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SCREENING AND IDENTIFICATION OF PROTEASE-PRODUCING MODERATELY HALOPHILIC BACTERIA FROM SALT FERMENTED FOODS

Miss Thanapun Taprig

A Thesis Submitted in Partial Fulfillment of the Requirements

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Department of Microbiology

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Thesis Title	SCREENING AND IDENTIFICATION OF PROTEASE-PRODUCING MODERATELY HALOPHILIC BACTERIA FROM SALT FERMENTED FOODS
By	Miss Thanapun Taprig
Field of Study	Industrial Microbiology
Thesis principal Advisor	Associate Professor Ancharida Akaracharanya, Ph.D.
Thesis Co-advisor	Associate Professor Somboon Tanasupawat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Faculty of Science
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

……Chairperson
(Associate Professor Suthep Thaniyavarn, Ph.D.)

……Thesis principal Advisor
(Associate Professor Ancharida Akaracharanya, Ph.D.)

……Thesis Co-advisor
(Associate Professor Somboon Tanasupawat, Ph.D.)

……External Member
(Wonnop Visessanguan, Ph.D.)

……Member
(Associate Professor Kanchana Juntongjin, Ph.D.)

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การคัดแยกและคัดกรองแบคทีเรียชอบเค็มปานกลางที่ผลิตโปรตีเอสจาก 30 ตัวอย่างอาหารหมักเค็ม (ปลาร้า, ปลาจ่อม, กะปิ, ปูดอง, น้ำปลา และ แมงดาดอง) ที่เก็บจากตลาดและโรงงาน ได้แบคทีเรีย 54 ไอโซเลท ซึ่งแสดงกิจกรรมสลายเคซีนบนอาหาร JCM No. 377 ที่เติม 1% skim milk และ 10% เกลือ จากผลการศึกษาลักษณะฟีโนไทป์และอนุกรมวิธานเคมีรวมทั้งการวิเคราะห์ลำดับเบสของยีน 16S rRNA สามารถแบ่งแบคทีเรียเหล่านี้ได้เป็น 7 กลุ่ม จัดเป็น *Halobacillus* 10 ไอโซเลต, *Virgibacillus* 24 ไอโซเลต, *Oceanobacillus* 4 ไอโซเลต, *Bacillus* 4 ไอโซเลต, *Idiomarina* 4 ไอโซเลต, *Salinivibrio* 5 ไอโซเลต และ *Halomonas* 3 ไอโซเลต ผลการศึกษาความคล้ายคลึงของลำดับเบสของยีน 16S rRNAของไอโซเลตที่เป็นตัวแทนกลุ่ม พบว่ากลุ่มที่ 1 TP4-4 มีความคล้ายคลึง กับ *Halobacillus trueperi* KCTC 3686^T (98.5%) TSN17 คล้ายคลึงกับ *Halobacillus salinus* JCM11546^T (96.8%) กลุ่มที่ 2 TKNR13-3 คล้ายคลึงกับ *Virgibacillus halodenitrificans* JCM 12304^T (99.3%) TCK24 คล้ายคลึงกับ *Virgibacillus dokdonensis* KCTC 3933^T (99.5%) กลุ่มที่ 3 TPR1-2 คล้ายคลึงกับ *Bacillus aquimaris* KCCM 14589^T (98%) กลุ่มที่ 4 TPS12 คล้ายคลึงกับ *Oceanobacillus picturae* LMG 19492^T (99%) กลุ่มที่ 5 TPS4-2 คล้ายคลึงกับ *Idiomarina loihiensis* DSM15497^T (99%) กลุ่มที่6 TM5-3 คล้ายคลึงกับ *Salinivibrio costicola* ATCC 33508^T (99%), และกลุ่มที่ 7 TKK10 คล้ายคลึงกับ *Halomonas alimentaria* KCCM 41042^T (97%) พบว่าแบคทีเรียสายพันธุ์ที่ทดสอบของสกุล *Halobacillus, Virgibacillus*, *Bacillus* และ *Oceanobacillus* มีกรด *meso*-diaminopimelic เป็นองค์ประกอบของผนังเซลล์ และพบ menaquinones ชนิด MK-7 ใน *Halobacillus* ค่า DNA G+C content ของแบคทีเรียที่แยกได้อยู่ในช่วง 35-53.73 mol% นอกจากนี้ได้ศึกษาความคล้ายคลึงของDNA ของแบคทีเรียในสกุล *Halobacillus* และ *Virgibacillus* เพื่อยืนยันการพิสูจน์เอกลักษณ์ด้วย

ค่า specific activity ของ โปรตีเอสของแบคทีเรีย 54 ไอโซเลต อยู่ในช่วง 0.0145- 4.229 หน่วยเอนไซม์/มิลลิกรัมโปรตีน แบคทีเรีย TKNR13-3 ซึ่งผลิตโปรตีเอสได้สูงสุด เจริญได้ดีที่สุดเมื่อเลี้ยงในอาหารที่มีเกลือ 10% และผลิตโปรตีเอสได้มากที่สุดเมื่อเจริญในระยะ stationary phase ในอาหาร JCM No. 377 ที่ เติม 2% yeast extract แทน casamino acids และเติมเกลือ 5% ที่ pH 6.5 อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 3 วัน โปรตีเอสมีแอคติวิตีสูงที่สุดที่ pH 8 มีเกลือ 15% และอุณหภูมิ 60 องศาเซลเซียส แสดงว่าเป็นเอนไซม์ที่ค่อนข้างชอบร้อน โปรตีเอสย่อยเคซีนนี้ประกอบด้วยโปรตีนที่มีขนาดน้ำหนักโมเลกุล 12, 21, 29, 39 และ 49 KDa. โปรตีเอสแอคติวิตีถูกยับยั้งอย่างมาก (77.62%) ด้วย chymostatin ดังนั้น โปรตีเอสของแบคทีเรีย TKNR13-3 นี้จึงจัดเป็น serine protease ชนิด chymotrypsin.

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	จุลชีววิทยาอุตสาหกรรม	ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
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THANAPUN TAPRIG: SCREENING AND IDENTIFICATION OF PROTEASE-PRODUCING MODERATELY HALOPHILIC BACTERIA FROM SALT FERMENTED FOODS. THESIS PRINCIPAL ADVISOR : ASSOC. PROF. ANCHARIDA AKARACHARANYA, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D., 101 pp.

An isolation and screening of protease-producing moderately halophilic bacteria from 30 salt fermented foods (pla-ra, pla-jom, shrimp paste, fermented crab, nam-pla and mang-da dong) collected from markets and factories, 54 isolates which exhibited caseinolytic activity on JCM No. 377 agar containing 1% skim milk and 10% NaCl were selected and identified. On the basis of their phenotypic and chemotaxonomic characteristics including phylogenetic analysis using 16S rRNA gene sequence, they were divided into 7 groups. Ten isolates were belonged to *Halobacillus*, 24 *Virgibaciilus*., 4 *Bacillus*, 4 *Oceanobacillus*, 4 *Idiomarina*, 5 *Salinivibrio* and 3 *Halomonas*. The similarity of 16S rRNA gene sequence revealed that representative strains of Group 1, TP4-4 was closely related to *Halobacillus trueperi* KCTC 3686^{T}(98.5%) and TSN17 was closely related to *Halobacillus salinus* JCM11546^{T} (96.8%). Group 2, TKNR13-3 was closely related to *Virgibcillus halodenitrificans* JCM 12304^{T} (99.3%) and TCK24 was closely related to *Virgibacillus dokdonensis* KCTC 3933^{T} (99.4%). Group 3, TPR1-2 showed 98% similarity to *Bacillus aquimaris* KCCM 14589^{T}. TPS12 in group 4 showed 99% similarity to *Oceanobacillus picturae* LGM 19492^{T}, TPS4-2 in group 5 showed 99% similarity to *Idiomarina loihiensis* DSM 15497^{T}, TM5-3 in group 6 showed 99% similarity to *Salinivibrio costicola* ATCC 33508^{T}and TKK10 in group 7 showed 97% similarity to *Halomonas alimentaria* KCCM 41042^{T}. Tested strains of *Halobacillus, Virgibcillus, Bacillus* and *Oceannobacillus* contained *meso*-diaminopimelic in cell wall peptidoglycan and menaquinone with 7 isoprene units (MK-7) was presented in *Halobacillus* strain. DNA G + C content of the tested strains ranged from 35-53.73 mol%. In addition, DNA-DNA hybridization experiments have been done to confirm the identification of *Halobacillus* and *Virgibcillus* strains.

Specificity protease activitiy of the 54 isolates ranged from 0.0145-4.229 U/mg protein. The highest protease producer, TKNR13-3, had maximum growth in the medium containing 10% NaCl and showed the highest protease production in stationary growth phase when cells were cultivated in JCM. No. 377 which casamino acids was replaced by 2% yeast extract and containing 5% NaCl, pH 6.5 and at 37 °C for 3 days. The optimum pH, salt concentration and temperature for crude protease activity were pH 8, 15% NaCl and 60 ºC. Thus, the enzyme was slightly or moderately thermophilic. Protein with molecular weight of 12, 21, 29, 39 and 49 KDa in crude protease exhibited caseinolytic activity.The protease activity was strongly inhibited (77.62%) by chymostatin. Therefore, the protease of strain TKNR13-3 was serine protease type chymotrypsin.

Department	Microbiology	Student' s Signature…….
Field of Study	Industrial Microbiology	Principal Advisor' s Signature……..
Academic Year	2007	Co-Advisor' s Signature……...

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LIST OF ABBREVIATONS

Abbreviations or symbols	*Term*
%	*percent*
^{o}C	*degree centigrade or Celsius*
µg	*microgram*
µ*l*	*microliter*
/	*per*
BIOTEC	*National Center for Genetic Engineering and Biotechnology*
DSM	*Deutsche Sammlung von Mikroorganismen*
JCM	*Japan Collection of Microorganisms*
LMG	*Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium*
kDa	*kilo dalton*
v/v	*volume by volume*
v/w	*volume by weight*

CHAPTER I

INTRODUCTION

Microbial proteases are well-studied group of hydrolases that catalyze the total hydrolysis of proteins. Besides their physiological importance, they constitute a class of enzymes of great application in commercial fields (Rao *et al*., 1998). These industrial processes are carried out under specific physical and chemical conditions which cannot always be adjusted to the optimal values required for the activity of the available enzymes. Although there are many microbial sources available for producing protease, only a few are recognized as commercial producers. For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature. Halophiles are the most likely source of such enzymes, because not only are their enzymes salt-tolerant, but many are also thermotolerant (Sanchez-Porro, 2003).

The halophilic bacteria are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing efforts of salts. They may be classified according to their salt requirement: slight halophiles grow optimally at 2-5% (w/v) NaCl; moderate halophiles grow optimally at 5-20% (w/v) NaCl; and extreme halophiles grow optimally above 20-30% (w/v) NaCl (Kushner, 1985). They are well adapted to hypersaline environments and have a number of novel molecular characteristics, such as halophilic exoenzyme that function in high salt concentration and could be of commercial interest and used in biodegradation processes. They have the advantage that most species are able to grow in wide range of salinities, in contrast to the more strict requirement of salt presented by halobacteria.

Although 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 change in extracellular osmolality and constitute an interesting group of microorganisms from biotechnological point of view because they can grow in absence NaCl until high NaCl concentration (Ventosa, 1998).

In recent years the isolation and characterization of proteases produced by these microorganisms have acquired enormous interest. The running of industrial processes at high salt concentrations requires the availability of protease showing optimal activities at such elevated salinity. The addition of such enzyme in laundry and dishwashing detergents has been of great importance. The treatment of agricultural waste and waste from food processing industries constitutes other areas of interest for halophilic hydrolytic enzyme. For example, a protease-producing moderate halophiles was isolated in north Taiwan can be used for deproteinization of crustacean wastes in the preparation of chitin and used for waste treatment from seafood industries (Yang *et al.*, 2000). So the screening of protease-producing halophilic bacteria is an interesting.

Previous studies, most of protease-producing halophilic bacteria were isolated from hypersaline enviroment such as desert, salt lakes, sediment of solar saltern, saline soils and thalassic while the protease-producing halophilic bacteria from traditional fermented food have not been extensively studied.

Salt fermented foods have a large proportion of salt, which allow various halophilic bacterias to thrive (Lopetcharat *et al*., 2001). Hence, salt fermented food is a good screening source for isolation of halophilic bacteria that show proteinase activity. There are several previous studies on proteinase from bacteria isolated from fish sauce, but few protease-producing bacteria from salt fermented foods. Growing either in the absence or up to 20% of NaCl made the moderately halophilic bacteria the most interesting halophilic bacterial group in this study.

The main objectives of this presence study were as followed:

1. To screen the protease-producing moderately halophilic bacteria from salt fermented foods.
2. To identify the protease-producing halophilic bacteria from salt fermented foods.
3. To maximize the protease production through optimization of the modified media and cultivation conditions.
4. To study some properties of crude protease

CHAPTER II

LITERATURE REVIEW

2. Proteases

Proteases, a proteolytic enzymes, are degradative enzymes which catalyze peptide bond cleavages resulted in total proteins hydrolysis (Fig. 2.1).

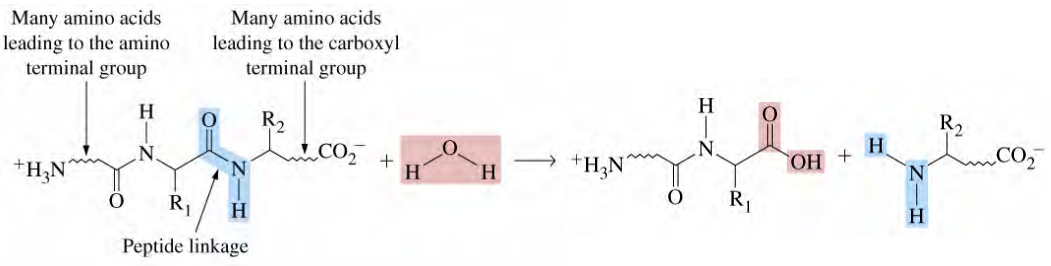

Fig. 2.1 Catalytic reaction of protease

Proteases are very important in food digestion as they breakdown the peptide bonds in protein foods to liberate amino acids. Additionally, proteases have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation.

2.1 Classification of protease

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified as 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 three major criteria (Barrett, 1994).

2.1.1 Type of reaction catalyzed. Proteases are grossly subdivided into two major groups, 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.

2.1.2 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. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the substilisin family which includes 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). 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. 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 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* sp.

- Cysteine/thiol proteases. Cysteine proteases was found in both prokaryotes and eukaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. 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. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Because they require a metal ion for their activity, so inhibited by metal chelating agents such as EDTA but not by sulfhydryl agents or DFP. Examples of enzyme in this group are carboxypeptidase, thermolysin, collagenase.

The threonine and glutamic acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (peptidases) or a water molecule (aspartic acid, metallo- and glutamic acid peptidases) nucleophilic so that it can attack the peptide carbonyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

2.1.3 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 2.1 Classification of protease characteristics

Protease category	**Type**	**Sub-types**	**Microbial occurrence**	**Conditions for optimal activity**
Exopeptidases (site of action)				
Aminopeptidases	Majority intracellular	–	Bacteria and fungi	–
Carboxypeptidases	Extracellular and intracellular	Serine carboxypeptidases, metallocarboxy peptidases, and cysteine carboxypeptidases	Bacteria and fungi	Variable
Endopeptidases (catalytic mechanism)				
Serine proteases	Extracellular and intracellular	Serine alkaline type—largest subgroup	Viruses, bacteria, and eukaryotes	Neutral and alkaline pH, with an optimum between pH 7 and 11, broad substrate specificities including esterolytic and amidase activity
Aspartic proteases	Extracellular and intracellular	Pepsin and rennin-like enzymes, acidic enzymes	Viruses, bacteria, and fungi	pH 3–4 and isoelectric points in the range of pH 3–4.5, molecular masses 30–45 kDa.
Cysteine/thiol proteases	Extracellular and intracellular	Papain-like; trypsin-like with preference for cleavage at the arginine residue; specific to glutamic acid; and others	Prokaryotes and eukaryotes	Normally, neutral pH But also, pH 4.9–8.4
Metalloproteases	Extracellular and intracellular	Neutral; alkaline; Myxobacter I; and Myxobacter II	Prokaryotes and eukaryotes	pH 4.0–10.0

2.2 Industrial applications of protease

Proteases have a large variety of applications, mainly in the detergent and food industries (Table 2.2). The worldwide requirement for enzymes for individual applications varies considerably.

Table 2.2 Usage of protease in an industry

	Sales (million US$)	Share of industrial proteinase market (%)
Detergent proteinases	140	89.2
Microbial rennets	12	7.6
Baking proteinases	3	1.9
Leather	1	0.6
Miscllaneous	1	0.7
Totals	157	100.0

2.2.1 Detergents. Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures.The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes.

Two microbial proteinases dominate detergent products today. While these appear as seven products from four suppliers, the microbial sources tend to be either *Bacillus licheniformis* or the alkalophilic Bacilli.

All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found compatible with commercial detergents used in India (Phadatare, *et al*., 1993) and retained 43% of its activity at 50°C for 50 min in the presence of Ca^{2+} (25 mM) and glycine (1 M) (Bhosale, *et al*., 1995).

2.2.2 Food industry. The use of proteases in the food industry dates back to antiquity.

- Dairy industry. The major application of proteases in the dairy industry is in the manufacture of cheese. The milk-coagulating enzymes fall into three main categories, (i) animal rennets, (ii) microbial milk coagulants, and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 to 40,000.

- Baking industry. Wheat flour is a major component of baking processes. It contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Endo and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Bacterial proteases are used to improve the extensibility and strength of the dough.

- Manufacture of soy products. Soybeans serve as a rich source of food, due to their high content of good-quality protein. Proteases have been used from ancient times to prepare soy sauce and other soy products. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds.

- Debittering of protein hydrolysates. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness.

- Synthesis of aspartame. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis of aspartame. Toya Soda (Japan) and DSM (The Netherlands) are the major industrial producers of aspartame.

2.2.3 Pharmaceutical Industry. The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures.

2.2.4 Leather Processing. Two operations in converting animal skins to leather use proteases, at least, to some extent: unhairing of hides and bating. Use of microbial enzymes in these steps can be characteized as, ‘limited’, for both, but for different reasons.

- Unhairing. For unhairing of hides, microbial serine proteases are competing against inexpensive chemicals: lime and sodium sulfide. The alkaline swelling and dehairing is very effective and reasonably fast (Aunstrup, 1980). The first option has led to an opening for microbial proteases, however the second option has been the more popular elective.

- Bating. Leather bating is the process which gives leather a degree of flexibility and suppleness. Erroneously, consumers often refer to this quality as leather 'softness'. Thus today, proprietary mixtures of chemicals and proteinases from *Aspergillus oryzae, Bacillus amyloliquefaciens or B. licheniformis* can be found in the bating house supply room.

2.2.5 Silver recovery. Alkaline proteases find potential application in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5 to 2.0 % (by weight) silver in its gelatin layers. Further, a continuous process for silver recovery was also reported on the basis of kinetic studies and mechanism of enzymatic hydrolysis of the gelatin layers on X-ray film and the resulting release of silver particles.

2.2.6 Waste treatment. Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. These proteases can solubilize proteins in wastes through a multistep process to recover liquid concentrates or dry solids of nutritional value for fish or livestock. The end product was a heavy, grayish powder with a very high protein content which could be used as a feed additive. Similarly, many other keratinolytic alkaline proteases were used in feed technology for the production of amino acids or peptides, for degrading waste keratinous material in household refuse , and as a depilatory agent to remove hair in bath tub drains, which caused bad odors in houses and in public places.

2.2.7 Other applications. Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure function relationship, in the synthesis of peptides, and in the sequencing of proteins. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

2.3 Sources of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms.

2.3.1 Plant proteases. The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth.

2.3.1.1 Papain. Papain is a traditional plant protease and has a long history of use (Schechler and Bweger, 1967). It is extracted from the latex of Carica papaya fruits. The performance of the enzyme depends on the plant source, the climatic conditions for growth, and the methods used for its extraction and purification.It is extensively used in industry for the preparation of highly soluble and flavored protein hydrolysates.

2.3.1.2 Bromelain. Bromelain is prepared from the stem and juice of pineapples. The major supplier of the enzyme is Great Food Biochem., Bangkok, Thailand. The enzyme is characterized as cysteine protease and is active from pH 5 to 9. Its inactivation temperature is 70°C, which is lower than that of papain.

2.3.1.3 Keratinases. Some of the botanical groups of plants produce proteases which degrade hair. Digestion of hair and wool is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems.

2.3.2 Animal proteases. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennin.

2.3.2.1 Trypsin. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Trypsin is used in the preparation of bacterial media and in some specialized medical applications.

2.3.2.2 Chymotrypsin. Chymotrypsin is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids. It is stored in the pancreas in the form of a precursor, chymotrypsinogen, and is activated by trypsin in a multistep process.

2.3.2.3 Pepsin. Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by

autocatalysis in the presence of hydrochloric acid. It exhibits optimal activity between pH 1 and 2, while the optimal pH of the stomach is 2 to 4. Pepsin is inactivated above pH 6.0.The enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids.

2.3.2.4 Rennin. Rennet is a pepsin-like protease (rennin, chymosin; EC 3.4.23.4) that is produced as an inactive precursor, prorennin, in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin or by its autocatalysis. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para-k-casein and C-terminal glycopeptide.

2.3.3 Microbial proteases. 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).

2.3.3.1 Bacteria. Most commercial proteases, mainly neutral and alkaline proteases, 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. 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.

2.3.3.2 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. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheesemaking industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. Fungal alkaline proteases are also used in food protein modification.

2.3.3.3 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) (Sanchez-Porro, 2003). 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.

Although there are many microbial sources available for producing protease, only a few are recognized as commercial producers. For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature. Halophiles are the most likely source of such enzymes, because not only are their enzymes salt-tolerant, but many are also thermotolerant. (Sanchez-Porro, 2003).

2.4 Halophilic bacteria

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts (Kushner, 1985).

2.4.1 Slight halophiles. The slight halophile grow optimally at 0.2– 0.85 mol/L 2–5% (w/v) NaCl, such as *Pseudomonas, Moraxella, Flavobacterium, Acinetobacter* and *Vibrio*, they isolated from sea fish and shell.

2.4.2 Moderate halophiles. The moderate halophiles grow optimally at 0.85–3.4 mol/L 5–20% (w/v)NaCl. Moderately halophilic bacteria constitute a large group of organisms encompassing a great diversity of bacteria. from the taxonomic point of view, constitute a very heterogeneous group of microorganisms which include species from different genera. Archae bacteria; Methanohalophilus, Halomethanococus, Gram-negative; *Deleya*, *Arhodononas, Chromohalobacter*, *Dichotomicrobium*, *Flavobacterium*, *Haloanaerobium*, *Halobacteroides*, *Haloincola*, *Halomonas*, *Halovibrio*, *Pseudomonas*, *Spirochaeta*, *Sporohalobacter*, *Vibrio*, *Volcaniella*, Gram-positive; *Halobacillus*, *Bacillus*, *Clostridium*, *Marinococcus*, *Micrococcus*, *Salinicoccus*, *Salibacillus*, *Lentibacillus*, *Virgibacillus*, and *Sporosarcina*, and facultative anaerobe; *Flavobacterium salegens* and *Arhodomonas aquaeolei*; Actinomycetes; Genera *Actinopolyspora.*

2.4.3.Extreme halophiles. The extreme halophiles grow optimally above 3.4–5.1 mol/L 20–30% (w/v)NaCl. The extremely halophilic archaeobacteria are well known because, they often were (and still are) simply called"red halophiles", such as Genera *Halobacterium*, *Haloferax*, and *Haloarcula*, *Halococcus*, *Natronomanas, Haloterrigena*. In contrast, nonhalophiles grow optimally at less than 0.2 mol / L 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.

2.5 Moderately halophilic bacteria

Bacillus

Most Gram-positive or Gram-variable, endospore-forming rods with moderately halophilic or halotolerant properties have previously been assigned to genus *Bacillus*, e.g. *Bacillus dipsosauri* (Lawson etal.,1996), *Bacillus halodurans* (Nielsen *et al*, 1995).

Halobacillus

Gram-positive, rod-shaped, endospore-forming, halophilic bacteria were isolated grew optimally in the presence of 2–10%(w/v) NaCl, poor grow thin the absence of NaCl and grew in the presence of less than 23%NaCl, cell wall peptidoglycan type A4b based on L-Orn–D-Asp predominant menaquinone, menaquinone-7 (MK-7). Cellular fatty acid profile branched fatty acids major fatty acids anteiso-C, 15:0 iso-C 15:0 and iso-C 16:0. DNA G+C content 45mol%. 16S rDNA sequences 97.4–98.4%to the type strains of *Halobacillus* species. DNA–DNA relatedness between 7.3–9.2%. *Halobacillus salinus* sp.nov. is proposed (Yoon *et a/*.,2003).

Table2.4 Differential phenotypic characteristics of *Halobacillus* species(Yoon *et al*., 2003)

Characteristic	Strain HS-3^T	*H. halophilus*	*H. litoralis*	*H. trueperi*
Cell morphology	Rods	Cocci or oval	Rods	Rods
Gram staining	+(v)	+	+	+
Colony colour	Pale orange-yellow	Orange	Orange	Orange
Growth at/in:				
45 ^{0}C	+	-	-	-
pH 5.0	+	-	-	-
25%NaCl	-	-	+	+
Hydrolysis of: Aesculin	+	-	-	-
Starch	-	+	-	-
Tween 80	+	-	-	-
Acid production from:				
D- Galactose	w	-	-	+
Maltose	+	-	+	+
D-trehalose	+	-	+	+
DNA G+C content	45	40-41	42	43

Virgibacillus

Cells of all species are endospore-forming rod-shaped. They were Gram-variable, motile, and slightly halophilic bacteria. Grew optimally at 37^{o}C and in the presence of 4–5%NaCl and were positive for catalase but negative for growth on D-arabinose and D-xylose. All contained meso-diaminopimelic acid in cell-wall peptidoglycan and MK-7 as the predominant menaquinone. Anteiso-C15:0, iso-C15:0, anteiso-C17:0 and iso-C 16:0 were major fatty acids. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids. DNA G+C content was 36.7 mol%. 16S rRNA gene sequences similarity of 95.3–98.7% *Virgibacillus* DNA–DNA relatedness range 8.4–17.5% novel *Virgibacillus* species *Virgibacillus dokdonensis* sp. nov. The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) with the transfer of *Bacillus pantothenticus* to *Virgibacillus pantothenticus*, *Virgibacillus proomii* (Heyndrickx *et al.*, 1999) and *Virgibacillus carmonensis*, *Virgibacillus necropolis* and *Virgibacillus picturae* (Heyrman *et al.*, 2003), were described. Two *Salibacillus* species (Wainø et al., 1999; Arahal *et al.*, 2000) have been reclassified in the genus as *Virgibacillus marismortui* and *Virgibacillus salexigens* (Heyrman *et al.*, 2003). Recently, *Bacillus halodenitrificans* was transferred to the genus as *Virgibacillus halodenitrificans* (Yoon *et al.*, 2004). Slightly halophilic Virgibacillus-like bacterial strain, DSW10^{T}. Comparative 16S rRNA gene sequence analyses most closely related *Virgibacillus* species Morphological, cultural, physiological and biochemical are shown inTable2.5 species description below meso-diaminopimelic acid diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinine found unsaturated menaquinone with seven isopreneunits(MK-7), and a minor amount of MK-6. Major components of the fatty acids branched fatty acids anteiso-C 15:0 (34.4%), iso -C 15:0 (19.4%), anteiso -C17:0(15.4%), iso-C16:0 (12.3%), iso-C 17:0 (7.2%),iso-C 14:0 (4.7%)andiso-C13:0(1.1%)and straight-chain fatty acid C 16:0 (2.4%). Major polar lipids tidylglycerol, phosphatidylethanolamine and unidentified phospholipids. DNA G+C content of 36.7 mol%.

Table 2.5 Differential phenotypic characteristics of *Virgibacillus* species(Yoon *et al*.. 2005)

Characteristic	1	2	3	4	5	6	7	8	9
Spore shape	S or E	S or E	S or E	E	E	E or S	E	E or S	E
Spore position	T or ST	T or ST	T or ST	C, ST or T	T or ST	ST	C, ST or T	T	T or ST
Gram staining	V	+	+	+	+	+	+	+	v
Pigmentation	-	-	-	-	-	Pink	-	-	-
Growth at/in:									
0.5%NaCl	+	w	w	-	-	-	w	w	v(-)
25%NaCl	-	-	-	w	-	-	-	-	v(+)
Hydrolysis of:									
Aesculin	+	+	+	+	+	w	-	w	v(-)
Casein	+	+	+	+	+	+	+	w	+
Gelatin	+	+	v	+	+	-	w	v	+
Acid production from:									
D- Galactose	+	-	+	w	-	-	-	w	+
D-Glucose	+	-	+	w	+	-	w	w	+
L-Rhamnose	-	+	v	-	-	-	-	-	-
D-trehalose	-	+	+	-	-	-	w	v	+
D-Mannitol	-	-	-	-	-	-	-	w	v(+)
DNA G+C content	36.7	36.9-38.3	36.8-37.0	36.3-39.5	39.0-42.8	38.9	37.3	39.5-40.0	38-39

Species: 1, *V. dokdonensis*; 2, *V. pantothenticus* ; 3, *V. proomii* ; 4, *V. salexigens* ; 5, *V. marismortui*; 6, *V. carmonensis* ; 7, *V. necropolis*; 8, *V. picturae*; 9, *V. halodenitrificans*. +, Positive; 2, negative; W, weakly positive; ND, not determined; V, variable; E, ellipsoidal; S, spherical; C, central; ST, subterminal; T, terminal; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PLs, unidentified phospholipids; APL, unknown aminophospholipid; tr, trace.

Oceanobacillus

Gram positive, motile by peritrichous flagella, spore forming rods. Endospore are subterminal or terminal and slightly swell the sporangia. Obligately aerobic, facultatively alkalophilic, and extremely halotolerant .Growth occur at temperature 15-42^{o}C. Colonies are circular.Chemoorganotrophic. The genus *Oceanobacillus*, with the single species *Oceanobacillus iheyensis*,was first described by Luetal.(2001), and *Oceanobacillus oncorhynchi* was subsequently proposed by Yumoto *et al*.(2005).

Table 2.6. Differential characteristics of *Oceanobacillus* species(Yumoto *et al*., 2005)

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Pigmentation	-	-	-	-	-	-	Pink	-	-	-	-
Spore shape	E	E	ES	ES	E	E	ES	E	ES	E	E
Spore position	T	TS	TS	TS	C,ST	TS	S	C,ST	T	TS	S
Growth at/in:											
0.5%NaCl	w	-	+	w	-	+	-	w	w	+	+
25%NaCl	-	w	-	-	w	-	-	-	-	-	-
Hydrolysis of:											
Gelatin	-	+	+	v	+	+	-	w	w	-	-
Aesculin	+	-	+	+	+	+	w	-	w	+	NT
Acid production from:											
D- Galactose	-	+	-	+	w	-	-	-	w	-	-
D-Glucose	w	+	-	+	w	+	-	w	w	+	+
L-Rhamnose	-	-	+	v	-	-	-	-	-	w	-
D-Trehalose	w	+	+	+	-	-	-	w	w	-	+
D-Turanose	-	-	+	-	-	-	-	-	w	w	-
DNA G+C content	41	38	37-38	37	36-40	39-43	40	37	40	36	38.5

Strains: 1, *V. koreensis* KCTC 3823^{T}2, *V. halodenitrificans* KCTC 3790^{T}; 3, *V. pantothenticus* KCTC 3539^{T}; 4, *V. proomii* KCTC 3822^{T}; 5. *V. salexigens* KCTC 3844^{T}; 6, *V. marismortui* KCTC 3845^{T}; 7, *V. carmonensis* KCTC 3819^{T}; 8, *V. necropolis* KCTC 3820^{T}; 9, *V. picturae* KCTC 3821^{T}; 10, *Oceanobacillus iheyensis* KCTC 3954^{T}; 11, *O. oncorhynchi* R-2^{T} (data from Yumoto et al., 2005). tr, Trace amount; –, not detected. The positions of double bonds can be located by counting from the methyl (v) end of the carbon chain; cis and trans isomers are indicated by the suffixes c and t.

Salinivibrio

The genus *Salinivibrio* is the third genus in the family *Vibrionaceae,* belonging to the γ-subclass of the class *Proteobacteri*a. This genus was transferred from *Vibrio costicola* based on the significant phenotypic and genotypic differences between this genus and other *Vibrio* species (Mellado *et al.*, 1996.). At present, the genus is monospecific, but it comprises three subspecies: *S. costicola* subsp. *costicola* (Smith, 1938), *S. costicola* subsp. *vallismortis* (Huang *et al*., 2000) and *S. costicola* subsp. *alcaliphilus*. (Romano *et al*., 2005). They were moderately halophilic bacteria which are distributed in salted meats, brines and hypersaline environments and grow in the presence of 0-20% NaCl.

Table 2.7 Differential characteristics of *Salinivibrio*, *Vibrio* and related genera (Mellado *et al*., 1996)

Characteristic	*Salinivibrio*	*Vibrio*	*Halovibrio*	*Deleya*	*Vocaniella*	*Arhodomonas*
Morphology	Curved rods	Curved rods	Curved rods	Rods	Short rods	Short rods
Pigmentation	None	Cream	Light brown	None	None	None
Growth at/in:						
0%NaCl	-	-	-	-	-	-
20%NaCl	+	-	+	+	+	+
Hydrolysis of:						
Gelatin	+	+	-	-	+	-
Starch	-	+	-	ND	-	-
Acid production from:						
D-Glucose	+	+	-	+	-	ND
Arginine	+	-	-	-	ND	-
Lysine	-	+/-	-	ND	ND	-
Ornithine	-	+/-	-	ND	ND	-
DNA G+C content	49.4-50.5	38-51	61	52-68	59.1-65.7	67

Idiomarina

The genus *Idiomarina* was first proposed by Ivanova *et al*. (2000) to accommodate two marine bacteria, *Idiomarina abyssalis* and *Idiomarina zobellii*. Since the description of the genus, the names of the species *Idiomarina baltica* (Brettar *et al*., 2003), *Idiomarina loihiensis* (Donachie *et al*., 2003), *Idiomarina fontislapidosi* (Martnez-Canovas et al., 2004), *Idiomarina ramblicola* (Martnez-Canovas *et al*., 2004) *Idiomarina seosinensis* (Choi&Cho,2005) their ability to grow with in abroad range of temperatures, pH values and NaCl concentrations (Martnez-Canovas *et al.*, 2004).

Table 2.8 Differential characteristics of *Idiomarina* and related species (Ivanova *et al*., 2000)

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Straight or curved rods	Straight or curved rods	Rods	Rods	Curved rods	Straight or curved rods	Slightly curved rods	Slightly curved rods
NaCl range (% w/v)	1-15	1-20	0.6-15	1.0-10	0.8-10	0.5-20	0.5-25	0.5-15
Temperature range (^{0}C)	4-45	4-40	4-30	4-30	8-46	4-46	4-45	15-40
Hydrolysis of:								
Aesculin	+	+	-	-	+	-	+	+
Casein	-	ND	ND	ND	ND	ND	+	+
Tyrosine	+	ND	+	+	ND	ND	-	-
Starch	-	ND	-	-	-	ND	-	-
Tween 80	+	+	ND	+	+	+	+	+
Acid production from:								
D-Glucose	-	-	-	-	w	-	-	-
Growth on:								
L-Arabinose	-	ND	-	-	+	ND	-	-
Maltose	-	ND	-	-	-	+	-	-
L-Alanine	-	+	+	+	-	+	-	-
L-Serine	-	-	-	-	-	+	-	-
DNA G+C content	45.1	45.0	50.4	48.0	49.7	47.4	46.0	48.7

Strains:1, *I. homiensis*; 2, *I. seosinensis* ; 3, *I. abyssalis* ; 4, *I. zobellii*; 5, *I. baltica*; 6, *I. loihiensis*; 7, *I. fontislapidosi*; 8, *I. ramblicola*.

Halomonas

A Gram-negative, moderately halophilic bacterial strain. Cell are non-motile and cocci or short rods. The predominant isoprenoid quinone is ubiquinone-9. The major fatty acids are C18:1 7c, C16:0, C19:0 cyclo 8c and C16:0 7c and/or iso 15:0 2OH. *Halomonas alimentaria* sp. nov. is proposed (Yoon *et al*., 2002).

Table 2.9 Phenotypic characteristics that differentiate the genus *Halomonas*

Characteristic	Strain YKJ-16^T	*H.halodenitrificans*	*H.cupida*	*H.pacifica*
Cell morphology	Cocci or short rods	Cocci or short rods	Straight rods	Straight rods
Nitrate reduction	+	+	+	-
Hydrolysis of:				
Aesculin	-	-	+	-
Casein	-	-	-	-
Gelatinn	-	-	-	-
Tyrosine	-	-	-	-
Tween 80	-	+	+	+
Urea	+	+	+	+
Xanthine	-	-	-	-
Growth at :				
4 ^{0}C	+	ND	-	-
40 ^{0}C	+	ND	-	+
45 ^{0}C	+	ND	-	+
Anaerobic growth	+	+	-	-
DNA G+C content	63	64-66	60-63	67-68

2.6 Novel species of halophilic bacteria reported from 2006

Hoon Yoon *et al* (2007) reported *Halobacillus campisalis* sp. nov., isolated from marine solar saltern in Korea. Gram-positive or variable, motile and coccoid or oval-shaped. It grew optimally at pH 7.0-8.0 and 37^{o}C. The 16S rRNA gene sequence similarity to the type strains was 97.7-98.6%. It contained MK-7 as the predominant menaquinone. The major fatty acids were anteiso-C15:0, anteiso-C17:0 and iso-C16:0. The DNA G+C content was 42.1 mol%.

Soto-Ramirez *et al* (2008) reported *Halobacillus mangrove* sp. nov. isolated from mangrove in Puerto Rico. He bacteria was moderately halophilic, spore-forming, Gram-positive, short-rod shaped. It was able to grow at NaCl concentrations in the range 5-20%(w/v) with optimum growth at 10%(w/v) NaCl. Growth occurred at temperatures of 10-50 degrees C(optimal growth at 33-35 degrees C) and pH 6.0-9.0(optimal growth at pH 7.0) The 16s rRNA

gene sequence was 99.2% similarity to the type strain. The DNA G+C content was 45.7 mol%, the major isoprenoid quinine was MK-7 and the cell-wall peptidoglycan was of the L-Orn-D-Asp type. Level of DNA-DNA hybridization with *H. dabanensis* JCM12772^{T}was 29% and below 70%to other recognized members of the genus *Halobacillus* .The major fatty acids were iso-C (16:0), anteiso-C anteiso (15:0), iso 14:0 and iso-C(14:0) and iso-C(15:0).

Chen *et al* (2008) reported *Virgibacillus kekensis* sp. nov. isolated from salt lake China. Gram-positive moderately halophilic, motile,strictly aerobic, endospore-froming, oxidase- and catalase-positive, rod-shaped. It grew in the presence of 0-25%(w/v) NaCl and at pH 6.0-10.0 degrees C; optimum growth was observed with 10%(w/v) NaCl and at pH 7.0 and 37 degrees C. It had meso-diaminopimelic acid as the diagnostic diamino acid, MK-7 as the predominant quinine, with a significant amount of MK-6, and anteiso-C(15:0), iso-C(14:0) and C(16:0) omega7c alcohol as major fatty acids. Major polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content was 41.8 mol%. The 16S rRNA gene sequences similarities to the type strains were 94.9-97.3%.

Quesada *et al* (2007) reported *Virgibacillus olivae* sp. nov. isolated from waste wash-water from processing of Spanish-style green olives. The bacteria was Gram-positive, spore-forming rods.It was able to utilize cellubiose, glucose, mannose and rhamnose as carbon sources. The major cellular fatty acids were iso-C15:0, anteiso-C15:0, iso-C17:0 and anteiso-C17:0. The 16S rRNA gene sequence was 99% to *Virgibacillus marismortui* 123^{T} . DNA-DNA relatedness to *V. marismortui* ATCC700626^{T} was less than 47%.

Kwon *et al* (2006) report *Idiomarina homiensis* sp. nov. isolated from seaahoresand in Korea. Cells were straight or slightly curved rods and fromed light-yellow colonies on marine agar medium. The major isoprenoid quinine wasubiquinone (Q-8) and the predominant cellular fatty acids were C15:10iso(19.3%), C17:1w9c iso(11.9%), C17:0 iso (10.9%), C18:1w7c(10.4%), C16:0(9.0%) and C16:1w7c and/or C15:0 iso2-OH(7.2%). The G+C content of the DNA was 45.1% mol.Analysis of 16S rRNA gene sequences revealed 94.3 to 95.5% similarities to the type strains.

CHAPTER III

MATERIALS AND METHODS

Instruments, materials, chemical reagents, and media

Name list of all instruments, materials, chemical reagents, and media are shown in Appendix A.

Methods

3.1. Screening of protease-producing halophilic bacteria

3.1.1 Screening of protease-producing halophilic bacteria on agar plate

A total of 40 samples of salt fermented foods were collected from markets in Thailand. Samples collected in a plastic bag were diluted with 10%(w/v) NaCl solution and screened for the desired bacteria not later than 24 hours after collection. Protease-producing bacteria were screened qualitatively by cultivating on halobacterium agar medium JCM No. 377 containing 10%(w/v) NaCl and 1%(w/v) skim milk, and incubated at 37 ºC for 7-10 days. Then, the colonies surrounded by clear zone were selected for further study.

3.1.2 Quantitative protease producing analysis(caseinolytic activity)

A loopful of 54 bacterial isolates that showed clear zone on halobacterium medium JCM No. 377. containing 10% (w/v) NaCl was inoculated in 125-ml Erlenmeyer flask containing 20 ml of halobacterium medium JCM No. 377 containing 10%(w/v) NaCl. After 3 days of incubation at 37°C on a rotary shaker operated at 200 rpm, the cell-free supernatant recovered by centrifugation at the speed of 10,000 rpm (13,300 g), 4°C for 20 min was used as crude enzyme for protease activity (caseinolytic activity) assay by method described by 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%(w/v) NaCl 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 10,000 rpm (13,300 g), 37°C for 10 min to remove precipitate.

The supernatant obtained(0.5 ml) was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of Folin-Ciocalteu reagent, then incubated at 37°C for 20 min. Absorbance at 660 nm of the color developed was measured. A correlation between an absorbance at 660 nm and tyrosine concentration was constructed for an estimation of tyrosine liberated.

One unit of protease was defined as an amount of the enzyme yielding an equivalent of 1 µg of tyrosine per minute under the defined assay conditions. Blank control was performed in the same manner except the enzyme was added after the addition of TCA solution.

Total protein in the supernatant was measured by the method described by Lowry *et al*. (1951). The detail of the analytical methods are described in Appendix C.

3.2. Identification methods

3.2.1 Morphological and cultural characteristics

Gram staining. Thin smear of bacterial cells prepared on a clean slide was heat fixed, covered with crystal violet solution for 30 sec, and rinsed with water. Next, the smear was covered with iodine solution for 30 sec, and rinsed with water, decolorized with 95%(v/v), ethanol washed with water and then counter stained with safranin for 30 min. The slide was blot dried and examined under microscope using oil immersion(1000x) objective len.

Colonial characteristics. An appearance of the above 3-7 days-old colony was record. Colonies grown on the halobacterium medium JCM No. 377 containing 10%(w/v) NaCl at 37°C for 1 day were examined for their characteristics as described by Barrow and Feltham(1993).

Motility and flagella stainning. Fresh cells were observed for their motility by wet mount under a microscope. The microscopic slide which was precleaned by the manufacturer and briefly flaming in order to confine, the stain with in two-thirds of the slide surface. An inoculating needle which touched to the top of an isolated colony was gently mixed with distilled water dropped in the confined area on slide. The suspension which did not have visible opalescence was spread over the staining area, tapped off onto a disinfectant-soaked gauze sponge, then air dried on a level surface. The spread cell suspension was stained by Forbes (1981) method, with 1 ml of stain at ambient temperature for 1 min, washed with tap water, counterstained with Hucker's modified Gram crystal violet for 1 min, washed, blotted dry, and examined under microscope using an oil immersion objective len by starting near to the wax line.

3.2.2 Physiological and biochemical characteristics

Oxidase test. A few drops of 1% (w/v) N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride were dropped on sterile filter paper disc. Bacterial cells grown on halobacterium medium JCM No. 377 containing 10% (w/v) NaCl at 37°C for 1 day was streaked across the moist area of the filter paper disc by sterlie loop. The apperance of dark-purple colour on the filter paper within 30 sec indicated positive reaction.

Catalase test. A small amount of pure culture was transferred from agar medium onto glass slide, immediately poured over with 3%(v/v) hydrogen peroxide(H_2O_2). The evolution of gas bubbles indicated a positive reaction.

Growth at different temperatures. Bacterial cells were inoculated on halobacterium agar medium JCM No. 377 and incubated at 37°C , 40°C and 50°C for 7 days, then growth was investigated.

Growth in different NaCl concentration. Bacterial cells were inoculated on halobacterium agar medium JCM No. 377 containing 0, 5, 10, 15, or 20%(w/v) NaCl and incubated at 37°C for 7 days, then growth was investigated.

Growth at different pH. Bacterial cells were inocubated into halobacterium broth JCM No. 377, which pH broth adjusted to 5, 6, 8 or 9 and incubated at 37°C for 7 days, then growth was investigated.

Nitrate reduction test. Baterial cells were inoculated into nitrate broth and incubated at 37°C for 7 days, then a drop each of Solution A and Solution B reagent for nitrate reduction test were added. Red colour formation within 5 min indicated negative reaction which nitrate was not reduced.

L- Arginine hydrolysis. Bacterial cells were inoculated onto arginine agar slant and incubated at 37°C for 7 days. Colour change of the indicator to red indicated a positive reaction.

Aesculin hydrolysis. Bacterial cells were inoculated into aesculin broth containing 10%(w/v) NaCl and incubated at 37°C for 5 days. Black colour formation which indicated a positive reaction was daily monitored.

Gelatin hydrolysis. Bacterial cells were inoculated on halobacterium agar medium JCM No. 377 containing 12%(w/v) gelatin instead of casamino acid and incubated at

37°C for 7 days, then poured over colonial surface with 5-10 ml of 30%(w/v) trichloroacetic acid. Clear zone surrounded the colony indicated a positive reaction.

Starch hydrolysis. Bacterial cells were inoculated on halobacterium agar medium JCM No. 377 containing 1%(w/v) soluble starch and incubated at 37°C for 7 days, then poured over the colony with Lugol's iodine solution. Clear zone surrounded the colony indicated a positive reaction. Area contained non-hydrolyses starch turned to blue colour.

Tyrosine hydrolysis. Bacterial cells were inoculated on halobacterium agar medium JCM No. 377 containing 0.5%(w/v) tyrosine instead of casamino acid and incubated at 37°C for 7 days. Clear zone surrounded the colony indicated a positive reaction.

Deoxyribonuclease (DNase) activity. Bacterial cells were inoculated on a DNase test agar medium and incubated at 37°C for 7 days, then poured over the colony with 1 N HCl. Clear zone surrounded the colony indicated a positive reaction.

Indole test. Bacterial cells were inoculated into tryptone broth and incubated at 37°C for 7 days, then 4 drops of Kovacs' reagent were added on the top of the culture. Red colour formation indicated a positive reaction.

Acid from carbohydrates. An acid formation from carbon sources was performed by using modified marine oxidation-fermentation medium or MOF as described by Leifson (1963), which various carbon sources at final concentration of 1% (w/v) was supplemented. The following 22 different carbon sources including L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, *myo*-inositol, maltose, D-mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose and D-xylose were test. The media were pH adjusted to 7.2 and supplemented with 0.2%(w/v) phenol red solution as an indicator. The medium (2 ml) was aliquoted into test tube. Two drops of bacterial cells suspensed in saline solution were inoculated into the medium and incubated at 37ºC for 7 days. Colour change from orange to yellow indicated a positive reaction. Cell growth and acid formation were daily monitored.

3.2.3 Chemotaxonomic characteristics

Cell wall analysis. Approximately 3 mg of dried cell prepared as described above were hydrolysed with 1 ml of 6 N HCl at 100 ºC for 18 hours. After cooling, the hydrolysate was filtered and the filtrate was dried by rotary evaporator. The dried material was dissolved in 1 ml of distilled water and repeated drying. The dry residue was redissolved in 0.3 ml of distilled water and analysed by thin-layer chromatography(TLC). The sample(3 µl) was applied on plastic cellulose TLC plate (Merck No. 5577, E. Merck, Darmstadt, FRG), developed with the solvent system of methanol-water-6N HCl-pyridine (80:17.5:1.5:10, V/V) which took approximately 3 hours or more. One microlitre of 0.01 M DL-diaminopimelic acid (Sigma Chemical Co., St. Louis, Mo., USA) was applied as a standard. The spots were visualized by spraying with 0.2%(w/v) ninhydrin in water-saturated n-buthanol followed by heating at 100 ºC for 5 min. Diaminopimalic acid (DAP) isomers appeared as dark-green spots with R_f 0.29 (LL-isomer) and 0.24(*meso*-and DD-isomer). 3-Hydroxy- DAP appeared below the *meso*-isomer with approximate R_f of 0.02. Spots of other amino acids run faster than DAP (R_f = 0.37-0.08). The DAP spot gradually disappeared in a few hours.

Quinone analysis. A 300 ml of bacterial culture grown in halobacterium medium JCM No.377 broth containing 10%(w/v)NaCl on a rotary shaker (200 rpm) at room temperature for 1 day was harvested by centrifugution at 4^{o}C, 12,000 rpm (9,200 g)(10 min) washed with sterile distilled water and lyophilized. The dried cells were suspended in chloroform-methanol (2:1, V/V) and shaked for 3 hours to extract isoprenoid quinone. After separation of cells by filration, the filtrate was concentrated to dryness under reduced pressure by a rotary evaporator. Crude quinone obtained was dissolved in small amout of acetone, analysis by thin-layer chromatography by spotted on siliga-gel glass plate (20x20 cm, E. Merk, Silica gel 60F254, Art.6715) and developed with a solvent system of petroleum and diethyl ether (85:15, V/V). Standard quinones were included. The quinone spots were visualized by UV light at 254 nm. The R_f of menaquinone was 0.4. Menaquinone band was scraped off and extracted with acetone. The purified quinones were analysed by HPLC (Shimadza model LC-3A) using µ-Bondapak C_{18} column (Water Associates, Milford, Mass., USA) eluted by methanol-isopropanol (1:1, v/v) at 1.2 ml/min flow rate. Number shown in an abbreviation of menaquinone (e.q. MK-7, MK-6, etc.) indicated number of isoprene unit in side chain.

DNA G+C base composition. Bacterial DNA isolation was performed by method recommended by Saito and Miura (1963). Briefly, log phase cell of bacterial (100 ml) grown in halobacterium medium JCM. No.377 broth containing 10%(w/v) NaCl at 37^{o}C (200 rpm) for 1 day were harvested by centrifugation at 4^{o}C, 12,000 rpm (9,200 g)(10 min) and washed twice with 10 ml of saline-EDTA. Bacterial cells were suspended in 0.1M Tris-buffer pH 9.0 containing 10mg/ml lysozyme in Saline-EDTA and 10%(w/v) sodium dodecyl sulfate (SDS) to induce cell lysis. After well mixing the suspension was heated at 60°C for 10 min. The cell suspension changed from turbid to opalescent and became very viscous indicated cell lysis. Phenol-chloroform (1:1 v/v) (4 ml) was added, mixed by vortexing at least 30 sec and centrifuged at 4^{o}C, 12,000 rpm (9,200 g) (10 min) for 10 min. The supernatant was then transferred to a small beaker. DNA was precipitated by adding cold 95%(v/v) ethanol into the supernatant. The DNA precipitate was spooled with a grass-rod, rinsed with 70%(v/v) then 95% (v/v) ethanol and air dried. The DNA was then dissolved in 5 ml of 0.1 x SSC, mixed with RNase A solution (0.3 ml) and incubated at 37 °C for 20 min. Then 0.5 ml of 10 x SSC and 2 ml of phenol-chloroform were sequentially added, mixed by vortexing for 1 min and centrifuged at 4^{o}C, 12,000 rpm (9,200 g) (10 min) for 10 min. The upper layer was transferred to new sterile tube. The DNA was precipitated by adding cold 95%(v/v) ethanol and the DNA precipitate was spooled with a glass-rod rinsed with 70%(v/v) then 95% (v/v) ethanol. After air dried, the DNA was dissolved in 5 ml of 0.1 x SSC. Purity and quality of the DNA solution were determined from the ratio of adsorbance at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur and Doty (1962). DNA G+C composition was determined by Tamaoka and Komagata (1984) method. DNA (0.5-1.0 g/l; $A_{260\ nm}$ = 10-20) was boiled in boiling water for 5 min, and cooled in ice water. The DNA solution (10 µl) was transferred to new eppendorf tube, mixed with nuclease P1 (EC 3.1.3.30)(10 µl) and incubated at 50 ºC for 1 hour. Then, alkaline phosphatase (EC 3.1.3.1) solution(10 µl) was added and incubated at 37 ºC for 1 hour. The DNA G+C content of the hydrolysate was analyzed by HPLC. The HPLC conditions used are shown in Table 3.1.

Table 3.1 HPLC conditions for DNA G+C content analysis

Column	Nakarai Cosmosil packed column 5C_{18}(150X4.6 mm)
Column temperature	Room temperature
Sample	5-10 µl
Eluent	0.2 M $NH_4H_2PO_4$-acetonitrile(20:1, v/v)
Flow rate	1ml / min
Detector wave length	270 nm

3.2.4 16S rRNA gene sequence and phylogenetic analysis

The 16S rRNA gene was PCR amplified using 9F (5'GAGTTTGATCCTGGCTCA G'3 , *Escherichia coli* numbering) and 1541R (5'AAGGAGGTGATCCAGCC'3) as forward and reverse primers, respectively. The amplified 16S rRNA gene sequence was analyzed by automated DNA sequencer ABI PRISM 377 Genetic analyser (Applied Biosystems) using the following primers: 339F(5'CTCCTACGGGAGGCAGCAG'3), 785F(5'GGATTAGATACCC TGGTAGTC'3), 1099F(5'GCAACGAGCGCAACCC'3), 357R(5'CTGCTGCCTCCCGTAG'3) and 802R (5'TACCAGGGTATCTAATCC'3). The sequence was multiply aligned with the CLUSTAL X program (version 1.83; Thompson *et al*., 1997), then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbor-joining method (Saito and Nei, 1987) in MEGA program version 4 (Kumar *et al.*, 1999). The confidence value of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The sequence similarity value among the closest strains were calculated manually after pairwise alignment obtained. Gap and ambiguous nucleotides were eliminated from the calculations.

DNA-DNA hybridization labelled with photobiotin

DNA-DNA hybridization; Under optimal condition, 100 µl portion of heat-denatured, purified DNA solution of unknown and type strains (10µg of DNA /1ml) in phosphate-buffered saline comtaining 0.1 M $MgCl_2$ were incubated for 2 hours at 37ºC in microdilution plates (Nunc Corp., Denmark). Photobiotinylation of DNA was performed, by meaning that 10 µg /1 ml of photobiotin and an equql volume of DNA solution (10 µg of DNA / 1 ml) were mixed in an eppendorf tube and then irradiated with sunlamp(500W) for 25 min. After irradiation, free photobiotin was removed by n-butanol extraction. The biotanylation of DNA was used immediately for hybridization experiment.

For quantitative detection of biotinylated DNA in microdilution wells, 200 µl of a prehybridize solution (20xSSC, 5%Denhadt solution, 50% formamide) containing 10 µg of denatured salmon sperm DNA / ml was added to microdilution plates and then incubated at 39°C for 1 hr. The prehybridize solution was discarded and replaced with 100 µl portions of hybridization mixture (20xSSC, 5% Denhadt solution, 3%dextrane sulfate, 50% formamide, 10 µg ofdenatured salmon sperm DNA / ml) contraining 10 µg of bitinytlated DNA. The microplates

were then covered with aluminium foil, and incubated for overnight (16 hours) at 39°C. After hybridzation, the microdilution well were washed three times 200 µl of 0.2 x SSC buffer. A 100 µl of streptavidin peroxidase conjugate solution (Boehringer Mannheim Germany) was added to the wells, and the preparations were incubated at 37°C for 30 min. After incubation, the wells were washed three times with PBS-buffer. Then the enzyme solution was discarded and 100 µl of substrate 3,3',5.5' – tetramethyl benzidine – H_2O_2 solution (Wako,Japan) was added to each well. The plates were inculated at 37°C for 10 min. The reaction was stopped with 2 M H_2SO_4 and the colour intensity was measured with Microplate Reader Model 3350 (Bio-Rad, CA,USA) at a wavelength 450 nm. The homology values for the DNA-DNA hybridization were calculateted. In practice, a DNA-homology above 70% indicates a relationship in the species level reported by Wayne et al., 1987.

3.3 Optimization of crude protease production

One loopful of strain TKNR13-3 grown in 50 ml of halobacterium medium JCM No. 377 containing 10% (w/v) NaCl in 250 ml Erlenmyer flask and incubated with shaking (200 rpm) at 37ºC for 2 days was used as inoculum.

A 2.5 ml of the inoculum was inoculated into 50 ml of the same medium in 250 ml Erlenmyer flask and incubated at the same above condition for 7 days. Protease production was quantified by the method described by Hiraga *et. al*. (2000). Hammarsten casein (Research organics, Germany) dissolved in 50 mM Tris-HCl, pH 7.5 containing 10% (w/v) NaCl, at 1.33% (w/v) was use as substrate. A 0.5 ml of cell-free supernatant was incubated with 1.5 ml of the substrate solution at 37º C for 60 min. The reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 4^{o}C, 12,000 rpm (9,200 g)(10 min) for 10 min. The resulting supernatant (0.5 ml) was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of Folin-Ciocalteu reagent, incubated at 37º C for 20 min and absorbance at 660 nm was determined. The amount of tyrosine liberated was quantified from linear relation curve plotted between various concentration of tyrosine and absorbance at 660 nm . Protein concentration was analyzed by the method described by Lowry *et. al*. (1951) using bovine serum albumin as standard. The influence of nutrients, yeast extract concentration, NaCl concentration, initial pH, and incubation temperature on protease production were determined by varying each parameters . An optimal condition of prior experiment was used as the basis in the latter experiment to optimize the conditions.

3.4 Characterization of crude protease

Protease produced by strain TKNR13-3 at the above optimal conditions was used in this study. The protease activity was determined by the method described above at various pH, temperature and NaCl concentration. The effect of various kind of protease inhibitors including 10 μM E-64, 2 mM EDTA, 10 μM leupeptin, 2 mM PMSF, 1 μM pepstatin and 0.1 mg/ml trypsin from soybean on protease activity was analyzed after incubation of an equal volume of crude protease and the above protease inhibitors at room temperature (26º - 28ºC) for 30 min.

The TKNR13-3 strain was cultivated in modified JCM no. 377 medium containing 2%(w/v) yeast extract instead of 0.5%(w/v) casamino acid, 5% NaCl, pH 6.5 at 37°C. Culture broth after 3 days of cultivation was centrifuged at 12000 xg for 10 min at 4 °C. Supernatant was precipitated by the addition of ammonium sulfate to 80% saturation and allowed to stand at 4 °C overnight. The resulting precipitate was collected by centrifugation at 10,000 xg for 20 min at 4 °C. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.5 and dialyzed against the same buffer. Sample was subjected to electrophoresis and stained for protease activity by the modified of Garcia-Carreno *et al*.(1993). Protein (100 μg) were loaded into the gel made of 4% stacking gel and 12% separating gel and then subjected to electrophoresis at a constant voltage of 75 V using a mimi vertical Hoefer apparatus. After electrophoresis, the gels were immersed in 100 mL of 1.675% (w/v) sodium caseinate in 50 mM Tris-HCl, pH 7.5 for 1 h at 0 °C to allow the substrate to penetrate into the gels. The gels were then immersed in 1.675% sodium caseinate in 50 mM Tris-HCl 10% NaCl pH 7.5 at 0 °C for 1 h. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol and acetic acid. Development of clear zones on blue background indicated proteolytic activity.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of protease-producing halophilic bacteria

4.1.1 Screening of protease-producing halophilic bacteria on agar plate

From 40 salt fermented food samples collected in Thailand, the moderately halophilic bacteria were isolated from only 30 samples. Disappearance of the desired bacteria in the other 10 samples might due to an aging of the samples. Among the 54 isolates that exhibited protease activity on skim milk agar plate containing 10%(w/v)NaCl (Table 4.1), 6 isolates were isolated from pla-jom, 25 isolates were isolated from pla-ra, 15 isolates were isolated from shrimp paste, 5 isolates were isolated from Fermented crab, 1 isolate was isolated from nam-pla and 2 isolates were isolated from mang-da dong.

Table 4.1 Sample, sample code, sample location, date of isolation and isolate no.

Product / sample code	Sample location	Date of isolation	Isolate no.
Pla-jom/ TP4	Nakhonayok	April 22 , 2006	TP4-4, TP4-5, TP4-6, TP4-7
Pla-jom/TP4			TP4-1, TP4-3
Pla-ra /TPS12	Suphanburi	November 15, 2006	TPS12,TPS12-1,TPS12-2
Pla-ra / TPS4	Suphanburi	November 15, 2006	TPS4-2, TPS4-1
Pla-ra /TPK	Kalasin	November 15, 2006	TPK1, TPK2
Pla-ra /TPP1	Petchaburi	September 19, 2007	TPP1-1
Pla-ra /TPP2	Petchaburi	Septempber 19, 2007	TPP2-2
Pla-ra /TPSK2	Sukotai	Septempber 19, 2007	TPSK2-2, TPSK2-3
Pla-ra /TPPN1	Pisanulok	Septempber 19, 2007	TPPN1-1, TPPN1-2
Pla-ra /TPNS1	Nakhonsawan	Septempber 19, 2007	TPNS1-2
Pla-ra /TPR1	Ratchaburi	Septempber 19, 2007	TPR1-1, TPR1-2
Pla-ra /TPN1	Nan	April 22, 2006	TPN1
Pla-ra /TPC5	Chonburi	November 15, 2007	TPC5-1, TPC5-2, TPC5-3, TPC5-4, TPC5-5
Pla-ra/ J	Samutsakhon	March 18, 2006	J1, J4
Pla-ra/CC	Chachoengsao	February 23, 2002	CC7-1
Shrimp paste /TS17	Nan	April 22, 2006	TSN17, TSN17-2, TSN17-4
Shrimp paste /TS17	Nan	April 22, 2006	TSN2
Shrimp paste /TKK3	Samutsakhon	June 28, 2006	TKK3-7
Shrimp paste /TKK8	Samutsakhon	June 28, 2006	TKK8
Shrimp paste /TKK9	Samutsakhon	June 28, 2006	TKK9
Shrimp paste /TKK10	Samutsakhon	June 28, 2006	TKK10
Shrimp paste /TKNR12	Nakhonratchasima	Septempber 19, 2007	TKNR12-2,TKNR12-8,
Shrimp paste /TKNR13	Nakhonratchasima	Septempber 19, 2007	TKNR13-1,TKNR13-3, TKNR13-5,
Shrimp paste /TKNR14	Nakhonratchasima	September 19, 2007	TKNR14-1, TKNR14-2
Fermented crab /TCN4	Nan	April 22, 2006	TCN4
Fermented crab /TCN24	Nan	April 22, 2006	TCN24, TCN24-1
Fermented crab /TCR11	Ratchaburi	Septempber 19, 2007	TCR11-1,TCR11-2
Nam-Pla /RF	Rayong	February 2, 2002	RF1-2
Mang-Da Dong (TM)	Samutsakhon	June 28, 2007	TM5-2-2
Mang-Da Dong (TM)	Samutsakhon	June 28, 2007	TM5-3
Total			54

4.1.2 Quantitatively protease activity assay

To measure protease production quantitatively, each isolate was grown in halobacterium medium JCM No. 377 containing 10%(w/v)NaCl at 37^{o}C (200 rpm) for 3 days. Cell-free supernatant of the cultures were analyzed for protease produced and protein content.

Strain TKNR13-3 produced maximal protease (0.55 units/ml). As shown in Fig.4.1, strain TKNR13-3 also produced maximal specific protease activity (4.23 units/ mg protein). Range of protease produced by the halophilic bacteria isolated was 0.01 – 4.23 units/mg protein. Fifteen isolates produced 0.01 -1.2 units/mg protein, 30 isolates produced 1.2-2.9 units/mg protein and 9 isolates produced 3- 4.23 units/mg protein. The strain TKNR13-3 was selected for further studies on optimal conditions for protease production and protease characterization.

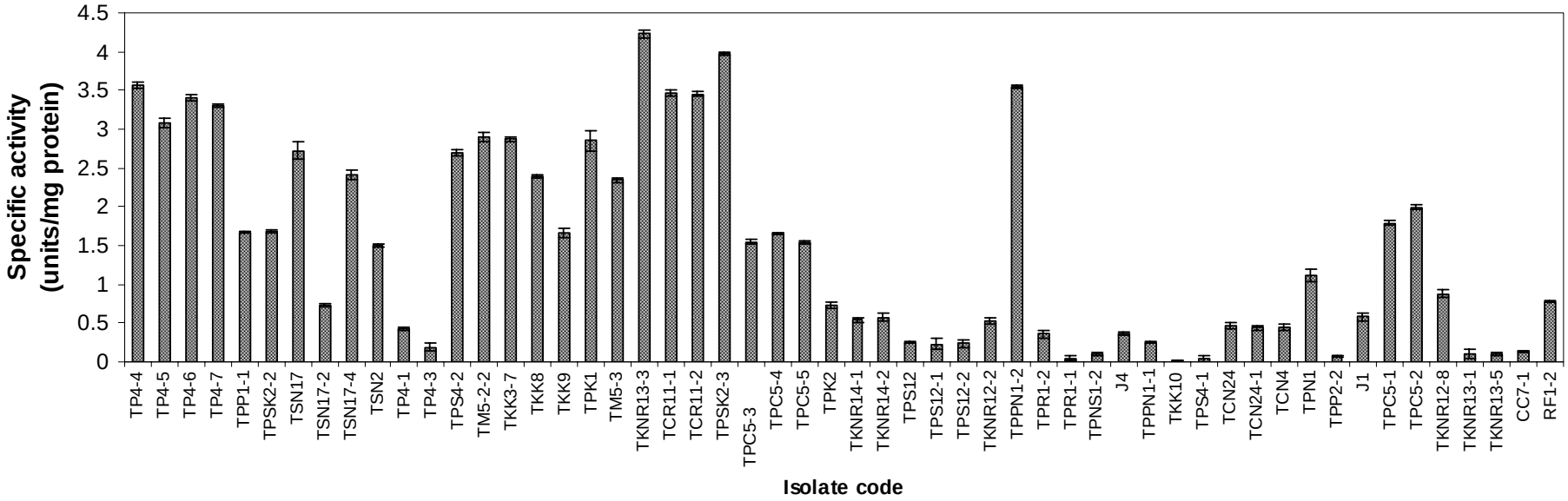

Fig. 4.1 Protease production in cell-free supernatant of the halophilic bacteria isolates.

4.2 Identification of halophilic isolates

On the basis of morphological, cultural, physiological, biochemical, and chemotaxonomic characteristics including 16S rRNA gene sequence and phylogenetic analysis**,** 54 isolates of the protease-producing halophilic bacteria were divided into 7 groups (Tables 4.2, 4.3, 4.4, and 4.5)

4.2.1 Morphological and cultural characteristics

Most of isolates were rod-shaped, aerobic, Gram-positive bacteria (except strain TP4-1, TP4-3, TPS4-2, TM5-2-2, TKK3-7, TKK8, TKK9, TPK1, TM5-3, TPS4-1, TPK2, and TKK10 were Gram-negative). According to colony morphology, Gram straining and cell shape, grow in NaCl, 54 isolated were divided into 7 groups. Group 1 isolates showed circular, yellow, smooth, slightly irregular, slightly raised. Group 2 appeared circular, slight irregular, raised, cream, creamy-grey, and milky white colonies. Group 3 translucent 5, 6 and 7 were Gram-negative bacteria. Colony morphology were variable characteristics as shown in Table 4.2.

4.2.2 Physiological and biochemical characteristics.

All of them grew in the medium containing 10-15%(w/v) NaCl, at pH 6 to pH 8 and hydrolysed DNA, casein and gelatin. Urease and indole test were negative. Most of them were positive for catalase, oxidase, and grew at 40 and 50 ºC, and produced acid from glucose, D-cellobiose, D-fructose, glycerol, D-galactose, maltose, and D-ribose but not from melezitose Nitrate reduction, hydrolysis of L-arginine, L-tyrosine, starch and Tween 80 were variable characteristics as shown in Table 4.3. (Table 4.4). All the isolates were moderately halophilic bacteria based on their optimal growth in 5-10%NaCl.

Table 4.2 Morphological and cultural characteristic of halophilic isolates.

Isolate code	Colony morphology	Cell shape	Gram	Skim milk (10% NaCl) agar medium	
				Colonial diameter(cm)	Protease activity (clear zone)
Group 1 : TP4-4	Circular, smooth, slightly	Rods	+	0.3-0.4	+++
TP4-5	irregular, raised, yellow				++
TP4-6	colonies.				++
TP4-7					++
TPP1-1					+++
TPSK2-2					++
TSN17-2					++
TSN17-4					++
TSN17					++
TSN2					+
Group 2 : TKNR13-3	Circular, slight irregular,	Rods	+	0.2-0.3	++++
TCR11-1	raised, translucent, cream				+++
TCR11-2	colonies.				++
TPSK2-3					++
TPC5-4					+++
TPC5-5					++
TPC5-3					+
TKNR12-2					+
TKNR14-1					+
TKNR14-2					+
TPR1-1					+
TPNS1-2					+
J4					+
CC7-1					+
TPPN1-1					+
TCN24					+
TCN24-1					+
TCN4					+
TPN1					+
TPP2-2					+
J1					+
TPC 5-1					+
TPC 5-2					+
TKNR12-8					+

Table 4.2 (cont.) Morphological and cultural characteristic of 7 groups of the bacteria isolates.

Isolate No.	Colony morphology	Cell shape	Gram	Skim milk (10% NaCl) agar medium	
				Colonial diameter (cm)	Protease activity (clear zone)
Group 3: TPR1-2	Circular, smooth, raised, brown cream colonies.	Rods	+	0.2-0.3	+
RF1-2					+
TKNR13-1					+
TKNR13-5					+
Group 4: TPS12	Circular, raised, light cream-beige colonies.	Rods	+	0.1-0.2	++
TPS12-1					++
TPS12-2					++
TPPN1-2					++
Group 5: TP4-1	Opaque, raised, light yellowish colonies.	Rods	-	0.2-0.3	+
TP4-3					+
TPS4-2					++
TM5-2-2					++
Group 6: TKK3-7	Circular, convex, opague, cream colonies.	Rods	-	0.3-0.5	+++
TKK8					+++
TKK9					++
TPK1					+++
TM5-3					+++
Group 7: TKK10	Circular, brown cream colonies.	Rods	-	0.3-0.5	+
TPS4-1					+
TPK2					+

Symbols: ++++; strong, +++; good, ++; moderately, +; weak, -; non

Table 4.3 Physiological and biochemical characteristics of the isolates.

Isolate No.	Gram	Endospore	Oxidase test	Catalase test	Growth at pH				Growth at °C		Growth in %NaCl					Hydrolysis						MR	VP	Nitrate reduction	Citrate utilization
					5	6	8	9	15°C	50°C	0%	1%	2%	15%	20%	Starch	Tween 80	Gelatin	Casein	Tyrosine	l-arginine				
TP4-4	+	+	+	+	-	+++	++	+	+	+	+	+	+++	+	+	+	-	+	+	-	-	-	-	-	-
TP4-5	+	+	+	+	-	+++	++	++	+	+	+	++	++	++	+	+	-	+	+	-	-	-	-	-	-
TP4-6	+	+	+	+	-	+++	+++	++	+	+	+	+	++	+	+	+	-	+	+	-	-	-	-	-	-
TP4-7	+	+	+	+	-	+++	++	++	+	+	+	++	++	++	+	+	-	+	+	-	-	-	-	-	-
TPP1-1	+	+	+	+	-	++	++	++	+	+	+	+++	++	+	+	+	-	+	+	-	-	-	-	-	-
TPSK2-2	+	+	+	+	-	++	++	++	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-
TSN17-2	+	+	+	+	-	+	+++	++	+	-	-	-	+	++	+	+	-	+	+	-	-	-	-	-	-
TSN17-4	+	+	+	+	-	+	+++	++	+	-	-	-	+	+	++	+	-	+	+	-	-	-	-	-	-
TSN17	+	+	+	+	-	+	+	+	-	-	-	-	-	++	+	+	-	+	+	-	-	-	-	-	-
TSN2	+	+	+	+	-	+	+++	++	-	-	-	-	+	+	++	+	-	+	+	-	+	-	-	-	-

Table 4.3 (cont.) Physiological and biochemical characteristics of the isolates.

Isolate No.	Gram	Endospore	Oxidase test	Catalase test	Growth at pH				Growth at °C		Growth in %NaCl					Hydrolysis						MR	VP	Nitrate reduction	Citrate utilization
					5	6	8	9	15°C	50°C	0%	1%	2%	15%	20%	Starch	Tween 80	Gelatin	Casein	Tyrosine	l-arginine				
TKNR13-3	+	+	+	+	+	++	+++	++	+	-	+	+	++	+++	+	-	-	+	+	-	-	-	-	+	-
Tcr11-1	+	+	+	+	+	++	+++	++	+	-	+	++	++	+	+	-	-	+	+	-	-	-	-	+	-
Tcr11-2	+	+	+	+	+	++	+++	++	+	-	+	++	++	+	+	-	-	+	+	-	-	-	-	+	-
TPSK2-3	+	+	+	+	+	++	++	++	+	-	-	++	++	+++	+	-	-	+	+	-	-	-	-	+	-
TPC5-3	+	+	+	+	-	++	++	++	-	-	-	++	++	+	-	-	-	+	+	-	+	-	-	+	-
TPC5-4	+	+	+	+	-	+	+	+	-	+	-	+	++	+	-	-	+	+	+	-	+	-	-	+	-
TPC5-5	+	+	+	+	-	++	++	++	-	-	-	+	+	+	-	-	-	+	+	-		-	-	+	-
TKNR 12-2	+	+	-	+	-	-	+	+++	-	+	-	+	+	++	-	-	+	+	+	-	-	-	-	-	-
TKNR14-1	+	+	+	+	-	+	+	+	-	+	-	+	++	+	-	-	+	+	+	-	+	-	-	+	-
TKNR14-2	+	+	+	+	-	+	+	+	-	+	-	+	++	+	-	-	+	+	+	-	+	-	-	+	-
TPR1-1	+	+	+	+	+	+++	++	++	-	+	+	+++	+++	+	-	-	-	+	+	-	+	-	-	+	-
TPNS1-2	+	+	+	+	+	+++	+++	+++	-	+	+	++	+++	+++	-	-	-	+	+	-	-	-	-	+	-
CC7-1	+	+	+	+	+	+	++	++	-	+	+	++	++	+	-	-	-	+	+	-	-	-	-	-	-
TPPN1-1	+	+	-	+	+	++	++	+	-	+	+	++	+++	++	-	-	-	+	+	-	-	-	-	+	-
J4	+	+	+	+	+	+++	++	++	-	-	+	++	+++	++	-	-	-	+	+	-	-	-	-	+	-

Table 4.3 (cont.) Physiological and biochemical characteristics of the isolates.

Isolate No.	Gram	Endospore	Oxidase test	Catalase test	Growth at pH				Growth at °C		Growth in %NaCl					Hydrolysis						MR	VP	Nitrate reduction	Citrate utilization
					5	6	8	9	15°C	50°C	0%	1%	2%	15%	20%	Starch	Tween 80	Gelatin	Casein	Tyrosine	l-arginine				
TCN24	+	+	+	+	-	w	+++	+++	-	+	+	++	+++	+	+	-	+	+	+	-	+	-	-	-	-
TCN24-1	+	+	+	+	-	w	+++	+++	-	+	+	++	+++	+	+	-	+	+	+	-	+	-	-	-	-
TCN4	+	+	+	+	-	w	+++	+++	-	+	+	++	+++	+	+	-	+	+	+	-	+	-	-	-	-
TPN1	+	+	+	+	-	++	+++	+++	-	+	+	+++	+++	++	+	-	-	+	+	-	+	-	-	-	-
TPP2-2	+	+	+	+	-	++	++	++	-	+	+	++	++	+	+	-	+	+	+	-	-	-	-	-	-
J1	+	+	+	+	-	++	++	++	-	+	+	++	+++	++	+	-	+	+	+	-	+	+	-	-	-
TPC5-1	+	+	+	+	-	+++	+++	++	-	+	-	++	++	++	-	-	-	+	+	-	+	-	-	-	-
TPC5-2	+	+	+	+	-	++	++	++	-	+	-	++	++	++	+	-	-	+	+	-	-	-	-	-	-
TKNR12-8	+	+	+	+	-	+	+++	+++	-	+	+	++	+++	+	+	+	+	+	+	-	+	-	-	-	-
TPR1-2	+	+	+	+	-	++	+	-	-	+	+	++	+++	+	-	-	-	+	+	-	-	-	-	+	-
RF1-2	+	+	+	+	-	+	++	++	-	-	+	++	++	+	-	-	-	+	+	-	+	-	-	+	-
TKNR13-1	+	+	+	+	+	+	++	+	-	-	+	+	++	++	+	-	-	+	+	-	-	-	+	+	+
TKNR13-5	+	+	+	+	+	+	++	++	-	-	+	++	++	-	-	-	+	+	+	-	-	-	+	-	-

Table 4.3 (cont.) Physiological and biochemical characteristics of the isolates.

Isolate No.	Gram	Endospore	Oxidase test	Catalase test	Growth at pH				Growth at °C		Growth in %NaCl					Hydrolysis						MR	VP	Nitrate reduction	Citrate utilization
					5	6	8	9	15°C	50°C	0%	1%	2%	15%	20%	Starch	Tween 80	Gelatin	Casein	Tyrosine	l-arginine				
TPS12	+	+	+	+	-	+	++	++	+	+	+	+	++	++	+	-	-	+	+	-	-	-	-	-	-
TPS12-1	+	+	+	+	-	+	++	++	+	+	+	+	++	++	+	-	-	+	+	-	-	-	-	-	-
TPS12-2	+	+	+	+	-	+	++	++	+	+	+	+	++	++	+	-	-	+	+	-	-	-	-	-	-
TPPN1-2	+	+	+	+	-	-	++	++	+	+	+	+	+	++	-	-	-	+	+	-	-	-	-	-	-
TP4-1	-	+	+	-	-	-	+++	+++	+	+	-	-	+	++	-	-	+	+	+	-	+	-	+	-	-
TP4-3	-	+	+	+	-	++	++	++	+	+	-	-	+	+++	-	-	+	+	+	-	+	-	-	-	-
TPS4-2	-	+	+	+	-	+	+++	+++	+	-	-	-	-	+	-	-	+	+	+	-	+	-	-	-	-
TM5-2-2	-	+	+	+	-	++	+++	+++	+	-	-	-	++	+++	-	-	+	+	+	-	+	-	+	-	-
TKK3-7	-	+	+	+	+	++	++	+++	+	-	-	-	+	+++	+	-	-	+	+	+	+	-	+	-	+
TKK8	-	+	+	+	+	++	++	+++	+	-	-	-	+	++	+	-	-	+	+	+	+	-	+	-	+
TKK9	-	+	+	+	+	++	+++	+++	+	-	-	-	+	+++	+	-	-	+	+	+	+	-	+	-	+
TPK1	-	+	+	+	+	+++	+++	+++	+	+	-	-	+	+++	++	-	-	+	+	+	+	-	+	-	+
TM5-3	-	+	+	+	-	+++	+++	+++	+	+	-	-	+	+++	++	-	-	+	+	+	-	-	+	-	-
TPK2	-	-	-	+	-	+++	+++	+++	+	-	+	++	++	++	-	+	-	+	+	-	+	-	-	-	+
TKK10	-	-	+	+	-	+++	+++	++	+	-	+	+	+	+	++	-	+	+	-	-	+	-	-	-	+
TPS4-1	-	-	+	+	-	+++	+++	+++	+	-	+	+	+	++	+++	-	+	-	-	-	-	-	-	+	+

Symbols: +; positive, -; negative

Table 4.4 Acid from carbohydrates of isolates.

Isolate	L-arabinose	D-cellubiose	D-fructose	D-galactose	D-glucose	Glycerol	Inulin	Lactose	myo-Inositol	Maltose	D-mannitol	Mannose	Melibiose	Melizitose	Raffinose	Rhamnose	D-ribose	Salicin	Surcose	D-sorbitol	D-trehalose	D-xylose
TP4-4	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TP4-5	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TP4-6	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TP4-7	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TPP1-1	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+	-
TPSK2-2	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TSN17-2	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	+	-	-	+	-	+	-
TSN17-4	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-
TSN17	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	+	+	-	+	-
TSN2	-	-	+	+	+	-	+	-	-	+	+	+	-	-	-	-	-	+	+	-	+	+
TKNR13-3	-	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TCR11-1	-	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TCR11-2	-	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TPSK2-3	-	-	+	+	+	+	-	+	-	+	-	+	-	-	-	-	+	-	+	-	-	-

Table 4.4 (cont.) Acid from carbohydrates of isolates .

isolate	L-arabinose	D-cellubiose	D-fructose	D-galactose	D-glucose	Glycerol	Inulin	Lactose	myo-Inositol	Maltose	D-mannitol	Mannose	Melibiose	Melizitose	Raffinose	Rhamnose	D-ribose	Salicin	Surcose	D-sorbitol	D-trehalose	D-xylose
TPC5-3	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+
TPC5-4	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+
TPC5-5	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+
TKNR12-2	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-
TKNR14-1	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+
TKNR14-2	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+
TPR1-1	-	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	-	+	+	-	-	-
TPNS1-2	-	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	-	+	+	-	-	-
CC7-1	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J4	-	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-
TPPN1-1	-	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-

Table 4.4 (cont.) Acid from carbohydrates of isolates .

isolate	L-arabinose	D-cellubiose	D-fructose	D-galactose	D-glucose	Glycerol	Inulin	Lactose	myo-Inositol	Maltose	D-mannitol	Mannose	Melibiose	Melizitose	Raffinose	Rhamnose	D-ribose	Salicin	Surcose	D-sorbitol	D-trehalose	D-xylose
TCN24	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-
TCN24-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
TCN4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
TPN1	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-
TPP2-2	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-
J1	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-
TPC5-1	-	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-
TPC5-2	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-
TKNR12-8	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-
TPR1-2	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
RF1-2	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-
TKNR13-1	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-
TKNR13-5	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-

Table 4.4 (cont.) Acid from carbohydrates of isolates.

isolate	L-arabinose	D-cellubiose	D-fructose	D-galactose	D-glucose	Glycerol	Inulin	Lactose	myo-Inositol	Maltose	D-mannitol	Mannose	Melibiose	Melizitose	Raffinose	Rhamnose	D-ribose	Salicin	Surcose	D-sorbitol	D-trehalose	D-xylose
TPS12	-	-	+	+	+	-	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	+
TPS12-1	-	-	+	+	+	-	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	+
TPS12-2	-	-	+	+	+	-	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	+
TPPN1-2	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TP4-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
TP4-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
TPS4-2	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
TM5-2-2	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TKK3-7	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TKK8	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-
TKK9	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	-	+	-	+	-
TPK1	-	-	+	-	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-
TM5-3	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-
TPS4-1	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-
TPK2	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+
TKK10	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-

Symbol: +; positive, -;negative

4.2.3 Chemotaxonomic characteristics

The results of cell wall analysis of 12 representative strains of each group revealed that 8 strains, TSN17, TKNR13-3, TPC5-4, TPS12, TPR1-1, TCK24 TPR1-2, and TKNR13-5, contained *meso*-diaminopimelic acid as the diagnostic diamino in the cell wall peptidoglycan, except strains, TP4-4, TM5-3, TKK10 and TPS4-2. The predominant menaquinone was menaquinone with seven isoprene units (MK-7) for 4 tested strains, TP4-4, TSN17, TSN17-2 and TSN17-4. The genomic DNA G+C contents ranged from 35 to 53.73 mol%.

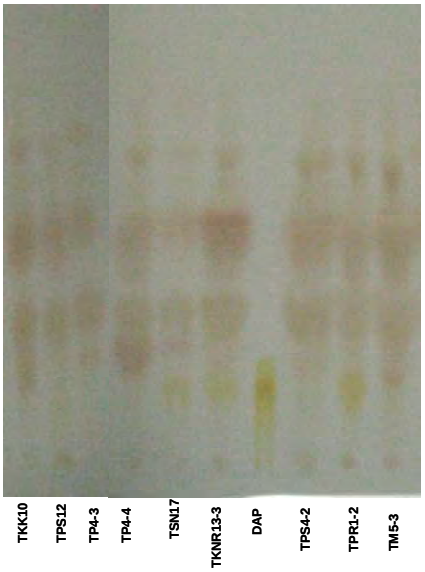

Fig 4.2 Thin Layer Chromatograph of diaminopimelic acid in cell wall of representative strains of each 7 different groups

4.2.4 16S rRNA gene sequence and phylogenetic analysis

The 16S rRNA gene sequence of representative strainsTP4-4, TSN17, TCK24 and TKNR13-3, comprised of 500 were analysed. The 16S rRNA gene sequence similarity of strain TP4-4 (Group 1A) and the type strain *Halobacillus trueperi* KCTC3686^T was 97%. TSN17 (Group 1C) with type strain *Halobacillus salinus* JCM11546^T was 97%. TCK24 (Group 2D) with type strain *Virgibacillus dokdonensis* KCTC3933^T was 99% while TKNR13-3 (Group 2A) with the type strain *V. halodenitrificans* DSM 10037^T (=JCM 12304^T) was 99.5%.

The similarity of strain TPC5-4 (510bp) in group 2B with *V. pantotheticus* LGM19492^T was 94%, TPR1-1(517 bp) in group 2C was 95% with *V. marismortui* DSM12325^T, TPR1-2 (503 bp) in group 3 was 98% with *Bacillus aquimaris* KCCM 14589^T, TPS12 (507 bp) in group 4 was 99% with *Oceanobacillus picturae* LMG 19492^T, TPS4-2(494 bp) in group 5 was 99% with *Idiomarina Ioihiensis* DSM15497^T, TM5-3(522 bp) in group 6 was 89% with *Salinivibrio costicola* ATCC 33508^T, and TKK10 (499 bp) in group 7 was 99% with *Halomonas alimentaria* KCCM41042^T (Table 4.5).

Table 4.5 Distribution and identification of the representative strains

Group	Isolate no.	% Similarity	Genera
1A	TP4-4 (500 bp)	97	*Halobacillus trueperi*
1B	TSN17-2 (435 bp)	98	*Halobacillus trueperi*
1B	TSN17-4 (524 bp)	97	*Halobacillus trueperi*
1C	TSN17(500bp)	97	*Halobacillus salinus*
2A	TKNR13-3 (500bp)	99.5	*Virgibacillus halodenitrificans*
2A	TCR11-1 (500bp)	99	*Virgibacillus halodenitrificans*
2B	TPC5-4 (510bp)	94	*Virgibacillus pantotheticus*
2C	TPR1-1 (517bp)	95	*Vigibacillus marismortui*
2D	TCN24 (500bp)	99	*Virgibacillus dokdonensis*
3	TPR1-2 (503bp)	96	*Bacillus aquimaris*
4	TPS12 (507 bp)	99	*Oceanobacillus picturae*
5	TPS4-2 (494bp)	99	*Idiomarina Ioihiensis*
6	TM5-3 (522bp)	89	*Salinivibrio costicola*
7	TKK10 (499bp)	99	*Halomonas alimentaria*

Characterization of the isolates

Group1. Group 1A contained 6 isolates TP4-4, TP4-5, TP4-6, TP4-7, TPP1-1 and TPSK2-2. They were spore forming, Gram-positive rods. Colonies were circular, smooth, slightly irregular, raised, yellow colonies. They were positive for catalase, oxidase, hydrolysis of casein, gelatin and starch but negative for MR-VP reaction, citrate utilization and nitrate reduction. They could grow at pH 6.0-9.0, at 15-50^{o}C, and in 0-20% NaCl. TP4-4 showed 98.5% sequence (1529 bp) similarity to *Halobacillus trueperi* KCTC 3686^{T} base on 16S rRNA gene sequence. They were identified as *Halobacillus trueperi* KCTC3686^{T} and differentiated from *Halobacillus trueperi* KCTC 3686^{T} (Yoon *et al*., 2003) as shown in Table 4.5. These isolates were found in pla-jom and pla-ra .

Group 1B contained 2 isolates TSN17-2 and TSN17-4. They were spore forming, Gram-positive rods. Colonies were circular, smooth, slightly irregular, raised, yellow colonies. They were positive for catalase and oxidase hydrolysis of casein, gelatin and starch but negative for MR-VP reaction, citrate utilization, nitrate reduction. (Tables 4.3). They could grow at pH 6.0-9.0 and at 15-40^{o}C in 2-20% NaCl. TSN17-4 showed 97% sequence (500 bp) similarity to *Halobacillus trueperi* KCTC3686^{T} base on 16S rRNA gene sequence. They were identified as *Halobacillus trueperi* KCTC 3686^{T} and differentiated from *Halobacillus trueperi* KCTC 3686^{T} as shown in Table 4.6. These isolates were found in shrimp paste.

Group 1C contained 2 isolates TSN17 and TSN2. They were spore forming Gram-positive rods. Colonies were circular, smooth, slightly irregular, raised, pale yellow colonies. The isolates in this Group showed differentiated characteristics from *Halobacillus salinus* JCM 11546^{T} as shown in Table 4.6. the representative strain TSN17 in Group showed 96.7% sequence (1529 bp) similarity to *Halobacillus salinus* JCM 11546^{T} (Yoon et al., 2003) base on 16S rRNA gene sequence. The isolates in this Group showed differentiated characteristics from *Halobacillus salinus* JCM 11546^{T} as shown in Table 4.6. These isolate were isolated from shrimp paste.

Table 4.6 Differential characteristics of strain in Group 1 and *Halobacillus* species.

Characteristics	Group 1A (6)	TP4-4	TSN17-4	*H. trueperi* KCTC3686^T	Group 1C (2)	TSN17	*H. salinus* JCM11546^T
Anaerobic growth	+	+	+	ND	-	-	-
Growth at :							
pH 5	-	-	-	-	-	-	+
Growth in : 0%NaCl	+	+	-	+	-	-	+
Hydrolysis of :							
Starch	+	+	+	-	+	+	-
Tween 80	-	-	-	-	-	-	+
Acid from :							
D-Mannitol	+(-1)	+	-	+	+	+	-
Cell wall type (DAP)	-	-	-	-	+	-	-
G+C (%mol)		47		43		43	45

Symbol: +; positive, -; negative, ND; data not shown, numbers in parentheses indicate the number of isolates showing the reaction.

Table4.7 DNA-DNA relatedness of *Halobacillus* strains

	Strain	%DNA relatedness with labeled strains
		TP4-4
Group IA	TP4-4	100
	TP4-5	70
	TP4-6	107
	TP4-7	105
	TPP1-1	76
Group 1B	TSN17-2	45
	TSN17-4	15.6
H. trueperi KCTC 3686^T		11.0
		TSN17
Group IC	TSN17	100
Group IB	TSN17-2	16.3
	TSN17-4	22.4

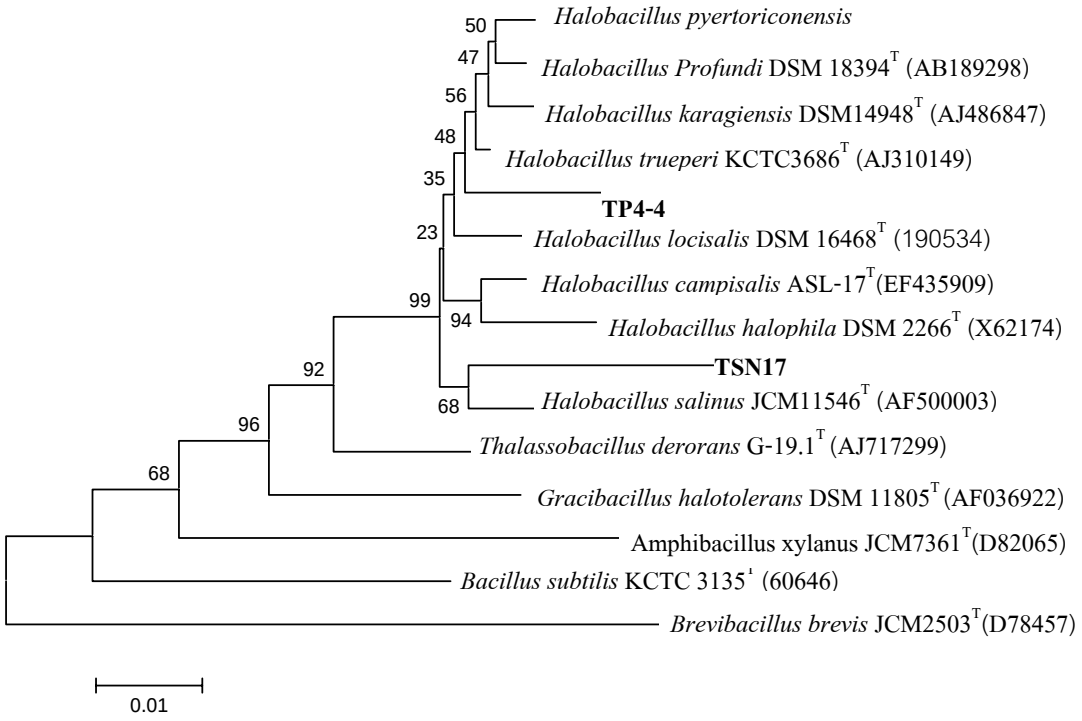

Fig. 4.3 Neighbour-joining-tree showing the phylogenetic position of strain TP4-4 and TSN17, *Halobacillus* species and related taxa based on 16S rRNA gene sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replication

Table 4.8 Percentage similarities of TP4-4 , TSN17, *Halobacillus* species and relate taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 TP4-4	100														
2 TSN17	96	100													
3 *H.salinus*	97..3	96.8	100												
4 *Halobacillus trueperi*	98.5	96.7	98.3	100											
5 *Gracilibacillus boraciitolerans*	94.4	93.7	94.7	95.3	100										
6 *Thalassobacillus devorans*	96	95	96.4	97.1	96	100									
7 *Halobacillus campisalis*	97.5	96.2	98.3	98.8	95.1	97	100								
8 *Halobacillus halophila*	96.7	95.5	97.5	98.1	94.4	96.4	98.5	100							
9 *Halobacillus locisalis*	97.9	96.6	98.3	98.9	95	96.4	98.3	97.6	100						
10 *Halobacillus puertoricone*	98	96.5	97.9	99.2	95.4	97.2	98.5	97.8	98.5	100					
11 *Halobacillus profundi*	98.2	96.6	98	99.3	95.2	96.7	98.5	97.9	98.5	99.3	100				
12 *Halobacillus karajiensis*	97.8	96.3	97.8	99.3	95	96.7	98.2	97.7	98.2	99	99.1	100			
13 *Brevibacillus brevis*	87.6	86.7	87.8	88.5	87.9	88.4	88.1	87.3	88.3	88.3	88.4	88	100		
14 *Amphibacillus xylanus*	90.8	90.1	91.1	91.5	91.9	91.8	91.7	91.5	91.6	91.3	91.3	91.1	87.5	100	
15 *Bacillus subtilis*	92	91.1	92.5	92.9	92.5	92.7	92.2	91.6	93.3	92.9	92.5	92.4	89	89.5	100

Group 2. Group 2A contained 4 isolates, TKNR13-3, TCR11-1, TCR11-2 and TPSK2-3 . Colonies were circular, slight irregular, raised, translucent, cream colonies. They grew in anaerobic condition but not at 50^{o} C. Positive for nitrate reduction but negative for citrate utilization. Acid produced from D-fructose, Lactose, D-manitol but not L-arabinose, raffinose, salicin and D-xylose. The strain TKNR13-3 showed 99% sequence (1550 bp) similarity to *Virgibacillus halodenitrificans* JCM 12304^{T}(Yoon et al., 2004) base on 16S rRNA gene sequence. All of them were identified as *V. halodenitrificans* based on DNA-DNA relatedness (Wyne et al., 1987) (Table). These isolates were isolated from shrimp paste, fermented crab and pla-ra.

Group 2B contained 6 isolates, TPC5-4, TPC5-3, TPC5-5, TKNR12-2, TKNR14-1 and TKNR14-2. Colonies were low convex, circular, slight irregular, creamy-grey colour. They no grew at pH5, in 0%NaCl. They showed negative for Voges-Proskauer reaction and citrate utilization but positive for nitrate reduction and hydrolysis of tween 80. Acid production from L-arabinose, D-xylose but not produce from raffinose and lactose. TPC5-4 showed 94% sequence (510 bp) similarity to *Virgibacillus pantotheticus* $IAM11061^{T}$ base on 16S rRNA gene sequence. These isolates were found in pla-ra and shrimp paste.

Group 2C contained 5 isolates, TPR1-1, TPNS1-2, J4, CC7-1 and TPPN1-1. Colonies were circular, smooth, slightly irregular, slightly raised, cream colour.They showed negative for Voges-Proskauer reaction and citrate utilization but positive for nitrate reduction. Acid produced from D- cellubiose, D-fructose, D-glucose and myo-Inositol No acid production from L-arabinose, raffinose, lactose and D-xylose. TPR1-1 showed 95 % sequence (517 bp) similarity to *V . marismortui* $DSM12325^{T}$ base on 16S rRNA gene sequence. All of them were identified as *V. marismortui* based on DNA-DNA relatedness (Wyne et al., 1987). These isolates were found in pla-ra.

Group 2D contained 9 isolates, TCN24, TCN24-1, TCN4, TPN1, TPP2-2, J1, TPC5-1, TPC5-2 and TKNR12-8. Colonies were Irregular, flat, translucent, raised, milky white colonies. They showed positive for hydrolysis of tween 80 but negative for Voges-Proskauer reaction and nitrate reduction. Acid produce from L-arabinose and salicin but produce from lactose and D-xylose. TCN24 showed sequence (1525 bp) similarity 99.5% to *V. dokdonensis* $KCTC 3933^{T}$ base on 16S rRNA gene sequence. These isolates were found in fermented crab, pla-ra and shrimp paste.

Table 4.9 Differential characteristics of strains in Group 2 and *Virgibacillus* sp.

Characteristics	Group 2A (4)	TKNR13-3	Group 2B (6)	TPC5-4	Group 2C (5)	TPR1-1	Group 2D (9)	TCN24	*V.halodenitrificans* DSM1037^T	*V.paantothenticus* IAM11061^T	*V.marismortui* DSM12325^T	*V.dokdonensis* KCTC3933^T
Growth at												
at pH 5.0	+	+	-	-	+	+	-	-	-	**ND**	**ND**	-
Growth in 0% NaCl	**+(-1)**	+	-	-	+	+	**+(-2)**	+	+	-	-	+
Anaerobic growth	+	+	-	-	+	+	+	+	+	+	-	+
Hyhrolysis of												
starch	-	-	-	-	-	-	-	-	-	**ND**	**ND**	+
Utilization of citrate	-	-	-	-	-	-	-	-	**ND**	+	**ND**	-
Nitrate reduction	+	+	**+(-1)**	+	**+(-1)**	+	-	-	+	+/-	**ND**	**ND**
Acid from												
L-Arabinose	-	-	**+(-1)**	+	-	-	**+(-7)**	-	-	-	-	-
Raffinose	-	-	-	-	-	-	**+(-7)**	-	+	-	+	-
Salicin	-	-	-	-	**+(-1)**	+	+	+	**ND**	+	**ND**	+
Galactose	+	+	-	-	-	-	**+(-1)**	+	+	+	-	+
Lactose	+	+	-	-	-	-	**+(-1)**	+	-	+/-	+	+
D-Xylose	-	-	**+(-1)**	+	-	-	-	-		-	**ND**	-

Symbol: +; positive, -; negative, ND; data not shown, numbers in parentheses indicate the number of isolates showing the reaction.

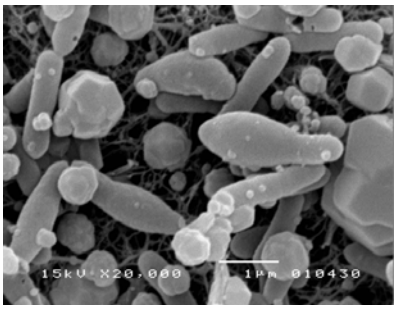

Fig. 4.4 Scanning electron micrograph of TKNR13-3 grown on JCM no. 377 agar at 37 ^{o} C for 5 days.

Table4.10 DNA-DNA relatedness of *Virgibacillus* strains

Strain		%DNA similarity with labeled strains JCM 12304^{T}
Group 2A	TKNR13-3	93.4
	TPSK2-3	66.2
	TCR11-1	79.2
	TCR11-2	79
V. halodenitrificans JCM 12304^{T}		100
		KCTC 3687^{T}
Group 2C	TPR1-1	107
	TPNS1-2	87.4
	CC7-1	80
	J4	105
	TPPN1-1	70.3
V. marismortui KCTC 3687^{T}		100

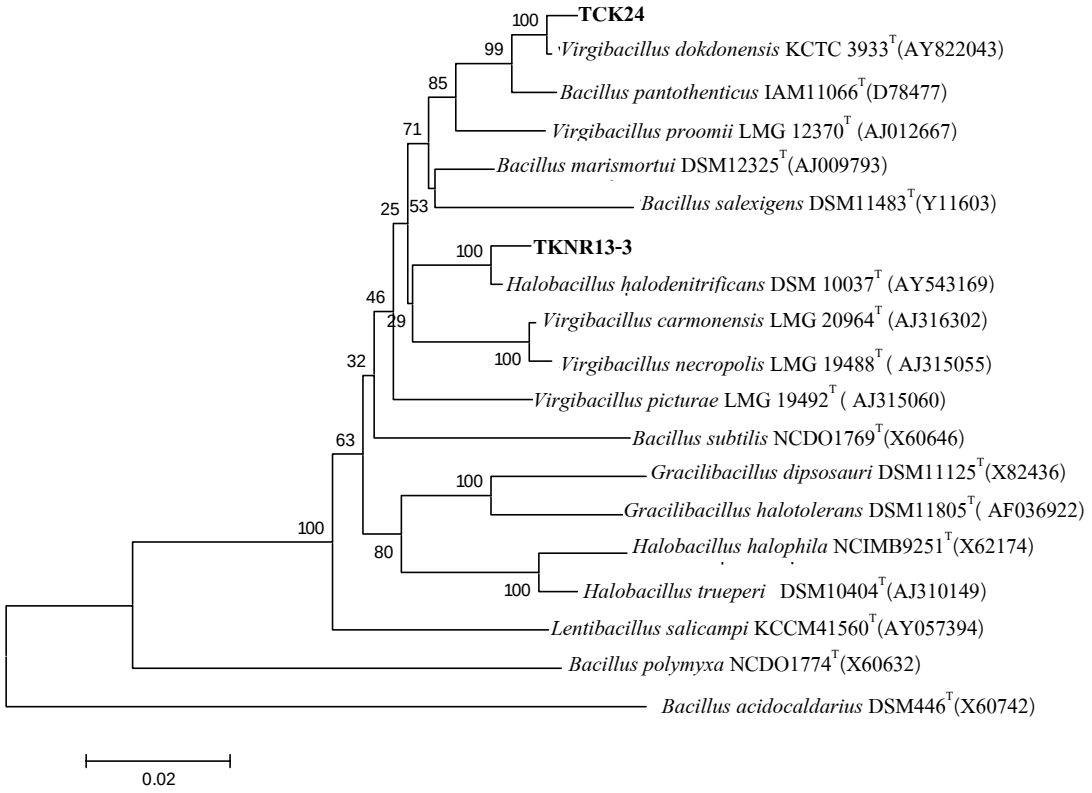

Fig. 4.5 Neighbour-joining-tree showing the phylogenetic position of strain TCK24, TKNR13-3, *Virgibacillus* species, and related taxa based on 16S rRNA gene sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replication

Table 4.11 Percentage similarities of TKNR13-3, TCK24, *Virgibacillus* species and related taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 TCK24	100																		
2 TKNr13-3	96.1	100																	
3 *V.dokdonensisDSW-10*	99.4	96.5	100																
4 *Virgibacillus pantothenticus*	98.4	96.3	98.8	100															
5 *Virgibacillus proomii*	96.8	96	97.3	97.5	100														
6 *Virgibacillus marismortui*	96.9	97	97.3	97.5	97.5	100													
7 *Bacillus salexigens*	95	95.1	95.4	95.3	95.6	96.4	100												
8 *Bacillus halodenitrificans*	96.3	99.3	96.7	96.8	96.4	97.6	95.8	100											
9 *Virgibacillus carmonensis*	95.7	96.8	96.1	96	96.5	96.9	95.3	96.9	100										
10 *Virgibacillus necropolis*	95.5	96.6	95.9	95.8	96.3	96.6	95	96.7	99.6	100									
11 *Virgibacillus picturae*	95.3	96.5	95.8	95.7	95.8	96.9	94.7	96.7	95.8	95.7	100								
12 *Lentibacillus salicampi*	94	94.2	94.5	94.7	94.5	94.9	93	94.8	94.7	94.8	93.7	100							
13 *Gracilibacillus dipsosauri*	92.9	93.6	93.4	93.3	94	93.6	92.2	94.2	93.4	93.3	93.8	92.6	100						
14 *Gracilibacillus halotolerans*	93.4	93.6	93.8	93.7	94.3	93.8	92.6	94	94	93.7	94.2	92.9	96	100					
15 *Halobacillus halophila*	94	94.1	94.4	94.5	94.2	94.5	92.2	94.3	94.1	93.8	93.8	92.3	93.2	93.7	100				
16 *Halobacillus trueperi*	94.2	94.7	94.7	94.6	94.6	95.4	93.3	94.9	94.8	94.7	94.6	92.9	94.3	94.8	98.2	100			
17 *Bacillus subtilis*	94.1	94.3	94.6	93.6	94.1	94.6	92.9	94.7	94.1	93.8	94.4	92	93.3	92.9	92.3	93.3	100		
18 *Bacillus polymyxa*	87.8	87.8	88.3	88.2	89.1	88.7	87	88.1	89.4	88.9	88.4	88.3	86.7	88.1	87.3	87.8	87.8	100	
19 *Bacillus acidocaldarius*	83.1	83.6	83.5	83.4	84.2	83.9	81.9	84.1	84.3	83.9	83.4	83.6	82.6	83.1	83.3	83.9	82.7	83.5	100

Group 3 had 4 isolates, TPR1-2, RF1-2, TKNR13-1, TKNR13-5. Colonies were circular, smooth, raised, brown cream colonies.They showed positive for Voges-Proskauer reaction and nitrate reduction. No acid production from L-arabinose, raffinose, lactose and D-xylose. The isolates in this group could be differentiated from *Bacillus aquimaris* KCCM 14589^T by grew at pH 9, hydrolysis of starch, acid produced from salicin and D-galactose. TPR1-2 showed 98 % sequence (503 bp) similarity to *Bacillus aquimaris* KCCM 14589^T(Yoon et al, 2003) base on 16S rRNA gene sequence . These isolates were found in pla-ra, nam-pla and shrimp paste.

Table 4.12 Differential characteristics of strains in Group 3 and *Bacillus* species.

Characteristics	Group 3 (4)	TPR1-2	*B.aquimaris* KCCM 14589^T	*B.marisflavi* KCCM 14588^T
Growth at 50° C	+(-3)	+	-	-
at pH 5.0	-	-	-	+
pH9.0	+(-1)	-	-	+
Growth with 0% NaCl	+	+	w	+
Hyhrolysis of :				
Starch	-	-	+	-
Tween 80	+(-3)	-	+	-
Acid from :				
D-Mannitol	-	-	-	+
Raffinose	-	-	-	w
Salicin	+	+	-	+
D-galactose	+(-1)	+	-	w
D-Xylose	-	-	-	+

Symbol: +; positive, -; negative, w; weakly positive, numbers in parentheses indicate the number of isolates showing the reaction.

Group 4 had 4 isolates, TPS12, TPS12-1, TPS12-2 and TPPN1-2. Colonies were circular, raised, light cream-beige colonies. They grew at 50°C but not in anaerobic condition.They showed negative for Voges-Proskauer reaction, starch, tyrosine hydrolysis and nitrate reduction. Acid produced from Galactose, D-manose and D-trehalose but not produced from L-arabinose, raffinose but produce from lactose and D-xylose. The isolates in this group could be differentiated from *Oceanobacillus picturae* LMG19492^T by grew at 50°C and anaerobic condition. TPS12 showed 99 % sequence (507 bp) similarity to *Oceanobacillus picturae* LMG19492^T (Lee et al, 2006) base on 16S rRNA gene sequence. These isolates were found in pla-ra.

Table 4.13 Differential characteristics of strains Group 4 and *Oceanobacillus* species.

Characteristics	Group 4 (4)	TPS12	*O.picturae* IGM19492^T	*O.itheyensis* HTE83^T	*O.oncorhynchi* R-2^T
Growth at 50° C	+	+	-	-	-
Anaerobic growth					
Hyhrolysis of :	-	-	+	**ND**	**ND**
Gelatin					
Acid from :	+	+	**w**	-	-
L-Arabinose					
L-Rhamnose	**+(-1)**	+	**w**	-	-
D-Mannose	-	-	-	**w**	-
D-Trehalose	+	+	**w**	+	+
	+	+	**w**	-	+

Symbol: +; positive, -; negative, ND; data not shown, numbers in parentheses indicate the number of isolates showing the reaction.

Group 5 had 4 isolates, TPS4-2, TM5-2-2, TP4-1 and TP4-3. Colonies were opaque, raised, light yellowish colonies . The isolates in this group could be differentiated from *Idiomarina* species by growth at 50° C**,** 0%NaCl and 20%NaCl as shown in Table 4.7 (Martınez-Ca novas et al, 2004). The representative strain in Group 3, TPS4-2 showed 99% sequence (494 bp) similarity to *Idiomarina loihiensis* DSM15497^T(Donachie et al, 2003) base on 16S rRNA gene sequence. These isolates were found in Pla-ra, Mang-Da Dong and Pla-jom.

Table 4.14 Differential characteristics of strain TPS4-2 in Group 5, and *Idiomarina* sp.

Characteristics	Group 5 (4)	TPS4-2	*I. loihiensis* DSM 15497^T	*I. fontislapidosi* LMG 22169^T
Growth at/in				
0% (w/v) NaCl	-	-	-	+
20%NaCl	-	-	+	+
50° C	+(-2)	-	-	-
Hydrolysis of :				
Starch	-	-	ND	-
Gelatin	+	+	ND	+
Tween80	+	+	+	+
Acid from:				
Glucose	+(-2)	-	-	-
Glycerol	+(-3)	-	+	ND

Symbol: +; positive, -; negative, ND; data not shown, numbers in parentheses indicate the number of isolates showing the reaction.

Group 6 contained 5 isolates, TM5-3, TKK3-7, TKK8, TKK9 and TPK1.Colonies were circular, convex, opague, cream colonies. They were Gram-negative bacteria and grew in anaerobic condition and grew at 2-20% NaCl. They showed positive for gelatin hydrolysis, VP reaction and citrate.No acid production from L-arabinose, raffinose, lactose, and D-xylose. TM5-3 showed 89% sequence(522 bp) similarity to *Salivibrio costicola* ATCC33508^T.(Mellado et al, 1996) base on 16S rRNA gene sequence. These isolates were found in Mang-Da Dong, shrimp paste and Pla-ra.

Table 4.15 Differential characteristics of TM5-3 in group 4 and *Salinivibrio* sp.

Characteristics	Group 6 (5)	TM5-3	*S. costicola* ATCC 33508^T
Growth at :			
pH 5	+(-1)	-	+
pH 9	+	+	+
Growth in :			
0%NaCl	-	-	-
20%NaCl	+	+	+
Hydrolysis of :			
Starch	-	-	-
Gelatin	+	+	+

Symbol: +; positive, -; negative, numbers in parentheses indicate the number of isolates showing the reaction.

Group 7 contained 3 isolates, TKK10, TPS4-1 and TPK2. Colonies were circular, brown cream colonies. They showed positive for nitrate reduction, citrate utilization and hydrolysis of tween 80 but negative for Voges-Proskauer reaction and nitrate reduction. They grew in 0%NaCl but not at 50 ^{o}C and pH 5. Acid produce from lactose but not from L-arabinose, raffinose, and D-xylose. TKK10 showed 97 % sequence(499 bp) similarity to *Halomonas alimentaria* KCCM41042^{T}(Yoon et al, 2002) base on 16S rRNA gene sequence. These isolates were found in shrimp paste and Pla-ra.

Table 4.16 Differential characteristics of strain TKK10 in Group7 and *Halomonas* sp.

Characteristics	Group 7 (3)	TKK10	*H. alimentaria* KCCM 41042^{T}	*H. pacifica* DSM4742^{T}
Nitrate reduction	+(-2)	-	+	-
Growth at 50^{o} C	-	-	-	ND
at pH 5.0	-	-	+	ND
Growth with 0% NaCl	+	+	-	ND
Anaerobic growth	+	+	+	-
Hydrolysis of :				
Tween 80	+(-1)	+	-	-
Tyrosine	-	-	-	+

Symbol: +; positive, -; negative, ND; data not shown, numbers in parentheses indicate the number of isolates showing the reaction.

In this study, total of 54 isolates exhibited caseinolytic activity on JCM No. 377 agar with 10% NaCl containing 1% skim milk and showed protease activities ranged from 0.0145-4.229 U/mg protein. They were distributed in salt fermented foods (pla-ra, pla-jom, shrimp paste, fermented crab, nam-pla and mang-da dong). They were identified based on the phenotypic and chemotaxonomic characteristics including the phylogenetic analysis using 16S rDNA sequences. They were divided in to 7 groups, TP4-4 (Group 1A) was closely related to *H. trueperi* KCTC 3686^{T}(98.5% sequence similarity) and TSN17 (Group 1B) to *H. salinus* JCM11546^{T} (96.8%). TKNR13-3 (Group 2A) was closely related to *V. halodenitrificans* JCM 12304^{T} (99.3%). TPC5-4 in group 2B was closed to *V. pantotheticus* LMG19492^{T} (94%), TPR1-1in group 2C was closely

related to *V. marismortui* KCTC 3867^{T} (95%), and TCK24 (Group 2D) was closely related to *V. dokdonensis* KCTC 3933^{T} (99.4%). TPR1-2 in group 3 showed 98% similarity to *Bacillus aquimaris* KCCM 14589^{T}. TPS12 in group 4 showed 99% similarity to *Oceanobacillus picturae* LGM 19492^{T}, TPS4-2 in group 5 showed 99% similarity to *Idiomarina Ioihiensis* DSM15497^{T}, TM5-3 in group 6 showed 99% similarity to *Salinivibrio costicola* ATCC 33508^{T}and TKK10 in group 7 showed 97% similarity to *Halomonas alimentaria* KCCM 41042^{T}. Some of the isolates seem to be the novel species, therefore, DNA-DNA hybridization experiments should be done to compare them with all the type strains such as in *Halobacillus* and related genera.

4.3 Optimization of protease production

Optimization of protease production of the best protease producing strain, TKNR13-3, was carried out in halobacterium medium JCM No. 377 containing 10%(w/v) NaCl and incubated at 37^{o}C (200 rpm). The influence of several factors including incubation time, medium composition, NaCl concentration, initial pH and incubation temperature on protease production were studied. Protease activity in cell-free supernatant and growth were determined. Growth was monitored from an absorbance at 600 nm. An prior optimal condition was used as the basis for the latter experiment.

4.3.1 Effect of incubation time on protease production

The strain TKNR13-3 was cultivated in halobacterium medium JCM No. 377 containing 10% (w/v) NaCl and incubated at 37^{o}C (200 rpm) for 7 days. Growth and specific activity of protease produced were monitored daily. Maximum specific activity of protease (5.2 units/mg protein) was obtained after 3 days of incubation, while growth was maximum(A600 nm of 1.6) after 2 days. (Fig. 4.6).

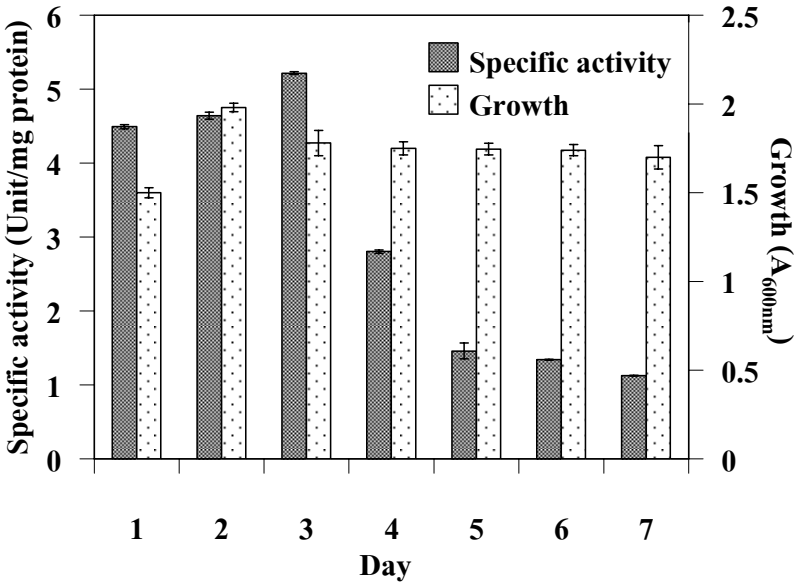

Fig. 4.6 Effect of incubation time on protease production and growth of strain TKNR13-3.

4.3.2 Effect of medium composition on protease production

The strain TKNR13-3 was cultivated in modified halobacterium medium JCM No. 168 containing 10% (w/v) NaCl and incubated at 37°C (200 rpm) for 3 days.The halobacterium medium JCM No. 377 was modified by using the following nutrients; soybean, skim milk, casein, yeast extract, gelatin, sodium caseinate at 0.5%(w/v) instead of casamino acid. Maximum protease production (0.9 units/ml) and growth (A600 nm of 1.9) were obtained in the modified medium containing yeast extract (Fig.4.7) An effect of yeast extract concentration in the modified halobacterium medium JCM No. 377 was investigated. Maximum protease production (1.75 units/ml) was obtained at 2% (w/v) yeast extract (Fig. 4.8).

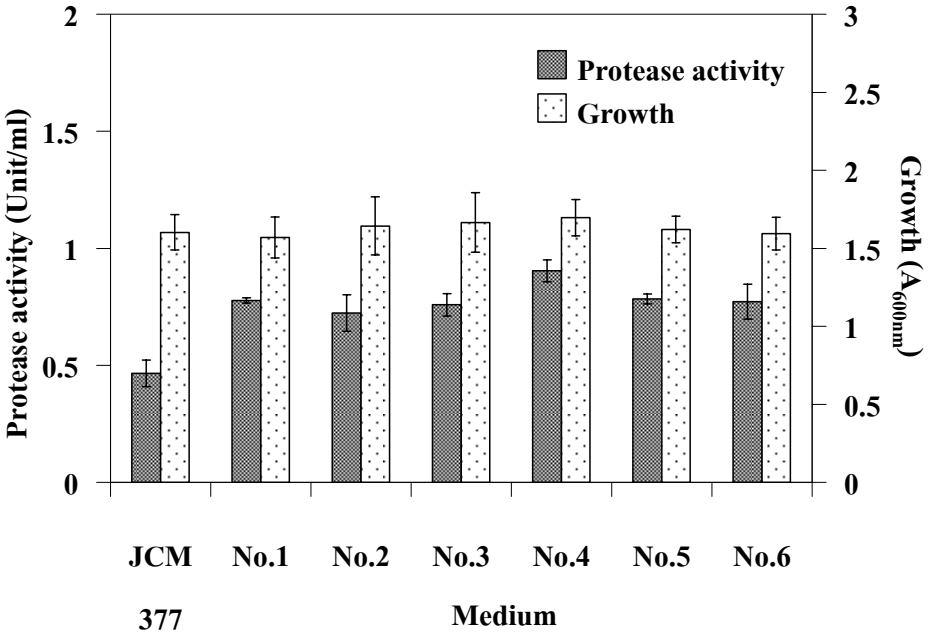

Fig 4.7 Effect of medium composition on protease production and growth of strain TKNR13-3 : modified JCM medium No. 377 by substitution of casamino acid with soybean (No.1), skim milk (No.2), casein (No.3), yeast extract (No.4), gelatin (No.5), sodium caseinate (No.6) at 0.5%(w/v), respectively .

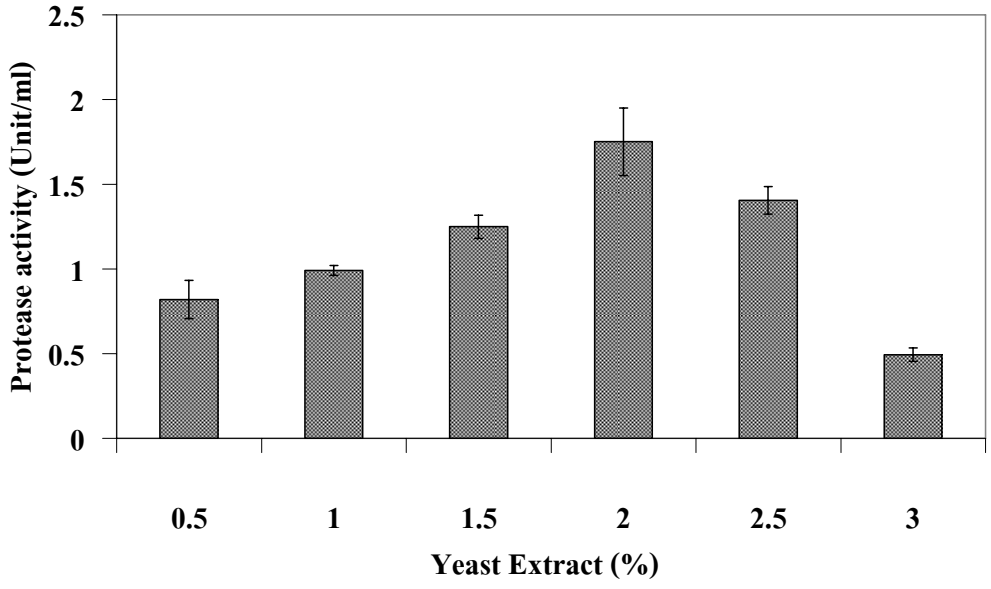

Fig.4.8 Effect of yeast extract concentration on protease production of strain TKNR13-3

4.3.3 Effect of NaCl concentration and cultivation time on protease production

The strain TKNR13-3 was cultivated in the modified halobacterium medium JCM No.377 containing 2% (w/v) yeast extract and various concentration of NaCl (0, 5, 10, 15 or 20% (w/v)) and incubated at 37ºC for 3 days with shaking (200 rpm). Maximum protease production (7.7 U/mg protein) was obtained in the medium containing 5% (w/v) NaCl (Fig.4.9) after 3 days of incubation. The result indicated that the strain TKNR13-3 is a moderate halophilic bacterium.

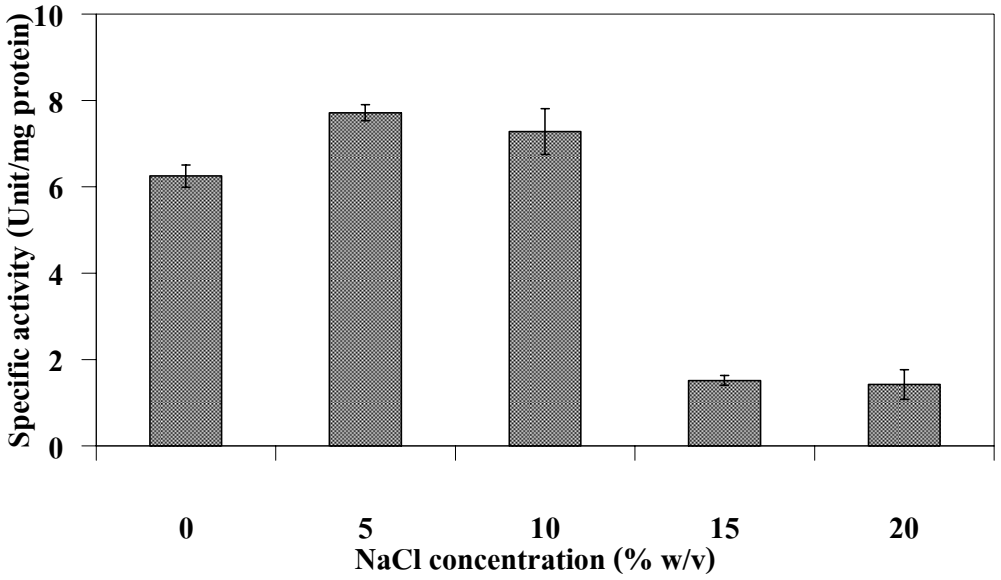

Fig. 4.9 Effect of NaCl concentration on protease production of strain TKNR13-3

4.3.4 Effect of initial pH on protease production

The strain TKNR13-3 was cultivated in the modified halobacterium medium JCM No.377 containing 2%(w/v)yeast extract and 5% (w/v) NaCl which was adjusted to pH 5.0, 6.0, , 6.5, 7.0, 7.5, 8.0, 9.0 and incubated at the same above condition for 3 days. As shown in Fig.4.9, the optimal pH for protease production was 6.5.

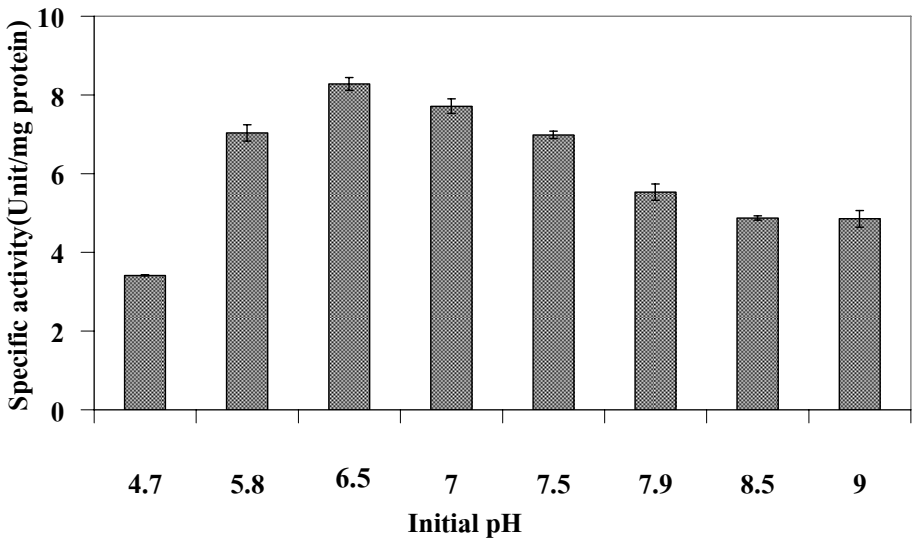

Fig.4.10 Effect of initial pH on protease production of strain TKNR13-3

4.3.5 Effect of incubation temperature on protease production

The strain TKNR13-3 was cultivated in the modified halobacterium medium JCM No.377 containing 2%(w/v)yeast extract and 5% (w/v) NaCl, pH 6.5 and incubated with shaking (200 rpm) at 30, 37, 45 or 50ºC for 3 days. As shown in Fig.4.11, the optimal temperature for protease production was 37ºC.

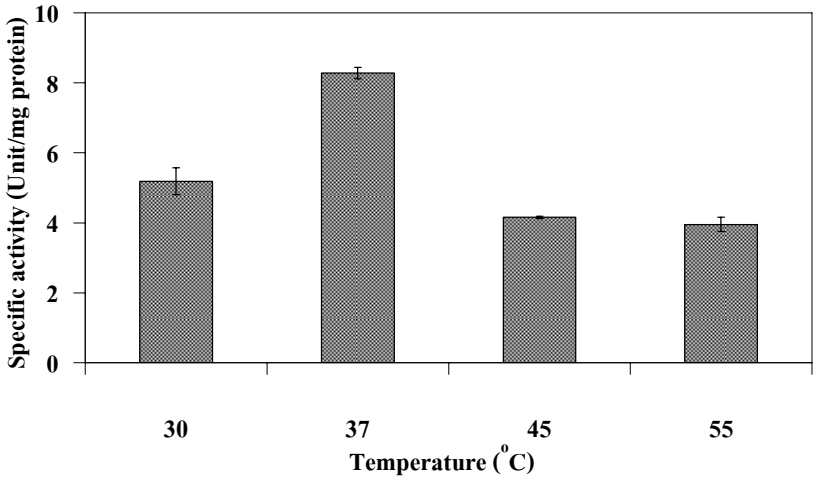

Fig.4.11 Effect of incubation temperature on protease production of strain TKNR13-3.

4.4 Characterization of crude protease

Some characteristics of crude protease produced by strain TKNR13-3 were determined using Hammersten casein as substrate and in the presence of 10% (w/v) NaCl.

Optimal pH: Protease activity assay was carried out over the pH range of 5 to 10 at 37ºC. Optimal pH for protease activity was 8 (Fig.4.12).

Optimal temperature: Protease activity (pH 8) was assayed at various temperature. Optimal temperature for protease activity was 60ºC (Fig. 4.13).

Optimal NaCl concentration : Protease activity was assayed at pH 8 and 60º C in the presence of various concentration of NaCl. Optimal concentration of NaCl for protease activity was 5% (w/v)(Fig.4.14).

Strain TKNR 13-3 produced protease at 4.23 units/mg protein and the enzyme was most active at pH 8, 60 ^{o}C and in the presence of 5%NaCl. *Virgibacillus marismortui*, moderately halophilic bacteria, from Pla-ra which produced almost the same amount of protease (4.38 units/mg protein). The protease reported was also most active in alkaline pH (pH 10) and in the presence of 5%NaCl. But the TKNR13-3 protease worked at higher temperature (60-70 ^{o}C) which was an adventage over the previous protease reported.

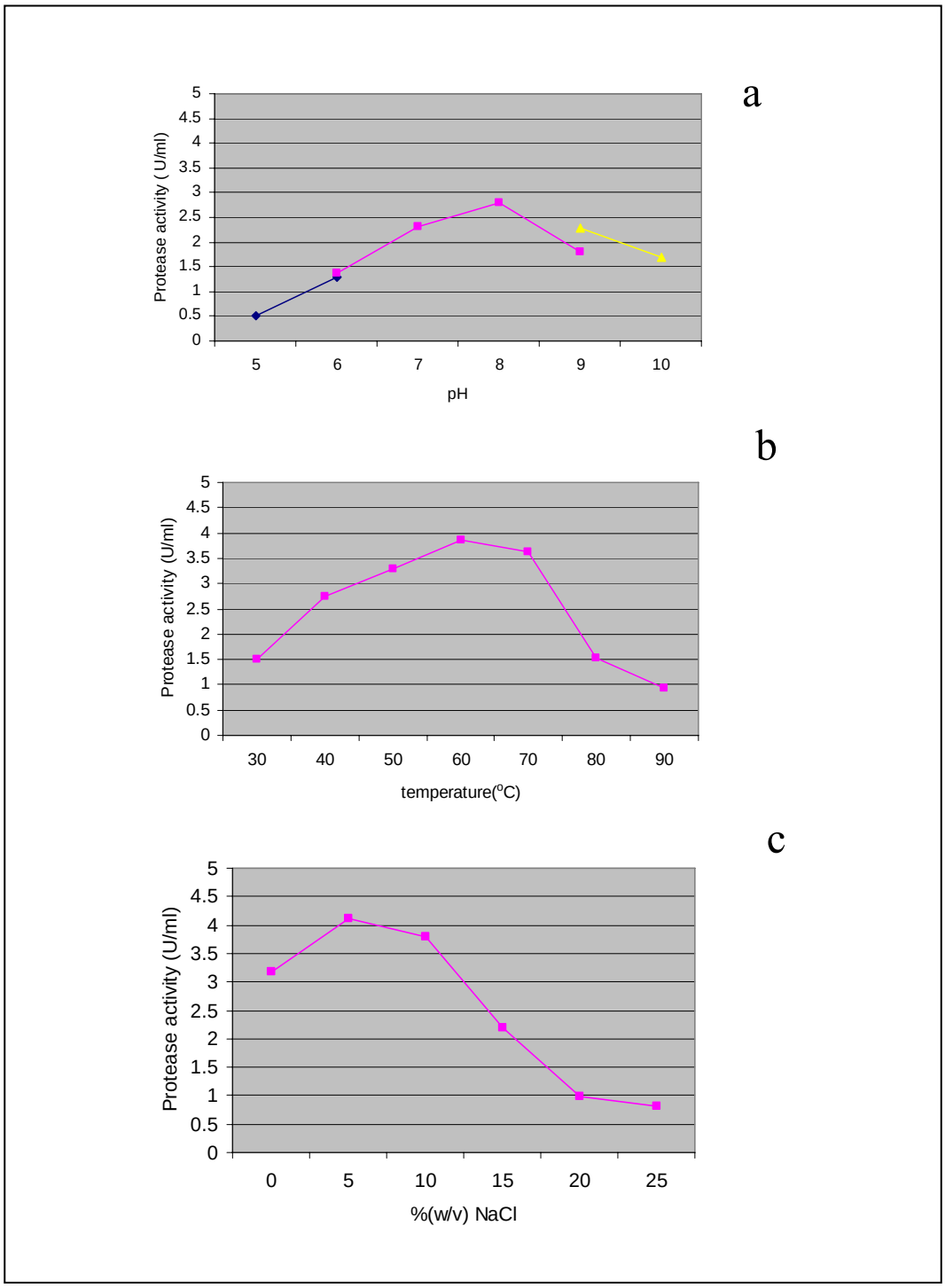

Fig.4.12 Effect of pH (a), temperature (b) and NaCl concentrations (c) on protease activity of strain TKNR13-3.

Crude protease incubated with various kind of protease inhibitors including 1mM PMSF, 1mM EDTA, 1 μM E-64, 1 μM pepstatin A, 10 μM Chymostatin, 10 μM leupeptin, 10 μM TLCK, 10 μM TPCK, 10 μM phosphoramidon and 0.1 g/l trypsin from soybean at room temperature (26-28 ^{o}C) for 60 min was assay for the remaining activity. Protease activity was measured in reaction mixture containing 10%(w/v)of NaCl concentration at pH 7.5 and 37^{o}C using Hammerstain casein as substrate. The enzyme activity was strong inhibited by chymostatin leupeptin and PMSF, 77.62%, 74.86% and 50.71%, respectively. The results suggest that the protease from strain TKNR13-3 were belong to serine type protease.Due to the highest inhibitor chymostatin, it was assumed to be chymotrypsin-type.

Table4.17 The effect of various kind of protease inhibitor on protease activity of TKNR13-3

Inhibitor	% inhibition	Specificity of inhibitor
control	0	
1mM PMSF	50.71	Serine protease
10 μM Leupeptin	74.86	Serine, cysteine protease
10μM Chymostatin	77.62	Chymotrypsin-like serine
10μM TLCK	25.52	Trypsin
10 μM TPCK	10.7	Chymotrysin
0.1g/l soybean trypsin inhibitor	10.93	Serine protease, trypsin
1mM EDTA	20.72	Metalloprotease
10μM Phosphoramidon	5.63	Metalloprotease
10 μM E-64	1.52	Papain, cysteine protease
1μM Pepstatin A	0.48	Aspartic protease

The crude extracellular protease of TKNR13-3 was identified by separation on gel electrophoresis followed by staining for proteolytic activity. Activity bands of protease were shown as clear zones on a dark background. Appearance of activity bands observed indicated the presence of proteases with the estimated molecular weights of 12, 21, 29, 39 and 49 KDa. Extracellular protease of TKNR13-3 were relatively small in comparison with the extracellular protease previously characterized from other moderately halophilic bacteria: 120 KDa from *Pseudomonas* sp. (A-14) (Duong *et al*. 1981), 38 KDa from *Pseudomonas* sp. CP76 (Sanchez-Porro *et al*. 2003), 100 KDa and 17 KDa from *Halobacillus thailanddensis* sp. nov.

isolated from fish sauce (Chaiyanan *et al*. 1999), 38 KDa from *Salinivibrio costicola* (Lama *et al*. 2005) , 18-81 kDa from *Virgibacillus* sp. SK37(Sinsuwan *et al*. 2007).

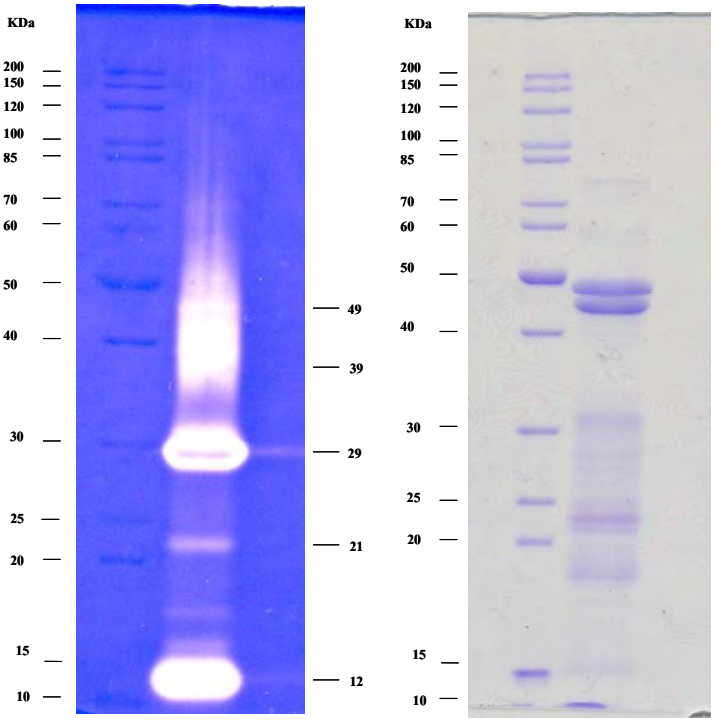

Fig.4.13 a) Activity stained substrate gel of crude extracellular protease of TKNR13-3 ,
b) SDS-PAGE analysis.

CHAPTER V

CONCLUSIONS

Fourty four halophilic protease producing bacteria comprising of 32 Gram- positive and 12 Gram-negative rod-shaped were isolated from pla-jom (6 isolates), pla-ra (25 isolates), shrimp paste(15 isolates), fermented crab (5 isolates), nam-pla(1 isolate) and mang-da dong (2 isolates) . They were divided into 7 groups based on their morphological, cultural, physiological and biochemical and chemotaxonomic characteristics including phylogenetic analysis 16S rRNA gene sequence. The isolates in Groups 1,2 ,3 and 4 were Gram-positive bacteria and in Groups 5,6 and 7 were Gram-negative bacteria.

- Group 1: contained 10 isolates. Their phenotypic characteristics were closed to *Halobacillus*. Some strains contained *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan. They had MK-7 as a major menaquinone. TP4-4 and TSN17 showed 98.5% sequence (1529 bp), 96.7% sequence (1529 bp) similarity to *Halobacillus trueperi* KCTC3686^T and *Halobacillus salinus* JCM 11546^T, respectively. They showed some different characteristics from the type strain of *Halobacillus trueperi* (Yoon *et al*., 2003) and they were the novel species as shown in Table 4.5 and Fig 4.4. These 10 isolates were found in pla-jom and pla-ra.

- Group 2: contained 24 isolates. Their phenotypic characteristics were closed to *Virgibacillus*. They contained *meso*-diaminopimelic acid in cell wall peptidoglycan. They had MK-7 as a major menaquinone. TKNR13-3 showed 99% sequence (1550 bp) similarity to *Virgibacillus halodenitrificans* JCM12304^T(Yoon *et al*., 2004). They were identified as *Virgibacillus*. These 24 isolates were found in shrimp paste, fermented crab and pla-ra.

- Group 3: contained 4 isolates. Their phenotypic characteristics were closed to *Bacillus*. TPR1-2 showed 98 % sequence (503 bp) similarity to *Bacillus aquimaris* KCCM 14589^T(Yoon *et al*, 2003 . These 4 isolates were found in pla-ra, nam-pla and shrimp paste.

- Group 4: contained 4 isolates. Their phenotypic characteristics were closed to *Oceanobacillus*. They contained *meso*-diaminopimelic acid in cell wall peptidoglycan. TPS12

showed 99 % sequence (507 bp) similarity to *Oceanobacillus picturae* LMG 19492^{T} (Lee *et al*., 2006). These 4 isolates were found in pla-ra.

- Group 5: contained 4 isolates. Their phenotypic characteristics were closed to *Idiomarina*. TPS4-2 showed 99% sequence (494 bp) similarity to *Idiomarina loihiensis* DSM15497^{T}(Donachie *et al*., 2003). These 4 isolates were found in pla-ra, mang-da dong and pla-jom.

- Group 6: contained 5 isolates. Their phenotypic characteristics were closed to *Salinivibrio*. TM5-3 showed positive for gelatin and tyrosine hydrolysis. TM5-3 showed 89% sequence(522 bp) similarity to *Salivibrio costicola* ATCC33508^{T}.(Mellado *et al*., 1996). These 5 isolates were found in mang-da dong, shrimp paste and pla-ra.

- Group 7: contained 3 isolates. Their phenotypic characteristics were closed to *Halobacills*. TKK10 showed 97 % sequence(499 bp) similarity to *Halomonas alimentaria* KCCM41042^{T}(Yoon *et al*., 2002). These 3 isolates were found in shrimp paste and pla-ra.

Among the 54 protease producing bacteria isolates which exhibited proteolytic clear zone, strain TKNR13-3 identified as *V. halodenitrificans* showed highest protease activity. Protease production of this strain was further optimized and some properties of the protease were characterized . Optimal conditions for protease of strain TKNR13-3 was produced maximum protease when grown in modified halobacterium JCM No. 377 medium without casamino acids containing 2% yeast extract and 5% (w/v) NaCl, pH 6.5 at 37º C for 3 days.

The optimum pH, temperature and NaCl concentration of the crude protease was pH 8, 60 ºC and 5 % (w/v) NaCl. The protease from strain TKNR13-3 were strongly inhibited (77.62%) by chymostatin .Therefore, the protease of strain TKNR13-3 were serine protease type chymotrypsin.

In this study, a lot of halophilic isolates distributed in salt femented foods. They were the moderately halophiles in which may play role in the fermentation of fish in high salt. It is still interesting to identify and characterize the enzyme activity of other isolates obtained in the study.

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APPENDICES

APPENDIX A

Instruments, materials, chemical reagents and media

1. Instruments and materials

- Analytical balance: Mettler Toledo model AG204, Urdorf, Switzerland.
- Autoclave: Tomy model SS-325, Tokyo, Japan.
- Centrifuges: Beckman Superspeed Centrifuge model Avanti J25, U.S.A; Eppendorf model 5430, Germany; and Sorvall: Superspeed Centrifuges model RC-5C, Plus and tabletop Centrifuges model RC-5C Plus, Newtown, USA.
- Circulating Water Bath: Techre model TE8 A, Cambridge, UK.
- Freezer Dryer: Savant model Super Modulya 233, NewYork, USA.
- Hot plate and stirrer: Thermolyne Crimarec2, Iowa, USA.
- Incubator: Memmert model BE500 (30°C, 37°C, 45°C, 50°C, and 55°C), Germany.
- Incubator shaker: New Brunswick Scientific model innova4300, U.S.A
- Magnetic stirrer: Ika model RO-10, Selangor, Malaysia.
- Microwave: Sanyo model EM-815FW, Japan.
- Oven: Contherm Digital Series incubator, Lower Hutt, New Zealand.
- pH Meter: Mettler Toledo model CH-8603, Switzerland.
- Pipetteman: Gilson, Villiers-Le-Bel, France.
- Precision balance: Mettler Toledo model PB3002, Urdorf, Switzerland.
- Refrigerator: Sharp model FC27 (-20°C), Japan and Deep Freezer REVCO model ULT1790-7-V12 (-80°C), USA.
- Shaking Water Bath: Memmert, model WB22 +SV1422, Germany.
- Spectrophotometer: Sherwood Scientific model259, Cambridge, UK.
- Stomacher:
- Vortex mixer: Barnstead/Thermolyne model M37610-26, Iowa, USA.

2. Chemicals (Analytical grade)

Chemicals	Company
Folin-Ciocalteu's phenol	Merck
Copper (II) sulfate pentahydrate	Sigma
Glucose	Merck
Hydrochloric acid	Merck
Sodium carbonate	Merck
Sodium citrate	Merck
Sodium hydroxide	Merck
Sodium postassium tartate	Merck
Trichloroacetic acid	Merck
tri-sodium citrate dihydrate	Merck
Ethylene diamine tetraacetic acid (EDTA)	Merck
Phenol red	Merck
Tyrosine	Sigma
Magnesium sulfate heptahydrate	Sigma
Sodium chloride	Carlo Erba
Trisma base	Merck
Sodium dodecyl sulfate	Fluka
Phenol	Carlo Erba
Chloroform	Mallinckrodt
Acetone	Merck
Methanol	Merck
Ethanol	Carlo Erba
L-arginine monohydrochloride	Fluka
L-glutamic acid sodium salt	BDH
Bovine serum albumin	Sigma

APPENDIX B

Culture media

All media were dispensed and steriled in autoclave at 121°C, 15 lbs/inch^2 for 15 min except media for acid from carbon sources test which was sterilized at 110°C, 15 lbs/inch^2 for 10 min.

1. 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	150	g
$FeCl_2.4H_2O$	0.362	g
$MnCl_2.4H_2O$	0.0362	g
Agar	20	g
Distilled water	1	L

Dissolved and adjusted pH to 7.2 with NaOH

2. Marine oxidation-fermentation medium (MOF)

Casitone(Difco)	1	g
Yeast extract	0.1	g
Ammonium sulfate	0.5	g
Tris buffer	0.5	g
Phenol red, 1.0% aq. solution	1	ml
Artificial sea water	1	L

Dissolved and adjusted pH to 7.5

3. L-Arginine agar medium

Peptone	1.0	g
NaCl	100	g
K_2HPO_4	0.3	g
Phenol red,1.0% aq.solution	1.0	ml
L(+)Arginine monohydrochloride	10.0	g
Agar	3.0	g
Distilled water	1	L

Dissolved, adjusted pH to 7.2 , distributed into tubes or screw-capped (6mm) bottles to a depth of about 16 mm(3.5ml).

4. Aesculin broth

Aesculin	1	g
Ferric citrate	0.5	g
NaCl	100	g
Peptone water	1	L

Dissolved the aesculin and iron salt in the peptone water, adjusted pH to 7.4 and sterilized at 115 ºC for 10 min.

5. Gelatin agar

JCM NO. 377 agar medium containing 10% (w/v) NaCl	
Gelatin	12% (w/v)

Dissolved the ingradients in distilled water, adjusted pH to 7.2.

6. Starch agar

JCM NO. 377 agar medium containing 10% (w/v) NaCl	
Starch	1% (w/v)

Dissolved the ingredient in distilled water and adjusted pH to 7.2.

7. Tyrosine agar

JCM NO.377 agar medium		
omitted casamino acid containing 10% (w/v) NaCl		
Starch	1% (w/v)	

Dissolved the ingredient in distilled water and adjusted pH to 7.2.

8. Deoxyribonuclease (DNase) media

DNase test agar (Difco)	42	g
Distilled water	1	L

Adjusted pH to 7.3 and heated to boiling to dissolve completely.

9. Tryptone water

Tryptone	5% (w/v)
NaCl	10% (w/v)

Adjusted pH to 7.2.

10. Nitrate broth

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

Dissolved and adjusted pH to 7.2.

APPENDIX C

Reagents

1. Determination of protein and soluble peptide

The protein and soluble peptide content were determined by the method of Lowry et al. (1951) using bovine serum albumin and tyrosine as standard, respectively.

1.1 Reagents

A: 2% (w/v) sodium carbonate in 0.1N NaOH

B: 0.5%(w/v) CuSO4 .5H2O in 1%(w/v) sodium citrate

C: 1 N Folin-Ciocalteu's phenol reagent

(2N Folin Phenol was diluted with distilled water to the final concentration of 1N, the solution was freshly prepared before use.)

D: Mixed 1 ml Reagent B with 50 ml Reagent A (or similar ratio) immediately before use.

1.2 Procedure

1. Placed 0.1 ml of proper dilution of culture broth (for protein determination) or clear supernatant of reaction mixture (for soluble peptide determination) in test tube.

2. Added 1 ml of Reagent D into the tube and vortexed immediately. Incubated at room temperature for 10 min

3. After 10 min of incubation, added 0.1 ml of Reagent C and vortexed immediately. Incubated at room temperature for 30 min.

4. Measured absorbance (OD) of the samples at 750 nm. Concentrations of protein or soluble peptide in the samples were compared to the standard curve of bovine serum albumin or tyrosine for

determination of protein or soluble peptide concentration, respectvely. Distilled water was used instead of sample in blank control.

1.3 Preparation of standard curve of tyrosine

Tyrosine at 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mM were prepared. The reaction mixtures for soluble peptide determination were carried out by the same procedure as described above. Measured absorbance at 750 nm were plotted against various concentrations of the tyrosine.

1.4 Preparation of standard curve of protein

Bovine serum albumin of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg/ml were. The reactions mixtures for protein concentration determination were carried out by the same procedure as described above. Measured absorbances at 750 nm were plotted against concentrations of the bovine serum albumin.

2. Flagella staining

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

Solvent was composed of 2.0 ml of 95% ethanol, 0.5 ml of glycerol, and 7.5 ml of Tris(hydroxymethyl)aminomethane buffer.

Dissolved the ingredients in the solvent.

3. Kovacs'reagent

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

Dissolved the aldehyde in the alcohol by gently warming in a water bath (about 50-55 ºC). Cooled and added the acid with care. Protected from light and stored at 4 C

4. Nitrate test reagent

Solution A

0.33%(w/v) sulphanilic acid in 5 N- acetic acid

Dissolved by gentle heating

Solution B

0.6%(w/v) dimethyl-α-napthylaminein 5 N-acetic acid

Dissolved by gentle heating

APPENDIX D

Reagents for DNA extraction and purification, DNA-DNA hybridization, and DNA base composition

1. Saline –EDTA(0.15M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA	37.22	g

NaCl and EDTA were dissolved in 1 L of ultra pure water and adjusted pH 8.0 by adding 1N HCl and then steriled by autoclaving at 121 ºC, 15 pounds/inch^2 pressure,for 15 min.

2. Phosphate-buffer saline (PBS)

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.12	g
Na_2HPO_4(anhydrous)	0.91	g
Distilled water	1	L

Dissolved and steriled by autoclaveing at 121 ºC, 15pounds/inch ^{2} pressure, for 15 minutes

3. 20 x SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	L

Dissolved, adjusted pH to 7.0 and steriled by autoclaveing at 121 ºC, 15 pounds / inch^2 pressure, for 15 minutes

4. 100 x Denhardt solution

Bovine serum albumin (Fraction V)	2	g
Polyvinylpyrrolidone	2	g
Ficoll 400	2	ml

Dissolved in 100 ml ultra pure water and stored at 4 ºC until used.

5. Salmon sperm

Salmon sperm DNA	10	mg per ml

10 mg of Salmon sperm DNA was dissolved in 10 Mm Tris + EDTA buffer pH 7.6 (1 ml), boiled for 10 min and then immediately cooled in ice. Sonicated the salmon sperm DNA solution for 3 min and stored at 4 ºC until used.

6. Prehybridization solution

100x Denhardt solution	2	ml
10 mg/ml Salmon sperm DNA	1	ml
20x SSC	10	ml
Formamide	50	ml
Distilled water	34	ml

Dissolved the ingredients, steriled and kept at 4 ºC.

7. Hybridization solution

Prehybridization solution	100	ml
Dextran sulfate	5	g

Dissolved and kept at 4 ºC.

For hybridization

8. Solution 1

Bovine serum albumin (Fraction V)	0.25	g
Triton X – 100	50	µl
PBS	50	ml

Dissolved the ingredients and kept at 4 ºC.

9. Solution 2

Streptavidin –POD conjugate	1	µl
Solution 1	4	ml

Dissolved just before used. The solution must be freshlv prepared.

10. Solution 3

3,3',5,5' Tetramethylbenzidine (TMB)		
(10 mg/ml in DMFO)	100	ml
0.3%(v/v) H_2O_2	100	ml
10% (v/v) DMFO in 0.1 M citric + 0.2 M Na_2HPO_4		
buffer pH 6.2	5	ml

Mixed the ingredients just before used. The solution must be freshly prepared.

11. Nuclease P1 solution

Dissolved Nuclease P1 (0.1 mg or 40 units) in 1 ml of 40 mM CH_3COONa + 12mM $ZnSO_4$, pH 5.3 and stored at 4 ºC .

12. Alkaline phosphatase solution

Alkaline phosphatase solution was prepared at 2.4 units/ml in 0.1 M Tris-HCl, pH 8.1.

APPENDIX E

Primers, 16S rDNA nucleotide sequences

1. Primers for 16S rDNA amplification and sequencing

9F	5'-GAGTTTGATCCTGGCTCAG-3’
1541R	5'-AAGGAGGTGATCCAGCC-3'
357R	5’-CTGCTGCCTCCCGTAG-3’
802R	5’-TACCAGGGTATCTAATCCC-3’
530F	5'-GTGCCAGCAGCCGCGG-3’

16S rDNA nucleotide sequences of strains

1. Strain TP4-4 (1529 bp)

CATTAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCG
GGAAGCGAGCTGATCCCTTCGGGGTGACGCTCGTGGAACGAGCGGCGAAAGGGGGGAGAAACAC
GTGGGCCAACCTGCCCTGTAAAGATCGGGATAACTCCCGGGAAACCGGGGCTAAATACCGGGTAA
TACTTTCTTTCGCATGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGG
TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAA
GTTCTGTTGTTAGGGAAGAACAAGTACCGTGCGAATAGAGCGGTACCTTGACGGTACCTAACGAG
GAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAAT
TATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGTAGCG
GTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCTGACG
CTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCGCACGCCGTAAACGG
ATGAGTGCTTAGGTGTTAAAAAATCTTCCACCCCTTAGTACTGAAGTTAACGCATTAAGCACTCCG
CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTACTTGACATCCTTGGACCTCCC
TAGAGATAGGGATTTCCCTTCGGGGACCAAGTGACAGGTGGTGCATGGTTGTCGTACAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATTTTAGTTGCCAGCATTCAGTT
GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAG
GTGTAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCC
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCC
GAAG

2. Strain TSN17 (1529 bp)

ATTAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTCCCTAATACATGCAAGTCGAGCGCGG
GAAGCGAGCTGATCCTCTTCGGAGGTGACGCTCGTGGAACGAGCGGCGGAAGGGTAAGTAACAC
GTGGGCCAACCCTGCCTGTAAGATCAGAAAAACCCCGGGAAACCGGAGCTAATGCCGGATTAAA
ACTTTTCTTCGCAGGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGAGCCACACTGGGATTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGATCGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGTTTTCGGATCGTAAA
GTTCTGTTGTTAGGGAAGAACAAGTACCGTGCGAATAGAGCGGCACCTTGACGGTACCTAACGAG

AAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
ATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTTTGATGTGAAAGCCCACGGCTCAACC
GTGGAGGGTCATTGGAAACTGGGGAACTTGAGGGCAGAAGAGGAGAGTGGAATTCCACGTGTAG
CGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCGGA
CGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACCCCGTAAACG
ATGAGTGCTAGGTGTTTAGGGGGCTTCCCACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCG
CCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTTGGACCACCCT
AGAGATAGGGTCTTCCCTTCGGGGACCAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTTAGTTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTG
TAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGG
AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
GGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCGCCG
AAGGTGGGACCAATGATTGGGGGGAAGTCGACTAGG

3. Strain TCN24 (1525 bp)

CCTAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGG
GAAGCAAGCAGATCTCCTTCGGGAGTGACGCTTGTGGAACGAGCGGCGGACGGGTGAGTAACAC
GTGGCCAACCTACCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGAAACAA
AGCGTCGCATGACGCAATGTTAAAAGGCGGCATATGCTGTCACTTACAGATGGGCCCGCGGCGCA
TTAGCTAGTTGGTGAGGTAAAGGCTCACCAACGCAACGATGCGTAGCCGACCTGAGAGGGTGGAA
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACTGAAAGTCTGACGGAGCAACGCCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACT
CTGTTGTTAGGGAAGAACAAGTGCCATTCGAATAGGTTGGCACCTTGACGGTACCTAACCAGAAA
GCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTTAACCGTGGA
GGGCCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACGGAAGCTG
AGGTGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACCCCCGTAAACGATGA
GTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGACCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACACCCCTAGAGAT
AGGGCATTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA

TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACT
CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGCAGCGAAGCCGCGAGGCCAAGC
AAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCA
CACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTG
GGACCAATGATTGGGGGGAAGTCGTACTAGG

4. Strain TKNR13-3 (1550 bp)

TTTGAGTTTTGCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGG
GAAGCAAGCTGATCCTCTTCGGAGGTGACGCTTGTGGAACGAAGCGGCGGACGGGGTGAGTTAAA
CACGTGGGCAACCTGCCTGTTAAGACTGGGATAACCCCGGGGAAACCGGGGCTAATACCGGATAA
TACTTTTCATCACCTGATGGAAAGTTGAAAGGTGGCTTCTTGCTACCACTTACAGATGGGCCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGG
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTTGATGAAGGTTTTCGGATCGTAAA
GCTTCTGTTGTTAGGGAAGAACCAAGTGCCGTTCGAATAGGGCGGCACCTTGACCGGTACCTAAC
CAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTTAAC
CGTGGAGGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAGTGCTAAGGGTGTTAGGGGGTTTTCCGCCCCCTTTAGTGCTGAAGTTAAACGCATTAAGC
ACTCCCCCTGGGGAGTACGGCCCGCAAGGCTGAAAACTCAAAAGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT
GCAATCGGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCA
GCTAGTGTCGTGAGGATGGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAG
CATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
ATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCAAA
ACCGCGAGGTCAAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTG
CATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAG
CCAGCCGCCGAAGGTGGGACCAATGATTGGGTGAAGTAATAATTCGTTGAGACCG

2. Primers for 16S rDNA amplification and sequencing

530F 5'-GTGCCAGCAGCCGCGG-3'

16S rDNA nucleotide sequences of strains

1. Strain TPS 4-2 (494 bp)

CTACNTCACTGTAACCGCTATTAACGATTACCCTTTCCTCACCACTGAAAGTGCTTTACAACCCGA
AGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTG
CTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGAACAGC
TAGGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCCGCTTGGGCTCATCTTTA
GGTGTGAGGCCCGAAGGTCCCCCACTTTGGTCCGTAGACATTATGCGGTATTAGCCACCGTTTCCA
GTGGTTGTCCCCCGCCTAAAGGCAAATTCCCAAGTCTTACTCACCCGTCCGCCGCTCGTCAGCAGA
GAAGCAAGCTTCTCTCTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTG
AGCCAGGATCAAACTA

2. Strain TM5-2-2 (500 bp)

ACGTCACTGTAACCGCTATTAACGACTACCCTTTCCTCACCACTGAAAGTGCTTTACAACCCGAAG
GCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCT
GCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAAACAGCTA
GGGATCGTCGCCTTGGTGAGCCTTTACCCCACCAACTAGCTAATCCCGCTTGGGCTCATCTTTAGG
TGTGAGGCCCGAAGGTCCCCCACTTTGGTCCGTAGACATTATGCGGTGTTAGCCACCGTTTCCAGT
GGTTGTCCCCCGCCTAAAGGGAAATTCCCAAGTCTTACTCACCCGTCCGCCGTTCGTCTGTAGAGA
AGCAAGCTTCTCTCTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCGGCGTTCAATCTGAG
CCAGGATCAAACTANCCCGGGGCGTTCACATCTAGCTTAACACACCGCCTACGTACGCTTTACGCN
NNNTAATTCCGATTAACGCTTGCACCCTCCGNANTACCGCNNNTGNTG

3. Strain J1 (500 bp)

GGNACCGTCAGGTGCCAACCTATTCGAATGGCACTTGTTCTTCCCTAACAACAGAGTTTTACGATC
CGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTA
CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTC
GGCTACGCATCGTTGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCT
GTAAGTGACAGCATATGCCGCCTTTTAACATTGCGTCATGCGACGCTTTGTTTCATCCGGTATTAG
CCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGTAGGTTGCCCACGTGTTACTCACCCGTCCGCC
GCTCGTTCCACAAGCGTCACTCCCGAAGGAGATCTGCTTGCTTCCCGCGCTCGACTTGCATGTATT
AGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTA

3. Strain TPSK2-3 (500 bp)

NNNNNNNNNNNNNNNNNGNACCGTCAGGTGCCGCCCTATTCGAACGGCACTTGTTCTTCCCTAA
CAACACATTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCC
ATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGC
CGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAA
TGCGCCGCGGGCCCATCTGTAAGTGGTAGCTAAAAGCCACCTTTCAACTTCTCATCAGGTGATGAA
AAGTATTATCCGGTATTAGCCCCGGTTTCCCGGGGTTATCCCAGTCTTACAGGCAGGTTGCCCACG
TGTTACTCACCCGTCCGCCGCTCGTTCCACAAGCGTCACCTCTGAAGAGGATCAGCTTGCTTCCCG
CGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTANNN

4. Strain TM5-3 (522 bp)

GNNNCTAACGTCAAGCCNTGCACCTATTAAGTACACCACCTTCCTCACTGCTGAAAGTGCTTNACA
ACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTCTCCCCCATTGTGCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAA
ACCAGCTAAGGATCGTCGCCTTGGTAAGCCATTACCTTACCAACTAGCTAATCCTAACTGGGCCCA
TCCCAACGCGATAGCTTACATGTAGAGGCCACCTTTGGTCCCTACTCGTAATGAACTAAGACATTA
TGCGTCAAACGGCTTTCCTCCTCGTCAGGGTAGGTTTCGAGACGTTACTCACCCGTCCGCCGCTCG
CCCGTCAGCAGATCCACGGAAAGTTCATTACCGGCATTTCCANTCGAGTTGCATGTGTTCGGCCTG
CCGGTGTTGTGCCAGCTGCCCGCGGATCAAACTAGCCAGGATCAAACTAT

5. Strain TPP1-1 (500 bp)

CCGTCAAGGTNCCGCTCTATTCGCACGGTACTTGTTCTTCCCTAACAACAGAACTTTNNNANCCGA
AGACCTTCATCGTTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTG
CTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGC
TACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCTGTA
AGTGATAGCGAGAAGCCATCTTTCAACTTTCCTTCATGCGAAAGAAAGTATTACCCGGTATTAGCC
CCGGTTTCCCGGAGTTATCCCGATCTTACAGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGC
TCGTTCCACGAGCGTCACCCCGAAGGGATCAGCTCGCTTCCCGCGCTCGACTTGCATGTATTAGGC
ACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACNN

6. Strain TCR11-1 (500 bp)

GNNNTNGNNCCGNNNNNTGCCGCCCTATTCGAACGGCACTTNTTCTTCCCTAACAACAGAGNTTT
ANGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGAT
TCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCT
CAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGC

CCATCTGTAAGTGGTAGCTAAAAGCCACCTTTCAACTTCTCATCAGGTGATGAAAAGTATTATC
CGGTATTAGCCCCGGTTTCCCGGGGTTATCCCAGTCTTACAGGCAGGTTGCCCACGTGTTACTCAC
CCGTCCGCCGCTCGTTCCACAAGCGTCACCTCCGAAGAGGATCAGCTTGCTTCCCGCGCTCGACTT
GCATGTATTAGGCACGCCGCCCGCGTTCGTCCTGAGCCAGGATCAAACTAAN

7. Strain TPR1-2 (503 bp)

GNNNNNGTACCGNCAGNTGCCGCCCTATTCGAACGGCACTTGTTCTTCCCTAACAACAGAGNTTT
ANNANNCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGAT
TCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCT
CAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGT
CCATCTGTAAGTGGTAGCGAAAAGCCACCTTTCAACAGTTCCTCATGCGAGGAATGAAGTTATCC
GGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACC
CGTCCGCCGCTGATCTCAGGGAGCAAGCTCCCATCGATCCGCTCGACTTGCATGTATTAGGCACGC
CGCCGGCGTTCGTCCTGAGCCAGGATCAAAC

8. Strain TPS12 (507 bp)

GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGCGCTCGCAGGCGGTCCTTTAAGTCTGATGTGAAATCTCGCGGCTCAACCGCGAA
CGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTG
AGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTC—
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGCTA

9. Strain TPC5-4 (510 bp)

NNNNNNNNNNNNNNGGNNAGTACCGTCAAGGTNCTGTCCTATTCGAACANTACTTGTTCTTCCCT
AACAANGNNNNNNNNNNNNNNACCTTCATCACTCACGCGGNNNNNNNNNNCTTTNNNCCATTGC
GGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCNNTCCCAGTGTGGCCGATC
ACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCTTTTATCTCACCAACTAGCTAATGCGC
CGCGGGCCCATCTGTAAGTGACAGCAAATGCCGCCTTTTAACTTCCCATCAGGCGATGAAAAGTG
TCATTCGGTATTAGCTCACGTTTCCGCGAGTTATCCCAATCTTACAGGCAGGTTGCCCACGTGTTA
CTCACCCGTCCGCCGCTCGTTCCACAAACGTCACCCCCGAAGGGGATCGATTTGCTTCCCGCGCTC
GACTTGCATGTATTAGGCACGCCGCCAGCGTTNGTCCTGAGCCAGGATCAAANNNNN

10. Strain TPP2-2 (500 bp)

NNNNNNNNNGNNNGGNACNGTCAAGGNGNNNNCTATTCGAATGGCACTTGTTCTTCCCTAACAAC
NNNNNNTTANNATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGNCAGACTTTCTTCCATTG
CGNAAGATTCCCTACTGCTGCCTCCCGTANGNGTCTGGGCCGTGTCTCANTCCCAGTGTGGCCGAT
CACCCTCTCNGGTCGGCTACTCATCGTTGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATGCG
CCGCGNGCCCNTCTGN

11. Strain TKK10 (499 bp)

GNNNNNACGGTCTCTCTGTGGGTGAGTCCTCCCTCGAGGGTATTGGCCCCGAGGATTTCTTCCCCA
CTGAAAGTGCTTTACAACCCGAAAGCCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCC
CATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTTCGGGCCGTGTCTCAGTCCCGATGTGG
CTGATCATCCTCTCAGACCAGCTACGGATCGCGGCCTTGGTGAGCCATTACCTCACCAACTAGCTA
ATCCGACATAGGCTCATCCGATAGCGCAAGGTCCGAAGATCCCCTGCTTTCTCCCGTAGGACGTAT
GCGGTATTAGCCTGGGTTTCCCCAGGTTATCCCCCACTACCGGGCAGATTCCTATGCATTACTCAC
CCGTCCGCCGCTCGACGCCTGGAAGCAAGCTTCCATCGTTTCCGCTCGACTTGCATGTGTTAGGCC
TGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCTTA

12. TKNR12-2 (520 bp)

NNNTTCNTGCTTCTGGTTAGGTACCGTCAAGGTACCGCCCTGTTCGAACGGTACTTGTTCTTCCCGT
AACAACAGAGCTTTACGATCCGAGGACCTTCTTCACTCACGCGGCGTTGCTCCGTCAGACTTTCGT
CCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTAGGCTCTTACCCCACCAACTAGCT
AATGCGCCGCGGGCCCATCTGCAAGTGATAGCACAAAGGCCATCTTTCAAACATGAATCATGCGA
TTCACGTCATCATCCGGTATTAGCCCCGGTTTCCCGGGGTTATCCCAGTCTTGCAGGTAGGTTGCC
CACGTGTTACTCACCCGTCCGCCGCTCGTTCCACAGACATTGTCCCCGAAGGGACGCTGTCTGCTT
CCCGCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCATGATCAAACTCT

13. TPC5-3 (333 bp)

NAAGCAGGCTGGCTTCTGGTTAGTACGTCAGGTACTGTCCTATTCGAACAATACTTGTTCTTCCCT
AACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGT
CCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCTTTTATCTCACCAACTAGCT
AATGCGCCGCGGGCCCATCTGTAAGTGACAGCAAATGCCGCCTTTTAACTTCCCATCAGGCAATG
AAAA

14. CC7-1 (394 bp)

TGNNCATACAAGTCTTCTGGTTAGTACGTCAGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCT
AACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGT
CCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCATTCCCAGTGTG
GCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTAGGCCGTTACCCTACCAACTAGCT
AATGCGCCGCGGGCCCATCTGTAAGTGACAGCCAAAAAGGCCGCCTTTCAACTTCTTGGCATGTA
CCAAAAAGTGTTATCCGGTATTAGACCCGGTTTCCCGGGGTTATACCAGTCTTACAGGCAAGTT

15. TSN17-2 (435 bp)

NNNGCNGACGGGCTCTCGTTAGGTACCGTCAGGTACCGCTCTATTCGCACGGTACTTGTTCTTCCC
TAACAACAGAACTTTACGATCCGAAGACCTTCATCGTTCACGCGGCGTTGCTCCGTCAGACTTTCG
TCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGT
GGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGC
TAATGCGCCGCGGGCCCATCTGTAAGTGATAGCAAGAAGCCATCTTTCAACTTTCCTTCATGCAAA
AGAAAGTATTACCCGGCATTAGCCCCGGTTTCCCGGGGTTATTCCGATCTTACAGGCAGGTTGCCC
ACGTGTTACTCACCCGTCCGCCGCTCGTTCCAAGAACTT

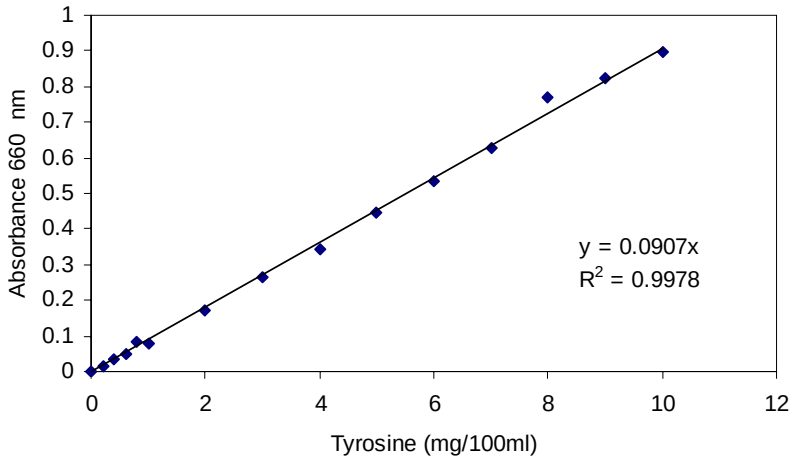
16. TSN17-4 (524 bp)

NNNNNNANCAGGCTCTCGTTAGGTACGTCAGGTACCGCTCTATTCGCACGGTACTTGTTCTTCCCT
AACAACAGAACTTTACGATCCGAAGACCTTCATCGTTCACGCGGCGTTGCTCCGTCAGACTTTCGT
CCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCT
AATGCGCCGCGGGCCCATCTGTAAGTGATAGCAAGAAGCCATCTTTCAACTTTCCTTCATGCGAAA
GAAAGTATTACCCGGCATTAGCCCCGGTTTCCCGGGGTTATTCCGATCTTACAGGCAGGTTGCCCA
CGTGTTACTCACCCGTCCGCCGCTCGTTCCACGAGCTTCACCCCAAAGGGATCCACTCGCTTCCCG
CGCTCGGGTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAAGATCAAACTATA

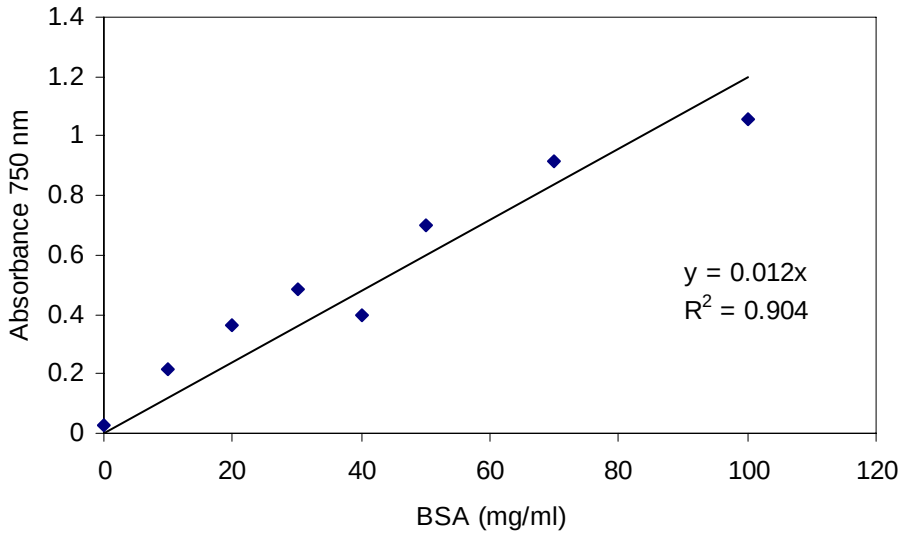
APPENDIX F

Standard curve of tyrosine and Bovine serum albumin(BSA)

1. Standard curve of tyrosine



2. Standard curve of Bovine serum albumin (BSA)



BIOGRAPHY

Miss Thanapun Taprig was born on December 29, 1983. She obtain a Bachelor of Science Degree in Microbiology from Kasetsart University, Bangkok, Thailand in 2006.

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