, HIB20-2 and SR5-3

HDS2-5 and HIB20-2 were cultivated in the JCM No. 169 with the specified nitrogen and carbon sources supplementation.   HDS2-5 had the maximum protease production 25 U/ml) in the medium containing 1% polypeptone (Figure 27).   SR5-3 isolate was cultivated in the JCM No. 377 with the specified nutrient supplementation. In contrast, all nutrients added to the basal medium did not enhance protease production of HIB20-2. For SR5-3, a variety of nutrients such as polypeptone, yeast extract, ami ami, sucrose, lactose, starch, and some free amino acids were supplemented in JCM 377 with different concentrations. The highest production of protease of the SR5-3 isolate (450 U/ml) was achieved during the late exponential growth phase (36 h) in the medium containing ami ami, aspartic acid, glutamic acid and glycine (Figure 28). Ami ami is a by-product from monosodium glutamate production. In addition to other free amino acids, it generally contains glutamic acid (4%). The addition of these amino acids, especially in the free form could effectively increase protease production. The result was in agreement with Izotova *et al*. (1983) and Stepanov *et al*. (1992). Since halophilic protease from *Halobacterium halobium* had remarkably high contents of glycine, aspartic acid, and glutamic acid, corresponding to 25% of the total numbers of residues in the enzyme, addition of such amino acids and nitrogen sources into the medium probably provided a sufficient amount of amino acids that were important for enzyme production. Moreover, acidic amino acids such as aspartic acid and glutamic acid possibly enabled cells to survive in the hypersaline habitats through the intracellular accumulation of potassium and evolution of proteins that were exceedingly rich in acidic amino acids (glutamate and aspartate) compared to basic amino acid (arginine and lysine) residues. Acidic residues results in a net negative charge on the protein surface that presumably prevents folding into the native (active) structure unless a cation counterion like K^+ is present (Martin *et al*., 1999). In contrast, protease production of SR5-3 isolate decreased drastically in the medium supplemented with sugars. Similar to Sanchez-Porro *et al*. (2003), the suppression could possibly due to a catabolic repression mechanism of extracellular enzyme production. Due to the highest protease production, 18-fold higher than that of HDS2-5, SR5-3 was therefore selected for further purification and characterization.
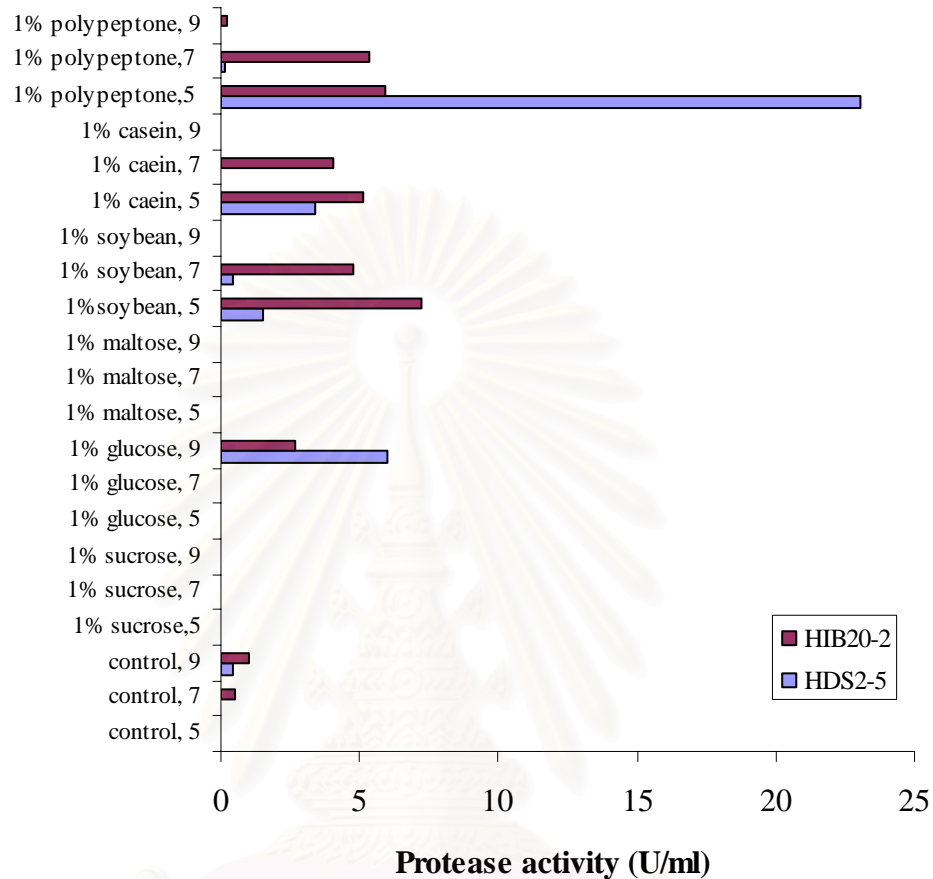
Figure 27. Effect of nitrogen and carbon sources to protease activity of HDS2-5 and HIB20-2. They were cultivated in the JCM No. 169 with the specified supplementation. The pH was adjusted to 7.2. Incubation was carried out with shaking (200 rpm) at 30ºC up to 9 days. Protease production was determined after 5, 7, 9 days of cultivation by using casein as substrate. Substrate dissolved in 25% NaCl containing 25 mM Tris-HCl pH 7.5. The reaction mixtures between crude enzyme and casein were incubated at 37°C for 60 min.
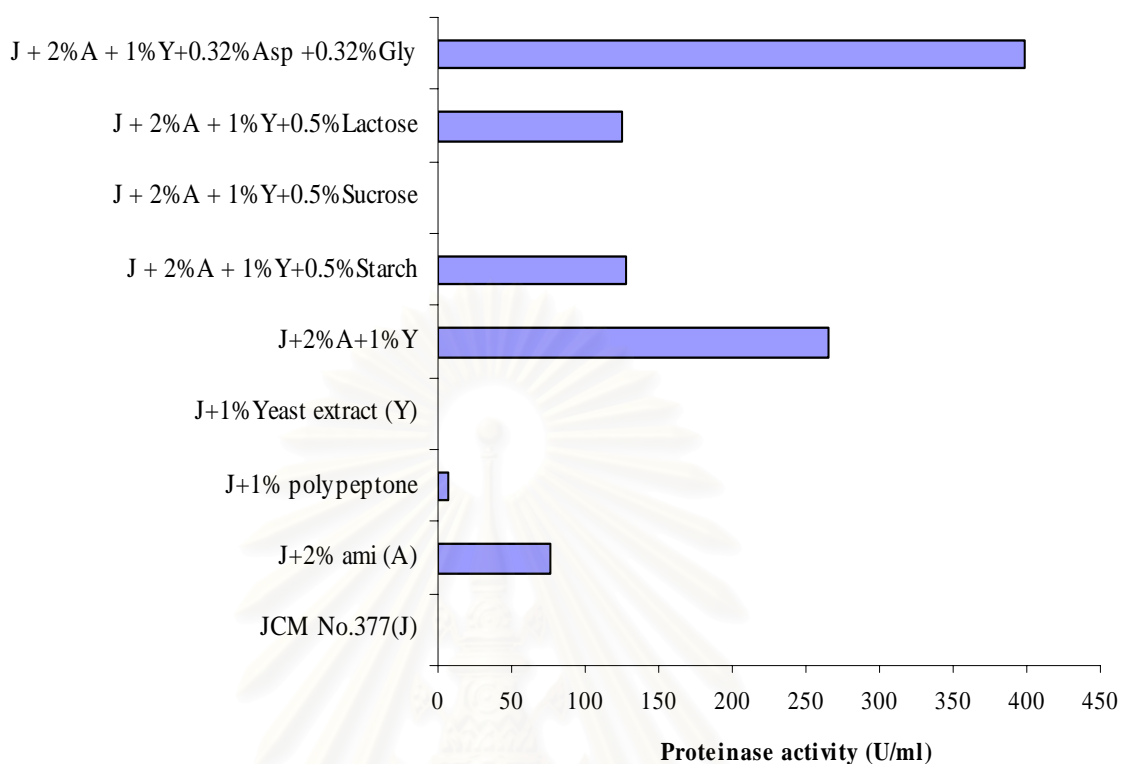
Figure 28. Effect of different nitrogen and carbon sources to the protease activity of SR5-3. SR5-3 was cultivated in the JCM No. 377 with the specified nutrient supplementation. The pH was adjusted to 7.2. Incubation was carried out with shaking (200 rpm) at 30ºC up to 2 days. Protease production was determined after 2 days of cultivation by using casein as substrate. Substrate dissolved in 25 mM Tris-HCl pH 7.5 containing 10% NaCl. The reaction mixtures between crude enzyme and casein were incubated at 37°C for 60 min.

5.   Purification and Characterization of crude SR5-3 protease

   5.1 Characterization of crude SR5-3 protease

      5.1.1 Effect of protease inhibitors of crude protease activity

      The supernatant of SR5-3 isolate was completely blocked by serine pretease inhibitors (Phenylmethylsulfonyl fluoride, PMSF) chymotrypsin inhibitor (chymostatin), and subtilisin inhibitor (α-microbial alkaline protease inhibitor, α -MAPI), while compounds such as trypsin inhibitor (leupeptin) did not inhibit the enzyme (Figure. 29). These results strongly suggest that the extracellular proteolytic activity corresponds to a serine protease. Among the serine proteases, it was assumed to be a chymotrypsin-type or a subtilisin-type, but not a trypsin-type.

      5.1.2 Effect of NaCl concentration on crude protease activity

      Effect of salt was observed at various concentrations of NaCl (0 to 30%) using both casein and Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-4-methyl-coumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA) as substrates. Suc-Ala-Ala-Pro-Phe-MCA is the synthetic substrate specific for chymotrypsin-type protease. With the increasing NaCl concentrations to 25-30%, more than 60% of the protease activity against casein decreased while in case of MCA-peptides, the protease activity increased about 1.4-fold (Figure 30). The reasons could be that, at higher salt concentrations, casein becomes less hydrophobic and the original conformation or folding is lost and hence making the substrate unavailable for proteolytic activity. In the higher salt concentrations small peptides retained their original conformations and were not affected by the way that the folding changes (Capiralla *et al*., 2002).
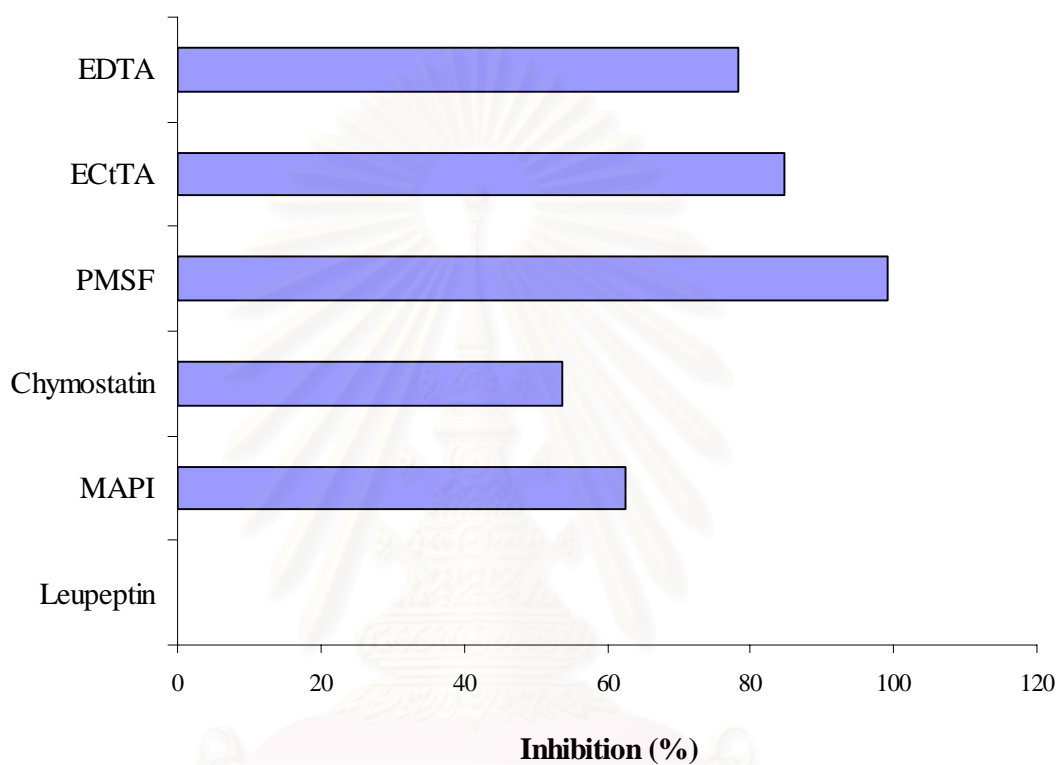
Figure 29. Effect of protease inhibitor on protease activity of supernatant of SR5-3.
The protease activities were determined by preincubation between crude
enzyme and protease inhibitors at 37°C for 10 min. After that, 10%
NaCl containing casein solution were added in the reaction mixtures and
incubated at 37°C for 60 min.
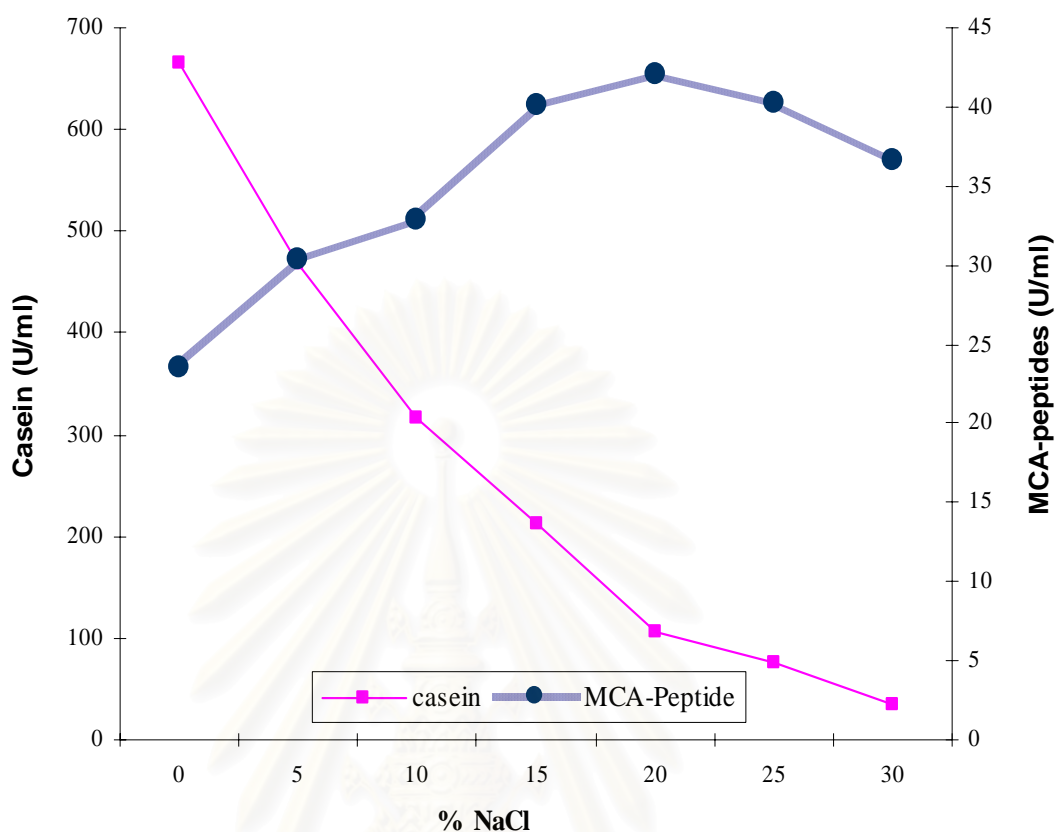
Figure 30. Effect of NaCl concentration on protease activity of crude supernatant of
SR5-3 by using casein and MCA-peptides. Protease production was
determined at 2 days of cultivation by using casein as substrate. Substrate
dissolved in 25 mM Tris-HCl, pH 7.5, containing various NaCl
concentration (0-30%). The reaction mixtures between crude enzyme and
casein were incubated at 37°C for 60 min. In case of MCA-peptides,
crude enzyme and 25 mM Tris-HCl containing various NaCl
concentration (0-30%) were preincubated at 37°C for 20 min. Then
MCA-peptides were added in the reaction mixtures and incubated at 37°C
for 30 min.

5.2 Purification of SR5-3 protease

Purification of SR5-3 protease was accomplished by two-step separation with ammonium sulfate precipitation followed by Bacitracin-affinity column chromatography (Table 30). Culture filtrate collected at 48 h with a total activity of 390 U/ml was used for the purification. Two peaks with protease activity were resolved by using Bacitracin-affinity column chromatography (Figure 31A). Peak A showed 13.3 U/mg of protein for casein substrate and 0.7 U/mg of protein for MCA-peptide substrate in the presence of 10% NaCl, while, Peak B showed 163 U/mg of protein for casein substrate and 82 U/mg of protein for MCA-peptide as a substrate under in the presence of 10% NaCl. Protease activity of peak A and peak B was suppressed in presence of high NaCl concentration by using casein as substrate. In case of MCA-peptide, protease activity of peak A was suppressed in the presence of NaCl, while that of peak B was increased about 2.5-fold in the presence of high NaCl concentration (20-30%). These data confirmed that peak A was a non halophilic protease or a salt inactivated-protease, while peak B is a halophilic protease or a salt activated-protease. Compared to the other previous studies, SR5-3 isolate probably was the first moderately halophilic bacteria to produce two types of proteases. The proteases from extreme halophiles have been studied and characterized but a few protease from moderate halophiles (Gimenez *et al*., 2000; Kamekura *et al*., 1993; Kamekura *et al*., 1996; Lama, *et al*. 2005; Qua et. al., 1981; Ryu *et al*., 1994; Schmitt *et al*., 1990; Studdert *et al*., 1997). Therefore, the halophilic proteases (peak B) were selected for purification and characterization. The protease (peak B) was purified 270-fold with a specific activity of 82 U/ mg protein and a final yield of 39%.

Purity and Molecular weight

Purity of the purified SR5-3 protease was evaluated by using SDS-PAGE. As shown in Figure 29B, the purified protease migrated as a single protein band with the molecular weight of 43 kDa (Figure 30B), corresponding to that determined by zymogram (Figure 30C). The results indicated that SR5-3 protease is the monomeric protein with a molecular weight of 43 kDa. The molecular weight of SR5-3 protease was closely to those of 49 kDa of serine protease from *Filobacillus* sp. RF2-5 (Hiraga *et al*., 2005).
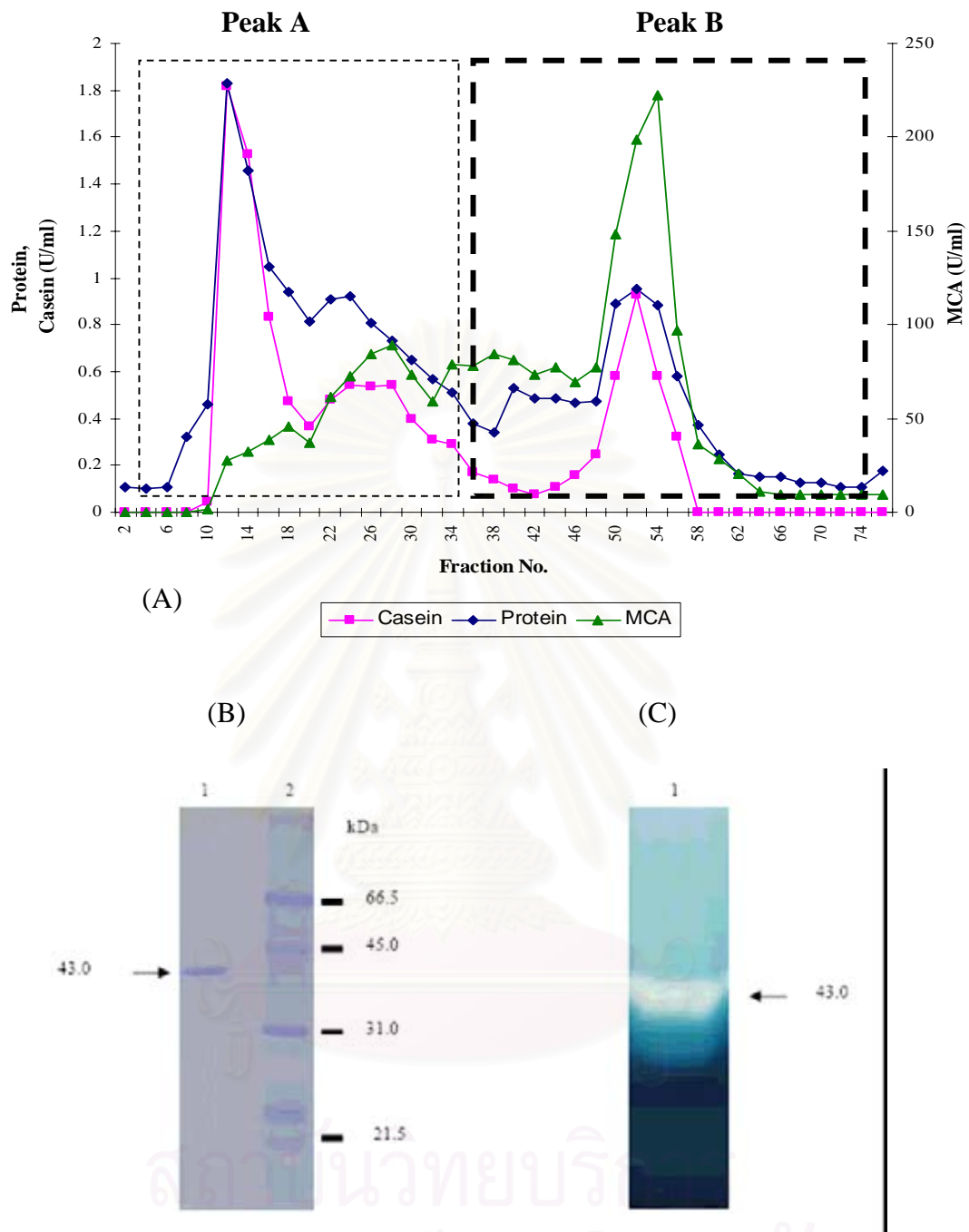
Figure 31. Bacitractin-sepharose profile(A) and SDS-polyacrylamide gel electrophoresis of the purified enzyme. 12.5% polyacrylamide gel was used for the analysis. (B) Stained with Coomassie brilliant blue R250: lanes: 1: purified protease after Bacitracin-Sepharose column; 2: Marker: bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21.5 kDa); (C) Zymogram: lanes 1: purified protease after Bacitracin-Sepharose column.

Table 30. Summary of purification of the SR5-3 protease.

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Purification (-fold)	Yield (%)
Culture supernatant	8620	3160	0.4	1	100
Ammonium sulfate ppt.	600	1720	2.7	9	54
Bacitracin-Sepharose	15	1230	82	270	39

The protease activities were determined by using MCA-peptides and presence of 20% NaCl. The purified enzyme and 25 mM Tris-HCl containing 20% NaCl were incubated at 37°C for 20 min. After that, MCA-peptides solution were added in the reaction mixtures and incubated at 37°C for 30 min.

5.4 Amino-terminal sequence analysis

The *N*-terminal amino acid sequence of the purified protease was determined to be W-V-P-N-D-T-Y-Y-S-G-Y-Q-Y-G-P-. Screening of the *N*-terminal amino acid sequence to the DDBJ databases revealed that the most similar enzyme was a 36.9 kDa-serine protease from *Bacillus* sp. Ak.1, with 87% identity (Maciver *et al*., 1994).

5.5 Characteristics of the purified SR5-3 protease

The purified protease from the SR5-3 isolate showed the maximal activity at pH 10.0 and 50°C (Figure 32A and 33A). The enzyme was stable in a broad pH range from pH 5.0-8.0 (Figure 32A) and was also stable up to 50 °C at pH 7.5 (Figure 33B, closed triangles). Addition of $CaCl_2$ to the enzyme solution enhanced the stability up to 60 °C at pH 7.5 (Figure 33B, closed circles). In order to determine the type of protease, the following protease inhibitors were used: serine protease inhibitors, PMSF, L-1-chloro-3-[-4-tosylamido]-7-amino-2-hepanone HCl (TLCK), chymostatin, leupeptin, and α-MAPI; cysteine protease inhibitors, L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E-64); and a metalloprotease

inhibitor, EDTA-2Na. Among them, PMSF, chymostatin, and MAPI were found to inhibit the protease activity almost completely, while leupeptin and E-64 showed 10-15% inhibition (Table 31). Therefore, the protease from the SR5-3 was identified to be a serine protease, based on its inhibition profile. Among the serine proteases, it was assumed to be a chymotrypsin-type or a subtilisin-type, but not a trypsin-type.

Effects of NaCl on the enzyme activity were analyzed at various concentration of NaCl (0 to 30%) using *N*-succinyl-Ala-Ala-Pro-Phe-MCA or casein as a substrate. The peptidase activity increased about 2.5-fold in the presence of 20-35% NaCl compared with that of control (Figure 34A). In the case of casein as a substrate, the protease activity was suppressed in the presence of high content of NaCl (Figure 34A). One of the reasons why the enzyme activity against casein was decreased at higher salt concentrations was that casein becomes less hydrophobic and the original conformation or folding was lost, making the substrate unavailable as a target for proteolytic activity. The stability of the protease was drastically increased in the presence of 20-35% of NaCl, but it was completely lost under the conditions of low concentration of NaCl (Figure 34B). Thus, these data indicated that the 43 kDa-protease from *Halobacillus* sp. SR5-3 is a halophilic protease or is salt activated.

Table 31. Eeffect of various inhibitors on the purified SR5-3 protease.

Inhibitors	Final concentration	Inhibition (%)
PMSF	1 mM	99
Chymostatin	100 μM	93
α- MAPI*	50 μM	87
Leupeptin	10 mM	13
E-64	10 μM	15
TLCK**	1 mM	0
EDTA	1 mM	0
EGTA	1 mM	0
Phosphoramidon	10 μM	0

* MAPI, α- 50 mMmicrobial alkaline protease inhibitor

**TLCK, L-1-chloro-3-[-4-tosylamido]-7-amino-2 -hepanone HCl.
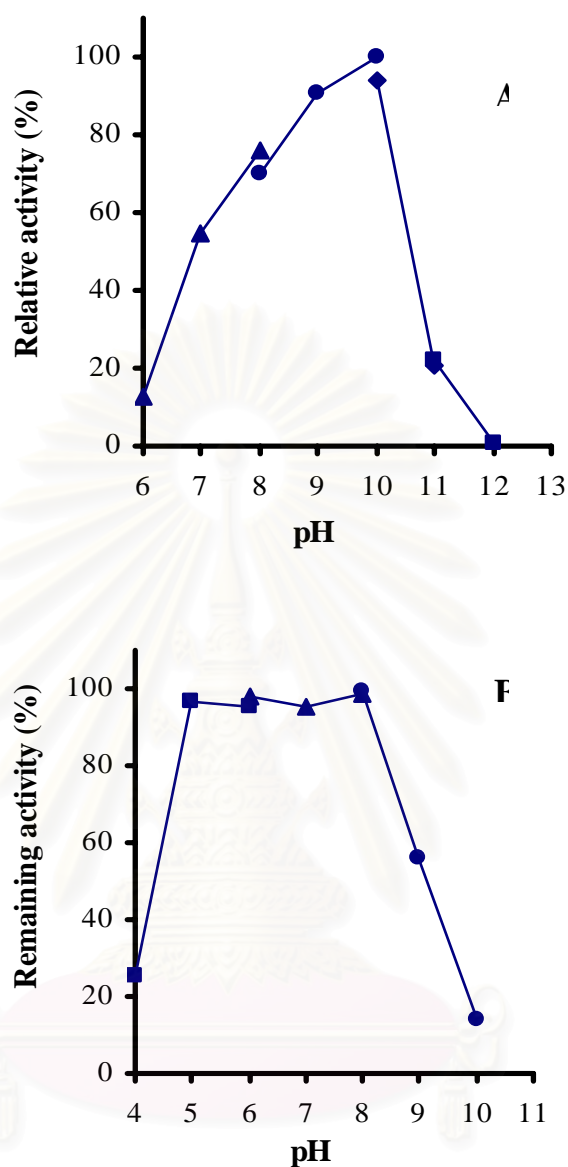
Figure 32. Effect of pH on the activity (A) and stability (B) of the purified SR5-3
protease. In the presence of 20% NaCl, Suc-Ala-Ala-Pro-Phe-MCA assay
was used for characterization of the protease. A, the buffers used were:
(▲) 25 mM KH_2PO_4/NaOH (pH 6.0-8.0); (●) 25mM H_3BO_3/ NaOH
(pH 8.0-10.0); (♦) 25mM $NaHCO_3$/NaOH (pH 10.0-11.0); (■) and 25
mM Na_2HPO_4/NaOH (pH 11.0-12.0). Maximal peptidase activity was
shown as 100%. B, the pH of the purified protease was adjusted at
various pHs by: (■) 25 mM citrate-NaOH (pH 4.0-6.0); (▲) 25 mM
KH_2PO_4/NaOH (pH 6.0-8.0); and (●) 25 mM H_3BO_3/ NaOH (pH 8.0-
10.0) buffer.
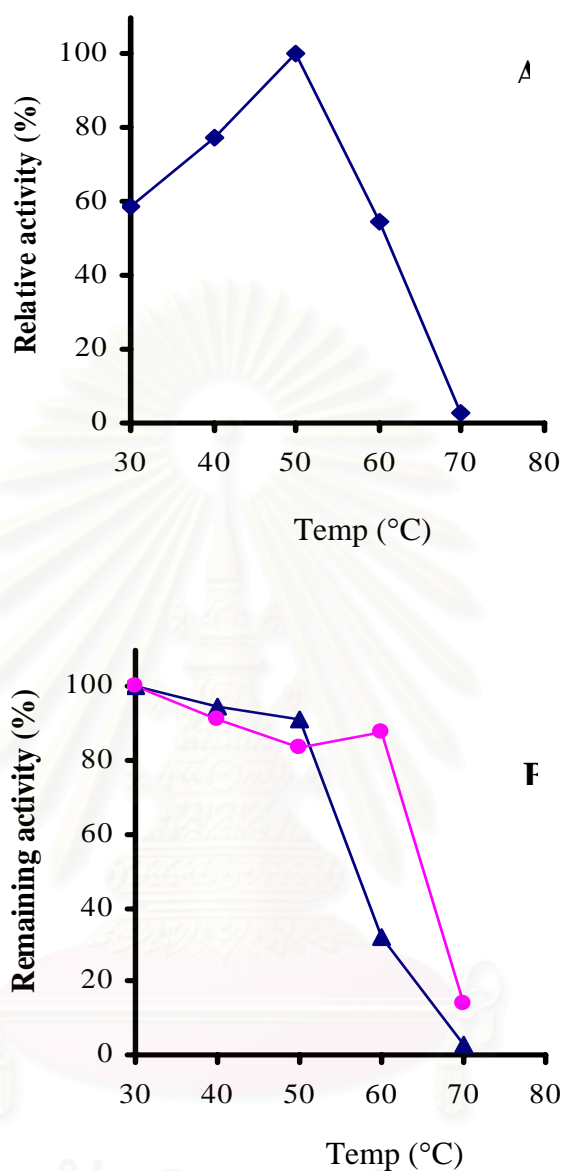
Figure 33. Effect of temperature on the activity (A) and stability (B) of the purified
SR5-3 protease. In the presence of 20% NaCl, Suc-Ala-Ala-Pro-Phe-
MCA assay was used for characterization of the protease. The enzyme
was incubated at 37°C for 3 h, then the residual peptidase activity was
measured at pH 7.5 for 20 min. A, peptidase activity was measured at 30-
70 °C and pH 7.5 for 20 min.　B, after heat treatment at various
temperatures (30 to 70 °C) and pH 7.5 for 2 h; (●) with 2 mM $CaCl_2$
(▲) without 2 mM $CaCl_2$ at pH 7.5, the remaining activity was measured
at pH 7.5 for 20 min.
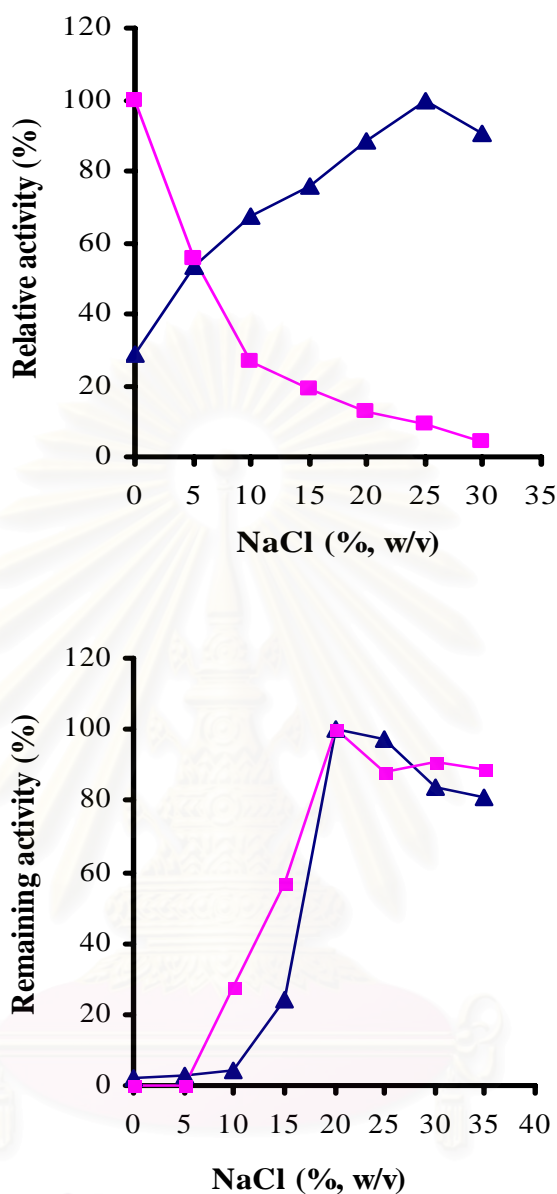
Figure 34. Effect of NaCl concentration on the activity **(A)** and stability **(B)** of the purified SR5-3 protease. **(A)** The activity of the purified enzyme was measured at various NaCl concentrations and pH 7.5 using casein (■) or Suc-Ala-Ala-Pro-Phe-MCA (▲) as substrate. **(B)** The purified enzyme was incubated at various concentration of NaCl at 37 °C and pH 7.5 for 24 h. Then the remaining activity was measured using casein (■) or Suc-Ala-Ala-Pro-Phe-MCA (▲) as substrate at pH 7.5. The relative protease/peptidase activity was defined as the percentage of activity with respect to the maximum protease/peptidase activity detected in the assay used.

5.6 Substrate specificity of the protease

A novel type of fluorescence energy transfer (FRET) combinatorial library, which was proven to be a sensitive and reliable substrate for determination of substrate specificity of proteases (Hiraga *et al*., 2005; Oda *et al*., 2005; Tunsakul *et al*., 2003) was used in order to determine the substrate specificity of the purified protease from *Halobacillus* sp. SR5-3. Fluorescent intensity measured after FRET-libraries were cleaved by the purified enzyme is the criteria used to specify the specificity at P1, P2 and P3 positions. FRETS-25Xaa-libraries (a total of 475 peptide substrates) were used to specify the specificity at P1 positions. The highest relative fluorescence activity, the second and the third, FRET-25Leu, FRET-25Gln and FRET-25-Ala, respectively, were proposed as for the preference at P1 position. (Figure 35A). The best two Xaa-containing substrate, FRET-25Leu (FRET-Zaa-Yaa-Leu) and FRET-25Gln (FRET-Zaa-Yaa-Gln) were used to specify the specificity at P2 and P3 positions. Yaa comprised five amino acids, Pro, Tyr, Lys, Ile and Asp, respectively, and Zaa also contained five amino acids, Phe, Ala, Val, Glu and Arg, respectively. Both Leu and Gln at Xaa-containing substrate showed the highest fluorescence intensity, when P2 position was Ile showing the fluorescence intensity form FRET-Zaa-Ile-Leu and FRET-Zaa-Ile-Gln series in Figure 35B and 35C. Therefore, the protease was found to prefer Leu, Gln and Ala at the P_1 position, and Ile at the P_2 position of the peptide substrate, respectively. The best two Yaa-Xaa-containing substrate, FRET-Zaa-Ile-Leu and FRET-Zaa-Ile-Gln, were used to specify the specificity at P3 positions. As for the preference at the P_3 position, the protease preferred to cleavage of Arg>Ala>Phe>Val>Glu in FRETS-Zaa-Ile-Leu based on the fluorescence intensity (Figure 35B), while Phe>Arg>Val>Ala>Glu in FREFS-Zaa-Ile-Gln of substrates, respectively (Figure 35C). One of the unique points in the substrate specificity of the purified protease was its preference of Ile at the P_2 position. This suggested that the S_2 subsite of the enzyme may be large and hydrophobic in nature (Oda *et al*., 2005). As shown in Figure 35A, the relative fluorescence activity of FRET-25-Met was nearly with FRET-25-Leu, FRET-25-Gln and FRET-25-Ala. Thus indicated that another unique point was the protease preferred Met residue at the P_1 position of the substrate as well as Leu, Gln, and Ala. There were few reports on serine protease which preferred Met at the P_1 position (Hiraga *et al.,* 2005).

(A)
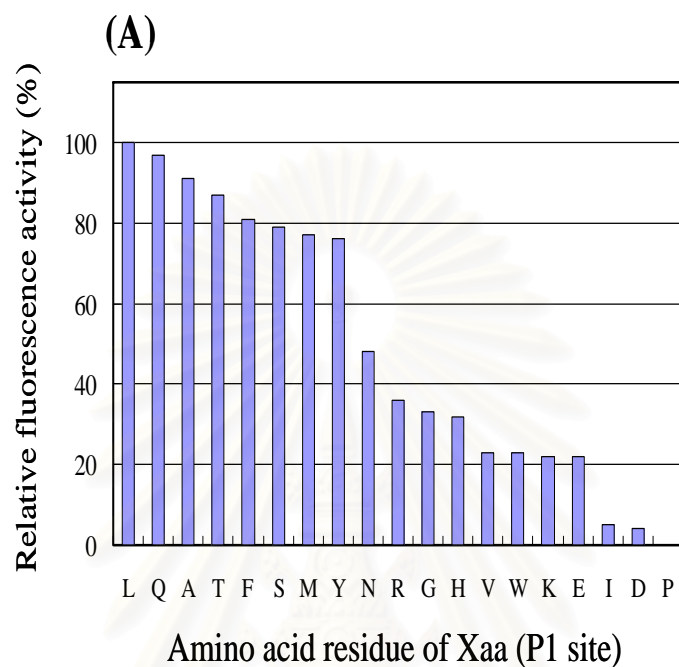
Figure 35A. Substrate specificity of the purified SR5-3 protease. (a) The P_1 preference. FRETS- 25Xaa-libraries (a total of 475 peptide substrates) were used for the primary screening. Upon the protease cleaved any peptide bond between D-A$_2$pr(Nma) and Lys (Dnp) in the substrate, the fluorescence increases in proportion to the release of Nma fluorophore from the initial Dnp quence (B) The P_2 and P_3 preferences.
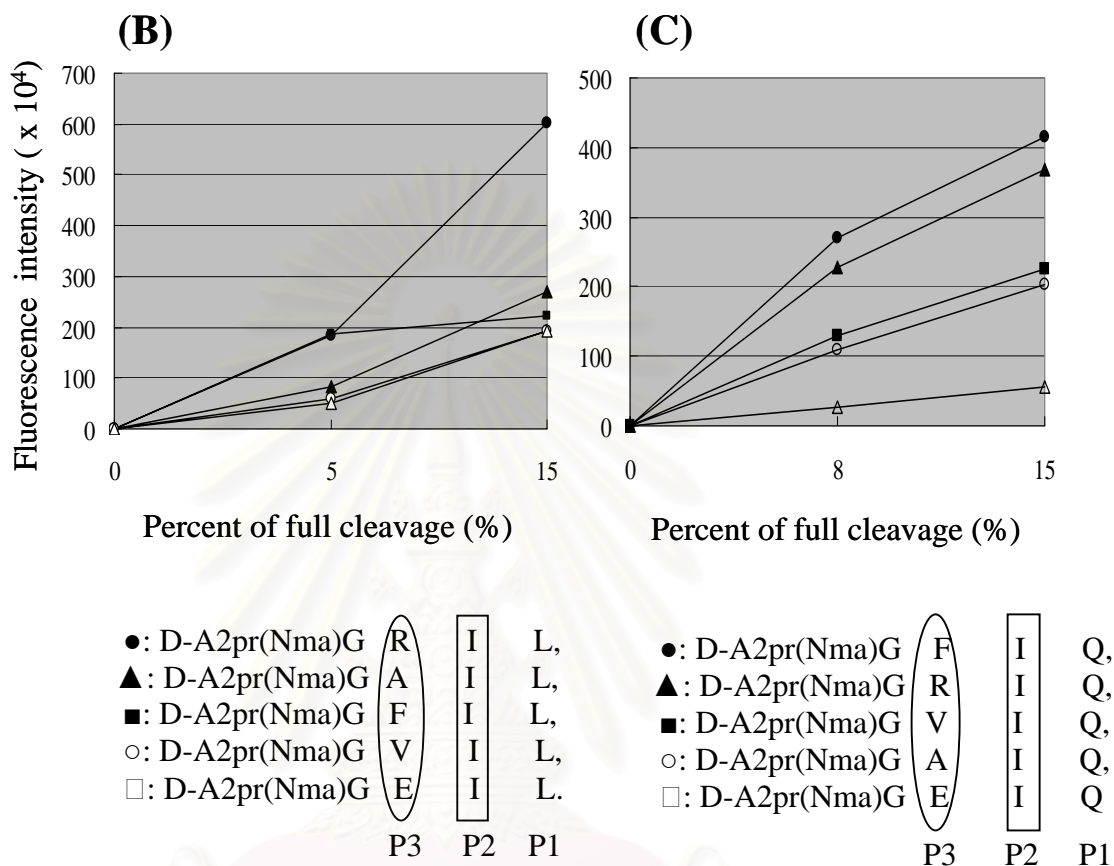
Figure 35B and 36C. The best two Xaa-containing substrate, FRETS-25L and FRET-25Q were used for the second screening of the P_2 and P_3 preferences. The cleaved products were subjected to LC-MS analysis. The chemical structure of the cleaved products was identified from the theoretical molecular weights.

Similar substrate specificity with slightly different preference at the P_1 (Phe, Met, and Thr) was observed in the halophilic protease from *Filobacillus* sp. RF2-5 isolated from fish sauce in Thailand (Hiraga *et al*., 2005)

The enzymatic properties of the SR5-3 protease were compared with a 36.9 kDa-serine protease from *Bacillus* sp. Ak.1, which showed the highest identity in the primary sequence among the proteases reported so far. Some differences were observed in their properties. For example, optimal pH and temperature of the SR5-3 protease were pH 10.0 and 50 °C using MCA-substrate in the presence of 20% NaCl and without Ca^{2+} ion, while the optimal pH and temperature of the protease from Bacillus sp. Ak.1 were pH 7.5 and 75 °C in the presence of 5 mM Ca^{2+}. The SR5-3 protease almost lost its activity after 2 h at 70 °C under the presence of 2 mM Ca^{2+} and 20% NaCl, whereas the protease from the Ak.1 retained 50% of the initial activity after 40 h at 75 °C in the presence of 5 mM Ca^{2+}. The SR5-3 protease was less thermostable than the Ak.1 protease.

Thus, a unique protease from a moderately halophilic *Halobacillus* sp. SR5-3 isolated from fish sauce made in Thailand was successfully and characterized. The uniqueness of the enzyme was: 1) the activity of the enzyme was enhanced about 2.5-fold by the addition of 20-35% NaCl, and the enzyme was highly stabilized by NaCl, and 2) the enzyme preferred Ile at the P_2 positions of substrates.

The protease described here, may be involved in the degradation of fish protein during fermentation at high salt concentration. In the future, the fermentation period of fish sauce (usually it takes about one year for fermentation) might be reduced by the addition of such moderately halophilic proteases from bacteria as *Halobacillus* sp. SR5-3 and *Filobacillus* sp. RF2-5, and extremely halophilic proteases.

CHAPTER V

CONCLUSION

In the course of investigation of halophilic bacteria presented in fish sauce (nam-pla), we have isolated 40 bacterial isolates and characterized them taxonomically. Twenty-seven isolates of rods and sixteen isolates of cocci were divided into eight groups based on their phenotypic and chemotaxonomic characteristics, DNA-DNA similarity and 16S rRNA sequencing. Group I (3 isolates), II (5 isolates), IV (1 isolate) and V (1 isolate) were cream, spore-forming moderate halophiles. Group I and II isolates contained anteiso-$C_{15:0}$ and phosphoglycerol (PG) including diphosphoglycerol (DPG) as the major fatty acid and polar lipids and MK-7 as major menaquinone. Group I isolate grew in a range from 3 to 25% NaCl and at 10 and 40 °C. They produced acid form cellobiose, D-fructose, D-glucose, glycerol, D-mannose, D-ribose, salicin and D-xylose, but not sucrose. They did not hydrolyzed tyrosine. Group I isolates were identified as *L. salicampi*. Group II isolates were non-motile and grew in a range from 3 to 30% NaCl. The maximum temperature for growth was 45 °C. They produced acid from D-glucose, glycerol, D-ribose, sucrose and D-xylose, but not cellobiose, D-fructose, D-mannose and salicin. These isolates were a new species of the genus *Lentibacillus*, for which a name *Lentibacillus juripiscarius* sp. nov. was proposed.

Group III isolates were Gram-negative, strictly aerobic, motile, non spore-forming rods. Hydrolysis of starch was positive but gelatin, casein and Tween 80 were negative. Acid is produced from several sugars such as arabinose, glucose and galactose. The DNA G+C content ranged from 63.1 to 63.5 mol% and contained *meso*-diaminopimelic acid. The major fatty acids were cyclo $C_{19:0}$ ω8c and $C_{16:0}$. They contained PG, DPG, phosphatidyl ethanolamine (PE) as major polar lipids. Group III isolates were identified as *Chromohalobacter salexigens*.

Group IV isolate (SR5-3) was strictly aerobic, white to cream yellow, motile short-rods. It produced from L-arabinose, D-fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, trehalose, and xylose and hydrolyzed gelatin,

tyrosine, and DNA. Urease was positive. Major polar lipids were PG, DPG. The G+C contents were 40.3 mol%. Isolate SR5-3 was a new species of *Halobacillus*

Group V isolate (RF2-5) was aerobic, spore-forming, motile and rods. It produced acid from D-fructose, D-galactose, D-glucose, sucrose, trehalose and xylose and hydrolyzed casein and gelatin. The peptidoglycan contained *meso*-DAP. The DNA G+C content was 36.5 mol%. It shared almost chemotaxonomic characteristics with *F. milosensis* JCM 12288[T] but differed in several phenotypic properties and cell-wall type. Group V isolate should be identified as a novel genus or species.

Group VI (10 isolates), Group VII (1 isolate) and Group VIII (15 isolates) were non spore forming extreme halophiles. Group VI isolates were rods, bright red in color and strictly aerobic. Cells of them lysed in distilled water. Polar lipids consisted of C_{20}–C_{25} derivatives of PGS, Me-PGP, PG and glycolipids (S-TGD-1, S-TeGD and TGD). Group VI isolates were identified as *H. salinarum*.

Group VII isolate was non-motile rods. Colonies was red. It did not grow in medium containing <20% NaCl. Hydrolysis of casein was positive. Acidification of the media was weak or not detectable. The polar lipids were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of PG and PGP-Me. Group VII isolate was identified as *H. saccharolyticus.*

Group VIII isolates were non-motile, aerobic cocci. Colonies were red in color. They produced acid from cellobiose, glucose, lactose, mannitol, raffinose and trehalose and utilized glutamic acid as single energy source. The maximum temperature for growth was 45°C. The diether lipids present were the diphytanyl derivative C_{20}:C20 and the phytanyl sesterterpanyl derivative C_{20}:C_{25}. PG and PGP-Me and unidentified glycolipids were present in greatest abundance and minor components. DNA G+C content ranged from 60.2 to 61.3 mol%. *Halococcus thailandensis* sp. nov. was proposed for this Group.

Among 40 isolates, Group IV (SR5-3) produced the highest clearzone on the skim milk plate and showed protease activity (0.29 U/ml) in the presence of 10% NaCl. The production of protease of this strain was completely dependent on the presence of amino acids such as glutamic acid, aspartic acid and glycine but not on the presence of sugar in JCM No. 377 medium. The purified enzyme was succeeded by one step of affinity chromatography, Bacitracin-Sepharose. Approximately 49 kDa of the purified protease was chymotrypsin-type or subtilisin-type. Its protease was highly stable in the presence of

20-35% NaCl. The protease activity was activated about 2.5-fold by the addition of 20-35% NaCl, respectively using Suc-Ala-Ala-Phe-Pro-MCA as a substrate. The enzyme was stable over a broad pH range from 5 to 10 at 37°C for 3h. The purified protease prefered hydroprobic amino acids at P1 position (Leu, Gln and Ala) and P2 position (Ile) by using FRETS combinatorial libraries. This suggested that the S_1 and S_2 subsite of the enzyme may be large and hydrophobic in nature. Therefore, during the protein hydrolysis, the purified protease from SR5-3 isolate was responsible for protein degradation in fish sauce fermentation when the pH drops to the acidic region during long term fermentation. According to the traditional fish sauce fermentation, the rate of production depends only on the activity of enzymes in the fish. Hence, rate of hydrolysis of fish proteins may be accelerated when the purified protease from *Halobacillus* sp. SR5-3 is added in the fish sauce fermentation.

Overall, this study suggested the occurrence of a variety of moderately and extremely halophilic bacteria at different stage during fish sauce fermentation. Protease-producing halophilic bacteria, *Lentibacillus juripiscarius*, *Halobacterium salinarum*, *Halobacillus* and *Filobacillus* isolates were predominantly found in the early stage of fermentation before gradually disappeared after 60 days of fermentation. Corresponding with the highest rate and extent of proteolysis, these organisms and their proteases should play an important role on fish protein liquefaction and the development of fish sauce characteristics in a subsequent stage. The non protease producing halophilic bacteria such as *Chromohalobacter salexigens*, *Lentibacillus salicampi, Halococcus saccharolyticus* isolates and *Halococcus thailandensis* were commonly found in the later stage. Although their contributions on the protein degradation were not evident, they could play a role on flavor development during ripening process. The findings in this study suggest the possibility to exploit these halophiles, both protease producing and non protease producing isolates, for future improvement of fish sauce fermentation.

REFERENCES

Amoozegar, M. A., Malekzadeh, F., Malik, K. A., Schumann, P., and Sproer, C. 2003. *Halobacillus karajensis* sp. nov., a novel moderate halophile. Int. J. Syst. Evol. Microbiol. 53: 1059–1063.

Aral, D. R., Teresa Garcia. M., Ludwig, W., Schleifer, K. H., and Ventosa, A. 2001a. Transfer of *Halomonas canadensis* and *Halomonas israelensis* to the genus *Chromohalobacter* as *Chromohalobacter canadensis* comb. nov. and *Chromohalobacter israelensis* comb. nov. Int. J. Syst. Evol. Microbiol. 51: 1443-1448.

Aral, D. R., Teresa Garcia. M., Vargas, C., Canovas, D., Nieto, J. J., and Ventosa, A. 2001b. *Chromohalobacter salexigens* sp. nov., a moderately halophilic species includes *Halomonas elongata* DSM 3043[T] and ATCC 33174[T]. Int. J. Syst. Evol. Microbiol. 51: 1456-1462.

Argos, P. 1987. A sensitive procedure to compare amino acid sequences. J. Mol. Biol. 193: 385-396.

Barett, A. J. 1994. Proteolytic enzymes: serine and cysteine peptidase. Method Enzymol. 244: 1-15.

Barrow, G. I., and Feltham, R. K. A. 1993**.** Cowan and Steel's manual for the identification of medical bacteria, 3rd edn. Cambridge: Cambridge University Press.

Burg, V. D. 2003. Extremophiles as a source for novel enzymes. Curr. Opin. Microbiol. 6: 213–218.

Capiralla, H., Hiroi, T., Hirokawa, T., and Maeda, S. 2002. Purification and characterization of a hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. Process Biochem. 38: 571-579.

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

Denner, E. B. M., McGenity, T. J., Busse, H.-J., Grant, W. D., Wanner, G., and Stan-Lotter, H. 1994. *Halococcus salifodinae* sp. nov., an archaeal isolate from an Austrian salt mine. Int. J. Syst. Bacteriol. 44: 774-780.

Duong Van Qua, Shimidu, U., and Taga, N. 1981. Purification and some properties of halophilic protease produced by a moderately halophilic marine *Pseudomonas* sp. Can. J. Microbiol. 27: 505-510.

Elazari-Volcan, B. 1957. Genus XII. *Halobacterium* Elazari-Volcani, 1940. In: Breed RS, Murray EGD, Smith NR (eds). Bergey's Manual of Determinative Bacteriology, 7th ed. Baltimore: The Williams & Wilkins Company, pp 207–212.

Ezaki, T., Hashimoto, Y., and Yabuuchi, E. 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39: 224-229.

Fitzgerald, P. M. D., Mckeever, B. M., Van Middlesworth, J. F., Springer, J. P., Heimbach, J. C., Chin-Tai Leu, Herber, K. W., Dixon, R. A. F., and Darke, P. L. 1990. Crystallographic analysis of a complex between human immunodeficiency virus type 1 protease and acetyl-pepstatin at 2.0 A resolution. J. Biol. Chem. 265: 14209-14219.

FDA, 1998. FDA and EPA guidance levels. In: Fish and Fishery Products Hazards and Controls Guide, 2nd Edition. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC, pp. 245-248, Appendix 5.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 783-791.

Flannery, W. L. 1956. Current status of knowledge of halophilic bacteria. Bacteriol. Rev. 20: 49-66.

Forbes, L. 1981. Rapid flagella stain. J. Clin. Microbiol. 3: 362-634.

Gimenez, M. I., Studdert, C. A., Sanchez, J. J. and De Castro, R. E. 2000. Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. Extremophiles. 4: 181-188.

Godfrey, T. and West, S. 1996. Industrial enzymology, 2nd ed, Macmilan Press, London, pp. 1-8.

Grant, W. D., and Larsen, H. 1990. Extremely halophilic archaeobacteria, order Halobacteriales ord.nov., In Bergey's Manual of Systematic Bacteriology, vol.3. eds. Staley, J.T., Bryant, M.P., Pfenning, N., and Holt, J.G. Baltimore, Md.: Williams & Wilkins. pp.2216-2233.

Gruber, C., Andrea Legat, A., Pfaffenhuemer, M., Radax, C., Weidler, G., Busse, H-J., and Stan-Lotter, H. 2004. *Halobacterium noricense* sp. nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of *Halobacterium* sp. NRC-1 as a strain of *H. salinarum* and emended description of *H. salinarum*. Extremophiles. 8:431–439.

Guzzo, J., Murgier, M., Filoux, A., and Lazdunski, A. 1990. Cloning of the *Pseudomonas aeroginosa* alkaline protease gene and secretion of the protease into the medium by *Escherichia coli*. J. Bacteriol. 172: 942-948.

Hiraga, K., Nishikata, Y., Namwong, S., Tanasupawat, S., Takada, T., and Oda, K. 2005. Purification and characterization of serine proteinase from halophilic bacterium, *Filobacillus* sp. RF2-5. Biosci. Biotechnol. Biochem. 69: 38-44.

Hiraga, K., Suzuki, T., and Oda, K. 2000. A novel Double-headed proteinaceous inhibitor for metalloproteinase and serineprotease. J. Biol. Chem. 275(33): 25173-25179.

Krung Thai Bank. 2005. Industry rating information: Fish sauce (Online). Available: http://www.cb.ktb.co.th/prod/brnew.nsf/0/15e7339cbed7df3e47256fd3002e3994?OpenDocument&Click= [2005, October 21]

Holt, J. G., Krieg, N. R., Sneath, P-H, A., Staley, J. T. and Williams, S. T. 1994. Bergey's Manual of Determinative Bacteriology, ninth edition. Extremely halophilic, aerobic archaeobacteria (Halobacteria). eds. Hensyl, W. R. pp. 739-746. Baltimore, Md.: Williams & Wilkins.

Hucker, G. J., and Conn, H. J. 1923. Method of Gram staining. Tech. Bull. N.Y. St Agric. Exp. Sta. No. 93: 3-37.

Izotova, L. S., Strongin, A. Y., Chekulaeva, L. N., Sterkin, V. E., Ostoslavskaya, V. I. Lyublinskaya, L. A., Timokhina, E. A., and Stepanov, V. M. 1983. Purification and properties of serine protease from *Halobacterium halobium*. J. Bacteriol. 155: 826-830.

Jeon, C. O., Lim, J.–M, Lee, G. S., Lee, J.–M., Xu, L.–H., Jiang, C.-L., and Kim, C.-J. 2005. *Lentibacillus salarius* sp. nov., isolated from saline sediment in China and emended description of genus *Lentibacillus*. Int. J. Syst. Evol. Microbiol. 55: 1339-1343.

Kamekura, M., and Seno, Y. 1993. Partial sequence of the gene for a serine protease from a halophilic archaeum *Haloferax mediterranei* R4, and nucleotide sequence of 16S rRNA encoding genes from several halophilic archaea. Experimentia. 49: 503-513.

Kamekura, M., Seno, Y., and Dyall-Smith, M. 1994. Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei*: gene cloning, expression and structural studies. Biochimica Biophysica Acta. 1294: 159-167.

Kämpfer, P., and Kroppenstedt, R. M. 1996. Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol. 42: 989-1005.

Kim, J., and Dordick, J. S. 1997. Unusual salt and solvent dependence of a protease from an extreme halophile. Biotech. Bioeng. 55:471-479.

Komagata, K., and Suzuki, K-I. 1987. Lipids and cell-wall analysis in bacterial systematics. Method Microbiol. 19: 161-203.

Kumar, S., Tamura, K., Jakobson, I. B., and Nei, M. 2001. MEGA 2: Molecular Evolution Analysis software. Bioinformatics. 17: 1244-1245.

Kushner, D. J. 1978. Life in high salt and solute concentration: halphilic bacteria. In Kushner, D. J. (Eds). Microbial Life in Extreme Environment. Academic Press, New York, pp. 317-368.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680– 685.

Leifson, E. 1963. Determination of carbohydrate metabolism of marine bacteria. J. Bacteriol. 85: 1183-1184.

Lama, L., Romano, I., Calanddrelli, V., Nicolaus. B., and Gambacrota, A. 2005. Purification and characterization of a protease produced by an aerobic haloalkaliphilic species belonging to the *Salinivibrio* genus. Res. Microbiol. 156: 478-484.

Lim, J-M., Jeon, C. O., Song, S. M., Lee, J-C., Ju, Y. J., Xu, L. H., Jiang, C–L., and Kim, C–J. 2005. *Lentibacillus lacisalsi* sp. nov., a moderately halophilic bacterium isolated from a saline lake in China. Int. J. Syst. Evol. Microbiol. 55: 1805-1809.

Lopetcharat, K., Choi, Y. J., Park, J. W. and Daeschel, M. A. 2001. Fish sauce products and manufacturing: a review. Food Rev. Int. 17: 68-88.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Maciver, B., Mchale, R. H., Saul, D, J., and Bergquist, P. L. 1994. Cloning and sequencing of a serine proteinase gene from a thermophilic *Bacillus* species and its expression in *Escherichia coli*. Appl. Environ. Microbiol. 60: 3981-3988.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 232: 10035-10038.

Martin, D. D., Ciulla, R. A., and Rorberts, M. F. 1999. Osmoadaptation in Archaea. Appl. Environ. Microbiol. 65: 1815–1825.

Minnikin, D. E., O' Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A., and Parlett, J. H. 1984. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipid. J. Microbiol. Methods. 2: 233-241.

Mermelstein, L. D., and Zeikus, J. G. 1998. in: Edited by K. Horikoshi, W.D. Grant (Eds.) Extremophiles. Microbial life in extreme environments, Wiley-Liss, New York, 1998.

Montalvo-Rodriguez, R., Lopez-Garriga, J., Vreeland, R. H., Oren, A., Ventosa, A., and Masahiro Kamekura, M. 2000. *Haloterrigena thermotolerans* sp. nov., a halophilic archaeon from Puerto Rico. Int. J. Syst. Evol. Microbiol. 50: 1065–1071.

Montero, C. G., Ventosa, A., Rodríguez-Valera, F., Kates, M., Moldoveanu, N., and Ruiz-Berraquero, F., 1989. *Halococcus saccharolyticus* sp. nov., a new species of extremely halophilic non-alkaliphilic cocci. Int. J. Syst. Bacteriol. 12: 167-171.

Nieto, J. J., Vargas, C., and Ventosa, A. 2000. Osmoprotection mechanisms in the moderately halophilic bacterium *Halomonas elongata*. Rec. Res. Dev. Microbiol. 4: 43-45.

Norberg, P. and Hofsten, B. V. 1969. Proteolytic enzyme from extremely halophilic bacteria. J. Gen. Microbiol. 55: 251-256.

Oda K., and Murao, S. 1974. Purification and some properties of acid proteinase A and B of *Scytalidium lignicolum* ATCC 24568[T]. Agric. Biol. Chem. 38: 2435-2444.

Oda, K., Takahashi, T., Takada, K., Tsunemi, M., Ng, K. K., Hiraga, K. and Harada, S. 2005. Exploring the subsite-structure of vimelysin and thermolysin using FRETS-libraries. FEBS. Lett. 579: 5013-5018.

Oren, A. 2002. Halophilic microorganisms and their environments; cellular origin and life in extreme habitats. Boston Kluwer Academic Publishers.

Oren, A., Antonio Ventosa, A., Gutierrez, M. C., and Kamekura, M. 1999. *Haloarcula quadrata* sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). Int. J. Syst. Evol. Microbiol. 49: 1149–1155.

Oren, A., Gurevich, P., Renia, T. Gemmell, R. T., and Teske, A. 1995. *Halobaculum gomorrense* gen. nov., sp. nov., a novel extremely halophilic archaeon from the dead sea. Int. J. Syst. Evol. Microbiol. 45: 747–754.

Oren, A., Ventosa, A., and Grant, W. D. 1997. Proposed minimal standards for description of New taxa in the order *Halobacteriales*. Int. J. Syst. Evol. Microbiol. 47: 233-238.

Park, J-N., Fukumoto, Y., Fujita, E., Tanaka, T., Washio, T., Otsuka, S., Shimizu, T., Watanabe, K., and Abe, H. 2001. Chemical composition of fish sauces produced in southeast and east asian countries. J. Food Compos. Anal. 14: 113-125.

Rawling, N. D., and Barret, A. J. 1993. Evolutionary families of peptidases. Biochem. J. 290: 205-218.

Roebler, M., and Muller, V. 2001. Osmoadaptation in bacteria and archaea: common principles and differences. Environ. Microbiol. 3(12): 743-754.

Ryu, K., Kim, J. and Dordick, J. S. 1994. Catalytic properties and potential of an extracellular protease from an extreme halophile. Enzyme Microbiol. Technol. 16: 266-275.

Saisithi, P. 1994. Traditional fermented fish: fish sauce production. In: fisheries processing: biotechnological application. Martin, A. M. (ed). Chapman & Hall, London. pp. 111-131

Saito, H., and Miura, K. 1963. Preparation of transforming deoxyribonucleic acid for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Saitou, N., and Nei, M. 1987. The neighboring-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sanchez-Porro, C., Mellodo, E., Bertoldo, C., Antranikian, G. and Ventosa, A. 2003. Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudalteromonas* sp. strain CP76. Extremophiles. 7: 221-228.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101. Newark, DE: MIDI.

Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Volker, H., and Thomm, M. 2001. *Filobacillus milensis* gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu-type peptidoglycan. Int. J. Syst. Evol. Microbiol. 51: 425-431.

Schmitt. W., Rdest, U., and Goebel, W. 1990. Efficient high-performance liquid chromatographic system for the purification of a halobacterium serine protease. J. Chromatography. 521: 211-220.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.

Spring, S., Ludwig, W., Marquez, M. C., Ventosa, A., and Schleifer, K. H. 1996. *Halobacillus* gen. nov., with descriptions of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of Sporosarcina halophila to *Halobacillus halophilus* comb. nov. Int. J. Syst. Bacteriol. 46: 492–496.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Mora, R. R., Swings, J., Trüper, H. G., Vauterin, L., Ward, A. C., and Whitman, W. B. 2002. Report of the Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology. Int. J. Syst. Evol. Microbiol. 52: 1043-1047.

Stackebrandt, E., and Goebel, B. M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rDNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

Stan-Lotter, H., Pfaffenhuemer, M., Legat, A.., Busse, H-J., Christian Radax, C., and Gruber, C. 2002**.** *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. Int. J. Syst. Evol. Microbiol. 52, 1807–1814.

Stepanov, V. M., Rudenskaya, G. N., Revina, L. P., Gryaznova, Y.B., Lysogorskaya, E. N., Filippova, I. Y. and Ivanova, I. I. 1992. A serine protease of an archaebacterium, *Halobacterium maditerranei.* Biochem. J. 285: 281-286.

Studdert, C. A., De Castro, R. E., Seitz, K. H. and Sanchez, J. J. 1997. Detection and preliminary characterization of extracellular proteolytic activities of the haloalkaliphilic archaeon *Natronococcus occultus.* Arch. Microbiol. 168: 532-535.

Tamaoka, J. 1994. Determination of DNA Base Composition. In M. Goodfellow, and A. G. O'Donnell (eds.), Chemical Methods in Prokaryotic Systematics, pp. 463-470. Chichester: John Wiley and Sons.

Tamaoka, J., and Komagata, K. 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. 25: 25-128.

Tanskul, S., Oda, K., Oyama, H., Noparatnaraporn, N., Tsunemi, M., and Takada, K. 2003. Substrate specificity of alkaline serine proteinase isolated from photosynthetic bacterium, *Rubrivivax gelatinosus* KDDS1. Biochem. Biophys. Res. Commun. 309: 547-551.

Tanasupawat, S., Shida, O., Okada, S., and Komagata, K. 2000. *Lactobacillus acidipiscis* sp. nov., and *Weissella thailandensis* sp. nov., islated from fermented fish in Thailand. Int. Syst. Evol. Microbiol. 50: 1479-1485.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. Clustal W : improving the sensiyivty of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids Res. 22: 4673-4680.

Thongsanit , J., Tanasupawat, S, Keeratipibul, S. and Jatikavanich, S. 2002. Characteristics and identification of *Tetragenococcus halophilus* and *Tetragenococcus mariaticus* strains from fish sauce (Nam-Pla). J. lactic acid bacteria. 13(1): 46-52.

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

Ventosa, A., Gutierrez, M. C., Garcia, T., and Ruiz-Berraquero, F. 1989. Classification of *Chromohalobacter marismortui* in a new genus, *Chromohalobacter* gen. nov., as *Chromohalobacterium marismortui* comb. nov., nom. rev. Int. J. Syst. Evol. Microbiol. 39: 382-386.

Ventosa, A., Nieto, J. J. and Oren, A. 1998. Biology of moderately aerobic bacteria. Microbiol. Mol. Biol. Reveiws. 504-544.

Vreeland, E. H. and Hochstein, L. 1992. The ecology of halophilic bacteria. eds. Vreeland, E. H. and Hochstein, L. pp. 26-79, Boca Raton, Florida : CRC Press.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, A. D., Kandler, O., Hrichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Truper, H. G. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. 37: 463-464.

Yoon, J-H., Kang, K. H., Tae-Kwang Oh, T-K., Park, Y-H. 2004. *Halobacillus locisalis* sp. nov., a halophilic bacterium isolated from a marine solar saltern of the Yellow Sea in Korea. Int. J. Syst. Evol. Microbiol. 8: 23–28.

Yoon, J. –H., Kang, K. H., and Park, Y.–H. 2002. *Lentibacillus salicampi* gen. nov., sp. nov., a moderlately halophilic bacterium isolated from a salt field in Korea. Int. J. Syst. Evol. Microbiol. 52: 2043-2048.

APPENDICES

APPENDIX A

Culture Media

All media were dispensed and steriled in autoclave for 15 min at 15 pounds pressure (121 ℃) except for acid from carbon sources test which was sterilized at 10 pounds for (110 ℃) 10 min.

1. Halobacterium medium JCM No. 168

yeast extract	5	g
Casamino acid	5	g
Sodium glutamate	1	g
Tri-sodium citrate	3	g
$MgSO_4.7H_2O$	20	g
KCl	2	g
NaCl	200	g
$FeCl_2.4H_2O$	0.362	g
$MnCl_2.4H_2O$	0.0362	g
Agar	20	g
Distilled water	1000	ml

Adjust pH 7.2 with NaOH

2. Halobacterium medium JCM No. 169

yeast extract	10	g
Casamino acid	7.5	g
Tri-sodium citrate	3	g
$MgSO_4.7H_2O$	20	g
KCl	2	g
NaCl	250	g
$FeSO_4.4H_2O$	0.05	g
$MnSO_4.4H_2O$	0.2	g
Agar	20	g

Distilled water	1000	ml
Adjust pH 7.2 with NaOH		

3. Halobacterium medium JCM No. 377

yeast extract	5	g
Casamino acid	5	g
Sodium glutamate	1	g
Tri-sodium 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	1000	ml
Adjust pH 7.2 with NaOH		

4. Marine oxidation-fermentation medium (MOF)

Casitone(Difco)	1	g
Yeast extract	0.1	g
Ammonium sulfate	0.5	g
Tris buffer	0.5	g
NaCl	6.5 หรือ 16.5	g
Phenol red 0.001%(1.0 ml of0.1%aqueous per 100 ml of medium)		
Artificial sea water	1000	ml
Adjusted pH to 7.5		

5. L-arginine agar medium

Peptone	1.0	g
NaCl	100 or 200	g
K_2HPO_4	0.3	g
Phenol red,1.0% aq.solution	1.0	ml
L(+)arginine hydrochloride	10.0	g

Agar	3.0	g
Distilled water	1000	ml

Dissolve the solids in the water, adjust to pH 7.2 , distribute into tubes or screw-capped (6mm) bottles to a depth of about 16 mm(3.5ml).

6. Aesculin broth

Aesculin	1	g
Ferric citrate	0.5	g
NaCl	100 or 200	g
Peptone water	1000	ml

Adjust pH 7.4

Dissolve the aesculin and iron salt in the peptone water and sterilized at 115 ºC for 10 min.

7. Casein agar

JCM NO. 169 or 377 agar medium (omitted casamino acid)

Skim milk	1% (w/v)

8. Gelatin agar

JCM NO. 169 or 377 agar medium omitted casamino acid

Gelatin	10% (w/v)

Dissolve and adjust pH 7.2.

9. Starch agar

JCM NO. 169 or 377 agar medium

Starch	10% (w/v)

Dissolve and adjust pH 7.2.

10. Tyrosine agar

JCM NO. 169 or 377 agar medium (omitted casamino acid)

Tyrosine	50	g

Dissolve and adjust pH 7.2.

11. Deoxyribonuclease (DNase) media

DNase test agar (Difco)	42	g
Distilled water	1000	ml

Adjust pH 7.3 ± 0.2 and heat to boiling to dissolve completely.

12. Nitrate broth

Beef extract	10	g
Peptone	10	g
NaCl	100 or 200	g
Distilled water	1000	ml

Dissolve and adjusted pH to 7.2.

13. Indole test

Bactopeptone	10	g
NaCl	100 หรือ 200	g
Distilled water	1000	ml

Dissolve and adjusted pH to 7.2.

14. Tween 80 agar medium

JCM NO.169 or 377 agar medium

Tween 80	2	ml

Dissolve and adjust pH 7.2.

15. Urea agar medium

JCM NO. 169 or 377 agar medium (omitted casamino acid)

Urea	2% (w/v)

Dissolve and adjust pH 7.2.

APPENDIX B

Reagents and Buffers

1. Determination of protein and soluble peptide

The protein and soluble peptide content was measured by the method of Lowry *et al*. (1951) with bovine serum albumin and tyrosine as standard, respectively.

1.1 Reagents

A: 2% sodium carbonate in 0.1N NaOH

B: 0.5% $CuSO_4 .5H_2O$ in 1% sodium citrate

C: 1 N Folin-Ciocalteu's phenol reagent (2N Folin Phenol was diluted with distilled water to the final concentration in 1N, the solution should be freshly prepared before use.)

D: 1 ml Reagent B + 50 ml Reagent A (or similar ratio) Make up immediately before use.

1.2 Procedure

A. Place 0.1 ml of proper dilution of culture broth (for protein determination) or clear supernatant of reaction mixture (for soluble peptide determination)

B. Add 1 ml of Reagent D into the tube and vortex immediately. Incubate at room temperature for 10 min3. After the 10 min incubation, add 0.1 ml of Reagent C to sample and vortex immediately. Incubate 30 min at room temperature.

C. Absorbance (OD) of samples was measured at 750 nm. Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

1.3 Preparation of standard curve of tyrosine

Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mM were prepared from tyrosine. The reactions were carried out with the same procedure by using MCA-peptides. Fluorescence was plotted against various concentrations of standards.

1.5 Preparation of standard curve of tyrosine

Standards of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µM were prepared from stock AMC (1 mM in DMSO). The reactions were carried out with the same procedure as described previously. Absorbance was plotted against various concentrations of standards

1.4 Preparation of standard curve of protein

Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg/ml were prepared from bovine serum albumin. The reactions were carried out with the same procedure as described previously. Absorbances were plotted against concentrations of standards.

2. 6 N HCl

Conc. HCl	60	mL
Distiller water	60	mL

Add conc. HCl into the distilled water.

3. 2 N H_2SO_4

Conc. H_2SO_4	2	mL
Distilled water	34	mL

Add conc. HCl into the distilled water.

4. Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	mL

5. 5% Trichloroacetic acid

Trichloroacetic acid	5	g
Distilled water	100	mL

Add conc. HCl into the distilled water.

6. Reagent 1 for fatty acid analysis (Saponification reagent)

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

Dissolve NaOH pellets in Mili-Q water and add MeOH.

7. Reagent 2 for fatty acid analysis (Methylation reagent)

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

pH must be below 1.5.

8. Reagent 3 for fatty acid analysis (Extraction solvent)

n-Hexane (HPLC grade or n-Hexane 1000)	50	mL
Methyl-tert-Butyl Ether (HPLC grade)	50	mL

9. Reagent 4 for fatty acid analysis (base wash reagent)

Sodium hydroxide	1.2	g
Mili-Q water	100	Ml

10. **Reagent 5 for fatty acid analysis (Saturated sodium chloride)**

11. **Dittmer&Lester reagent**

   Solution A

MoO_3	4.011 g
25 N H_2SO_4	100 mL

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

   Solution B

Molybdenum powder	0.178 g
Solution A	50 mL

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

12. **Anisaldehyde reagent**

Ethanol	90	mL
H_2SO_4	5	mL
p-Anisaldehyde	5.0	mL
Acetic acid	1.0	mL

13. **Dragendorff's reagent**

   Solution A

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

Solution B

KI	40	g
Distilled water	100	mL

Before spraying, mix solution A (10 mL) plus solution B (10 mL) plus acetic acid (10 mL).

14. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

Dissolve by gentle heating in a fume hood.

***N,N*-dimethyl-1-naphthylamine solution**

N,N-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	mL

Dissolve by gentle heating in a fume hood.

Two drops of sulphanilic acid solution and three drops of *N,N*-dimethyl-1-naphthylamine into peptone nitrate broth inoculing with the test microorganisms.

15. Phenol:Chloroform (1:1 v/v)

Crystalline phenol was liquidified in water bath at 65 ˚C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle.

16. 100xDenhardt solution

Bovine serum albumin	2%
Polyvinylpyrrolidone	2%
Ficoll 400	2%

17. 0.5M EDTA (pH 8.0)

800 mL of distilled water, 186.1 g of disodium ethylenediaminetetraacetate.$2H_2O$ was added and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (20 g of NaOH pellets). The volume was adjusted to 1 litre. The solution was dispensed into aliquots and sterilized by autoclaving for 15 minutes at 15 lb/in^2.

18. 5M NaCl

To 800 mL of Distilled water, 292.2 g of sodium chloride was added and adjusted the volume to 1 litre with distilled water. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2.

19. 2xPBS

8 mM Na_2HPO_4

1.5 mM KH_2PO_4

137 mM NaCl

2.7 mM KCl

The 2xPBS was adjusted the pH to 7.0 with 1N NaOH or 1N HCL. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2.

20. 10 mg/mL Salmon sperm DNA

A 10 mg of Salmon sperm DNA was dissolved in 1 mL of 10 mM TE buffer pH 7.6. Boiling for 10 minutes, immediately cooling in ice and sonication for 3 minutes.

21. 3 M Sodium acetate pH 5.2

To 800 mL of distilled water, 408.1 g of sodium acetate was added and adjusted the pH to 5.2 with glacial acetic acid. The volume was adjusted to 1 litre. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2.

22. 10% Sodium dodecyl sulphate (SDS)

The stock solution of 10% SDS was prepared by dissolved 10 g of sodium dodecyl sulphate in 100 mL sterilized distilled water. Streilization is not required for the preparation of this stock solution.

23. 20xSSC

3 M NaCl

0.1 M Tri-sodiumcitrate

The 20xSSC was adjusted the pH to 7.0 with 1N NaOH. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2.

24. 1 M Tris-HCl pH 8.0

The 1M Tris was prepared by dissolving 121.1 g of Tris base in 800 mL of distilled water. The pH was adjusted to the desired value by adding conc. HCL (pH 8.0, 42 mL of HCl). The solution was cooled to room temperature before making final adjustment to the desired pH. The volume of the solution was adjusted to 1 litter with with distilled water and sterilized by autoclaving.

25. RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

Dissolve 20 mg of RNase A in 10 mL 0.15 M NaCl and heat at 95 ˚C for 5-10 minutes. Keep RNase A solution in -20 ˚C.

26. RNase T$_1$ solution

RNase T$_1$	80	μL
0.1 M Tris-HCl (pH 7.5)	10	mL

Mix 80 µl of RNase T$_1$ in 10 mL of 0.1 M Tris-HCl (pH 7.5) and heat at 95 ˚C for 5 minutes. Keep RNase T$_1$ solution in -20 ˚C.

27. Proteinase K

Proteinase K (Sigma)	4	mg
50 mM Tris-HCl (pH 7.5)	1	mL

Use freshly prepared solution.

28. Nuclease P$_1$ solution

Nuclease P1	0.1	mg
40 mM CH$_3$COONa+12 mM ZnSO$_4$ (pH5.3)	1	mL

Store at 4 ˚C.

29. Alkaline phosphatase solution

Alkaline phosphatase	2.4 units	
0.1 M Tris-HCl (pH 8.1)	1	mL

30. 0.1 M Tris-HCl buffer, pH 9

Tris	1.21 mg
Distilled water	100 mL

Adjust the pH to 9 with HCl.

31. TE buffer

10 mM Tris HCl (pH 8.0)

1 m M Na_2-EDTA (pH 8.0)

32. TE buffer + RNase A

TE buffer	960 mL
RNase A (2 mg/mL)	100 μL

33. Saline-Na_2 EDTA

0.1 M NaCl

50 mM EDTA.2Na (pH 8.0)

34. Reagent and buffer for DNA-DNA hybridization

34.1 Prehybridization solution

100xDenhardt solution	5	mL
10 mg/ml Salmon sperm DNA	1	mL
20xSSC	10	mL
Formamide	50	mL
Distilled water	34	mL

34.2 Hybridization solution

Prehybridization solution	100	mL
Dextran sulfate	5	g

34.3 Solution I

Bovine serum albumin (Fraction V)	0.25	g
Titron X-100	50	μL
PBS	50	mL

34.4 Solution II

Streptavidin-POD	1	μL
Solution I	4	mL

34.5 Solution III

3,3',5,5'-Tetramethylbenzidine (TMB) (10 mg/mL in DMFO)	100	μL
0.3% H_2O_2	100	μL
0.4 M Citric acid + o.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMFO	100	μL

34.6 2 M H_2SO_4

H_2SO_4	22	mL
Distilled water	178	mL

The solution was sterilized by autoclaving.

35. Ethidium bromide solution (10 mg/mL)

The ethidium bromide solution was prepared by dissolving 1 g of ethidium bromide in 100 mL of distilled water. The solution was stored in light-tight container at room temperature.

36. Gel loading buffer

0.025 g of bromophenol blue was dissolved in 20 mL of 15% glycerol.

37. Tris-acetate EDTA (TAE) buffer

1xTBE buffer was used as an electrophoresis buffer throughout the study. The working solution of 1xTBE buffer was prepared from stock solution of 5xTAE buffer, as followed.

Tris-base	5.4 g
Boric acid	2.75 g
Na_2-EDTA	0.47 g
Distilled water	100 mL

38. Agarose gel

Agarose	1.6 g
1xTBE buffer	

39. Flagella staining

Basic fuchisin	0.5 g
Tannic acid	0.2 g
Aluminium sulfate	0.5 g

Solvent was composed of a mixture of 2.0 of 95% ethanol, 0.5 ml of glucerol, and 7.5 ml of tris(hydroxymethyl)aminomethane(tris)buffer.

40. Kovacs'reagent

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

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

APPENDIX C

Primers, 16S rRNA Nucleotide sequences and chemotaxonomic characteristics

(polar lipids, quinones and fatty acid)

1.   Primers for 16S rRNA amplification and Sequencing

1.1 IS40-3

EB-10F	5'-AGTTTGATCCTGGCTC-3'
EB-1530R	5'-AAGGAGGTGATCCAGCC-3'
EB-520R	5'-ACCGCGGCTGCTGGC-3'
EB-530F	5'-GTGCCAGCAGCCGCGG-3'
EB-1100R	5'-AGGGTTGCGCTCGTTG-3'
EB-1110F	5'-GCAACGAGCGCAACCC-3'

1.2   RF2-5

9F	5'-GAGTTTGATCCTGGCTCAG-3'
1541R	5'-AAGGAGGTGATCCAGCC-3'
339F	5'-CTCCTACGGGAGGCAGCAG-3'
357R	5'-CTGCTGCCTCCCGTAG-3'
785F	5'-GGATTAGATACCCTGGTAGTC-3'
802R	5'-TACCAGGGTATCTAATCCC-3'
1099F	5'-GCAACGAGCGCAACCC-3'
1100R	5'-AGGGTTGCGCTCGTTG-3'

1.3   Archaea

D30F	5'-ATTCCGGTTCATCCTGC-3'
D56R	5'-GYTACCTTGTTACGACTT-3'
D33R	5'-TCGCGCCTGCGCCCCGT-3'
D34R	5'-GGTCTCGCTCGTTGCCTG-3'
B99R	5'-GTGTTACCGCGGCTGCTG-3'
X10R	5'-ACGGGCGGTGTGTRC-3'

2.   16S rRNA Nucleotide sequences

```
AGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCAGGTAA
TCGCCCTTCGGGGCGTGCGCCTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGG
GCAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGAT
GTCTTTCCTCGCATGAGGGAAGGCTGAAAGACGGCCTTTGTGCTGTCACTTACAGAT
GGGCCCGCGGCGCATTAGTTAGTTGGTGAGGTAAGAGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGC
GTGGTTCGAACAGGGCCATGCCTTGACGGTACCTGACCAGAAAGCCCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAATCTCGCGGCTTAACCGCG
AGCGGTCATTGGAAACTGGGAGGCTTGAGTACAGAAGAGGAGAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGTTGAGTGCTAGGTGTTAGGGGGTTTCCGCCCCTTTGT
GCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAGCGGCAGAGATGCCGTGTT
CCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGT
TGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAA
ATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGG
GAAGCGAAGCCGTGAGGTGTAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCA
GGCTGCAACTCGCCTGTATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCA
ACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTGGGGCCAATG
ATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGC
```

Figure 36. The 16S rRNA nucleotide sequence of IS40-3.

```
GGGGCTACCATGCAGTCGAGCGGTACAGGTCCAGCTTGCTGGATGCTGACGAGCGGC
GGACGGGTGAGTATGCATAGGAATCTACCCAGTCGTGGGGGATAACCTGGGGAAACC
CAGGCTAATACCGCATACGTCCTACGGGAAGAAAGCGGGGGCTCTTCGGACCTCGCG
CGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGGCTCACCAAGGC
GACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGTGGGGA
AAGAAGGCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAAAGCCCCGGGC
TCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGAGGAAGGTAGA
ATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGC
GGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGTCNACTAGCCGTTGGGTCCTTGNAG
GANTTAGTGGCGCAGTTACGCGATAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTT
AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACCCTTGACATCCTGCGAACCCGGAAGAGATTC
CGGGGTGCCTTCGGGAGCGCAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTT
GTGAAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCCTATTTGCCAGCG
ATTCGGTCGGGAACTCTAGGGNAANTGCCGGTGANAAACCGGAGGNTTGTGGGGACG
ACGTCAGTCATCATGGCCCTTACGGGTAGGGCTACACACGTGCTACAATGGCCGGTA
CAAAGGGTTGCGAAGCCGCGAGGTGAAGCCAATCCCAGAAAGCCGGCCTCAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGCATCAGA
ATGGCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGACTGCACCAGAAGTGGTTAGCCTAACTTCGGAGGGCGATCACCACGGTGTGGTT
CATGACTGGGGTGAAGTCGTAACAAGGTAACCA
```

Figure 37. The 16S rRNA nucleotide sequence of DS26-2.

```
GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGTGAAGCAGGTGGATCCC
TTCGGGGTGACACCTGTGGATCGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCT
GCCTGTAAGACTGGGATAACCCCGGGAAACCGGGGCTAATACCGGGTAATACCGGAC
TCCGCATGGAGTCTGGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGC
GGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAA
CGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTCAGGGAAGAACACGTACCGTTCG
AACAGGACGGTACCTTGACGGTACCTGACGAGGAAGCCCCGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGGAACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA
CTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTACTCGATGAGTGCTAGGTGTTAGGGGGCTTCCACCCCTTAGTGCTGAAGT
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCTTCGGCCACCCCTGGAGACAGGGGGTTCCCTTCGG
GGACCGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCCTGACCTTAGTTGCCAGCATTCAGTTGGGCACT
CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAA
GCCGCGAGGTGTAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAA
CTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGA
AGTCGGTGAGGTAACCTTTTTGGAGCCAGCCGCCGAAGGTGGGGCCAATGATTGGGG
TGAAGTCGTAA
```

Figure 38 . The 16S rRNA nucleotide sequence of SR5-3.

```
AAGCAGACTGAATCCTTCGGGAGGACGTCTGTGGAACGAGCGGCGGACGGGTGAGTA
ACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC
CGGATAACTCATCGGATCGCATGATCCGATGTTGAAAGATGGCTTCTAGCTATCACT
TACAGATGGGCCCGCGGCGCATTAGTTAGTTGGTGAGGTAACGGCTCACCAAGGCCA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCATCCGCAATGGACGAAAGTCTGACGGT
GCAACGCCGCGTGAGTGAGGAAGGCCTTCGGGTCGTAAAACTCTGTTGTTAGGGAAG
AACAAGTTCCGTTCGAATAGGGCGGAGCCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTA
TTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAATCTTGCGGCTC
AACCGCAAGCGGTCATTGGAAACTGGGGAACTTGAAGACAGAAGAGGAGAGCGGAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGG
CTCTCTGGTCTGTGCTTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCCACCC
TTAGTGCTGCAGTTAACGCAATAAGCACTCCGCCTGGGGAGTACGGGCGCAAGGGTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAGGTCTTGACATTCTCGGACCACCCTAGAGATAGG
GTCTTCCCTTCGGGGACCGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCAT
TCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCAACACACGTGCTACAATGGATGGTAC
AATGGGCTGCGAAACCGCGAGGTGAAGCAAATCCCAAAAAGCCATTCTCAGTTCGGA
TTGTAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGTGGATCAGCA
TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGT
TGGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCTAGCCGCCGAAGGTGGGAC
CAATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCA
ACCCTTNAT
```

Figure 39 . The 16S rDNA nucleotide sequence of RF2-5 .

```
TCCGATTTAGCCATGCTAGTTGTGCGGGTTTAGACCCGCAGCGGAAAGCTCAGTAAC
ACGTGGCCAAGCTACCCTGTGGACGGGAATACTCTCGGGAAACTGAGGCTAATCCCC
GATAACGCTTTGCTCCTGGAAGGGGCAAAGCCGGAAACGCTCCGGCGCCACAGGATG
CGGCTGCGGTCGATTAGGTAGACGGTGGGGTAACGGCCCACCGTGCCCATAATCGGT
ACGGGTTGTGAGAGCAAGAGCCCGGAGACGGAATCTGAGACAAGATTCCGGGCCCTA
CGGGGCGCAGCAGGCGCGAAACCTTTACACTGTACGAAAGTGCGATAAGGGGACTCC
GAGTGTGAAGGCATAGAGCCTTCACTTTTGTACACCGTAAGGTGGTGCACGAATAAG
ACTGGGCAAGACCGTGCCAGCCGCCGCGGTAATACCGGCAGTCCGAGTGATGGCCGA
TCTTATGGGCCTAAGCGTCCGTAAACTGGCTGAACAAGTCCGTTGGGAAATCTGTCC
GCTTAACGGGCAGGCGTCCAGCGGAAACTGTTCAGCTTGGGACCGGAAGACCTGAGG
GGTACGTCTGGGGTAGGAGTGAAATCCTGTAATCCTGGACGGACCGCCGGTGGCGAA
AGCGCCTCAGGAGAACGGATCCGACAGTGAGGGACGAAAGCTAGGGTCTCGAACCGG
ATTAGATACCCGGGTAGTCCTAGCTGTAAACGATGTCCGCTAGGTGTGGCGCAGGCT
ACGAGCCTGCGCTGTGCCGTAGGGAAGCCGAGAAGCGGACCGCCTGGGAAGTACGTC
TGCAAGGATGAAACTTAAAGGAATTGGCGGGGGAGCACTACAACCGGAGGAGCCTGC
GGTTTAATTGGACTCAAACGCCGGACATCTTCACCAGCCCCGACAGTAGTAATGACG
GTTCAGGTTGATGACCTTACCCGAGGCTACTGAGAGGAGGTGCATGGCCGCCGTCAG
CTCTGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCGCACTCCTAA
TTGCCAGCAGTACCCTTTGGGTAGCTGGGTACATTAGGTGGACTGCCGCTGCCAAAG
CGGAGGAAGGAACGGGCAACGGTAGGTCAGTATGCCCCGAATGGGCTGGGCAACACG
CGGGCTACAATGGTCGAGACAATGGGAAGCCACTCCGAGAGGAGGCGCTAATCTCCT
AAACTCGATCGTAGTTCGGATTGAGGGCTGAAACTCGCCCTCATGAAGCTGGATTCG
GTAGTAATCGCGTGTCAGCAGCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACC
GCCCGTCAAATCACCCGAGTGGGGTTCGGATGAGGCCGGCATGCACTGGTCAAACTG
GGCTCCGCAAGGGG
```

Figure 40 . The 16S rRNA nucleotide sequence of HDS2-5.

```
CTGCCGGAGGCTATTGCTATCGGGATCCGATTCAGTCATGCTAGTCGCACGGGCTCA
GACCCGTGGCGAATAGCTCCGTAACACGTGGTCAAACTACCCTCTGGACCGGGACAC
CCTCGGGAAACTGAGGCTAATCCTAGATACTGCTTTCATGTTGGAATACAGAAAGCC
GGAAATGGTCCGCCGCCGGAGGACGTGACTGCGGCCGATTAGGTAGACGGTGGGGTA
ACGGCCCACCGTGCCGATAATCGGTACGGGTTGTGAGAGCAAGAACCCGGAGACGGA
CTCTGAGACAAGAGTCCGGGCCCTACGGGCGCAGCAGACGCGAAACCTTTACACTG
CACGACAGTGCGATAAGGGGATCCCGAGTGCGAGGGCATACAGTCCTCGCTTTTCGT
GACCGTAAGGTGGTCTCAGAATAAGGGCTGGCTAAGACCGGTGCCAGCCGCCGCGGT
AATACCGGCAGCCAGAGTGATAGCCACTCTTATTGGCTAAAGCGTCCGTAGCTGGCC
GAACGGGTCCGTCGGGAAATCCACACGCTCAACGGGTGGGCGTCCGGCGGAAACCAG
TCGGCTTGGGACCGGAGGACCTGCGGGGTACGTCCGGGGTAGGAGTGAAATCCTGTA
ATCCCGGACGGACCGCCGGTGGCGAAAGCGCCGCAGGAGGACGGATCCGACAGTGAG
GGACGAAAGCTTGGGTCTCGAACCGGATTAGATACCCGGGTAGTCCAAGCCGTAAAC
GATGCTCGCTAGGTGTGGCGCAGGCTACGAGCCTGCGCTGTGCCGTAGGGAAGCCGA
GAAGCGAGCCGCCTGGGAAGTACGTCCGCAAGGATGAAACTTAAAGGAATTGGCGGG
GGAGCACTACAACCGGAGGAGCCTGCGGTTTAATTGGACTCAACGCCGGACATCTCA
CCGGCACCGACAATGTGCAGTGACGGTCAGTGTGATGAGCTACCTGAGCCATGAGAG
GAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACG
AGCGAGACCCGCATCCCTAATTGCCAGCAACACCCATGTGGTGGTTGGGTACATTAG
GGAGACTGCCGCTGCCAAAGCGGAGGAAGGAACGGGCAACGGTAGGTCAGTATGCCC
CGAATGTGCCGGGCTACACGCGGGCTACAATGGCCGAGACAGTGGGACGCTACCCCG
AGAGGGGACGCTAATCTCCTAACCTCGGTCGTAGTTCGGATTGCGGGTTGAAACCCA
CCCGCATGAAGCTGGATTCGGTAGTAATCGCATTTCAGAAGAGTGCGGTGAATACGT
CCCTGCTTCCTTGCACACACCGCCCGTCAAAGCACCCGAGTGAGGTCCGGATGAGGT
CGACGCAA
```

Figure 41 . The 16S rDNA nucleotide sequence of HRF6**.**

```
ATTCCGGTTGATCCTGCCGGAGGCGATTGCTATCGAAGTCCGATTCACCCATGCTAG
TTGTACGGGTTTAGACCCGTAGCAAATAGCTCCGTAACACGTGGTCAAACTACCCTC
TGGACCGGGATACCCTCGGGAAACTGAGGCCAATCCCAGATACTGCTTTCATGTTGG
AATACAGAAAGTCGGAAACGGTCCGCCGCCGGAGGACGTGACTGCGGCCGATTAGGT
AGACGGTGGGGTAACGGCCCACCGTGCCGATAATCGGTACGGGTTGTGAGAGCAAGA
ACCCGGAGACGGTATTTGAGACAAGATACCGGGCCCTACGGGCGCAGCAGGCGCGA
AACCTTTACACTGCACGCCAGTGCGATAAGGGGACCCCGAGTGCGAGGGCATACAGT
CCTCGCTTTTCGTGACCGTAAGAAGGTCTCAGAATAAGAGCTGGGCAAGACCGGTGC
CAGCCGCCGCGGTAATACCGGCAGCTCGAGTGATAGCCACTATTATTGGGCCTAAAG
CGTCCGTAGCCGGCCGAACGGGTCCGTCGGGAAATCCACCCGCTCAACAGGTGGACG
TCCGGCGGAAACCAGTCGGCTTGGGGCCGGGAGACCAGAAAGGTACGTCCGGGGTAG
GAGTGAAATCCCGTAATCCTGGACGGACCGCCGGTAGCGAAAGCGTCTCTGGAGAAC
GGACCCGACGGTGAGGGACGAAAGCTTGGGTCTCGAACCGGATTAGATACCCGGGTA
GTCCAAGCTGTAAACGATGCTCGCTAGGTGTGGCGTTGGCTACGAGCCAGCGCTGTG
CCGTAGGGAAGCCGAGAAGCGAGCCGCCTGGGAAGTACGTCCGCAAGGATGAAACTT
AAAGGAATTGGCGGGGGAGCACTACAACCGGAGGAGCCTGCGGTTTAATTGGACTCA
ACGCCGGACATCTCACCGGCACCGACAGTGTGCAGTGACAGTCAGTCTGATGGGCTT
ACTTGAGCCACTGAGAGGAGGTGCATGGCCGCCGTCAACTCGTACCGTGAgGCGTCC
TGTTAAGTCAGGCAACGAGCGAGACCCGCGTCCTTAATTGCCAGCAGCAGCCTTGTG
CTGGCTGGGTACATTAAGGAGACTGCCGTCGCTAAGACGGAGGAAGGAACGGGCAAC
GGTAGGTCAGTATGCCCCGAATGTGCCGGGCGACACGCGGGCTACAATGGCCGAGAC
AGTGGGACGCTACCCCGAGAGGGGGCGCTAATCTCCTAACCTCGGTCGTAGTTCGGA
TTGCGGGTTGAAACCCACCCGCATGAAGCTGGATTCGGTAGTAATCGCATTTCAGAA
GAGTGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCAAATCACCCGAGT
GAGGTCCGGATGAGGC
```

Figure 42. The 16S rDNA nucleotide sequence of HDB5-2.

```
ATTGCTATCGGGGTCCGATTCAGCCATGCTAGTTGTACGGGTTTAGACCCGTAGCAA
ATAGCTCCGTAACACGTGGTCAAACTACCCTCTGGACCGGAATACCCTCGGGAAACT
GAGGCTAATTCCGGATACTGCTTTCATGTTGGAACACAGAAAGTCGGAAACGGTCCG
CCGCCGGAGGACGTGACTGCGGCCGATTAGGTAGACGGTGGGGTAACGGCCCACCGT
GCCGATAATCGGTACGGGTTGTGAGAGCAAGAACCCGGAGACGGTATCTGAGACAAG
ATACCGGGCCCTACGGGGCGCANCAGGCGCGAAACCTTTACACTGCACGCCAGTGCG
ATAAGGGGACCCCGAGTGCGAGGGCATACAGTCCTCGCTTTTCGTGACCGTAAGAAG
GTCTCAGAATAAGAGCTGGGCAAGACCGGTGCCAGCCGCCGCGGTAATACCGGCAGC
TCGAGTGATAGCCACTATTATTGGGCTTAAAGCGTCCGTAGCCGGCCGAACAGGTCC
GTCTGGAAATCCACCCGCTCAACGGGTGGACGTCCGGCGGAAACCAGTCGGCTTGGG
GCCGGGAGACCAGAGAGGTACGTCCGGGGTAGGAGTGAAATCCCGTAATCCTGGACG
GACCGCCGGTAGCGAAAGCGTNTCTGGAGAACGGACCCGACGGTGAGGGACGAAAGC
TTGGGTCTCGAACCGGATTAGATACCCGGGTAGTCCAAGCTGTAAACGATGCTCGCT
AGGTGTGGCGTTGGCTACGAGCCAGCGCTGTGCCGTAGGGAAGCCGAGAAGCGAGCC
GCCTGGGAAGTACGTCCGCAAGGATGAAACTTAAAGGAATTGGCGGGGGAGCACTAC
AACCGGAGGAGCTTGCGGTTTAATTGGACTCAACGCCGGACATCTCACCGGCACCGA
CAGTGTGCAGTGACAGTCAGTCTGATGGGCTTACTTGAGCCACTGAGAGGAGGTGCA
TGGCCGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGAC
CCGCGTCCCTAATTGCCAGCAGCAGCCTTGTGCTGGCTGGGTACATTAGGGAGACTG
CCGTCGCTAAGACGGAGGAAGGAACGGGCAACGGTAGGTCAGTATGCCCCGAATGTG
CCGGGCGACACGCGGGCTACAATGGCCGAGACAGTGGGACGCTACCCCGAAAGGGGA
CGCTAATCTCCTAACCTCGGTCGTAGTTCGGATTGCGGGTTGAAACCCACCCGCATG
AAGCTGGATTCGGTAGTAATCGCATTTCAGAAGAGTGCGGTGAATACGTCCCTGCTC
CTTGCACACACCGCCCGTCAAATCACCCGAGTGAGGTACCGGATGAGGCCGGCGCAA
CGCCGGT
```

Figure 43. The 16S rRNA nucleotide sequence of HIS10-2.

Table 32. Polar lipid composition and glycolic analyses of the representative strains of moderately halophilic isolates.

Isolate no.	Polar lipid type			Unidentified glycolipids
	PG	DPG	PE	
BN2-2	+	+	-	+
DB8-4	+	+	-	+
KS333-2B	+	+	-	+
IS40-3	+	+	-	+
IS40-2	+	+	-	+
IS10-5	+	+	-	+
IO-1	+	+	-	+
PS9-2	+	+	-	+
DS26-2	+	+	+	+
KS87-5	+	+	+	+
SR5-3	+	+	-	+
RF2-5	+	+	-	-

Table 33. Polar lipid composition and glycolic analyses of the representative strains of extremely halophilic isolates.

Isolate no.	Diether core lipids		Polar lipid type			Glycolipids		
	$C_{20}C_{20}$	$C_{20}C_{25}$	PG	PGP-Me	PGS	S-TGD-1	S-TeGD	TGD-1
HDS2-5	-	+	+	+	+	+	+	+
HDB1-1	-	+	+	+	+	+	+	+
HIB20-2	-	+	+	+	+	+	+	+
HRF6	+	+	+	+	-	-	-	-
HDB5-2	+	+	+	+	-	-	-	-
HDS7-4	+	+	+	+	-	-	-	-
HDS4-1	+	+	+	+	-	-	-	-
HKS333-2	+	+	+	+	-	-	-	-

Abbreviations:

PG; Phosphoglycerol   DPG; Diphosphatidylglycerol

PE; Phosphatidylethanolamine   PGS; Phosphoglycerolsulfate

S-TGD-1; Sulfated-triglycerol diether (Gal is removed from S-TeGD)

S-TeGD; (1-*O*-[β-D-galactose-(3'-SO$_3$H)-(1'→6')-α-D-mannose-(3'→1')- α-D-

Galactofuranose)- (1'→2')- α-D- Glucose]-2,3-di-*O*-phytyl-*sn*-glycerol

TGD-1; Triglycosyl diether (desulfated product of S-TDG-1)

Table 34. Quinone system of the representative halophilic isolates.

Isolate no.	Quinone profies				
	MK-6	MK-7	MK-8	MK-(H$_2$)	Ubi-9
BN2-2	2.1	9.6.8			
DB8-4	2.5	97.1			
KS333-3B	1.6	95.9			
IS40-3	2.3	96.7			
IS40-2	2.65	96.43			
IS10-5	1.51	97.86			
IO-1	2.34	96.4			
PS9-2	2.65	95.8			
DS26-2					90.1
KS87-5					92.98
RF2-5	1.61	95.18	2.69		
HDS2-5		2.3	89.23	3.95	
HDB1-1	6.1	2.84	86.1	3.77	
HIB20-2		2.97	73.37	18.9	
HRF6			4.7	93.31	
HDB5-2	2.18		5.89	84.72	3.0
HDS4-1		2.44	7.54	85.79	1.77
HKS333-2	2.57		5.57	87.53	2.84
HIS10-2			9.12	84.63	2.65
HDS7-4			11.89	83.29	2.61
HB10-5			12.52	81.17	2.13

Table 35. Fatty acid compositions of the representative halophilic isolates.

Fatty acids	% Fatty acid of representative strains										
	BN2-2	DB8-4	KS333-2B	IS40-3	IS40-2	IS10-5	I0-1	PS9-2	DS26-2	KS87-5	RF2-5
Saturated fatty acid											
$C_{9:0}$			0.12								0.17
$C_{10:0}$			0.05						5.01	26.36	0.05
$3OH\text{-}C_{10:0}$									0.31	2.11	
$C_{12:0}$			0.3					0.14	7.47	19.67	0.28
$2OH\text{-}C_{12:0}$									0.13		
$3OH\text{-}C_{12:0}$									12.17	22.62	
$C_{13:0}$											0.04
$C_{14:0}$	0.16	0.16	0.15		0.19	0.21	0.27	0.25	0.57	0.71	0.06
$C_{15:0}$	0.09	0.11	0.1	0.13	0.23	0.2	0.39	0.13			1.25
$C_{16:0}$	0.69	0.76	0.83	0.84	1.05	0.88	1.24	1.25	31.93	10.16	0.11
$C_{17:0}$					0.17	0.23	0.08		0.07		
$C_{17:0}$ CYCLO									11.93	4.39	
$C_{18:0}$					0.05		0.09	0.06	1.12		
$C_{19:0}$ CYCLO (ω8c)									23.23	3.58	
Unsaturated fatty acid											
$iC_{16:1}$							13.98				
$C_{16:1(\omega 5c)}$									0.06		
$C_{16:1(\omega 7c)}$ alcohol	0.65			0.64	3.3	1.99	0.82	0.23			4.99
$C_{16:1(\omega 11c)}$			0.05				0.15				
$C_{18:1(\omega 5c)}$									0.05		
$C_{18:1(\omega 7c)}$									3.16	2.68	
$C_{18:3(\omega 6c)}$ (6,9,12)							0.1				
$C_{18:1(\omega 9c)}$	0.04										0.08

Table 35 (continued)

Fatty acid	% Fatty acid of representative strains										
	BN2-2	DB8-4	KS333-2B	IS40-3	IS40-2	IS10-5	I0-1	PS9-2	DS26-2	KS87-5	RF2-5
Branched fatty acids											
i-$C_{11:0}$											1.0
a-$C_{11:0}$					0.05	0.08	0.09				0.05
i-$C_{12:0}$											0.19
i-$C_{13:0}$											1.59
a-$C_{13:0}$	0.07	0.06	0.07	0.1	0.10	0.16	0.27	0.06			0.14
i-$C_{14:0}$	13.36	11.18	12.16	10.16	9.53	11.06	5.01	20.72			13.47
3OH-i-$C_{14:0}$											0.69
2OH-$C_{14:0}$											0.09
i-$C_{15:0}$	3.87		2.93	4.9	5.6	5.97	4.9	1.89			40.37
a-$C_{15:0}$	41.36	45.84	38.06	**45.08**	41.56	49.54	54.25	29.48		1.25	18.85
i-$C_{16:0}$	22.63	0.76	26.03	20.20	18.32	15.56		30.64			11.7
$C_{16:1\ \omega9c}$ alcohol		0.12									
$C_{16:1\ \omega11c}$					0.3	0.17					0.06
i-$C_{17:0}$	0.64	0.81	0.77	0.54	0.7	0.49	0.47	0.28			1.71
a-$C_{17:0}$	16.28	17.33	18.35	16.7	17.7	13.03	17.51	14.61			2.21
i-$C_{17:1\ \omega10c}$											0.16
i-$C_{18:1}$						0.06		0.17			
i-$C_{18:0}$	0.07	0.07	0.09	0.1	0.05		0.06				0.06
a-$C_{19:0}$				0.07				0.07			
11-Methyl 18:1 ($\omega7c$)									0.21		
$C_{20:2\ (\omega6,\ 9c)}$									0.05		
Summed feature 4				0.27							

**a** Values are percentages of total cellular fatty acids

b Summed features represent groups of one or two fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of the following fatty acids: 2-OH-i-C15:0 and/or C16:1($\omega7c$). Summed feature 6 contained one or more of the following fatty acids : C19:1 $\omega11c$/C19:1 $\omega9$.
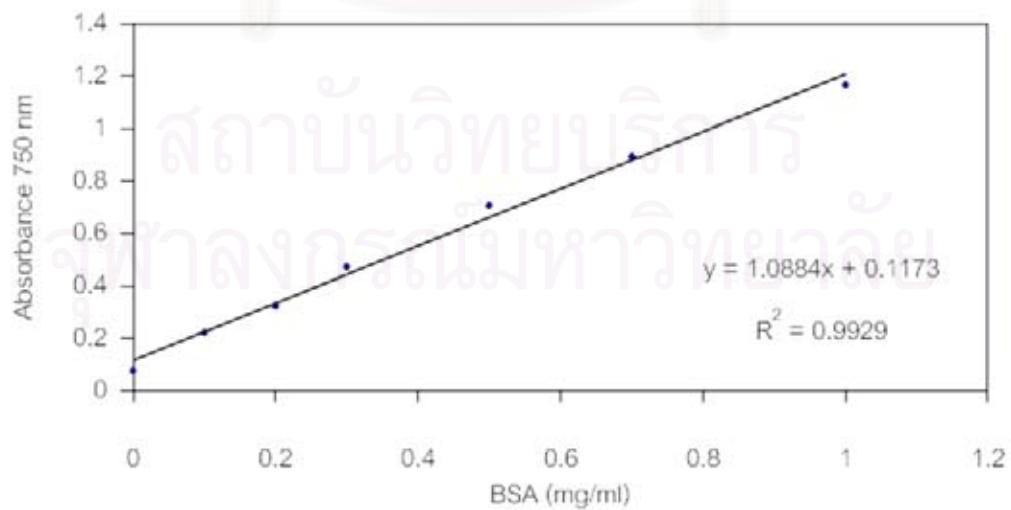
APPENDIX D

Standard curve of tyrosine, Bovine serum albumin(BSA) and AMC
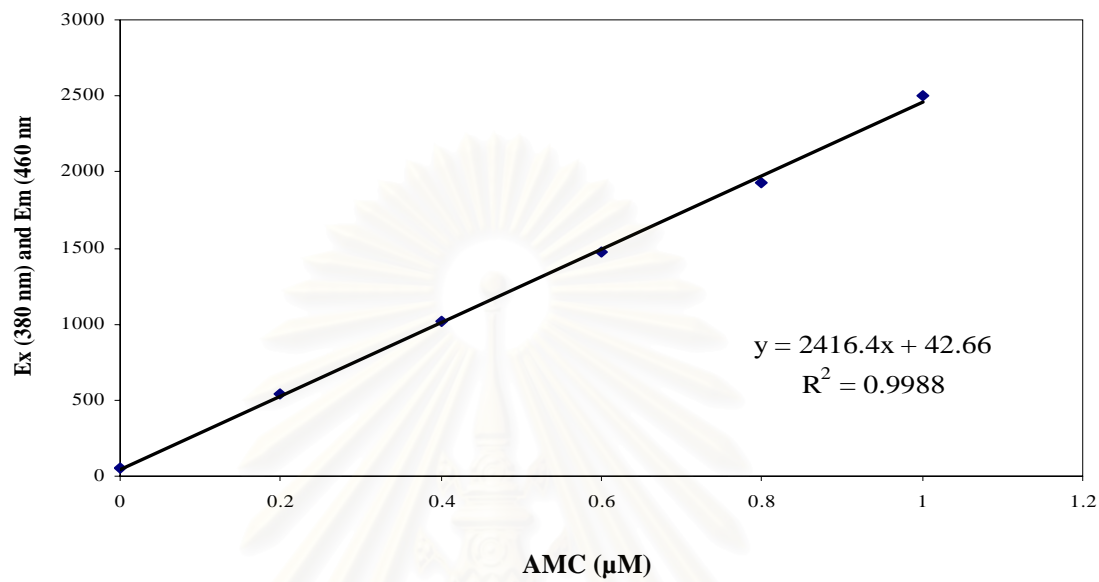
1. **Standard curve of tyrosine**

$y = 0.0972x + 0.0059$

$R^2 = 0.9998$
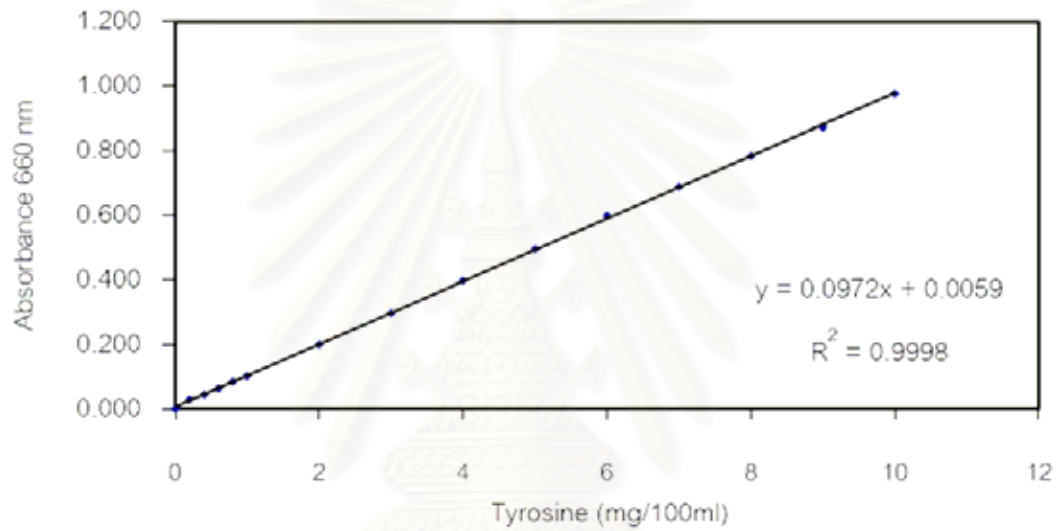
2. **Standard curve of Bovine serum albumin (BSA)**

$y = 1.0884x + 0.1173$

$R^2 = 0.9929$

3. Standard curve of AMC

$y = 2416.4x + 42.66$

$R^2 = 0.9988$

VITA

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Publications

1. Namwong, S., Tanasupawat, S, Smitinont, T., Visessanguan., W., Kudo, T., and Itoh, T. 2005. Characterization of *Lentibacillus salicampi* and *Lentibacillus juripiscarius* sp. nov. isolated from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. 55: 315-320.

2. Namwong, S., Hiraga, K., Takada, K., Tanasupawat, S., and Oda, K. 2006. **A** halophilic serine proteinase from *Halobacillus* sp. SR5-3 isolated from fish sauce: purification and characterization. Biosci. Biotechnol. Biochem. (In press).

3. Namwong, S., Tanasupawat, S., Visessanguan, W., Smitinonth, T., Kudo, T., and Itoh, T. *Halococcus thailandensis* sp. nov. from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. (submitted).