# การคัดกรองและพิสูจน์เอกลักษณ์ของ
# แบคทีเรียชอบเกลือที่ผลิตฮีสทามีนและอัลคาไลน์ฟอสฟาเทส

นางจารุวรรณ สิทธิพล

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# SCREENING AND IDENTIFICATION OF SELECTED HALOPHILIC BACTERIA PRODUCING HISTAMINE AND ALKALINE PHOSPHATASE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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จารุวรรณ สิทธิพล: การคัดกรองและพิสูจน์เอกลักษณ์ของแบคทีเรียชอบเกลือที่ผลิตฮีสทามีนและอัลคาไลน์ฟอสฟาเทส. (SCREENING AND IDENTIFICATION OF SELECTED HALOPHILIC BACTERIA PRODUCING HISTAMINE AND ALKALINE PHOSPHATASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร. สมบูรณ์ ธนาสุภวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.วรรณพ วิเศษสงวน, 167 หน้า

การคัดแยกแบคทีเรียที่ผลิตกรดแลคติคชอบเกลือจากอาหารหมัก 7 ชนิด ด้วยอาหารวุ้น MRS ที่เติมเกลือโซเดียมคลอไรด์ 10 เปอร์เซ็นต์ (น้ำหนักต่อปริมาตร) พบว่าแบคทีเรียที่คัดแยกได้ทั้ง 41 ไอโซเลต เป็นแบคทีเรียแกรมบวกรูปร่างกลม มีการจัดเรียงตัวเป็นสี่เซลล์ จากการศึกษาการย่อย 16s rRNA ของแต่ละไอโซเลตด้วยเอนไซม์ตัดจำเพาะ (endonuclease restriction enzyme) ชนิด *MboI* และ *AluI* และการจัดจำแนกชนิดของเชื้อด้วยลักษณะทางฟีโนไทป์ รวมทั้งการวิเคราะห์ลำดับเบสในช่วง 16S rRNA ของไอโซเลตตัวแทน สามารถจัดจำแนกเชื้อทั้ง 41 ไอโซเลตออกได้เป็น 2 กลุ่ม คือ กลุ่ม A และกลุ่ม B โดยกลุ่ม A ประกอบด้วย 22 ไอโซเลตเป็นแบคทีเรียในกลุ่ม *Tetragenococcus halophilus* และกลุ่ม B ประกอบด้วย 19 ไอโซเลตเป็น *T. muriaticus* เมื่อตรวจสอบการสร้างฮีสทามีนด้วยการวิเคราะห์ด้วยเครื่อง HPLC และตรวจหาลำดับเบสของยีน *hdc* (histidine decarboxylase) ในส่วน *hdc*A ซึ่งเป็นยีนโครงสร้างที่สำคัญในการผลิตเอนไซม์ฮีสทีดีนดีคาร์บอกซิเลสของแบคทีเรียแกรมบวกที่ผลิตฮีสทามีนหลายสายพันธุ์ พบว่ามีเพียงสายพันธุ์เดียวคือ KS87-14 ซึ่งจัดจำแนกเป็น *T. muriaticus* มีการผลิตฮีสทามีนในปริมาณที่สูงเป็น 10 เท่าของเชื้อ *T. muriaticus* สายพันธุ์มาตรฐานที่ได้เคยมีการรายงานไว้ แต่เมื่อเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน *hdc*A บางส่วนพบว่ามีความเหมือนกันกับยีน *hdc*A ของ *Staphylococcus* sp.

การคัดแยกแบคทีเรียชอบเกลือจากอาหารหมักที่เก็บจากตลาดและผลิตภัณฑ์ในครัวเรือน จำนวน 33 ตัวอย่าง ด้วยอาหารเลี้ยงเชื้อ Halobacterium JCM no. 377 สามารถแยกแบคทีเรียชอบเกลือปานกลางได้ 76 ไอโซเลต และมีเพียง 17 ไอโซเลต ที่ตรวจพบว่าสร้างเอนไซม์อัลคาไลน์ฟอสฟาเทสในอาหารเลี้ยงเชื้อ จากการจัดจำแนกชนิดของเชื้อด้วยลักษณะทางฟีโนไทป์ และอนุกรมวิธานเคมีรวมทั้งการวิเคราะห์ลำดับเบสในช่วง 16s rRNA สามารถแบ่งเชื้อ 17 ไอโซเลตได้เป็น 10 กลุ่ม และพิสูจน์เอกลักษณ์ได้เป็น *Staphylococcus saprophyticus* 3 ไอโซเลต, *S. napalensis* 3 ไอโซเลต, *S. sciuri* 1 ไอโซเลต, *Bacillus vietnamensis* 1 ไอโซเลต, *B. pumilus* 1 ไอโซเลต, *Virgibacillus halodenitrifican* 3 ไอโซเลต, *Oceanobacillus iheyensis* 1 ไอโซเลต, *Halobacillus mangrovi* 1 ไอโซเลต, *H. dabanensis* 2 ไอโซเลต และอีกหนึ่งไอโซเลตมีความใกล้เคียงกับ *Idiomarina zobellii* โดยอัลคาไลน์ฟอสฟาเทสถูกผลิตอยู่ในปริมาณ 10.08-81.5 ยูนิตต่อมิลลิลิตร และไอโซเลตที่ผลิตอัลคาไลน์ฟอสฟาเทสได้สูงสุดคือ TPS4-2 ซึ่งจัดว่ามีความใกล้เคียงกับ *Idiomarina zobellii* ลักษณะทางอนุกรมวิธานเคมีของสายพันธุ์ TPS4-2 ซึ่งมี ubiquinone-8 กรดไขมันที่เป็นส่วนประกอบหลักคือ iso-$C_{15:0}$ และ iso-$C_{17:0}$ ปริมาณ G+C เป็น 47.0 โมลเปอร์เซ็นต์ ผลการวิเคราะห์ลำดับเบสในช่วง 16s rRNA ใกล้เคียงกับ *Idomarina zobellii* DSM 15924$^T$ 98.7 เปอร์เซ็นต์ และมีความคล้ายคลึงของ DNA ต่ำเมื่อเทียบกับสายพันธุ์มาตรฐานของ *Idiomarina* และลักษณะของ $(GTG)_5$-PCR genomic fingerprint pattern มีความแตกต่างกับ fingerprint pattern ของสายพันธุ์มาตรฐานของ *Idiomarina* จึงเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Idiomarina piscisalsi*

การศึกษาการผลิตอัลคาไลน์ฟอสฟาเทสจากสายพันธุ์ TPS4-2$^T$ จัดเป็นเชื้อแบคทีเรียชอบเกลือปานกลางสายพันธุ์ใหม่และสามารถผลิตอัลคาไลน์ฟอสฟาเทสได้สูงที่สุดในกลุ่ม พบว่าเมื่อเลี้ยงในอาหาร Halobacterium JCM no.377 บนเครื่องเขย่าที่ความเร็วรอบ 200 รอบต่อนาที ที่อุณหภูมิ 37 องศาเซลเซียส TPS4-2$^T$ จะผลิตอัลคาไลน์ฟอสฟาเทสตั้งแต่ช่วงเริ่มต้นของการเจริญเติบโตในระยะ exponential และสร้างสูงสุดในช่วงเริ่มต้นของระยะ stationary ทั้งชนิดที่อยู่ในเซลล์และขับออกนอกเซลล์ ในเวลา 36 และ 54 ชั่วโมงของการเลี้ยงตามลำดับ เมื่อทำการเก็บตัวอย่างในทุก ๆ ชั่วโมงของการเลี้ยงเชื้อ พบว่าอัลคาไลน์ฟอสฟาเทสชนิดที่อยู่ในเซลล์มีปริมาณสูงกว่าชนิดที่ขับออกนอกเซลล์ ดังนั้นจึงเลือกที่จะทำการศึกษาคุณสมบัติของอัลคาไลน์ฟอสฟาเทสชนิดที่อยู่ในเซลล์ในขั้นตอนต่อไป อัลคาไลน์ฟอสฟาเทสที่บริสุทธิ์บางส่วนที่แยกได้จากคอลัมน์ gel filtration มีน้ำหนักโมเลกุล 53 kDa โดยมีค่า $V_{max}$ และ $K_m$ ต่อสาร *p*-nitrophenylphosphate เท่ากับ 28.01 µmol/min และ 0.09 mM ตามลำดับ ทำปฏิกิริยาได้ดีที่สุดในสภาวะที่มีความเข้มข้นเกลือโซเดียมคลอไรด์ 0.3 M ที่ pH 10.0 อุณหภูมิ 60 องศาเซลเซียส และมีเสถียรภาพสูงสุดที่สภาวะ pH 7-8.0 และที่อุณหภูมิ 37–55 องศาเซลเซียส กิจกรรมของเอนไซม์ถูกกระตุ้นให้สูงขึ้นเมื่อมีอิออนของ $Mg^{2+}$และ $Ca^{2+}$ ในปฏิกิริยา นอกจากนี้พบว่ากิจกรรมของเอนไซม์แปรผันตรงตามความเข้มข้นของเกลือโซเดียมคลอไรด์ในช่วง 0-0.3 M และเมื่อความเข้มข้นเพิ่มขึ้นถึง 1-4 M กิจกรรมของเอนไซม์ลดลงและคงอยู่ที่ 40% เอนไซม์ถูกยับยั้งด้วย EGTA ได้อย่างสมบูรณ์ จากการศึกษานี้พบว่าอัลคาไลน์ฟอสฟาเทสจากสายพันธุ์ TPS4-2 เป็น metalloenzyme ที่สามารถเร่งปฏิกิริยาได้ด้วยอิออนของ $Ca^{2+}$ และ $Mg^{2+}$

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

ปีการศึกษา 2554 ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

# # 507 6959933: MAJOR PHARMACEUTICAL CHEMISTRY AND NATURAL PRODUCTS
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JARUWAN SITDHIPOL: SCREENING AND IDENTIFICATION OF SELECTED HALOPHILIC BACTERIA PRODUCING HISTAMINE AND ALKALINE PHOSPHATASE. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: WONNOP VISESSANGUAN, Ph.D., 167 pp.

Forty-one tetrad-forming halophilic lactic acid bacteria were isolated from 7 kinds of fermented foods using MRS agar with 10% NaCl. These bacteria were Gram positive cocci arranged in tetrad form. On the basis of *MboI* and *AluI* digested 16S rRNA gene restriction fragment patterns and 16S rRNA gene sequence analysis of the representative isolates, 41 isolates could be divided into two groups (A, B). Twenty-two isolates (group A) were identified as *T. halophilus* while another nineteen isolates (group B) were identified as *T.muriaticus*. Histamine formation was determined using HPLC and the partial gene of histidine decarboxylase (*hdc*) was sequenced. Only one isolate, KS87-14 identified as *T. muriaticus*, prolifically formed histamine. It indicated that rare strains produced histamine. However, this strain produced histamine 10 times higher than the reported *T. muriaticus* strains. A structural gene of pyruvoyl dependent histidine decarboxylase (*hdc*A gene) found mostly in histamine-producing gram-positive bacteria, was detected in KS87-14. Alignment of the partial sequence of *hdc*A gene from KS87-14 to *hdc*A gene previously deposited in the database showed that it was similar to the s*taphylococcal hdc*A gene.

Seventy-six moderately halophilic bacteria were isolated from 33 samples of fermented fish and salted fish using halobacterium JCM no.377. Only 17 isolates exhibited extracellular ALP activity. Based on their phenotypic characteristics and 16S rRNA gene sequence analyses, these isolates were identified as *Staphylococcus saprophyticus* (3 isolates), *S. napalensis* (3 isolates), *S. sciuri* (1 isolates), *Bacillus vietnamensis* (1 isolates), *B. pumilus* (1 isolates), *Virgibacillus halodenitrifican* (3 isolates), *Oceanobacillus iheyensis* (1 isolates), *Halobacillus mangrovi* (1 isolates), *H. dabanensis* (2 isolates), and the last isolate was closely related to *Idiomarina zobellii*. They possessed ALP activities ranging from 10.08-81.50 U/ml. The isolate TPS4-2 showed the highest ALP activity. It grew optimally at 30-37$^{o}$C, pH 8 and in the presence of 10-15% (w/v) NaCl. The predominant ubiquinone was Q-8 and major cellular fatty acids were iso-$C_{15:0}$ and iso-$C_{17:0}$. DNA G+C content was 47.0 % mol. Based on 16S rRNA gene sequence analyses, TPS4-2$^{T}$ was closely related to *I. zobellii* DSM 15924$^{T}$ at 98.7%. It had low levels of DNA-DNA relatedness to the closest type strain *I. zobellii* DSM 15924$^{T}$ and its unique $(GTG)_5$-PCR genomic fingerprint pattern was different from all closely phylogenic type strains of *Idiomarina*, therfore it was proposed as *I. piscisalsi* nov. sp.

TPS4-2$^{T}$ was selected for further study due to its novel specie and high ALP production. TPS4-2$^{T}$ could produce both extracellular and intracellular ALP at the beginning of the exponential phase and the highest production was observed at the early stationary phase. TPS4-2$^{T}$ was cultivated in halobacterium JCM377 pH 8.0 at 37°C in rotary shaking at 200 rpm. Since intracellular ALP was found higher than extracellular ALP at every sampling hour, therefore, intracellular ALP was further characterized. The maximum intracellular ALP production was at 36 h. The partially purified ALP presented a molecular mass of 53 kDa, calculated by gel filtration. Using *p*-nitrophenylphosphate as substrate, the $V_{max}$ and $K_m$ values were 28.01 μmol/min and 0.09 mM, respectively. It had maximal activity in the presence of 0.3 M NaCl, pH 10.0, at 60°C. Stability was fully at pH 7.0-8.0 and 37–55°C. The presence of $Mg^{2+}$ and $Ca^{2+}$ could stimulate the ALP activity. In addition, the ALP activity was proportional to NaCl concentration in the range of 0-0.3 M. When NaCl concentration was increased 1-4 M, the ALP activity decreased and ceased at 40%. The EGTA could completely inhibit ALP activity. This study indicated that the ALP of TPS4-2$^{T}$ was metalloenzyme that can be activated by $Ca^{2+}$and $Mg^{2+}$ ions.

Field of Study: Pharmaceutical Chemistry and Natural Products  Student's Signature .....

Academic Year: 2011  Advisor's Signature .....

Co-advisor's Signature .....

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| | | |
|---|---|---|
| α | = | Alpha |
| ATCC | = | American Type Culture Collection, Maryland, U.S.A. |
| ALP | = | Alkaline phosphatase |
| ˚C | = | Degree celsius |
| DAP | = | Diaminopimelic acid |
| DDBJ | = | DNA Data Bank of Japan |
| DEAE | = | Diethylaminoethanol |
| DEAE FF | = | DEAE Sepharose Fast Flow |
| DNase | = | Deoxyribonuclease |
| DSM | = | Deutsche Sammlung von Mikroorganismen |
| E64 | = | L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane |
| EDTA | = | Disodiumethylenediaminetetraacetate |
| EMBL | = | European Molecular Biology Laboratory |
| g | = | Gram |
| GenBank | = | National Institute of Health genetic sequence database |
| h | = | Hour |
| HCl | = | Hydrochloric acid |
| HPTLC | = | High performance thin layer chromatograhphy |
| JCM | = | Japan Collection of Microorganisms |
| kDa | = | kilo Dalton |
| KOH | = | Potassium hydroxide |
| L | = | Liter |
| MEGA | = | Molecular Evolutionary Genetics Analysis |
| MeOH | = | Methanol |
| *meso*-DAP | = | *meso*-Diaminopimelic acid |
| M | = | molar |
| min | = | minute |
| µg | = | microgram |
| mg | = | milligram |

| | | |
|---|---|---|
| µl | = | microliter |
| ml | = | milliliter |
| µm | = | micrometer |
| mm | = | millimeter |
| NaCl | = | Sodium chloride |
| NaOH | = | Sodium hydroxide |
| NAG | = | N-acetly glucose amine |
| NAM | = | N-acetyl muramic acid |
| nm | = | Nanometer |
| % | = | Percent |
| PAGE | = | Polyacrylamide gel electrophoresis |
| PBS | = | Phosphate buffer saline |
| PCR | = | Polymerase chain reaction |
| PE | = | Phosphatidylethanolamine |
| PG | = | Phosphatidylglycerol |
| PMSF | = | Phenylmethylsulfonyl fluoride |
| *p*-NPP | = | Para-nitrophenylphosphate |
| rDNA | = | Ribosomal deoxynucleic acid |
| rRNA | = | Ribosomal ribonucleic acid |
| rpm | = | Round per minute |
| sec | = | Second |
| SDS | = | Sodium dodesylsulfate |
| sp. | = | Species |
| SSC | = | Standard sodium citrate |
| TCA | = | Trichloroacetic acid |
| TLC | = | Thin layer chromatography |
| TLCK | = | Tosyl-L-lysine chloromethyl ketone |
| TPCK | = | Tosyl-L-phenylalanine chloromethyl ketone |
| UV | = | Ultraviolet |
| v/v | = | volume / volume |
| w/v | = | weight / volume |

# CHAPTER I

## INTRODUCTION

Fermented fishery products, such as fish sauce (*nam-pla*), shrimp paste (*ka-pi*) and fermented fish (*pla-ra*), play important roles in the Thai cuisine. As these products containing over 10% (w/v) NaCl (Phithakpol, 1995), they can be used as an alternative to table salt and have been consumed daily by Thai people since ancient times. However, these products contain histamine, which is one of the most important biogenic amines causing toxicological effects such as allergy and inflammation (Santos, 1996; Satomi *et al*., 2008). Histamine is generated by decarboxylation of histidine through the microbial enzyme histidine decarboxylase (HDC) (Brink *et al*., 1990). Since the quantity of histamine in the products depends on the level of histidine, some products especially from Scombroid fish species containing high levels of free histidine in their muscle could be deteriorated in quality and safety through high levels of histamine. Histamine in fermented foods is generally caused by Gram-positive lactic acid bacteria, whereas that in raw fish products or Scombroid fish is caused mostly by Gram-negative enteric bacteria (Landete *et al*., 2007; Satomi *et al*., 2008). The ability of bacteria to decarboxylate histidine is highly variable. It depends not only on the species, but also on the fermentation process and the environmental conditions. For example, although the prolific histamine forming bacteria have been isolated from temperature-abused raw fish, they have rarely been isolated from fermented fish products (Kim *et al*., 2003). Only a few halophilic bacteria such as *Tetragenococcus* spp. have been isolated as prolific histamine-formers from fish sauce (Kimura *et al*., 2001; Yongsawatdigul *et al*., 2004; Taira *et al*., 2007, Satomi *et al*., 2008; Udomsil *et al*., 2010)

Histamine is difficult to destroy because it is heat stable and not detectable through organoleptic analysis by even trained panelists (Taylor, 1986). Except the gamma irradiation, no other food processing methods are available for histamine degradation (Kim *et al*., 2004). As histamine intoxication (Scombroid poisoning) is one of major health significances associated with ingestion of foods containing high

histamine. In the United States, the toxic level of histamine that poses a risk to health is set at 500 ppm, and the caution level is 50 ppm. The European Union has also set a level of 100-200 ppm for seafood, and Codex has also proposed regulations at this level (Lehane and Olley, 2000). The determination of histamine in foods is of great interest not only for the food safety aspect, but also as the indicators for quality of freshness or spoilage of foods (Awan *et al*., 2008). Therefore, the accomplishment of surveillance plans in order to avoid the propagation of contaminated products, as outlined by Italian Legislative Decree no. 531-30/12/1992 (Muscarella *et al*., 2005) requires accurate, reproducible, and fast analytical methods such as HPLC, one of the most popular or main laboratory methods and frequently reported for the separation and quantification of histamine (Saaid *et al*., 2009). In addition, molecular methods for detection and identification of food borne bacteria are becoming widely accepted as an alternative to traditional culture methods. One of these is the polymerase chain reaction (PCR) an important method for rapid, sensitive and specific detection of target genes. It can use to detect the histamine-producing bacteria through amplifying the gene encoding for the enzyme histidine decarboxylase (HDC).

On the other hand, the halophilic bacteria in fermented fish product have an advantage by generating alkaline phosphatases (ALP). ALPs catalyze the hydrolysis of monoesters of phosphoric acid in an alkaline environment. Their existence in many organisms ranging from bacteria to mammals indicate that ALPs are involved in fundamental biochemical processes (Zueva *et al*., 1993). Especially in prokaryotic organisms, ALPs might play an important role in the phosphate metabolism (Moura *et al*. 2001). ALP has become a useful tool in molecular biology laboratories by removing phosphates from DNA and preventing the DNA from ligating (Sun *et al*, 2007). In addition, ALPs are widely used as enzymatic labels in immunoassay for determining such as: herbicides, pesticides and biologically organic compound in the pharmaceutical and food industries (Muginova *et al*., 2007; Vieille and Zeikus 2001). In some applications, ALP with particular properties, *e.g.* active under the high salt concentration, high thermal stability and storage stability are required. At present, there are a lot of studies about ALP from bacteria and mammal, but there are a few studies on ALP from moderate halophilic bacteria.

As known, the moderate halophilic bacteria can produce several useful compounds such as enzymes, polymers and compatible solutes with beneficial properties which can facilitate their exploitation for biotechnological and industrial applications. In order to find a new source with significant ALP properties for commercial purpose, this work deals with the screening and identification of the moderate halophillic bacteria together with the purification and characterization of alkaline phosphatase of the selected novel strain isolated from the fermented food were performed. The targets of this investigation are to provide useful information of ALP from moderate halophillic bacteria for further application.

This work is divided into two parts. The first part is related to the systematically identification and study on the possible role of various *Tetragenococcus* isolated from fishery products on histamine accumulation using two techniques, HPLC method for investigating histamine-producing ability of *Tetragenococcus* grown in histidine broth and multiplex PCR method for detecting histidine decarboxylase gene The second part is to identify the moderate halophillic bacteria including the purification and characterization of alkaline phosphatase of the selected novel strain isolated from fermented food.

**The main objectives of this investigation are as follows:**

1. To screen and to identify histamine and alkaline phosphatase producing halophilic bacteria from fermented fishery products based on the phenotype and genotypic characteristics.

2. To detect histamine decarboxylase (*hdc*) gene and histamine production of selected halophilic isolates.

3. To purify and characterize the alkaline phosphatase of the selected halophilic isolates.

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Halophilic bacteria

### 2.1.1 Physiology of halophilic bacteria

Halophilic bacteria can thrive in hypersaline environments which distributed all over the world, in natural brines, coastal and deep-sea, underground salt mines and artificial salterns. They can balance the osmotic forces that accompanied to their life in a high solute environment by accumulating inorganic ion and organic compounds intracellulary that referred as compatible solutes (Margesin and Schinner, 2001). These compounds counteracted the tendency of the cell to become dehydrated under high osmotic strength by placing the cell in positive water balance with its surroundings and can be accumulated compatible solutes at high concentrations without interfering with cellular metabolism (Moral, Severin, Romos-Cormenzana, Truper and Galinski, 1994). Halophile excrete compatible solutes from their cytoplasm or uptake from the medium to achieve osmotic balance (Robert, 2000).

Cell wall of halophilic bacteria is stabilized by sodium ions. In low sodium environment, the cell wall of extremely halophilic bacteria is broken down. Sodium ion ($Na^+$) bound to outer surface of cell wall that is vital for maintaining cellular integrity. Extremely halophilic bacteria is similar to Gram negative bacteria but cell wall was quite different. In cell wall of extremely halophilic bacteria do not contain peptidogycan, but comprises of glycoprotein. The negative charges are contributed by the carboxyl groups of amino acids in the cell wall. Glycoprotein is shielded by $Na^+$. When $Na^+$ is decreased, the negatively charged regions of the proteins actively extrude each other and cell lyses (Margesin and Schinner, 2001). Cytoplasmic protein of halophilic bacteria contains very low levels of hydrophobic amino acids which probably represents an evolutionary adaptation to highly ionic cytoplasm of halophilic. The ribosome also requires high $K^+$ levels for theirs stability while ribosome of nonhalophiles requires no $K^+$. It indicated that the halophilic bacteria are highly adapted, both internally and externally, to living in highly ionic environment (Ventosa

*et al.,* 1998). Lipid membrane of halophilic bacteria has a low $H^+$ and $Na^+$ permeability at high salt concentration. Vossenberg *et al*. (1999) showed that pH and the salt concentration influenced on the proton and sodium ion permeability in lipid membrane of the halophiles. The proton permeability in lipid membrane of halophlies is independent of the salt concentration and is essentially constant between pH 7-9. Thus, the membranes of halophiles are steady over a wide range of salt concentrations, slightly alkaline pH and are well adapted to halophilic conditions.

Several halophiles are found on salted food, such as salted fish, meat and other fermented food products. Villar *et al*. (1985) found that *Pediococcus halophilus* was a dominant bacterium at the end process of anchovies. *Halomonas salina* was isolated from fully cured wet and dry bachalao (dried salted codfish) that contains about 19% salt by Vihelmsson *et al*., 1966. *Pseudomonas beijerinckii* and *Halomonas halodenitrificans* were isolated from salted beans preserved in brine and meat curing brines, respectively. Moreover, *Halobacterium* sp., *Halococcus* sp., *Halobacillus* sp. SR5-3, *Filobacillus* sp. RF2-5, *Lentibacillus salicampi*, *L. halophilus, L. juripiscarius, Piscibacillus salipiscarius*, *V. dokdonensis*, *V. halodenitrificans*, *V. marismortui*, *V. siamensis*, *B. vietnamnensis*, *Chromohalobacter salexigens*, *Salinivibrio siamensis, Gracilibacillus thailandensis* were distributed in Thai fish sauce (*nam-pla*), fermented fish (*pla-ra*),salted fishes and other fermented food product (Thongthai and Suntinanalert, 1991; Hiraga *et al*., 2005; Namwong *et al*., 2005, 2006; Sirilak *et al*., 2006; Sinsuwan *et al*., 2008; Chamroensaksri *et al*., 2008, 2009 and Tanasupawat *et al*., 2006, 2007, 2010).

### 2.1.2 Systematic of halophilic bacteria

They are classified according to their salt requirement for growth and their behavior toward salt to be three groups: 1) halotolerant, 2) moderately halophilic and 3) halophillic archea (Ventosa *et al*., 1998). Halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance in salts sometimes dependent on nutritional factors and environmental.

The salt requirement and tolerance properties of halophilic bacteria are highly variable according to the growth temperature and the nature of nutrients available (Ventosa *et al*., 1998). They can produce a variety of colonial characteristics from pigmented to non-pigmented according to the salt concentration in media. Because of the diverse physiology of halophilic bacteria is no steady, thus it make many problems affected to the accuracy of conventional method for halophilic bacterial systematics (Kushner, 1993). Therefore, a polythetic view involving the combination of conventional and modern bacterial systematics is needed for halophilic bacterial systematics. Whenever the chemical data and/or the molecular data disagreed with taxonomic clusters produced by phenotypic means, preference should be provisionally given to the latter clusters until further research resolves the discrepancy (Vreeland, 1993b). Nowadays, the halophilic bacterial identification are used the phenotypic and chemotaxonmic characterization along with 16S rRNA gene sequences analysis. (Stan-lotter *et al*., 2002 and Yoon *et al*., 2003).

#### 2.1.2.1. Extremely halophilic bacteria

Extremely halophilic bacteria grow optimumlly at 3.4-5.1 M (20-30%) NaCl and will not grow at low salt concentration. They are classified into the class *Halobacteria;* order *Halobacteriales* and family *Halobacteriaceae* (Grant et al., 2001). Cells are rods, coccus or aggregation of involution forms from disks to triangle. Colonies are various shades of red due to the presence of $C_{50}$ carotenoids (bacterioruberins). They lack muramic acid-containing peptidoglycan in the cell envelope. Cytoplasmic membrane is composed of phytanyl ether lipids. They inhabit hypersaline environments such as salt lakes, soda lakes and salterns. The family *Halobacteriaceae* consists of 33 genera as follows: *Halobacterium*, *Haladaptatus, Halalkalicoccus*, *Halarchaeum*, *Haloarcula, Halobaculum*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halogranum, Halogeometricum*, *Halolamina* , *Halomarina*, *Halomicrobium*, *Halonotius*, *Halopelagius, Halopiger*, *Haloplanus*, *Haloquadratum, Halorhabdus*, *Halorubrum*, *Halosimplex, Halovivax*, *Halostagnicola*, *Haloterrigena, Natrialba*, *Natrinema, Natronoarchaeum, Natronobacterium, Natronococcus*, *Natronolimnobius, Natronomonas and Natronorubrum.*

### 2.1.2.2. Moderately halophilic bacteria

Moderately halophilic bacteria grow optimally at 0.85-3.4 M (5-20% w/v) and require at least 0.5 M (~3% w/v) NaCl for growth. They constitute very heterogeneous groups. In general, most halophiles within the bacteria are moderate rather than extreme halophiles. Moderately halophilic eubacteria are both heterotrophs and phototrophs. The heterotrophs include gram-negative and gram-positive moderate halophiles. Gram-negative species of moderately halophilic bacteria are *Deleya halophila*, *Desulfohalobium retbaense*, *Desulfovibrio halophilus*, *Flavobacterium halmephilum*, *Haloanaerobacter chitinovorans*, *Haloanaerobacter saccharolytica*, *Haloanaerobium praevalens*, *Halobacteroides halobius*, *Halomonas halodenitrificans*, *Halomonas halodurans*, *Halomonas elongata*, *Halomonas eurihalina*, *Halomonas subglaciescola*, *Paracoccus halodenitrificans*, *Pseudomonas beijerinckii*, *Pseudomonas halophila*, *Sporohalobacter lortetii*, *Sporohalobacter marismortui*, *Spirochaeta halophila,* and *Salinivibrio costicola*. Species of gram-positive moderate halophiles are *Micrococcus halobius*, *Sporosarcina halophila*, *Marinococcus halobius,* and *Marinococcus albus*.

The lipid side chains are fatty acids i.e., saturated fatty acid ($C_{13:0}$, $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$) and unsaturated fatty acids (iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$). The major polar lipids present in most species are phosphoglycerol (PG) and diphosphoglycerol (DPG). Additional types of lipids may occur such as are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), diphosphatidylglycerol (cardiolipin, CL) and glycolipids. Generally the content of negatively charged phospholipids (PC, CL) increases at the expense of neutral phospholipids (PE) as salinity increases. Cell wall peptidoglycans contain peptides and glycans, N-acetyl (glycolyl) muramic acid (NAM) and N-acetylglucosamine (NAG). Some of the amino acids are only found in cell walls, but not in other cellular proteins i.e., D-amino acids, e.g D-alanine and diaminopimelic acid (DAP).

**Genus *Virgibacillus*** This genus was firstly proposed by Heyndrickx *et al.* (1998). The members of genus *Virgibacillus* are Gram positive rods. Cells arrangement occurrs singly, pair, short or long chain. Colonies are small, circular, low convex and slightly transparent to opaque. They show catalase positive, motile and produce endospores. Spores are usually spherical to ellipsoidal and locate at terminal or subterminal position. Additional, they can hydrolyze gelatin, aesculin and casein. They produce acid from a variety of carbohydrate, depending on species. They grow between 15$^{o}$C and 50$^{o}$C with the optimum temperature at about 28$^{o}$C or 37$^{o}$C. Theirs growth are stimulated by concentration of NaCl range from 4-10%. The DNA G + C contents are 36-43 mol%. Cell wall peptidoglycan contains *meso*-diaminopimelic acid type in their structure. Nowadays, the genus *Virgibacillus* comprises 25 species as following: *V. pantothenticus,V. proomii, V. salexigens*, *V. carmonensis*, *V. necropolis*, *V. marismortui*, *V. halodenitrificans*, *V. dokdonensis*, *V. koreensis*, *V. olivae*, *V. halophilus*, *V. chiguensis*, *V. kekensis*, *V. salaries*, *V. arcticus*, *V. salinus*, *V. sediminis*, *V. byunsanensis* , *V. subterraneus*, *V. xinjiangensis*, *V. alimentarius, V. siamensis*, *V. soli, V. alimentarius* and *V. campisalis* (Niederberger *et al*., 2009; Chen *et al*., 2009; Carrasco *et al*., 2009; Kämpfer *et al.,* 2011, Tanasupawat *et al*., 2010; Wang *et al*., 2010; Kim *et al.,* 2011; Kim *et al.,* 2011; Lee *et al.,* 2012), The differential characteristic of *Virgibacillus* species are shown in Table 2.1.

**Table 2.1** Characteristics of species belonging to the genus *Virgibacillus*

| **Strain** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spore shape | E,S | E,S | E,S | E | E | E,S | E | E | E | E | E,S | E,S | E,S | E |
| Spore position | ST | T,ST | T,ST | T,ST | ST | T | T | T,ST | C,T,ST | T,ST | T,ST | T,ST | ST | C,T,ST |
| Anaerobic growth | - | + | + | + | - | - | + | - | - | - | + | + | - | + |
| Temp. range | 10-40 | 15-55 | 15-50 | 10-45 | 5-45 | 10-50 | 10-45 | 15-50 | 10-40 | 20-45 | 15-50 | 15-50 | 10-50 | 15-50 |
| Growth at/in: | | | | | | | | | | | | | | |
| 0 % NaCl | - | + | + | - | + | + | - | - | W | + | - | - | - | - |
| 20% NaCl | - | + | + | + | - | + | + | + | - | + | + | - | + | + |
| pH 10 | ND | - | - | - | + | + | - | - | ND | - | - | - | + | + |
| Hydrolysis of: | | | | | | | | | | | | | | |
| Starch | ND | - | + | - | ND | + | ND | - | ND | + | ND | | - | - |
| Casein | + | + | + | + | ND | - | ND | + | + | + | + | + | + | + |
| Gelatin | - | + | + | + | + | - | - | + | W | + | + | V | W | + |
| Aesculin | W | + | + | - | ND | - | + | + | - | + | + | + | + | + |
| Nitrate reduction | + | + | - | + | + | + | - | + | + | + | V | - | - | + |
| Acid from: | | | | | | | | | | | | | | |
| D-Glucose | - | + | + | + | + | + | W | + | W | - | - | + | + | W |
| myo-Inositol | - | ND | + | - | - | - | - | - | - | - | - | | ND | - |
| D-Fructose | - | + | + | + | + | - | + | + | W | + | - | + | + | W |
| D-Galactose | - | + | + | + | W | - | - | - | - | - | - | + | - | W |
| D-Mannose | - | + | + | + | + | - | - | + | W | - | - | + | + | - |
| L-Rhamnose | - | ND | - | - | - | - | - | - | - | - | + | V | - | - |
| Major polar lipid | DPG, PG | DPG, PG, PE,PLs | DPG, PG, PE,PLs | DPG, PG, PLs | ND | DPG, PG, PLs | DPG, PG, PLs | DPG, PG, PE,PLs | DPG, PG, PLs | ND | DPG, PG, PE,PLs | DPG, PG, PE,PL | DPG, PG, PE | DPG, PG, PLs |
| DNA G+C content (mol%) | 38.9 | 37.3 | 36.7 | 38 | 42.6 | 41.8 | 41 | 39-42.8 | 37 | 33.4 | 36.9-38.3 | 36.8-37 | 37.3 | 36-40 |

1, *V. carmonensis;; 2, V. chiguensis; 3, V. dokdonensis; 4, V. halodenitrificans; 5, V. halophilus; 6, V. kekensis; 7, V. koreensis; 8, V. marismortui; 9, V. necropolis; 10, V. olivae; 11, V. pantothenticus; 12, V. proomii; 13, V. salaries; 14, V. salexigens*;

+, Positive reaction; –, negative reaction; W, weakly positive reaction; V, variable reaction; ND, not determined

**Table 2.1** (Cont.1) Characteristics of species belonging to the genus *Virgibacillus*

| **Strain** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** | **25** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spore shape | E | E,S | E.S | E | E,S | | E,O | E.S | E | E | S |
| Spore position | C,ST | T,ST | T | ST | T,ST | | T | T | ST | T,ST | T |
| Anaerobic growth | + | + | ND | - | - | - | - | + | + | - | + |
| Temp. range | 0-30 | 15-40 | 10-45(30) | 10-55 | 8-52 | 10-40 | 4-40 | 10-50 | 15-40 | 4-40 | 15-40 |
| Growth at/in: | | | | | | | | | | | |
| 0.5 % NaCl | + | - | + | - | + | + | + | + | + | + | + |
| 20% NaCl | - | - | + | - | - | + | - | + | - | + | + |
| Hydrolysis of: | | | | | | | | | | | |
| Starch | ND | - | - | - | - | - | + | - | ND | ND | ND |
| Casein | + | - | - | - | + | - | - | - | W | ND | - |
| Gelatin | + | - | + | + | - | - | - | - | + | ND | - |
| Aesculin | ND | + | - | + | - | + | - | + | - | ND | - |
| Nitrate reduction | + | + | + | + | + | + | + | + | - | ND | + |
| Acid from: | | | | | | | | | | | |
| D-Glucose | W | + | + | - | + | + | - | + | + | - | - |
| D-Fructose | W | + | - | + | + | + | W | + | - | - | - |
| D-Galactose | ND | + | - | + | - | + | + | + | - | - | - |
| D-Mannose | W | ND | - | + | - | - | - | + | - | - | - |
| L-Rhamnose | ND | ND | - | - | ND | + | ND | + | + | - | - |
| Major polar lipid | DPG, PG, PLs | DPG, PG, GL,PLs | DPG, PG PLs | DPG, PG, PLs | DPG, PG | DPG, PG, PLs | DPG, PG, PLs | DPG, PG, GL | DPG, PG, PLs | DPG, PG, PLs | DPG, PG, PLs |
| DNA G+C content (mol%) | 38.2 | 38.8 | 39.5 | 40.9 | 44.5 | 38.8 | 37.6 | 37.1 | 38 | 37 | 39.5 |

15, *V. arcticus* (Niederberger *et al*., 2009)*;* 16, *V. salinus* (Carrasco *et al*., 2009)*;* 17, *V. zhanjiangensis* (Peng *et al*., 2009);
18, *V. sediminis* (Chen *et al*., 2009); 19, *V. xinjiangensis*; 20, *V. salinus* (Carrasco *et al.,* 2009); 21, *V. byunsanensis;*
22, *V. subterraneus* (Wang *et al*., 2010); 23, *V. siamensis* (Tanasupawat *et al*., 2010); 24, *V. alimentarius* (Kim *et al*., 2011);
25*, V. campisalis*; (Lee *et al*., 2012) ; +, Positive reaction; –, negative reaction; W, weakly positive reaction;
V, variable reaction; ND, not determined.

**Genus *Halobacillus***

The genus *Halobacillus* was firstly proposed by Spring *et al.* (1996) through the reclassification of *Sporosarcina halophila* as *Halobacillus halophilus* and the description of two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*. The members of genus *Halobacillus* are Gram-positive, spore-forming, rod-shaped or spherical to oval cells, strictly aerobic, and chemoorganotrophic. Catalase, oxidase and DNase are positve and urease is negative. Nitrate is not reduced to nitrite. They can motile by flagella. Endospores resist at 75°C for at least 10 min. The Voges-Proskauer reaction is negative. The DNA G+C content ranges from 40 to 43 mol%.

*Halobacillus* can be separated clearly from other related genera based on the cell-wall peptidoglycan type based on L-Orn-D-Asp (Spring et al., 1996), with the exception of that for *H. campisalis* and *H. seohaensis*, which is based on *meso*-diaminopimelic acid. In present, the genus *Halobacillus* is divided into 18 species namely, *Halobacillus halophilus, H. trueperi and H. litoralis (Spring et al., 1996), H. salinus* (Yoon *et al*., 2003), *H. karajensis* (Amoozegar *et al*., 2003), *H. locisalis* (Yoon *et al*., 2004), *H. yeomjeoni* (Yoon *et al*., 2005), *H. dabanensis* and *H. aidingensis* (Liu *et al*., 2005), *H. profundi* and *H. kuroshimensis* (Hua *et al*., 2007), *H. campisalis* (Yoon *et al*., 2007), *H. faecis* (An *et al*., 2007), *H. mangrovi* (Soto-Ramirez *et al*., 2008), *H. seohaensis* (Yoon *et al*., 2008), *H. alkaliphilus* (Romano *et al*., 2008), *H. hunanensis* (Peng *et al*., 2009) and *H. salsuginis* (Chen *et al*., 2009). The differential characteristics of *Halobacillus* species are shown in Table 2.2.

**Table 2.2** Characteristics of species belonging to the genus *Halobacillus*.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Cell morphology | Cocci or oval-shaped | Rods | Rods | Rods | Rods | Rods |
| Flagellation | Single peritrichous | Peritrichous | Peritrichous | Absent | Peritrichous | Single |
| Spore shape/position | S/C | E,S/C,ST | E,S/C,ST | E,S/C,ST | E | E |
| Colony colour | Orange | Orange | Orange | Cream or white | Pale orange–yellow | Light orange–yellow |
| Max. temp for growth (°C) | 40 | 43 | 44 | 49 | 45 | 42 |
| Growth at: | | | | | | |
| 4 °C | - | - | - | - | - | - |
| pH 5.0 | - | - | - | - | + | + |
| pH 5.5 | - | - | - | - | + | + |
| 0.5% NaCl | - | + | + | - | + | - |
| 25% NaCl | - | + | + | - | - | - |
| Hydrolysis of: | | | | | | |
| Aesculin | - | - | - | + | + | + |
| Casein | + | - | - | + | + | - |
| Gelatin | + | + | + | + | + | - |
| Starch | + | - | - | + | - | + |
| Tween 80 | - | - | - | - | + | - |
| Acid production from | | | | | | |
| D-Fructose | - | + | + | + | + | + |
| D-Galactose | - | + | + | - | W | - |
| Maltose | - | + | + | + | + | - |
| Sucrose | - | + | + | - | + | + |
| D-Xylose | - | - | - | - | - | - |
| Cell-wall type | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp |
| DNA G+C content (mol%) | 40.1-40.9 | 42 | 43 | 41.3 | 45 | 44 |

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

**Table 2.2** (Cont.1) Characteristics of species belonging to the genus *Halobacillus.*

| Characteristic | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|
| Cell morphology | Long rods filamentous | Rods | Cocci or oval-shaped | Rods | Rods | Rods |
| Flagellation | Single | Peritrichous | Peritrichous | Peritrichous | Absent | Absent |
| Spore shape/position | E/C,ST | E/C,ST | S/C | E/C,ST | E,S/C | E,S/C, |
| Colony colour | Light yellow | Orange | Light yellow | Cream to Orange | Orange | Yellow–orange |
| Max. temp for growth (°C) | 48 | 40 | 41 | 50 | 45 | 48 |
| Growth at: | | | | | | |
| 4 °C | - | - | + | - | - | - |
| pH 5.0 | - | - | - | + | - | - |
| pH 5.5 | - | - | + | + | + | + |
| 0.5% NaCl | + | + | + | + | + | + |
| 25% NaCl | - | - | - | + | - | + |
| Hydrolysis of: | | | | | | |
| Aesculin | - | - | + | - | ND | + |
| Casein | + | + | + | + | ND | + |
| Gelatin | + | + | - | - | + | + |
| Starch | - | - | + | + | ND | + |
| Tween 80 | + | + | - | - | ND | + |
| Acid production from | | | | | | |
| D-Fructose | - | + | + | + | + | + |
| D-Galactose | - | - | + | - | + | - |
| Maltose | + | + | - | + | + | + |
| Sucrose | + | + | + | + | + | + |
| D-Xylose | - | - | - | + | - | - |
| Cell-wall type | L-Orn-D-Asp | L-Orn-D-Asp | DAP | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp |
| DNA G+C content (mol%) | 42.9 | 42.2 | 42.1 | 41.4 | 46.5 | 42.1 |

Strain 7, H. yeomjeoni (Yoon *et al*., 2005); 8, H. aidingensis (Liu *et al*., 2005); 9, H. campisalis(Yoon *et al*.,2007);
10, H. dabanensis (Hua *et al*., 2007); 11, H. faecis (An *et al*., 2007); 12, H. kuroshimensis(Hua *et al*., 2007);
+, positive; - negative; ND, no data; NG, no growth. E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal

**Table 2.2** (Cont.2) Characteristics of species belonging to the genus *Halobacillus*

| Characteristic | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|
| Cell morphology | Rods | Cocci | Rods | Rods | Rods | Rods |
| Flagellation | Absent | ND | ND | Single | peritrichous | Peritrichous |
| Spore shape/position | E,S/C, | S | ND | E/C,ST | E, ST | E/ST |
| Colony colour | Pale yellow | Pale Orange | Cream | Yellowis hwhite | Cream | Cream |
| Max. temp for growth (°C) | 47 | 45 | 50 | 38 | 40 | 45 |
| Growth at: | | | | | | |
| 4 °C | - | - | - | + | - | - |
| pH 5.0 | - | - | - | - | - | |
| pH 5.5 | + | - | - | - | - | |
| 0.5% NaCl | + | + | - | - | - | - |
| 25% NaCl | + | - | - | - | + | - |
| Hydrolysis of: | | | | | | |
| Aesculin | + | ND | - | - | ND | + |
| Casein | + | ND | + | + | - | - |
| Gelatin | - | ND | + | - | + | + |
| Starch | + | ND | + | + | - | + |
| Tween 80 | + | ND | + | - | + | - |
| Acid production from | | | | | | |
| D-Fructose | + | + | - | - | - | - |
| D-Galactose | - | - | - | - | - | - |
| Maltose | + | + | - | - | - | + |
| Sucrose | + | + | - | + | - | + |
| D-Xylose | + | + | - | + | - | - |
| Cell-wall type | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | *meso*-DAP | *meso*-DAP | *meso*-DAP |
| DNA G+C content (mol%) | 43.3 | 43.5 | 45.7 | 39.3 | 41.8 | |

Strain 13, H. profundi (Hua *et al*., 2007); 14, H. alkaliphilus (Romano *et al*., 2008); 15, H. mangrovi (Soto-Ramirez *et al*., 2008); 16, H. seohaensis (Yoon *et al*., 2008); 17, H. hunanensis (Peng *et al*., 2009); 18, H. salsuginis(Chen *et al*., 2009) ;
+, positive; - negative; ND, no data; NG, no growth. E, Ellipsoidal; S, spherical; C, central; T, terminal; ST, subterminal

**Genus *Oceanobacillus***

The members of genus *Oceanobacillus* are Gram-positive, motile by peritrichous fagella, spore-forming rods. Endospores are subterminal or terminal and slightly swell the sporangia. They are obligately aerobic, facultatively alkaliphilic, chemoorganotrophic and halophilic bacterium. Colonies are round and growth occur at temperature 15-42$^{o}$C. The genus *Oceanobacillus* was first described by Lu *et al*. (2001) to accommodate an aerobic, rod-shaped, spore-forming halophilic bacterium, *Oceanobacillus iheyensis*. At the present, the genus *Oceanobacillus* comprised: *O. iheyensis* from a deep-sea environment, *Oceanobacillus caeni* from wastewater, *O. picturae* from a painting, *O. profundus* from deep-sea sediment, *O. chironomi* from a chironomid egg mass *O. oncorhynchi subsp. oncorhynchi* from the skin of a rainbow trout, *O. oncorhynchi subsp. incaldanensis* from an algal mat and *O. kapialis* from fermented shrimp paste (*ka-pi*) (Namwong *et al.,* 2009) For *O. iheyensis*, endospores are ellipsoid and colonies are creamy white. Growth occurs at 0-21% (w/v) NaCl, with optimum growth at 3% w/v NaCl and temperatures of 15-42$^{o}$C (optimum 30$^{o}$C). The pH range for growth is 6.5-10 (optimum 7.0-9.5). Hydrolyzes gelatin, casein, Tween 40, and Tween 60, but not hydrolyze starch. α-D-glucose, maltose, D-mannose, and turanose are assimilated. The main menaquinone type is MK-7. The G+C content is 35.8%. Predominant fatty acids are $C_{15:0}$ anteiso, $C_{15:0}$ iso and $C_{14:0}$ iso. The differential characteristics of *Oceanobacillus* species are shown in Table 2.3.

**Table 2.3** Characteristics of species belonging to the genus *Oceanobacillus.*

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Colony size (mm) Spores | 0.3-2.7 | 0.1-1.5 | NA | 2-3 | NA | NA | NA | NA |
| Shape | O | ES | E | E | E | E | - | E |
| Position | T | T | T, S | S | T | C | - | T, S |
| Temperature range for growth ($^{o}$C) | 8-43 | 8-40 | 15-42 | 15-40 | 15-42 | 20-45 | 10-40 | 12-46 |
| pH range for growth | 6.0-9.0 | 6.0-9.0 | 6.5-10.0 | 9.0-10.0 | 6.5-9.5 | 6.0-9.0 | 6.5-9.5 | 6.5-10.0 |
| NaCl for growth (w/v, %) | | | | | | | | |
| Optimum NaCl | 6-14 | 6-12 | 3 | 7 | 1-3 | 2-5 | 10 | 1-3 |
| Range NaCl | 0.5-24.0 | 0-22 | 0-21 | 0-22 | 0-14 | 0-10 | 5-20 | 0-11 |
| Hydrolysis of: | | | | | | | | |
| Aesculin | - | - | + | + | + | - | + | W |
| Gelatin | + | W | + | - | + | - | + | - |
| Casein | + | - | + | - | + | NA | + | - |
| Acid from: | | | | | | | | |
| L-Arabinose | + | - | - | NA | - | + | NA | NA |
| D-Fructose | + | - | + | + | + | + | NA | NA |
| Glycerol | - | + | + | NA | + | + | NA | NA |
| Maltose | + | - | + | + | + | + | NA | NA |
| D-Mannitol | + | - | W | - | + | W | NA | NA |
| D-Mannose | + | + | + | + | + | NA | + | - |
| Melibiose | - | - | - | + | - | W | NA | NA |
| Methyl α-D-glucoside | - | + | NA | NA | NA | NA | NA | NA |
| Trehalose | - | - | - | + | + | NA | NA | NA |
| DNA G+C content (mol%) | 39.7 | 40 | 36 | 38.5 | 40.2 | 33.6 | 40.1 | 38.1 |

Strains: 1*, O. kapialis* SSK2-2$^{T}$; 2, *O. picturae* KCTC 3821$^{T}$ ; 3, *O. iheyensis* JCM 11309$^{T}$; 4, *O. oncorhynchi subsp. oncorhynchi* JCM 12661$^{T}$ ; 5, *O. profundus* KCCM 42318$^{T}$ ; 6, *O. caeni* KCTC13061$^{T}$; 7, *O. oncorhynchi subsp. incaldanensis* DSM 16557$^{T}$; 8, *O. chironomi* DSM 18262$^{T}$.
E, ellipsoidal; ES, ellipsoidal or spherical endospores; O, oval; C, central; S, subterminal; T, terminal.
+, Positive; -, negative; w, weak; NA, not available. All data from Namwong *et al*., 2009.

**Genus *Idiomarina***

The genus *Idiomarina* was firstly proposed by Ivanova et al., 2000. The genus *Idiomarina*, family *Idiomarinaceae*, class *Gammaproteobacteria*, as proposed by Ivanova *et al*., (2004) according to the phylogenetic relationships of marine Alteromonas like bacteria. The family *Idiomarinaceae* contained two genera, *Idiomarina* and *Pseudidiomarina*, which *Pseudidiomarina* was proposed by Jean *et al*., (2006). Recently, the genera *Idiomarina* and *Pseudidiomarina* were combined into a single genus Idiomarina, on the basis of the absence of distinguishing phenotypic and chemotaxonomic characteristics (Taborda *et al*., 2009, 2010).

The members of genus *Idiomarina* are Gram-nagative, strictly aerobic, heterotrophic and mesophilic eubacteria. Cells are rod or slightly curved rods. Colonies are slightly yellowish-colored and non pigment. Optimum pH for growth is 7.0 and they can grow over wide range of 0.5%-25% (W/V) NaCl. Most motile by presence of polar-flagella. Catalase and oxidase are both positive. They can utilize some of carbohydrates, organic acids as carbon sources. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. High contents of cellular fatty acids are iso- and anteiso-branched; $C_{15:0}$ iso, $C_{17:0}$ iso, $C_{17:0}$ anteiso, $C_{17:1}$isoω9*c*3-OH. A major isopreniod quinone is Q-8. The DNA G+C contents ranges from 45 to 54 mol%.

All members have been isolated from saline environments (Taborda *et al*., 2009). Such as: *I. salinarum (*from a marine solar saltern), *I. seosinensis* (from hypersaline water of a solar saltern) and *I. abyssalis* (from deep seawater of Pacific Ocean). Presently, *Idiomarina* comprises of 20 species namely, *I. abyssalis*, *I. zobellii* (Ivanova *et al*., 2000), *I. baltica* (Brettar *et al*., 2003), *I. loihiensis* (Donachie *et al*., 2003), *I. fontislapidosi* (Martínez-Cánovas *et al*., 2004), *I. ramblicola* (Martínez-Cánovas *et al*., 2004), *I. seosinensis* (Choi and Cho 2005), *I. homiensis* (Kwon *et al*., 2006), *I. salinarum* (Yoon *et al*., 2007), *I. taiwanensis* (Jean *et al*., 2006) *I. sediminum* (Hu and Li, 2007), *I. donghaiensis* and *I. maritima* (Wu *et al*., 2009) *I. marina* (Jean *et al*., 2009), *I. tainanensis* (Jean *et al*., 2009) , *I. insulisalsae* (Taborda *et al*., 2010), *I. aestuarii* (Park *et al*. 2010), *I. xiamenensis* (Wang *et al*., 2011)*, I. aquimaris* (Chen *et al*., 2011), *I. maris* (Zhang *et al*., 2012). The differential characteristics of *Idiomarina* species are listed in Table 2.4.

**Table 2.4** Characteristics of species belonging to the genus *Idiomarina*.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Cell morphology | Rods | Rods | Slightly ovoid rods | Slightly curved rods | Slightly curved rods | Slightly curved rods | Rods |
| Flagellation | Polar | Single polar, Fimbrae | Single or subpolar | | Polar | Polar | Single polar |
| Temp. for growth (°C) | 4-30 | 4-30 | 4-46 | 8-46 | 4-45 | 15-40 | 4-40 |
| Growth at: | | | | | | | |
| pH | 6.0-9.0 | 5.5-9.5 | ND | ND | 5-10 | 5.0-10.0 | 6.0-10.0 |
| NaCl (%) | 0.6-15 | 1-10 | 0.5-20 | 0.8-10 | 0.5-2 | 0.5-15 | 1-20 |
| Hydrolysis of: | | | | | | | |
| Aesculin | - | - | ND | + | + | + | + |
| Casein | + | ND | ND | ND | - | + | - |
| Starch | - | - | - | - | - | - | - |
| Tween 80 | + | - | ND | + | + | + | + |
| Growth on: | | | | | | | |
| L-Arabinose | - | - | ND | + | - | - | ND |
| Maltose | - | - | + | - | - | - | ND |
| Melibiose | - | - | ND | ND | ND | ND | ND |
| Glycerol | - | - | + | - | - | - | - |
| L-Alanine | + | + | + | - | - | - | + |
| L-lysine | - | - | ND | ND | - | - | ND |
| L-phenylalanine | - | - | ND | ND | - | - | ND |
| Major fatty acid type | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched |
| DNA G+C content (mol%) | 50 | 47.4 | 47.4 | 49.7 | 45.1 | 48.7 | 45.0 |

Strain 1, *I. abyssalis* (Ivanova *et al*., 2000); 2, *I. zobellii* (Ivanova *et al*., 2000); 3, *I. loihiensis* (Donachie *et al*., 2003); 4, *I. baltica* (Brettar *et al*., 2003); 5, *I. fontislapidosi* (Martínez-Cánovas *et al*., 2004); 6, *I. ramblicola* (Martínez-Cánovas *et al*., 2004); 7, *I. seosinensis* (Choi and Cho, 2005) ; +, positive; - negative; ND, no data; NG, no growth

**Table 2.4** (Cont.1) Characteristics of species belonging to the genus *Idiomarina*.

| Characteristic | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|
| Cell morphology | Rods | Rods | Rods | Rods | Rods | Rods | Slightly curved rods |
| Flagellation | Single polar | None | Single polar | None | Peritrichous | None | Peritrichous |
| Temp. for growth (°C) | 4-45 | 15-42 | 4-42 | 15-40 | 15-45 | 10-42 | 10-45 |
| Growth at: | | | | | | | |
| pH | 6.0-9.0 | 5-6 | 6.0-8.0 | 6.0-10.0 | 6.5-10.0 | 6.0-10.0 | 6.5-10 |
| NaCl (%) | 1-15 | 0.5-11 | 4-40 | 0.5-15 | 0.5-10 | 0.5-15 | 0.5-15 |
| Hydrolysis of: | | | | | | | |
| Aesculin | + | + | + | + | - | - | - |
| Casein | - | - | - | + | + | - | - |
| Starch | - | - | - | - | - | - | - |
| Tween 80 | + | + | + | + | + | - | + |
| Growth on: | | | | | | | |
| L-Arabinose | - | - | + | ND | - | - | - |
| Maltose | - | + | - | + | - | - | - |
| Melibiose | - | - | + | - | - | - | - |
| Glycerol | - | - | - | - | - | - | - |
| L-Alanine | - | + | + | + | - | - | - |
| L-lysine | ND | ND | ND | - | - | - | ND |
| L-phenylalanine | - | + | - | + | ND | - | ND |
| Major fatty acid type | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched |
| DNA G+C content (mol%) | 45.1 | 49.3 | 53.9 | 50.0 | 45.5 | 46.6 | 45.2 |

Strain; 8, *I. homiensis* (Kwon *et al*., 2006); 9, *I. taiwanensis* (Jean *et al*., 2006); 10, *I. salinarum* (Yoon *et al*., 2007); 11, *I. sediminum* (Hu and Li, 2007); 12, *I. donghaiensis* (Wu *et al*., 2009); 13, *I. marina* (Jean *et al*., 2009); 14, *I. maritima* (Wu *et al*., 2009); +, positive; - negative; ND, no data; NG, no growth

**Table 2.4** (Cont.2) Characteristics of species belonging to the genus *Idiomarina*

| Characteristic | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|
| Cell morphology | Rods | Rods | Rods | Rods | Rods | Slightly curved rods |
| Flagellation | None | None | Single polar | Single polar | Single polar | Single polar |
| Temp. for growth (°C) | 10-42 | 10-37 | 10-45 | 4-42 | 10-45 | 4-42 |
| Growth at: | | | | | | |
| pH | 6-10 | 5.0-10.0 | 6.0-10.0 | 6-10 | 6.0-11.0 | 6.0-11.5 |
| NaCl (%) | 0.5-15 | 1-10 | 1-12 | 0-15 | 0.5-15 | 0.1-15 |
| Hydrolysis of: | | | | | | |
| Aesculin | - | + | - | + | + | - |
| Casein | - | + | - | ND | - | + |
| Starch | - | - | - | - | - | - |
| Tween 80 | - | + | - | - | + | ND |
| Growth on: | | | | | | |
| L-Arabinose | - | ND | ND | ND | ND | - |
| Maltose | - | ND | - | ND | + | - |
| Melibiose | - | ND | - | ND | - | - |
| Glycerol | - | ND | - | ND | + | - |
| L-Alanine | - | ND | + | ND | + | - |
| L-lysine | - | - | ND | ND | ND | ND |
| L-phenylalanine | - | ND | - | ND | + | ND |
| Major fatty acid type | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched | Iso-branched |
| DNA G+C content (mol%) | 46.9 | 56.4 | 51.6 | 50.4 | 51.1 | 50.4 |

Strain 15, *I. tainanensis* (Jean *et al*., 2009) ; 16, *I. aestuarii* (Park *et al*., 2010) ; 17, *I. insulisalsae* (Taborda *et al*., 2010) ; 18, *I. xiamenensis* (Wang *et al*., 2011); 19, *I. aquimaris* (Chen *et al*., 2011); 20, *I. maris* (Zhang *et al*., 2012);
+, positive; - negative; ND, no data; NG, no growth

**Genus *Tetragenococcus***

The moderately halophilic lactic acid bacteria, genus *Tetragenococcus* is classified within the family *Enterococcaceae,* which belong to the order *Lactobacillales* in the class *Bacilli* (Garrity and Holt, 2001). The genus was created by Collins *et al.* (1990) after reclassifion *of Pepdicoccus halophilus as T. halophilus.* This genus was found in soy sauce, miso, soy paste, brined anchovies, fish sauce, shrimp paste, fermented mustard, kim-chi and sugar thick juice samples. Colonies are small and non pigmentation, non-spore forming Gram positive cocci. Catalase and oxidase are both negative, optimum pH and temperature are 7.0-8.0 and 25-35 °C. They produces L(+)-lactic acid from glucose, but D (-)-lactic acid depends on species-specific. Cellular fatty acid contains long-chain saturated monounsaturated, and cycropropane-ring types, cis-vaccennic acid (ω7 isomer) (Collins *et al.*, 1990). Type of peptidoglycane is Lys-D-Asp (Satomi *et al*., 1997). The G+C contents are 34-36 mol%. The both species, *T. halophilus* and *T. muriaticus* are homofermentative with no $CO_2$ production from glucose

At present, this genus comprises five species. The first specie *T. halophilus* was proposed by Collins *et al.* in 1990 and is separated to two subspecies as, *T. halophilus* subsp. *flandriensis* and *T. halophilus* subsp. *halophilus* by Justé *et al*. in 2012, the second and other species are *T. muriaticus* (Satomi *et al.*, 1997), *T. solitarius* (Ennahar & Cai, 2005), *T. koreensis* (Lee *et al.*, 2005), and *T. osmophilus* (Justé *et al*., 2012), respectively. The differential characteristics of *Tetragenococcus* species are showned in Table 2.5. This genus can thrive at high salt concentration of 25% (w/v) NaCl. The specie *T. muriaticus* has been found as a potent halophilic histamine former from Japanese traditional fermented fish sauce (squid liver sauce) by Satomi *et al*. in 1997. Many researchers found the distribution of this specie in other fermented products and it was widely distributed in mixed populations with *T. halophilus* in fermented products like Japanese fermented puffer fish ovaries. Also they found that the ability of this strain to produce histamine (Kimura *et al*., 2001). Previously, Karnop (1988) isolated a halophilic bacterium as the main histamine former in semi-preserved anchovies and identified it as *Pediococcus halophilus*, which reclassified to *T. halophilus*. Therfore, both of *T. halophilus* and *T. muriaticus* play important roles in histamine formation in salted food products.

**Table 2.5** Characteristic of species belonging to the genus *Tetragenococcus*.

| Characteristic | *T. halophilus* ATCC 33315$^T$ | *T. koreensis* ATCC 35924$^T$ | *T. muriaticus* JCM 10006$^T$ | *T. osmophilus* IAM 1676$^T$ | *T. solitarius* DSM 5634$^T$ |
|---|---|---|---|---|---|
| Cell morphology | Spherical, pairs | Cocci | Tetrads, pairs | Cocci | Ovoid |
| Peptidoglycan | Lys-D-Asp | Lys-D-Asp | Lys-D-Asp | nd | nd |
| Oxidase | - | - | - | - | - |
| Growth at 45 (°C) | - | - | - | - | + |
| Optimum NaCl (%) | 5-10 | 2-5 | 7-10 | nd | 7-10 |
| NaCl range (%) | nd | 0-8 | 1-25 | 0-25 | 6.5 |
| Nitrate reduction | - | - | - | nd | nd |
| Lactic acid production (homofermentative) | He | Ho | Ho | He | Ho |
| Growth on MRS | - | + | - | - | + |
| Fermentation of sugars: | | | | | |
| L-Alabinose | + | - | - | - | - |
| D-Cellulose | + | - | - | nd | + |
| D-Galactose | + | + | - | - | + |
| Maltose | + | + | - | + | + |
| D-Xylose | + | + | - | - | - |
| Mannitol | - | + | + | + | + |
| Xylitol | - | + | - | - | - |
| DNA G+C content (mol%) | 34-36 | 38.3 | 36.5 | 36.7 | 38 |

Source : Satomi *et al.*, 1997; Ennahar & Cai, 2005; Lee *et al.*, 2005; Justé *et al*. 2012; +, positive; - negative; nd, no data; Ho, homofermentative; He, heterofermentative

#### 2.1.2.3. Halotolerant bacteria

Halotolerant bacteria, are tolerant high salinity but do not require salt for growth. Horikoshi and Grant (1998) defined the slight halophile can resist over wide range of 0-3 M NaCl concentration, but grow optimally in range of 0-1 M NaCl concentration. Whereas, non-halophiles grow optimally at less than 0.2 M NaCl. Halotolerant organisms can grow both in the absence and high concentration of salt, such as *Staphyllococcus, Bacillus, Vibrio, Jeotgalicoccus*, *Pseudomonas, Moraxella, Flavobacterium and Acinetobacter.* However, many researchs found *Staphyllococcus* sp. and *Bacillus* sp. grew in 15% NaCl and optimally in 1% NaCl; they are defined as halotolerant bacteria.

### 2.1.3 Application of halophilic bacteria

Moderately halophilic bacteria have the potential for interesting and promising applications. As known, the halophilic bacteria living in fructuating salinity environments have developed several strategies to survive during osmotic up- and downshift. The majority of moderately halophilic respond to an external salinity increase by accumulation of low molecular weight organic compound. They can produce compound of industrial interest such as compatible soluted (trehalose, glycerol, proline, ectoines, glycine, betaines, hydroxyectoine and ectoines), enzymes, and polymers, they also exhibit benificial physiological properties which can facilitate their exploitation for commercial applications. A considerable amount of effort has been dedicated to the study of salt-tolerant extra- and intracellular enzymes of the moderately halophilic bacteria, especially enzymes in biotechnological processes. These include hydrolases (amylases, nucleases, phosphatases, and proteases), which are currently of commercial interest. Alkaline phosphatase (ALP) is one of interesting halophilic enzymes that has been extensively studied and reported from extremely halophilic archea. The study on the ALP from moderately halophilic bacteria are still limited to *Vibrio* and *Halomonas* species. Therefore, the investigation of ALP from newly isolated moderately halophilic bacteria is interesting to be a new potential source or new poperty of ALP.

**2.2 Histamine in fermented fishery products**

Histamine (β-imidazolylethylamine) is an important biogenic amine having low molecular weight and biological activities in various processes such as neurotransmission and numerous brain functions, secretion of pituitary hormones, regulation of gastrointestinal and circulatory functions and inflammatory reactions (Leurs *et al*., 1995). Histamine is derived from the amino acid histidine decarboxylated by enzyme histidine decarboxylase (HDC: EC 4.1.1.22) from microbial organisms (Brink *et al*., 1990) and is founded in many kinds of food such as wine, cheeses, fermented meat (Suzzi and Gardini, 2003; Lehane and Olley, 2000), fermented vegetables (Tsai *et al*., 2005a), fermented fishery products (Kimura *et al*., 2001), fish sauce (Sato *et al*., 1995, Yongsawatdigul *et al*., 2004), shrimp paste (Tsai *et al*., 2006) and salted anchovies (Hernandez–Herrero *et al*., 1999), fermented fishery products (Kimura *et al*., 2001), fish sauce (Sato *et al*., 1995, Yongsawatdigul *et al*., 2004). Its quantity in these foods depends on the protein content of the raw materials and the microbial activity (Suzzi and Gardini, 2003; Lehane and Olley, 2000). Low amount of histamine in foods is not harmful to health unless large amounts are consumed, or the natural mechanism for the catabolism of the histamine of the individual is inhibited. Typical symptoms through histamine overdose observed in certain individuals are included vomiting, sweating, headache and hyper- or hypotension (Lehane and Olley, 2000). The Food and Drug Administration (FDA) established an advisory level of 500 ppm to be hazardous to human health (FDA, 1998). Above this level, humans begin to encounter the allergy-like symptoms of the disease. However, the toxic dose of histamine per individual is difficult to determine and depends on the efficiency of the detoxification mechanisms of the person (Taylor, 1986, 1989).

Early detection of histamine producing bacteria is accomplished by PCR of the gene encoding for the enzyme histidine decarboxylase (HDC). Bacterial HDC have been characterized in different organisms and divided to two enzyme families according to their sequences and characteristics completely difference. First, pyridoxal phosphate-dependent HDC are found in Gram-negative bacteria belonging to various species (i.e., *Morganella morganii, Enterobacter aerogenes, Photobacterium phosphoreum, Raoultella planticola*, etc). Second, pyruvoyl-

dependent HDC are found in Gram-positive bacteria and especially lactic acid bacteria implicated in food fermentation or spoilage, such as *Clostridium perfringens, Tetragenococcus muriaticus, Oenococcus oeni, Lactobacillus hilgardii, and Lactobacillus buchneri,* among others.

Many reports showed that especially fish contain several kinds of microbial organism producing histamine such as; *Morganella (Proteus) morganii, Klebsiella pneumoniae* and *Hafnia alvei* isolated from fish incriminated in *S*combroid poisoning (Taylor and Speckard, 1983), *Proteus vulgaris, P. mirabilis, Enterobacter aerogenes, Enterobacter cloacae, Serratia fonticola, S. liquefaciens* and *Citrobacter freundii* generally known as histamine-former in fish (Lopez-Sabater *et al.*, 1996; Tsai *et al.*, 2004), halotolerants bacteria such as *Staphylococcus spp., Vibrio spp.* and *Pseudomonas* III/IV-NH isolated from fermented salted sardine and fish products, *S. epidermidis, S. xylosus, K. oxytoca, E. cloacae, Pseudomonas cepaciae,* and *Bacillus spp*. from salted Spanish anchovies (Hernandez *et al*., 1999), *Pantoea* spp. and *E. cloacae* isolated from salted mackerel in Taiwan (Tsai *et al*., 2005b). Although prolific histamine forming bacteria have been isolated from temperature abused raw fish, they have rarely been isolated from fermented fish products, such as fish sauce and fish paste. Only a few halophilic bacteria, such as *T. muriaticus*, have been isolated as prolific histamine-formers from fish sauce.

## 2.3 Bacterial alkaline phosphatases

Alkaline phosphatase (ALP) (EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called *dephosphorylation*. As the name suggests that alkaline phosphatases are most effective in alkaline environment. It is sometimes used synonymously as basic phosphatase (Tamás, 2002).

### 2.3.1 Alkaline phosphatase reaction

Alkaline phosphatase (ALPs) catalyze several types of phosphoryl and sulfuryl transfer reactions through the formation of a phophoseryl intermediate (Weiss *et al*., 1988), often with enormous rate accelerations (Galperin *et al*., 1998; Galperin

and Jedrzejas, 2001). The step of ALPs reaction involves the following, first phosphomonoester was coordinated with two $Zn^{2+}$ atom. Next, serine at active site was deprotonated and activated with $Mg^{2+}$ which was coordinated with hydroxide. Then the active serine was attacked with nucleophilic forming a covalent enzyme-phosphate intermediate and releasing the free alcohol. Finally, $Zn^{2+}$-coordinated with hydroxide and hydrolyzed the enzyme-phosphate intermediate to generate phosphate. The hydrolysis reaction was catalyzed by ALPs are shown in Figure 2.1 (A, B).

A


B

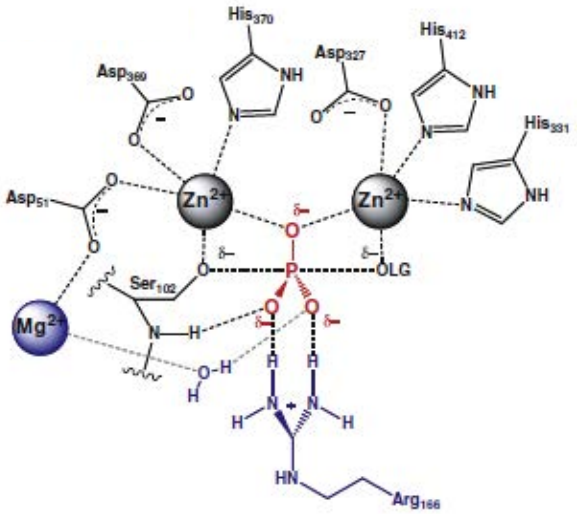


**Figure 2.1** Catalytic reaction of ALPs (A); ALP efficiently catalyzes phosphate hydrolysis and (B); The position of the leaving group is indicated by LG, and the second substituent of phosphodiesters is indicated by R (Bobyr *et al*., 2012).

The conserved ALPs core fold and the conserved active site generating three divalent metal ions are coherent with the essentially identical overall catalytic mechanism of ALPs. However, differences were observed in the metal ions involved in catalysis (such as $Zn^{2+}$ can be replaced by $Co^{2+}$, $Ca^{2+}$ amd $Mn^{2+}$) and in two structural elements modulating protein properties. These variable and optional modules enlarge the dimer interaction surface, which has been characterized as

critical for protein stability, catalytic ability and allosteric regulation of ALPs (Wende *et al*., 2010).

### 2.3.2 Structure of alkaline phosphatase

There are several classes of alkaline phosphatases depending on the presence or absence of metal ion cofactors such as zinc, magnesium, or iron. Some phosphatases must be under regulation at various levels and therefore have structural features allowing specific interactions with selected partners. Other phosphatases seem to be rather non-specific both in terms of substrate specificity and role. The active site of ALPs consists of two zinc and one magnesium ions (Figure 2.2). The $Zn^{2+}$ ion at the M1 site is hexa-coordinated in a distorted octahedral arrangement by Asp273 (bidentate), His277, His465, and two oxygen atoms from Sul610 (or possibly a phosphate ion). The zinc ion at the M2 site is penta-coordinated by Asp12, S65, Asp315, His316, and one oxygen of Sul610. The $Mg^{2+}$ ion at the M3 site is octahedrally hexa-coordinated by Asp12, Thr118, Glu268, and three waters. The residues involved in the first sphere of metal binding are conserved in the ALPs. All three metal ions take part in the catalysis and the magnesium ion is also important for stability. The latter abstracts the proton from the nucleophilic serine through generation of a metal-bound hydroxide ion. This mechanism is assumed to be a general one. Addition of zinc to many ALPs is inhibitory but can be reactivated with magnesium for optimal activity at alkaline pH. The different effects may be due to sub-optimal coordination of the magnesium-bound hydroxide ion in the initial nucleophilic attack by different ions (Helland *et al*., 2009).

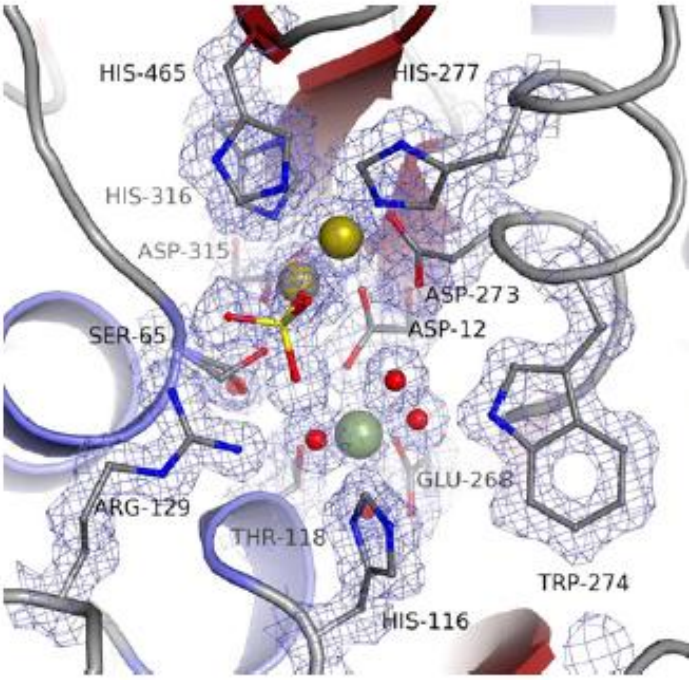


**Figure. 2.2.** The active site of ALPs, including the catalytic zinc (gold spheres) and magnesium (green sphere) ions, along with the sulphate (red and yellow ball-and-stick) (Helland *et al*., 2009).

### 2.3.3 Microbial alkaline phosphatase

On the fact that phosphate cannot be synthesized by microbes, makes all alkaline phosphatases (ALPs) more important for their survival and the cell obtains inorganic phosphate from nucleic acids, phosphorylated sugars, proteins, etc. Therefore, many bacteria have complex mechanisms for regulation of synthesis and activities of phosphatases that gives them competitive advantage especially in nutrient deficient conditions. ALPs can be isolated from variety of microorganisms including *Escherichia coli*, *Pseudomonas*, *Aerobactor*, *Bacillus* sp., *Thermotoga neapolitana*, *Thermus thermophilus* (Pantazaki *et al*., 1998), *Bacillus stearothermophilus*, , but only few reports on ALPs from halophillic species *e.g. Halobacterium halobium* (Bonet *et al*., 1994), *Halobacterium cutirubrum* (Fitt and Baddoo 1979), *Haloarcular marismortui* (Goldman *et al*., 1990), *Aeropyrum pernix* (Helianti *et al*., 2007) and *Pyrococcus abyssi* (Zappa *et al*., 2001). Previously information on microbial ALPs are summarized in Table 2.6.

**Table 2.6.** Microorganisms reported to produce ALPs

| **Microbial species** | **Reference** |
|---|---|
| *Vibrio alginolyticus* | Hayashi *et al*., 1973 |
| *Halobacterium cutirubrum* | Fitt and Baddoo, 1979 |
| *Vibrio cholera* | Roy *et al*., 1982 |
| *Haloarcular marismortui* | Goldman *et al*.,1990 |
| *Halobacterium halobium* | Bonet *et al*.,1994 |
| *Thermotoga neapolitana* | Dong and Zeikus, 1997 |
| *Thermus thermophilus* | Pantazaki *et al*., 1998 |
| *Bacillus intermedius* S3-19 | Sharipova *et al*., 1999 |
| *Pyrococcus abyssi* | Zappa *et al*., 2001 |
| *Bacillus sphaericus* | Dhaked *et al*., 2005 |
| *Neurospora crassa* | Bogo *et al*., 2006 |
| *Aeropyrum pernix* K1 | Helianti *et al*., 2007 |
| *Rhizopus microsporus* var. *rhizopodiformis* | Junior *et al*., 2008 |
| *E.coli* EFRL 13 | Qureshi *et al*., 2010 |
| *Bacillus* sp. | Mahesh *et al*., 2010 |
| *Bacillus licheniformis* MTCC1483 | Pandy and BaniK, 2011 |

### 2.3.4 Properties of Alkaline phosphatase

Most microbial ALPs are located in the periplasmic space (the space between the cytoplasmic membrane and the outer membrane) of cell wall of bacteria (Cheng and Costerton, 1973; Bhatti *et al.,* 1976; McNicholas and Hulett, 1977; Malamy and Horecker, 1961; Thompson and McLeod, 1974; Doonan and Jensen, 1977). The majority of ALPs reported from microorganisms occur intracellularly such as *E. coli*, *Halomonas* sp., *Vibrio* sp., *Bacillus licheniformis*, *Aeropyrum pernix* K1 (Sharipova et al., 2000; Guimaräes *et al*., 2003; Helianti *et al*., 2007; Ishibashi *et al.,* 2011).

However, some reported ALPs such as Psychrophilic *Arthrobacter* (Prada *et al.,* 1996), an halophilic archaebacterium *Haloarcular morismortui* (Goldman *et al*., 1990), a psychrotolerant *Bacillus sphaericus* (Dhaked *et al*., 2005) were found to produce extracellular ALPs. Additionally, *Rhizopus microspores* can produced ALPs both intracellular and extracellular enzyme and has optimum temperature and pH at

65 °C and pH 8.0 respectively (Junior *et al.*, 2008). ALP in *B. licheniformis* was localized in regions on the inner size of plasma membrane in log phase and it secretes more than 80% of ALP in medium at the stationary phase. The region of ALP varied depending on the growth and cultured age (McNicholas and Hulett, 1977). In addition, *Aspergillus caespitosus* secreted the highest ALP when this fungi was cultivated in media supplemented with wheat raw and sugar cane (Guimaräes *et al*., 2003).

Intracellular ALP from *E.coli* is the most intensively studied in structure and catalytic properties aspects. It has been served as the basis for comparison to enzymes with similar homology. *E. coli* ALP is a homodimeric metalloenzyme and the monomer comprises 449 amino acid residues and lacks carbohydrate and fatty acid moieties. Many mammalian ALPs have also been studied. The human ALPs are classified into four groups (isozymes) named after the tissue which predominantly contains each type: the intestinal, the placental, the germ cell, and lastly the liver, bone, kidney, or tissue non-specific alkaline phosphatases. Usually the commercial ALPs are produced from *E.coli* or Calf intestine (Quresh *et al*., 2010).

### 2.3.6 Characterization of purified alkaline phosphate

General purification methods reported for ALP were precipitated with ammonium sulfate before subjected to ion exchange chromatography by using DEAE-Sephadex A25column, affinity column containing L-histidyldiazobenzylphosphonic acid linked to agarose, and further purified by gel filtration chromatography to measure molecular weight (MW) and checked the purity of active fraction by SDS-PAGE (10-12.5% acrylamide). For example, in 2000, Hauksson and teams were purified ALP from *Vibrio* sp. by an affinity column (2.5x2 cm) containing L-histidyldiazobenzylphosphonic acid linked to agarose and followed using an ion-exchange column (DEAE-Sepharose). An ALP was purified 151 fold with 54% yield from the culture medium using a single step affinity chromatography procedure on agarose-linked L-histidyldiazobenzylphosphonic acid. The active enzyme was a 55± 6 kDa monomer. The enzyme had optimal activity at pH 10 and exhibited heat-labile with a half-life of 6 min at 40°C and 30 min at 32°C.

In 2005, Dhaked and teams purified extracellular ALP from *B. sphaericus* using ammonium sulfate treatment followed by DEAE-Sephadex A25 (Pharmacia, USA) and gel filtration chromatography (Bio-gel A column (Bio-rad, USA). It was found that the enzyme was purified 11.1 fold with a total yield of 15.8% with the molecular weight approximated of 76 kDa.

In 2007, Helianti and teams purified and characterized thermostable native alkaline phosphatase from an aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 by applied into an affinity chromatography column (C 1.0/10 Pharmacia column containing L-histidyldiazobenzylphosphonic acid-agarose (Sigma) as a matrix. It was observed that this purified enzyme was predicted as a homodimeric structure with a native molecular mass of about 75 kDa and monomer of about 40 kDa. It has been demonstrated as a very thermostable ALP which can retaining activity at 76% at 90°C for 5.5h and 67% at 100°C for 2.5h. Beside that, these enzymes were increased in addition of exogenous Mg (II), Ca (II), Zn (II), and CO (II).

In 2008 Junior and teams found that *Rhizopus microspores* can produced ALP both intracellular and extracellular types. Both of these were purified by DEAE-cellulose and ConA sepharose chromatography before applied to gel filtration, it was found that intra- and extracellular ALP were purified 5.0 and 9.3 fold, respectively. These enzymes were predicted as dimeric structures of about 57 kDa for both forms. Optimum temperature and pH for both phosphatases were 65 °C and pH 8.0, respectively. $Mn^{2+}$, $Na^{+}$ and $Mg^{2+}$ stimulated the activity, while $Al^{3+}$ and $Zn^{2+}$ activated only the extracellular form. In 2010, Mahesh and teams purified and characterized ALP from *Bacillus spp.* by DEAE-Cellulose ion exchange chromatography. It was observed that the enzyme has molecular weight approximately 84 kDa which its activity was enhanced by $Mg^{2+}$ up to 66% and 80% activity was inhibited by EDTA. The characteristics of ALPs from some microorganisms are listed in Table 2.7.

### 2.3.7 Application of alkaline phosphate (ALP)

The importance of ALP in clinical medicine and molecular biology has made it a popular subject for scientific study and commercial utility. ALPs have been extensively used in the diagnostics, immunology and molecular biology as

biochemical markers in quantitative measurements of disease, linked enzymes in ELISA (Sun *et al*., 2007; Muginova *et al*., 2007; Baranov *et al*., 2008) and also used in nonradioactive detection techniques, probing, blotting and sequencing systems (Nilgiriwala *et al*., 2008; Sasajima *et al*., 2010).

Microbial alkaline phosphatases have been intensively studied and used in medical research, biotechnological and various practical applications, such as used to reveal the presence of target detector enzyme complexes using chromogenic or chemiluminescent compounds in Cloning experiments, Gene sequencing, blotting, and nonisotopic probing (Dong and Zeikus 1997), used in Enzyme-linked immunosorbent assays (ELISA) in the pharmaceutical (Zueva *et al*.1993) and used to operate in photochemical or chromogenic systems in Western, Southern, or Northern analysis. Morever, ALPs are good models to study the thermal stabilization mechanism of metal ion-dependent catalysis at high temperature. ALP has been conventionally used in food industry as index of adequate pasteurization and the detection of alkaline phosphatase activity of thermally treated liquid milk products has become a common procedure for milk quality control (Rankin *et al.*, 2010). In agricultural application, the phosphate solubilizing microorganisms especially *Bacillus megaterium* var. phosphaticum with name phosphobacterin is used as biofertilizers and bioinoculants in order to increase the availability of phosphate to plants by mineralizing organic phosphate compounds and rendering inorganic phosphorus compounds.(Ponmurugan and Gopi, 2006)

**Table 2.7** Microrganism and some alkaline phosphatase characteristics

| Microrganisms | Molecular weight (kDa) | Optimum pH | Optimum Temperature ($^{o}$C) | Reference |
|---|---|---|---|---|
| *Thermotoga neapolitana* | 87 | 9.5 | 85 | Dong and Zeikus, 1997 |
| *Thermus caldophilus* GK24 | 108 | 7.4 | 80 | Park et al., 1999 |
| Chicken intestine | 67 | 9.8 | 30 | Sharma *et al*., 2000 |
| *Pyrococcus abyssi* | 54 | 11 | 70 | Zappa *et al*., 2001 |
| *Aspergillus caespitosus* | 134.8 | 8.5 | 70 | Guimarães et al.,2003 |
| *Bacillus sphaericus* | 76 | 7.0 | 25 | Dhaked *et al*., 2005 |
| *Neurospora crassa* | 137 | 9.5 | 65 | Bogo *et al*., 2006 |
| *Aeropyrum pernix K1* | 75 | 10.0 | 95 | Helianti *et al*.,2007 |
| *Rhizopus microsporus var.rhizopodiformis* | 118 | 8.0 | 65 | Junior *et al*., 2008 |
| *Halobacterium salinarum* | 45.3 | 10.5 | 37 | Wende et al., 2009 |
| *Bacillus spp.* | 84 | 8.8 | 65 | Mahesh *et al*., 2010 |

# CHAPTER III

# EXPERIMENTAL

## 3.1 Halophilic lactic acid bacteria producing histamine

### 3.1.1 Sample collection and isolation of halophilic lactic acid bacteria

The salted fermented foods; fermented fish, fish sauce, shrimp paste, *nam-budu, pla-chom, kung-chom* and *tai-pla* including fermented soy bean, soy sauce, were obtained from various provinces in Thailand. The moderate halophilic lactic acid bacteria were isolated by a pour plate technique using MRS (De Man, Rogosa, & Sharpe, 1960) agar with 10% (w/v) NaCl. Incubation conditions were at 30$^{o}$C for 3-5 days. The colonies on medium plate were selected and purified. The pure cultures were maintained both in lyophilized form and on medium slant kept at 4°C for further study.

### 3.1.2 Identification methods

The colonies grown on MRS containing 10% NaCl after 3 days incubation at 30°C were examined for their morphological, cultural, physiological, and biochemical characteristics.

#### 3.1.2.1 Morphological characterization

Cell morphology, cell arrangement, Gram staining and colonial appearance were examined on the 3 days culture grown on MRS with 10% (w/v) NaCl incubated for 30 $^{o}$C for 3 days as described by Barrow and Feltham (1993).

#### 3.1.2.2 Physiological and biochemical characterization

**3.1.2.2.1 Catalase test** A small amount of pure growth was transferred on the slide. Hydrogen peroxide ($H_2O_2$) 3% was immediately dropped onto a portion of a colony on the slide. The evolution of gas bubbles indicating a positive test was observed.

**3.1.2.2.2 Oxidase test** Each colony was streaked on MRS containing 10% NaCl and incubated at 30°C for 1-3 day. A few drops of 1% tetramethyl-p-phenylenediamine were dropped on sterile filter paper disc. The test

culture was smeared across the moist paper disc with sterlie loop. The apperance of dark-purple colour on paper within 30 sec. denoted a positive reaction.

**3.1.2.2.3 Nitrate reduction** The cultured cells were inoculated in nitrate broth (Appendix A-2) with the cultures and incubated at 30$^{o}$C for 3-5 days. A drop of each Solution A and Solution B reagent (Appendix A-3) was added. When nitrite is present a pink colour developed within 5 minutes.

**3.1.2.2.4 Hydrolysis of L-arginine** The cultured cells were inoculated on the arginine agar slant (Appendix A-4) and incubated for 3-5 days. A positive reaction is shown by a colour change of the indicator to red.

**3.1.2.2.5 Hydrolysis of casein** The cultured cells were inoculated on the casein agar medium (Appendix A-5) and incubated at 30°C for 3-5 days. The clear zones areas indicated the positive of hydrolysis.

**3.1.2.2.6 Hydrolysis of gelatin** The cultured cells were inoculated on the gelatin agar medium (Appendix A-6) and incubated at 30°C for 3-5 days. Flood the surface with 5-10 ml of 30% trichloroacetic acid, and the clear zones areas indicated the hydrolysis.

**3.1.2.2.7 Growth at different parameters** Temperatures range for growth (30, 37, 45 and 50°C) was tested using MRS agar (difco) with 10% (w/v) NaCl pH 7.0, initial pHs (4.0, 5.0, 6.0, 8.0 and 9.0) and concentrations of NaCl (0, 5, 10, 15, and 20% w/v) was done using MRS broth with 10% w/v as basal medium except the study at different concentrations of NaCl and pH was adjusted with sterile 0.1N NaOH and 0.1 N HCl. The growth examination was observed after 3 days incubation at 30$^{o}$C for cultured agar plate and observed by measuring the optical density at 600 nm for cultured broth and then optimal condition for growth was evaluated.

**3.1.2.2.8 Acid formation from carbohydrate** The acid production from carbon sources was tested in a basal medium of GYPB broth with 10% (w/v) NaCl. (Tanasupawat et al., 1998) (Appendix A-7) and the addition of appropriates carbon sources at final concentration 1% (w/v). The following 22 different carbon sources were used including L-arabinose, D-cellobiose, D-fructose, D-galactose,

D-glucose, glycerol, inulin, lactose, myo-Inositol, maltose, D-mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose, D-xylose. The media were adjusted to pH 7.2 and phenol red 0.2% solution (w/v) was added as an indicator solution. The 2 ml liquid was dispensed into a 11 mm diameter test tube. The broth was inoculated with a drop of bacterial cell suspensed in saline and incubated at 30ºC. Growth was recorded daily for up to 5 days. The positive results were shown by colour change of the indicator from red to yellow.

**3.1.2.3 Genotypic characterization**

**3.1.2.3.1 16S rRNA gene sequence analysis** Genomic DNA extraction and purification from all isolated bacteria was performed according to the method recommened by Saito and Miura (1963). The bacterial cell grown in MRS agar with 10 % (w/v) NaCl cultured at 30$^{o}$C to obtain the cell growth during logarithmic phase. The cell were harvested and followed the method as described in 3.2.2.4.1 (16S rRNA gene sequence analysis) from step I (isolation of genomic DNA), step II (amplification of 16s rRNA gene), step III (sequencing of 16s rRNA gene) to step IV (phylogenetic analysis), respectively. Except the purification in step I, the genomic DNA was purified by the DNeasy Tissue Kit (Qiagen GmbH, Germany).

**3.1.2.3.2 Digestions of 16s rRNA gene PCR products with restriction endonucleases** Genomic DNAs was extracted from cell grown in log phase and purified by the DNeasy Tissue Kit (Qiagen GmbH, Germany). The 16s rRNA genes were amplified by PCR with Taq DNA polymerase and primers 20F (5'-GAGTTTGATCCTGGCTCAG-3') the *Escherichia coli* numbering system (Brosius *et al*., 1981) and 1500R (5'-GTTACCTTGTTACGACTT-3'). The purified PCR products were separately digested with restriction endonucleases *Mbo*I and *Alu*I (Takara, Japan) and the digested reactions were incubated at 37$^{o}$C 24 h. The digested reaction products were analysed by 2.5 % (w/v) agarose gel electrophoresis and run at 150V for 1 h in 1X Tris-acetate running buffer (Satomi *et al*., 1997).

### 3.1.3 Determination of histamine formation

Production of histamine was quantified by growing each of isolated moderately halophilic bacteria in medium containing L-histidine monohydrochloride as a precursor amino acid. Each isolates was inoculated into 1 ml of histidine broth (Appendix A-8) and 100 µl of pre-cultured cells in histidine broth was inoculated into 10 ml of histidine broth and incubated at 30$^{o}$C for 6 days. Histamine was extracted from supernatant sample and derivatized by the method modified from Eerola *et al*. (Eerola *et al*., 1993) and introduced to HPLC analysis. Histamine formers (*Tetragenococcus muriaticus* JCM 10006$^{T}$, *T. muriaticus* JCM10007) and non-histamine formers (*T. halophilus* ATCC 33315$^{T}$ and *T. koreensis* KCTC3924$^{T}$) were used as reference strains.

### 3.1.4 Detection of histamine decarboxylase (*hdc*) gene

To confirm if all isolated strains have *hdc* genes, specific detection of partial *hdc* gene fragment was performed using PCR amplification with 3 oligonucleotide primer sets (Table 3.1) including JV16HC-JV17HC (Le Jeune et al., 1995), primers 106-107 (De Las Rivas et al., 2005) and HDC3-HDC4 (Coton and Coton, 2005), respectively. DNA amplification of partial *hdc* with JV16HC-JV17HC and primer set 106-107 were performed with the following cycling parameters: 95$^{o}$C 10 min, 30 cycles of 30 sec at 95°C, 30 sec at 52°C and 30 sec at 72°C and a final elongation step of 10 min at 72°C (De Las Rivas et al., 2005). PCR condition for HDC3-HDC4 primer set was as follows; 95$^{o}$C 5 min, 32 cycles of 95$^{o}$C 45 sec, 52$^{o}$C 45 sec, 72$^{o}$C 1 min 15 sec with a final extension at 72$^{o}$C for 5 min. The PCR products were visualized by electrophoresis with 1.5% (w/v) agarose gel (for primers set JV16HC-JV17HC and 106-107) and 0.8 % (w/v) agarose gel (for primers set HDC3-HDC4) stained with ethidium bromide.

**Table** 3.1 PCR Primers used for detecting histidine decarboxylase (*hdc*) gene.

| Primer name | Sequence 5'-3'[a] | Amplicon size (bp) | Described by |
|---|---|---|---|
| **Gram-positive bacteria** | | | |
| JV16 HD | AGATGGTATTGTTTCTTATG | 367 | Le Jeune et al., 1995 |
| JV17HD | AGACCATACACCATAACCTT | | |
| HDC3 | GATGGTATTGTTTCKTATGA | 440 | Coton and Coton, 2005 |
| HDC4 | CAAACACCAGCATCTTC | | |
| **Gram-negative bacteria** | | | |
| 106 | AAYTCNTTYGAYTTYGARAARGARG | 534 | De Las Rivas et al. 2005 |
| 107 | ATNGGNGANCCDATCATYTTRTGNCC | | |

[a] Y, C or T; R, A or G; W, A or T; D, G, A or T; N, A, C, G or T.

## 3.2 Study on Alkaline phoshatase producing moderate halophilic bacteria

### 3.2.1 Sample collection and isolation of moderate halophilic bacteria

The thirty-three fermented fish and salted fish samples were collected from local markets in various provinces of Thailand including Kalasin (KL), Mahasarakham (MK), Rayong (RY), Nakornsawan (NSW), Sukho Thai (SK) and Samut Songkhram (SMK). Isolation of halophillic bacteria from these samples were done by spread plate technique on JCM no.377 medium (Appendix A-1) and incubated at 37$^{o}$C for 1-3 days. The colonies on medium plate were selected and purified. The pure cultures were maintained both in lyophilized form and on medium slant kept at 4°C for further study.

### 3.2.2 Identification methods

The colonies on JCM no. 377 containing 10% NaCl after 1 day incubation at 37°C were examined for their characteristics (Barrow and Feltham, 1993). Morphological, cultural, physiological, and biochemical characteristics of the isolates were determined using the methods described by Barrow and Feltham (1993), Leifson (1963) and Namwong (2005) along with several supplementary tests.

#### 3.2.2.1 Morphology characterization

Cell morphology, cell arrangement, Gram staining, colonial appearance and flagella staining (Appendix A-9) were examined on 3-5 days cultured grown on JCM no. 377 containing 10% (w/v) NaCl incubated for 37 $^{o}$C for 3 days (Barrow and Feltham, 1993; Forbes, 1981)

**3.2.2.2 Physiological and biochemical characterization**

**3.2.2.2.1 Oxidase, Catalase, L-Arginine and gelatin hydrolysis, nitrate reduction and Growth at different parameters** were done in the same manner that above described in 3.1.2.2 but the basal medium used JCM no. 377 containing 10% NaCl except the study at different concentrations of NaCl and adjusted pH with sterile 0.1 N NaOH and 0.1 N HCl. The growth examination was observed after 3-5 days incubation at 37$^{o}$C.

**3.2.2.2.2 Aesculin hydrolysis**. Inoculated aesculin broth (Appendix A-10) containing 10% NaCl was examined daily up to 5 days for blacking of hydrolysis.

**3.2.2.2.3 Starch hydrolysis**. Inoculate in the JCM no. 377 (Appendix A-11) containing 1% soluble starch and incubated plates at 37°C for 5 days. Flood the plate with Lugol's iodine solution then the medium turns blue where starch has not been hydrolysed; clear colourless zones indicated the hydrolysis.

**3.2.2.2.4 Tyrosine hydrolysis**. Bacterial cell were inoculated on the JCM no.377 medium agar (Appendix A-12) containing 0.5% tyrosine but omitted casamino acid and incubated at 37°C for 5 days. Clear zone indicate areas of the tyrosine hydrolysis.

**3.2.2.2.5 Tween 80 hydrolysis.** Bacterial cell were inoculated on the JCM no.377 medium agar containing 2% tween 80 but omitted casamino acid (Appendix A-13) and incubated at 37°C for 5 days. Clear zone indicate areas of the tyrosine hydrolysis.

**3.2.2.2.6 Indole test**. Inoculate the cultures in tryptone broth (Appendix A-14); and incubated at 37$^{o}$C for 5 days. After incubation, it was tested by adding 4 drops of Kovacs' reagent (Appendix A-15), using *iso*-amyl alcohol as solvent.

**3.2.2.2.7 Acid from carbohydrate**. The fermentation of carbon sources was investigated by Biolog GN2 plate as described by Choi & Cho (2005). Briefly, cells preparation of the tested strain for Biolog GN2 plate was grown in log phase in JCM no.377 agar plate at 30-37°C. The fresh cell was suspened in the

inoculating fluid buffer supplemented with 1 M NaCl and the cell suspension was adjusted turbidity until the optical density at 600 nm ($OD_{600}$) of 0.3. The microplates were inoculated with 150 µl of the cell suspension per well for each strain and incubated at 30°C. The results were read visually after incubation for 1, 2, 3 and 4 days by microplate reader according to manufacturer instruction.

**3.2.2.2.8 API ZYM test** Enzymatic activity profile was determined by API ZYM strip (bioMérieux) according to manufacturer instruction. Briefly, fresh bacterial cell was suspened in normal saline solution and was adjusted the turbidity of cell suspension equal to a McFarland No.4. After that distributed the cell suspension into API ZYM strips and incubated at 37$^{o}$C in dark incubation box with normal atmospheric condition. After 6 h of incubation, reagents A and B were added to each cupule following the manufacturer's direction. The reactions were developed for 5 min under a powerful light source. After light exposure, the reaction were read and recorded on the result sheet. A value ranging from 0-5 was assigned, corresponding to the color developed: 0 represents to a negative reaction, values 1, 2, 3, 4 and 5 represent to positive reaction depending on the level of intensity that means value 5 represents to a reaction of maximum intensity (Table 3.2).

**Table 3.2** Reading table of API ZYM

| No | Enzyme assayed for | Substrate | pH | Result | |
|---|---|---|---|---|---|
| | | | | Positive | Negative |
| 1 | Control | | | Colorless | |
| 2 | Alkaline phosphatase | 2-naphthyl phosphate | 8.5 | Violet | |
| 3 | Esterase (C4) | 2-naphthyl butyrate | 6.5 | Violet | |
| 4 | Esterase lipase (C8) | 2-naphthyl caprylate | 7.5 | Violet | |
| 5 | Lipase (C14) | 2-naphthyl myristate | 7.5 | Violet | |
| 6 | Leucine arylamidase | L-leucyl-2-naphthylamide | 7.5 | Orange | |
| 7 | Valine arylamidase | L-valyl-2-naphthylamide | 7.5 | Orange | |
| 8 | Cystine arylamidase | L-cystyl-2-naphthylamide | 7.5 | Orange | Colorless or very pale yellow* |
| 9 | Trypsin | N-benzoyl-DL-arginine-2-naphthylamide | 8.5 | Orange | |
| 10 | α– Chymotrypsin | N-glutaryl-phenylalanine-2-naphthylamide | 7.5 | Orange | |
| 11 | Acid phosphatase | 2-naphthyl phosphate | 5.4 | Violet | |
| 12 | Naphthol-AS-BI-phosphohydrolase | Naphthyl-AS-BI-phosphate | 5.4 | Blue | |
| 13 | α– galactosidase | 6-Br-2-naphthyl-aD-galactopyranoside | 5.4 | Violet | |
| 14 | β– galactosidase | 2-naphthyl-bD-galactopyranoside | 5.4 | Violet | |
| 15 | β– glucuronidase | Naphthol-AS-BI-bD-glucoronide | 5.4 | Blue | |
| 16 | α– glucosidase | 2-naphthyl-aD-glucopyranoside | 5.4 | Violet | |
| 17 | β–glucosidase | 6-Br-2-naphthyl-bD-glucosaminide | 5.4 | Violet | |
| 18 | N-acetyl-b-glucosaminidase | 1-naphthyl-N-acetyl-bD-glucosaminide | 5.4 | Brown | |
| 19 | α- mannosidase | 6-Br-2-naphthyl-aD-mannopyranoside | 5.4 | Violet | |
| 20 | α-fucosidase | 2-naphthyl-aL-fucopyranoside | 5.4 | Violet | |

* Colorless or color of the control if the strip has been exposed to an intense light source after addition of the reagents; if the strip has not been exposed to intense light, a very pale yellow color is obtained.

**3.2.2.2.9 Susceptibility to antibiotics** The sentivity to antibiotics was tested using the disc-diffusion method (Bauer *et al*., 1966). After cell suspensions were spreaded on halophilic medium agar and the antibiotic paper discs (6 mm in diameter) were placed on the medium. The effects of the antibiotics on cell growth or inhibition zones were observed after 3 days of incubation at 37°C. The strain was considered susceptible when the inhibition zone extened more than 3 mm beyond the antibiotic disc (Jean *et al*., 2009, Chen *et al*., 2011)

**3.2.2.3 Chemotaxonomic characterization**

**3.2.2.3.1 Diaminopimelic acid analysis.** Dried cells (10 mg) were hydrolyzed with 6N HCl at 100 °C for 18 h. The hydrolyzed solution was filtered and evaporated. The 400 μL of distilled water was added into dried sample. The solution was loaded onto cellulose HPTLC plate no.5787 and developed with MeOH: $H_2O$: 6 N HCl: Pyridine (80:26:4:10). Finally, the cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol for detection (Komagata and Suzuki, 1987).

**3.2.2.3.2 Cellular fatty acids.** Dried cells (40 mg) were put into screw-cap tube and added with 1 mL of reagent 1 (Appendix A-16.1). This suspension was shaken well before heating at 100 °C for 30 min and then cooling to room temperature in water. The reagent 2 (Appendix A-16.2) was added into the suspension and mixed for 5 to 10 sec with vortex mixer. The suspension was heated at 80 °C for 10 min and cooled to room temperature in water. Then, reagent 3 was added (Appendix A-16.3) and the suspension was mixed for 10 min and was transferred the upper layer to another tube. The reagent 4 (Appendix A-16.4) was added into the suspension and mixed for 5 min. Addition of the reagent 5 (Appendix A-16.5) may required if the suspension became to emulsion form. The upper layer was analysed cellular fatty acid using gas chromatrgraphy (Minnikin et al., 1984).

**3.2.2.3.3 Quinone analysis**. The isoprenoid quinone was extracted from suspension of freezed-dried cells (20 mg: 2 ml) by using chloroform-methanol (2:1, V/V) in flask and gentltly shaked for 6 hours. After filtration, the filtrate was concentrated until dry on the rotary evaporator and was dissolved in a small amout of acetone. Actone solution was applied to thin-layer chroatography on a 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 should also be

included. The quinone spots can be visualized by UV light at 254 nm. The $R_f$ of menaquinone was 0.4. The band of menaquinone was scraped off and extracted with acetone. The purified quinones were examined by HPLC (Shimadza model LC-3A). μ- Bondapak $C_{18}$ column (water Associates, Milford, Mass., USA) was employed and eluted by methanol-isopropanol (1:1, v/v) with flow rate 1.2 ml/min. The abbreviation (e.g. MK-7, MK-6, etc.) used for menaquinone indicated the number of isoprene unit in the side chain.

**3.2.2.3.4 Determination of DNA base composition** Bacteria in each group was extracted and purified genomic DNA according to the method recommened by Saito and Miura (1963). The guanine-plus-cytosine (G+C) content was determined in DNA base composition by the HPLC method as described by Tamaoka and Komagata (1984). In briefly, 10 μl of purified DNA (≈0.5-1 mg/ml) was heated in boiling water for 5 min and immediately cooled on ice. DNA was denature and then hydrolyzed into nucleosides using 10 μl of nuclease P1 solution (Appendix B-1.1) in water bath at 50°C for 1 h. After incaubation, 10 μl of alkaline phosphatase solution (Appendix B-1.2) was added and incubated at 37 ºC for 1 h. The hydrolyzed DNA was analyzed by the reverse-phase HPLC (high-performance liquid chromatography on Nakarai Cosmosil packed column $5C_{18}$ (150x4.6 mm), eluted using 0.2 M $NH_4H_2PO_4$ : acetonitrile (20:1, v/v) as mobile phase at flow rate 1 ml/min and detected at wave length 270 nm. An equimolar mixture of nucleotides was used as the quantitative standard for analysis of DNA base composition.

**3.2.2.4 Genotypic characterization**

**3.2.2.4.1 16S rRNA gene sequence analysis**

**Step I: Isolation of genomic DNA** Genomic DNA isolation and purification from bacteria in each group was performed according to the method recommened by Saito and Miura (1963). Briefly, the bacterial cell grown in JCM no.377 medium containing 10% NaCl cultured at 37°C, 1 day to obtain the cell growth during logarithmic phase. The cell were harvested by scraping and washed twice with 2 ml of saline-EDTA buffer pH 8.0. (Appendix B-1.3) Cells were lysed using lysozyme in 0.1 M Tris-buffer pH 9.0 (Appendix B-1.4) and 10% sodium dodecyl sulfate (SDS) (Appendix B-1.5). The mixed suspension was heated at 60°C for 10 min. After the cells were lysed, the cell suspention was changed from turbid to

opalescent and became very viscous. Following the addition of 4 ml of phenol-chloroform (1:1 v/v) (Appendix B-1.6), the mixture was mixed for at least 30 sec., then centrifuged at 12,000 g for 10 min. The supernatant was transferred into a small beaker. Then cold 95% ethanol was added into supernatant to precipitate DNA. DNA was pooled using a glass-rod, and rinsed with 70% (v/v) ethanol, 95% (v/v) ethanol and air dried. DNA was dissolved in 0.5 ml of 0.1 x SSC (Appendix B-1.7). RNase A solution (50 µl) was added (Appendix B-1.8) and the DNA solution was incubated at 37 °C about 20 min for the purification. After adding 0.5 ml of 10 x SSC, 1 ml of phenol-chloroform were mixed by vortex for 1 min and centrifuged at 12,000 g for 10 min. The upper layer was transferred to another tube. The DNA was precipitated by adding cold 95% ethanol and DNA was spooled with a grass-rod then rinsed with 70% (v/v) ethanol and 95% (v/v) ethanol. After air dried, DNA was dissolved in 0.5 ml of 0.1 x SSC. The purity and quality of DNA solution were determined from the ratio between adsorbance value at 260 and 280 nm ($A_{260}/A_{280}$) as described by Marmur and Doty (1962).

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

**Step III : Sequencing of 16S rRNA gene** The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp®PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96°C followed by 25 cycles of 10 sec at 96°C (denaturing of DNA), 5 sec at

50°C (primer annealimg), and 4 min at 60°C (polymerization). Sequencing for each sample was carried out in both forward and reverse directions.

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

**3.2.2.4.2 DNA-DNA hybridization** DNA labeling probe with photobiotin was done by mixing 10 µl of purified DNA solution (1 mg/mL) and 15 µl of photobiotin solution (1 mg/mL) in an Eppendorf tube and then the mixture was irradiated with sunlamp for 30 min on ice. After irradiation, the excess photobiotins were removed by the addition of 100 µl of 0.1 M Tris-HCl buffer pH 9.0 and 100 µl of n-butanol. The upper layer was removed. A 100 µL of n-butanol was added and mixed well and removed the upper layer. The biotinylated DNA solution was boiled for 15 min and immediately cooled in ice. The solution was sonicated for 3 min and dissolved with hybridization solution (Appendix B-2.1). DNA-DNA hybridization solution was performed by Ezaki, Hashimoto, and Yabuuchi (1989). 100 µL of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno$^{TM}$Plate: MaxiSorp$^{TM}$surface) and fixed by incubation at 37 °C for 2 h. After incubation, the DNA solution was removed. 200 µL of a prehybridization solution (Appendix B-2.2) was added to microdilution wells. The microdilution plate was incubated at hybridization temperature, 50°C (group X), for 5 h. The prehybridization solution was removed from the wells and replaced with 100 µL of a hybridization mixture

containing biotinylated DNA. After hybridization, the microdilution wells were washed three times with 200 µl of 0.2xSSC buffer. Then 200 µL of solution I (Appendix B-2.3) was added to microdilution wells and incubated at 30 °C for 10 min. Solution I was removed from the wells and replaced with 100 µL of solution II (Appendix B-2.4). The microdilution plate was incubated at 37 °C for 30 min. After incubation, the microdilution plate was washed for three times with 200 µL of PBS (Appendix B-2.5). 100 µL of solution III (Appendix B-2.6) was added, and the plate was incubated at 37 °C for 10 min. The enzyme reaction was stopped with 100 µL of 2M $H_2SO_4$. The absorbance was measured at 450 nm with Microplate Reader (Microplate Manager$^R$ 4.0 Bio-Rad Laboratories, Inc) and calculated for the value of percentage DNA homology (Tanasupawat et al., 2000).

**3.2.2.4.3 Repetitive sequence based polymerase chain reaction (rep-PCR) fingerprinting** Rep-PCR fingerprinting as described by Versalovic *et al*. (1994) was carried out with $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') and the PCR and electrophoresis condition were modified from Chokesajjawatee *et al*. (2008). The reactions were performed in a Thermo cycler (DYAD ALD 1244, MJ research Inc., Massachussets). Amplicons were separated on 1% LE Seakem® agarose (BME, Rockland, ME, U.S.A) in 0.5X TBE at 120 V for 2 h and 40 min prior to stain in 0.5X TBE containing 5 µg/ml ethidium bromide (Sigma, USA) for 5 min. and destained in tap water for 20 min, with shaking. The gel image was captured by using an image scanner Typhoon 9410 (Amersham Biosciences). The resulting fingerprints were analysed by using a pattern analysis software package, Gel ComparII version 4.5 (Applied Maths BVBA, Belgium). The similarities of the fingerprint patterns were calculated using Dice coefficient and the unweighted-pair group method with average linkages (UPGMA-unweighted pair group method arithmetic averages). The different dendrograms were visually interpreted to set the delineation level separately for each species.

### 3.2.3 Screening of ALP-producing bacteria

**3.2.3.1. Primary screening** To select ALP-producing isolate, All 76 isolated moderately halophilic bacteria from 3.2.1 were screened by growing on Heart Infusion agar (Difco) containing 0.01 % phenolphthalein bisphosphate tetrasodium salt (Sigma) supplemented with 10% NaCl (w/v) as the method described by Barber & Kuper (1951). After incubation at 37°C for 1-2 days, all pink colonies were selected as potential ALP-producing isolates. These ALP-producing isolates were futher studied on taxonomy identification and ALP-producing secondary screening.

**3.2.3.2 Secondary screening** To confirm their quantitatively ALP activity, a loopfull of the selected ALP-producing strain on primary screening was inoculated into 5 ml of JCM No.377 broth and incubated in a rotary shaker at 37$^{o}$C (200 rpm) for 24 h for the seed culture. The seed cultured broth (0.5 ml) was inoculated (1%) into 50 ml of JCM No.377 broth medium in 250 ml Erlenmeyer flask (duplicate) and incubated as above mentioned conditions. Cell-free supernatant was collected by centrifugation at 4$^{o}$C, 10,000 rpm (13,300 g) for 15 min and used for crude extracellular ALP assay. The precipitated cell was suspended in 50 mM Tris-HCl pH 8.0 and then the cell suspension was sonicated on ice by sonicator 20 second 5 times. Cell suspension was centrifuged at 35,000 rpm for 30 min. After discarded debris cell, this supernatant was used for crude intracellular ALP assay. The ALP-producing isolate exhibited high ALP activity, was selected to further study on ALP production, purification and characterization.

**3.2.3.3 Alkaline phosphatase (ALP) activity assay** ALP activity was assayed according to the method described by Helianti *et al.,* (2007). Reaction mixture composing of 1.0 ml of 10 mM *p*-nitrophenylphosphate (*p*-NPP) (Sigma) in 0.2 M Tris-HCl buffer pH 10.0, and 0.1 ml of the crude enzyme was incubated at 37$^{o}$C for 15 min. The reaction was stopped with 1 ml of 1M NaOH and its absorbance was measured at 405 nm. Reaction of blank was done in the same manner except the enzyme was added after an addition of 1 M NaOH solution. An absorbance at 405 nm plotted against concentration of standard *p*-nitrophenol solution was used as standard curve. One unit (U) of alkaline phosphatase (ALP) is defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute under the defined assay

condition. Enzyme assay was performed in triplicate and the results were represented in average values with standard deviation.

**3.2.3.4 Preparation of standard curve of *p*-nitrophenol** Standard solution of 0, 5, 10, 20, 30, 40, 50 and 100 µmol/ml were prepared from *p*-nitrophenol and were done in the same manner of ALP assay reaction

**3.2.3.5 Protein determination** Protein concentration was estimated by Lowry method (Lowry *et al*., 1951) using BSA as standard (Appendix C-2).

### 3.2.4 Determination of the growth and ALP production

The selected strain was maintained in 20% (v/v) glycerol and kept at -80$^{o}$C, was cultured on JCM no.377 agar plate and incubated at 37°C for 1-2 days. For enzyme production, the inoculum was prepared by inoculating a loop full of fresh pure culture from JCM no. 377 agar plate into 5 ml of seed culture broth in 15 ml test tube and incubating in rotary shaker at 37°C, 200 rpm for 24 h to obtain an inoculum culture with an absorbance at 620 nm of 5. (≈$10^{8}$ CFU/ml)

To determine growth and ALP production kinetics, the selected strain was freshly cultured and inoculated on JCM no. 377 medium with 1% initial inoculum ($A_{620}$≈ 5) in 250 ml Erlenmeyer flasks containing 50 ml of medium and cultured in rotary shaker at 37°C, 200 rpm for 72 h. Samples taken at 2 h interval were assayed in cell for intracellular ALP activity and in cell-free supernatant for extracellular ALP activity. The growth was monitored by measuring an absorbance at 620 nm.

### 3.2.5 Characterization of crude intracellular ALP

A 10 ml of the inoculum was inoculated (5%) in 200 ml of JCM no. 377 broth in 1 L Erlenmyer flask and incubated in rotary shaker at 37°C, 200 rpm for optimum ALP-producing time. The optimal condition of prior experiment was used as the basis in the latter experiment to optimize the conditions. Cells at optimum cultivation were collected by centrifugation at 4$^{o}$C, 10,000 rpm (13,300 g) for 15 min. The precipitated cells were suspended in 50 mM Tris-HCl pH 8.0 and then sonicated on ice by sonicator 20 second paused 40 second (5 times). After that cell suspension was centrifuged at 35,000 rpm for 30 min 4$^{o}$C. After dicarded debris cell, this supernatant was used as crude intracellular ALP for characterization.

**3.2.5.1 Effect of temperature on ALP activity and stability.**

ALP activity of crude intracellular enzyme was assayed at different temperatures at 37, 45, 50, 55, 60, 65, 70 and 75°C in 0.2 M Tris HCl buffer pH 10.0 for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed. To study the effect of temperature on enzyme stability, the enzyme was incubated at different above mentioned temperatures ranging from 37-70°C for 1 h in water bath, after that the enzyme was immediately cooled in ice, and the remaining activity was assayed in 0.2 M Tris HCl buffer pH 10.0 at optimum temperature ($60^oC$) for 15 min and was expressed as a percentage of the activity before incubation.

**3.2.5.2 Effect of pH on ALP activity and stability**.

ALP activity of crude intracellular was assayed over the pH range of 7.0-12.0 using 0.2 M Tris HCl buffer at pH 7-11 and 0.1 M glycine/NaOH buffer at pH 11-12 at $60^oC$ for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed. The effect of pH on stability was perfomed by incubating enzyme in buffer with pH values ranging from 7-11 at 25°C for 1 h. The remaining activity was assayed in 0.2 M Tris HCl buffer at optimum pH (pH 10.0) $60^oC$ for 15 min and was expressed as a percentage of the activity before incubation.

**3.2.5.3 Effect of NaCl concentration on ALP activity.**

To study the effect of NaCl concentration, ALP activity was assayed by 0.2 M Tris HCl buffer in the presence of different final concentrations of NaCl (0, 1, 1.5, 2, 2.5, 2.8, 3.1, and 3.5 M) at $60^oC$, pH 10.0 for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed**.**

**3.2.5.4 Effect of inhibitor on ALP activity.**

The effect of various inhibitors on ALP activity was determined by incubating the crude intracelluar enzyme with an equal volume of enzyme inhibitor solution to obtain the final concentration designated; 10 μM chymostatin, 1 μM E-64, 1 mM Disodiummethylenediaminetetraacetate (EDTA), 1 mM Ethylene glycol tetraacetic acid (EGTA), 10 μM leupeptin, 1 mM phenylmethane sulfonyl fluoride (PMSF), 2 μM pepstatin A, 0.1mg/ml soybean trypsin inhibitor (SBTI), 10 μM Tosyl-L-lysine chloromethyl ketone (TLCK) and 10 μM Tosyl-L-phenylalanine

chloromethyl ketone (TPCK). The mixture was allowed to stand at room temperature (25$^{o}$C) for 2 h. Thereafter, the remaining activity was measured at 60$^{o}$C, pH 10.0 for 15 min and calculated percent of the inhibition. Control was done in the same manner, but deionized water was used instead of inhibitors.

**3.2.5.5 Effect of metal ion on ALP activity**

The effect of metal ion was investigated by using $CuSO_4$, $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$. The crude intracellular enzyme was assayed activity in the presence of cation such as $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ with a final concentration of 5 mM in 0.2 M Tris HCl buffer at optimum conditions (pH 10.0 at 60°C for 15 min). The relative activity was expressed as a percentage of the highest ALP activity assayed.

### 3.2.6 Purification of ALP from TPS4-2$^{T}$

#### 3.2.6.1 Preparation of crude intarcellular enzyme

The inoculum was inoculated (5%) in 200 ml of JCM no. 377 broth in 1 L Erlenmyer flask and incubated in rotary shaker at 37°C, 200 rpm. Cultured broth after 36 h cultivation was centrifuged at 13,300 g for 15 min at 4°C. The precipitated cells were collected and suspened in 50 mM Tris-HCl buffer pH 8.0 (1 g wet cell: 5 ml) and then sonicated on ice by sonicator 20 second paused 40 second (5 times). After cells were lysed, cell suspension was centrifuged at 35,000 rpm for 30 min 4$^{o}$C. The supernatant was used as crude intracellular ALP for purification and characterization. The supernatant containing the intracellular enzyme was concentrated nearly 4-times using Amicon Ultra-10K device concentration with membrane having molecular weight cut-off 10 kDa and subsequently was diafiltered against two volumes of 50 mM Tris-HCl, pH 8.0.

#### 3.2.6.2. Purification procedure

All following purification steps were carried out at 4°C. HiTrap DEAE FF column (Ion exchange chromatography) was performed on Fast Protein Liquid Chromatography (FPLC) of the ÄKTA explorer purification system (GE Healthcare Bio-science, Uppsala, Sweden) with UV detector. The concentrated crude intracellular enzyme was filtrated through a 0.2 micron filter and was injected into HiTrap DEAE FF column (0.7 x 2.5 cm) (GE Healthcare), which was equilibrated

with approximately five bed volumes of 50 mM Tris-HCl, pH 8.0. The sample was eluted with stepwise salt gradient of 0-1 M NaCl in 50 mM Tris-HCl, pH 8.0 at a flow rate of 2.5 ml/min. Eluted proteins of each fraction were monitored by absorbance at 280 nm ($A_{280}$) and collected in 2.5 ml aliquots for further analysis. Each fraction was assayed for ALP activity and the active fractions were pooled and further purified by Superose 12 10/300 size exclusion column.

Pooled fractions with ALP activity from the HiTrap DEAE FF column were concentrated by Amicon Ultra-10K devices with 10,000 MWCO prior to size exclusion chromatography using Superose 12 10/300 GL column (1.0 x 30 cm) (GE Healthcare). The column was previously equilibrated with two bed volumes of 50 mM Tris-HCl containing 0.15 M NaCl. The sample was loaded onto the column and eluted with isocratically of 50 mM Tris-HCl containing 0.15 M NaCl at a flow rate of 0.1 ml/min. Eluted proteins of each fraction were monitored by absorbance at 280 nm ($A_{280}$) and collected in 0.5 ml aliquots for ALP activity analysis. The fractions with ALP activity were pooled and used for further characterization. The purity of the purified enzyme was analyzed by polyacrylamide gel electrophoresis.

**3.2.6.3 Polyacrylamide gel electrophoresis (PAGE)**

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenol blue). The samples (15 μl) were loaded on the gel made of 4% stacking and 12.5% separating gels (Appendix C-3.7, 3.8) and subjected to electrophoresis at a constant current of 20 mA per gel using an ATTO AE-6530 Dual mini-slab system. After electrophoresis, the gels were stained with silver staining.

Native-PAGE was performed using 12.5% separating gels in a similar manner with SDS-PAGE, except that the addition of SDS and reducing agent was omitted (Appendix C-3.1-3.8).

**3.2.6.4 Activity staining**

The enzyme sample was electrophoresed in native-PAGE. After electrophoresis, the gel was immersed in 0.2 Tris HCl buffer pH 10.0 containing 1mM *p*-nitrophenylphosphate as substrate for 10 min at room temperature with gentle shaking to allow the substrate to penetrate into the gels. Due to ALP catalytic reaction

with *p*-nitrophenylphosphate, *p*-nitrophenol and inorganic phosphate were released as product. The yellow color band of *p*-nitrophenol was developed within 5-10 min. and the released inorganic phosphate was stained by the malachite green according to the method as described by Queiroz-Claret and Meunier, 1993. In briefly, 1.5 ml of the gel coloration reagent (Appendix C-3.10) was added into 10 ml buffer which the gel immersed after the yellow band occurred. The gel was fixed and conserved in 25% ethanol (v/v) containing 2% glycerol (w/v).

### 3.2.7 Characterization of the partially purified ALP from strain TPS4-2$^T$

#### 3.2.7.1 Determination of molecular weight

The molecular weight of the partially purified ALP was determined using size exclusion chromatography on Superose 12 10/300 GL column (1.0 x 30cm) (GE Healthcare), at 4°C using FPLC on the ÄKTA explorer purification system (GE Healthcare). The protein solution was passed through the column equilibrated with approximately two bed volumes of 50 mM Tris-HCl, pH 8.0 containing 0.15M NaCl and then eluted with isocratically of the same buffer at a flow rate of 0.1 ml/min.

The ALP from TPS4-2$^T$ separated on size exclusion chromatography was estimated for its molecular weight by plotting relative partition coefficient ($K_{av}$) against the logarithm of molecular weight of the protein standards. The elution volume ($V_e$) was measured for each protein standard and the purified ezyme. Void volume ($V_o$) was estimated by the elution volume of blue dextran ($M_r$ 2,000,000). The standards used included thyroglobulin (bovine) ($M_r$ 670,000), γ-globulin (bovine) ($M_r$ 158,000), ovalbumin (chicken) ($M_r$ 44,000), myoglobin (horse) ($M_r$ 17,000) and vitamin $B_{12}$ ($M_r$ 1,350).

#### 3.2.7.2 Optimum temperature and thermal stability

ALP activity of the partial purified enzyme was assayed at different temperatures at of 37, 45, 50, 55, 60, 65, 70 and 75°C in 0.2 M Tris HCl buffer pH 10.0 for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed. For thermal stability, the partially purified enzyme was incubated at various above mentioned temperatures ranging from 37-70°C for 1 h after that immediately cooled in an ice bath, and the remaining activity was assayed in

0.2 M Tris HCl buffer at 60°C, pH 10.0 for 15 min and the result was expressed as a percentage of the remaining activity after incubation.

**3.2.7.3 Optimum pH on activity and stability**

ALP activity of the partial purified enzyme was assayed over the pH range of 7.0-12.0 using 0.2 M Tris HCl buffer at pH 7.0-11.0 and 0.1 M glycine/NaOH buffer at pH 11.0-12.0 at 60°C for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed. The pH stability experiment was conducted by incubating the in buffer with pH values ranging from 7.0-11.0 at 25°C for 1 h and measuring residual activity under the optimum assay conditions (60°C, pH 10.0 for 15 min).

**3.2.7.4 Effect of salt concentration on partially purified ALP activity**

To study the effect of NaCl concentration, the partial purified enzyme was diafiltered against ten volumes of 50 mM Tris-HCl buffer pH 8.0 at 4 °C. Then, the ALP activity was assayed in 0.2 M Tris HCl buffer in the presence of different final concentrations of NaCl (0, 0.05, 0.1, 0.2, 0.3, 0.5, 1, 2, 3, and 4 M) at 60°C, pH 10.0 for 15 min. The relative activity was expressed as a percentage of the highest ALP activity assayed.

**3.2.7.5 Effect of inhibitors on the partially purified ALP activity**

The effect of various inhibitors on the partial purified ALP activity was determined by incubating the partial purified enzyme with an equal volume of enzyme inhibitor solution to obtain the final concentration designated (10 μM chymostatin, 1 μM E-64, 1 mM EDTA, 1 mM EGTA, 10 μM leupeptin, 1 mM phenylmethane sulfonyl fluoride (PMSF), 2 μM pepstatin A, 0.1mg/ml soybean trypsin inhibitor (SBTI), 10 μM TLCK and 10 μM TPCK). The mixture was allowed to stand at room temperature for 1 h. Thereafter, the remaining activity was measured at 60°C, pH 10.0 for 15 min and calculated percent of the inhibition. The control was performed in the same manner except that deionized water was used instead of inhibitors.

**3.2.7.6 Effect of metal ion on the partial purified ALP activity**

The effect of metal ion was investigated by using $CuSO_4$, $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$. The partial purfied enzyme was assayed activity in the

presence of cation such as $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ with a final concentration of 1 mM in 0.2 M Tris HCl buffer under optimum conditions (pH 10.0 at 60°C for 15 min). The relative activity was expressed as a percentage of the highest ALP activity assayed**.**

**3.2.7.7 Kinetic analysis on the partially purified ALP**

The ALP activity was assayed with different final concentration of *p*-nitrophenylphosphate ranging from 0.1-4 mM under optimum conditions (60°C, pH 10.0 for 15 min). The values of Michalis-Menten constant ($K_m$), maximal velocity ($V_{max}$) were graphically determined and calculated according to Hanes-Woolf plots using the initial velocity of the reaction. The values of catalysis constant ($K_{cat}$) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, that [E] is the active enzyme concentration.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Histamine producing halophillic lactic acid bacteria

### 4.1.1 Halophillic lactic acid bacteria isolates and sources of samples

Forty-one of moderated halophillic bacteria were isolated from 7 types of traditional thai fermented foods (fermented fish: nam-pla, nam-budu, tai-pla, pla-chom, fermented shrimp: ka-pi, kung-chom: fermented soy bean: soy sauce) available in various provinces of Thailand as shown in Table 4.1

**Table 4.1** Location, isolate number, and number of isolate

| Source | Province | Isolate code | No. of isolates |
|---|---|---|---|
| Fish sauce (*nam-pla*) | Samutprakarn | IB20-7, IS40-7, IS60-1, KS11-1,KS18-2,KS35-4, KS87-1, KS54-9, KS87-14 | 9 |
| Kung-chom | Bangkok | PH136 | 1 |
| Nam Budu | Songkhla | PH88, A80 | 2 |
| Pla-chom | Surin | PH27, PH65, PH155 | 3 |
| Shrimp paste (ka-pi) | Phangnga | SP34-3, SP36-1, SP37-2, SP39-3, SP40-1, SP43-1 | 6 |
| Shrimp paste | Songkhla | KM1-1, KM1-2, KM1-3, KM1-4, KM1-5, KM1-6, KM 2-2, KM3-1, KM3-2, KM3-3, KM3-4, KM4-1, KM6-1, KM6-2, KM6-3, KM6-4 | 16 |
| Soy sauce mash | Samutsakorn | SO45-46, SO25-6, SO45-1 | 3 |
| Tai-pla | Narathivas | PH120 | 1 |
| Total | | | 41 |

### 4.1.2 Morphological, physiological and biochemical characteristics

These isolated moderated halophillic bacteria were Gram-positive, facultatively anaerobic, non-spore forming cocci, non-motile, arranged in pairs or tetrads. Colonies on MRS agar plate supplemented with 10% (w/v) NaCl were white, circular low convex with entire margin and nonpigmented and approximately 0.6-1

mm in diameter. All strains produced L-lactic acid homofermentatively from glucose. Catalase, oxidase, nitrate reduction and gelatin hydrolysis were all negative. Optimum pH range and temperature were 7-9 and 30°C. Cells were able to grow in aerobic and anerobic jar. Most of the isolates were able to grow at 40°C (but not at 45°C), in the presence of 5-25% (w/v) NaCl at pH ranging from 6-9 and produced acid from glucose, D-galactose, D-maltose, D-mannose and D-ribose. The more detail physiological and biochemical characteristics were described as below and shown in Table 4.3.

### 4.1.3 Identification of 16s rRNA PCR products with restriction endonucleases digestion and 16s rRNA gene sequencing and phylogenic analysis

These strains were divided into two groups, groups A and B on the basis of 16S rRNA gene restriction patterns obtained in *Alu*I and *Mbo*I digestions (Figure 4.1).

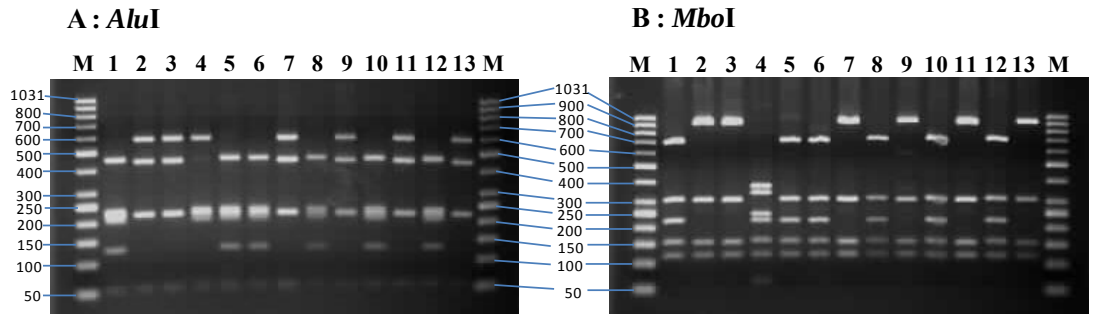


**Figure 4.1** Restriction pattern of 16S rRNA gene PCR product of isolates assigned to the genus *Tetragenococcus*. The restriction analysis was made for isolates assigned to the genus *Tetragenococcus* with restriction endonucleases *Alu*I (A) and *Mbo*I (B). For estimation of digestion fragments, 50bp DNA markers were used in 2.5% the agarose gel electrophoresis. Lane 1: *T. halophilus* ATCC 33315$^T$, lane 2: *T. muriaticus* JCM 10006$^T$, lane 3: *T. muriaticus* JCM 10007, lane 4: *T. koreensis* 3924$^T$, lane 5, 6, 8, 10, 12 : representative isolates of group A (SP37-2, SP36-1, KS35-4, KS87-1, PH155), lane 7, 9, 11, 13: representative isolates of group B (A80, KS87-14, KS54-9, KM1-5), M : 50 bp DNA marker.

Group A was composed of 22 isolates (Table 4.2). The digestion patterns of SP37-2, SP36-1, KS35-4, KS87-1 and PH155 were representative isolates of group A as shown in Figure 4.1. *Alu*I and *Mbo*I digestions of these isolates showed the same restriction patterns as that of *T. halophilus* ATCC 33315$^T$. *Alu*I digested products contained 5 bands of 130, 220, 230, 240 and 450-bp fragments while *Mbo*I digested products showed 5 bands of 120, 155, 225, 310 and 700-bp fragments, respectively. (Figure 4.1). In a phylogenic tree based on 16S rRNA gene sequences, SP37-2 and KS87-1 isolates were located within the cluster of *T. halophilus* (Figure 4.2) and had 99.4 to 99.5% pair-wise 16S rRNA gene sequence similar to that of type strain *T. halophilus*.

**Table 4.2 Identification based on 16S rRNA gene restriction analysis**

| Isolate no. (Species) | Restriction pattern by digestions with | |
|---|---|---|
| | ***ALu*I** | ***Mbo*I** |
| **Group A (*T. halophilus*)** | | |
| IB20-7, IS40-7, IS60-1, KS11-1, KS18-2, KS35-4, KS87-1, PH27, PH65, PH155, PH88,PH120, PH136, SO45-46, SO25-6, SO45-1, SP34-3, SP36-1, SP37-2, SP39-3, SP40-1, SP43-1 | 130, 220, 230, 240 and 450-bp fragments | 120, 155, 225, 310 and 700-bp fragments |
| **Group B (*T. muriaticus*)** | | |
| KM1-1, KM1-2, KM1-3, KM1-4, KM1-5, KM1-6, KM 2-2, KM3-1, KM3-2, KM3-3, KM3-4, KM4-1, KM6-1, KM6-2, KM6-3, KM6-4 A 80, KS54-9, KS87-14 | 220, 450 and 600-bp fragments | 120, 155, 310 and 950-bp fragments |

Biochemical properties of the strains in group A were studied in more detail. The isolates produced acid from D-cellobiose, glucose, D-galactose, D-mannose, D-ribose and salicin. Most strains of this group produced acid from amygdalin, L-arabinose, glycerol, maltose, D-manitol, D-melizitose, α-methyl-D-glucoside, sucrose, trehalose and D-xylose but did not produce acid from lactose, D-melibiose,

raffinose, rhamnose, D-sorbitol and L-sorbose, and showed positive reaction to hydrolysis of L-arginine. Most of them showed negative reaction to hydrolysis of casein and grew on MRS with 50-200 (g/l) NaCl at pH range 6.0-9.0 and at 30-40$^{o}$C (Table 4.3). These biochemical characteristics were similar to *T. halophilus* ATCC 33315$^{T}$. Therefore, these isolates were identified as *T. halophilus*. (Satomi *et al*., 1997)

Group B comprised of 19 isolates (Table 4.2). The digestion patterns of A80, KS87-14, KS54-9 and KM1-5 were representative isolates of group B as shown in Figure 4.1.These isolates gave the same restriction patterns as *T. muriaticus* JCM 10006$^{T}$, when digested with restriction endonucleases *Alu*I and *Mbo*I, which showed 220 450 and 600-bp fragments and 120, 155, 310 and 950-bp fragments, respectively (Figure 4.1). In a phylogenic tree based on 16S rRNA gene sequences, KS87-14 and KM1-5 isolates of group B were located within the cluster of *T. muriaticus* (Figure. 4.2) and had 99.0-99.6% pair-wise 16S rRNA gene sequence similar to that of type strain *T. muriaticus*. The isolates produced acid from glucose, D-manitol and D-mannose but did not produce acid from lactose and amygdalin. Most of this group produced acid from D-cellobiose, D-galactose, maltose, D-melizitose, D-ribose and sucrose but did not produce acid from L-arabinose, glycerol, D-melibiose, α-methyl-D-glucoside, raffinose, rhamnose, salicin, D-sorbitol, L-sorbose, D-trehalose and D-Xylose. Most of isolates in group B showed positive reaction to hydrolysis of casein but did not hydrolyze L-arginine and grew on MRS with 50-250 g /l NaCl at pH range 6.0-9.0 and at 30-40$^{o}$C (Table 4.3). Therefore, these isolates were identified as *T. muriaticus*. (Satomi *et al*., 1997)

In this study, phenotypic characteristics of the group A isolates *T. halophilus* that differentiated from group B isolates, *T. muriaticus* were the hydrolysis of L-arginine, hydrolysis of casein and acid production from amygdalin, glycerol and α-methyl-D-glucoside. However, both species showed heterogeneous sugar fermentation (Hanagata *et al*., 2003) which made it difficult to identify the two species. Therefore, the molecular method based on 16S rRNA gene restriction patterns would be more useful to clearly separate and to classify all new isolated to these type strains as shown in Figure 4.2.

**Table 4.3** Characteristics of isolates and related *Tetragenococcus* species.

| Characteristics | Group A (22) [a] | *T. halophilus* 33315$^T$ | Group B (19) | *T. muriaticus* 10006$^T$ | *T. koreensis* 3924$^T$ |
|---|---|---|---|---|---|
| Cell shape | | | Cocci in Tetrads | | |
| L-arginine hydrolysis | + | + | -(w1) | - | + |
| Casein hydrolysis | -(+2) | - | +(-5) | + | + |
| Growth in 0% NaCl | -(+3) | + | -(+3) | - | + |
| 20% NaCl | + | + | +(w1) | + | w |
| 25% NaCl | +(-5) | + | +(-6,w1) | + | - |
| Growth at pH 5 | -(+3) | - | - | - | + |
| 6 | + | + | + | w | + |
| Growth at 45°C | -(+4,w4) | - | -(w4) | - | w |
| Acid from: | | | | | |
| Amygdalin | +(-1,w3) | + | - | - | + |
| L-Arabinose | +(-5) | + | -(+3,w2) | - | - |
| D-Cellobiose | + | + | +(-3,w6) | + | + |
| D-Galactose | + | + | +(-2,w5) | + | + |
| Glycerol | +(-1,w8) | + | -(w2) | - | w |
| Lactose | -(+1,w7) | - | - | - | + |
| D-Maltose | +(-3w) | + | +(-1,w3) | + | + |
| D-Mannitol | +(-10) | - | + | + | + |
| D-Melibiose | -(+1,w13) | w | -(+5,w4) | - | - |
| D-Melezitose | +(8,w3) | + | +(-7) | - | + |
| α-Methyl-D-glucoside | +(-4,w12) | + | -(+2,w2) | w | w |
| Raffinose | -(+2,w7) | w | -(+1,w4) | - | - |
| Rhamnose | -(+3,w7) | w | -(+1,w5) | w | - |
| D-Ribose | + | + | +(-6,w7) | + | + |
| Salicin | + | + | -(-3,w11) | + | + |
| D-Sorbitol | -(+4,w4) | - | -(+1,w4) | - | - |
| L-Sorbose | -(+2,w2) | + | -(w2) | - | - |
| Sucrose | +(w1) | w | +(4,w4) | - | + |
| D-Trehalose | +(w1) | + | -(+2,w2) | - | + |
| D-Xylose | +(-8,w1) | w | -(w5) | + | + |

[a] Numbers in parentheses indicate the number of strains and the reaction of strain: +, positive reaction; w, weak reaction; -, negative reaction.

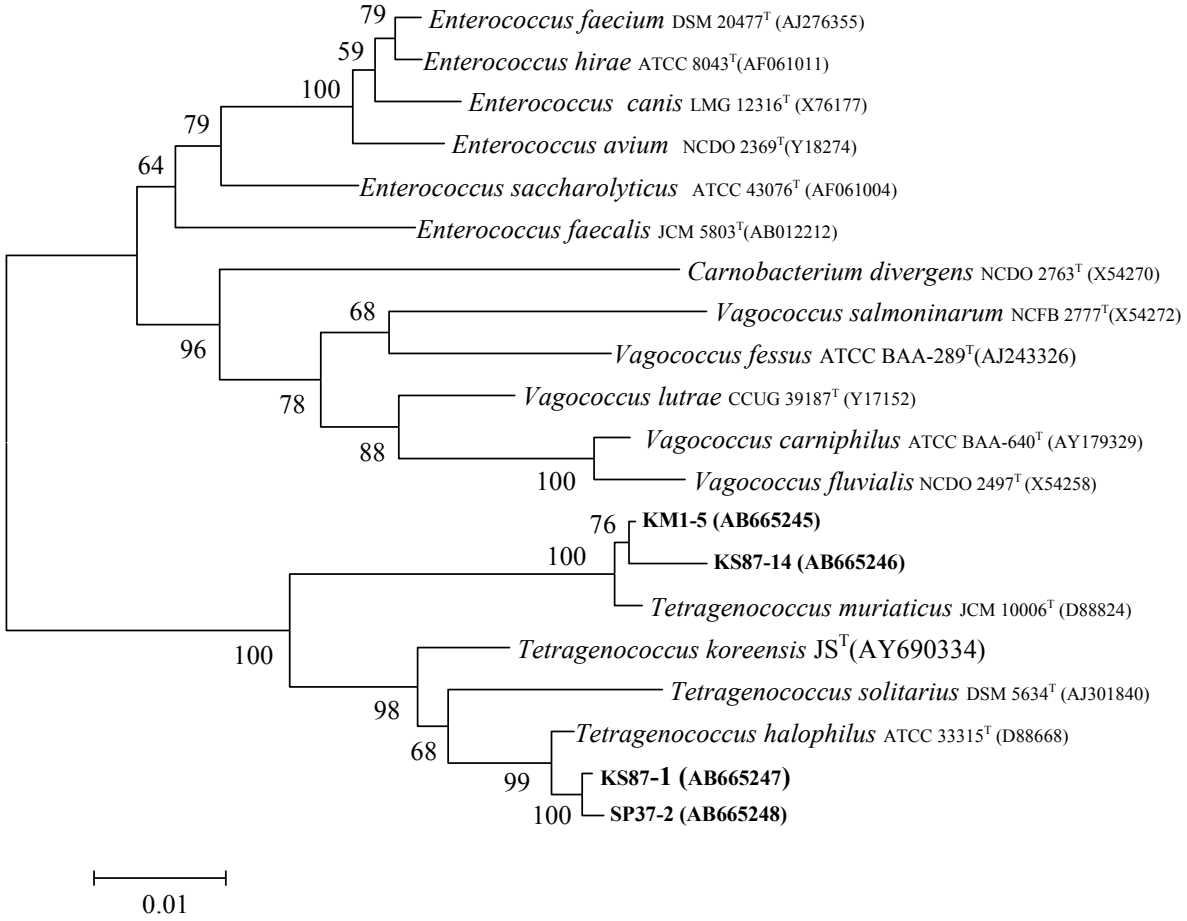


**Figure 4.2** Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between representative isolates in each group A (KS87-1, SP37-2), group B (KM1-5, KS87-14) and other related taxa. Number at nodes indicate bootstrap percentage above 50% derived from 1000 replications. Bar, 0.01 substitution per 100 nucleotide position.

### 4.1.4 Histamine production

All *Tetragenococcus* strains in group A and group B (with the exception of KS87-14) produced negligible amounts of histamine in the culture broth. Only the isolated KS87-14 was able to produce the highest level of histamine at 626 mg/l when compared with *T. muriaticus* JCM 10006$^T$ (78 mg/l) and *T. muriaticus* JCM 10007 (45 mg/l) that were used as reference strains and cultured under same condition for identification and histamine formation, respectively. *T. muriaticus* strains are widely distributed in fermented fish and reported to be the histamine producers (Satomi *et al*., 1997; Kimura *et al*., 2001; Kobayashi *et al*., 2000, Thongsanit *et al*., 2002). Histamine production in *T. muriaticus* was reported as the response to acidity stress. At pH 6.0, histamine production could be observed despite very weak growth (Kimura *et al*., 2001). Due to higher histamine producing ability of KS87-14, there was possibility that once the food was contaminated during manufacturing, it would grow and produce hazardous levels of histamine.

### 4.1.5 Histidine decarboxylase (*hdc*) gene

To detect the presence of histidine decarboxylase (*hdc*) gene, a PCR reaction was performed using 3 sets of primers available for *hdc* gene detection in Gram-positive bacteria. Corresponding with the histamine production, the positive amplification of *hdc* gene was observed in KS87-14 by using primers JV16HC and JV17HC giving an amplicon size of 367 bp (Figure 4.3) which was previously used to detect the *hdc*A, a structural gene of pyruvoyl dependent histidine decarboxylase of histamine-producing Gram-positive bacteria, in particular *Clostridium perfringens* (Recsei *et al*., 1983). Compared with previously published nucleotide sequences for the *hdc*A (Figure 4.4), the amplified fragment sequence of KS87-14 obtained showed conserved regions that appeared to be completely similar to the recently deposited *hdc*A gene sequences of *Staphylococcus epidermidis* TYH1 isolated from the fish-miso (Yokoi *et al*., 2011) and *Staphylococcus capitis* isolated from Spanish dry-cured ham (De Las Rivas *et al*., 2008). In contrast, the sequence was only 83% identical with the partially *hdc*A gene of *T. muriaticus* JCM 10006$^T$. The genes related to histamine production were constructed with four genes, *hdc*P, *hdc*A, *hdc*B, and *hdc*RS, and highly conserved among several lactic acid bacteria. Moreover, these

genes could be transferred beyond barrier of gener (Lucas *et al*., 2005). These results suggested the possibility of gene transfer among lactic acid bacteria and *Staphylococci* which were frequently reported as histamine formers in salted fish, fermented meat products and soy bean products.

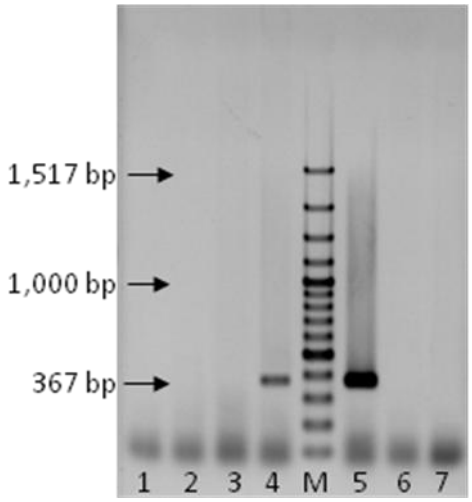


**Figure 4.3** PCR detection of the histidine decarboxylase gene (*hdc*) by using primer pair JV16HC and JV17HC giving an amplicon size of 367 bp. The reaction was performed by using distilled water as negative control (lane 1), and using chromosomal DNA from isolate SP 37-2 (lane 2), KS87-1 (lane 3), KS87-14 (lane 4), *T. muriaticus* JCM10006$^T$ (lane 5), *T. halophilus* ATCC 33315$^T$ (lane 6), KM1-5 (lane 7), and 100 bp ladder marker (lane M).

```
T.muriaticus JCM 10006T   --------------------------------------------------TAGATGGTATT 11
O.oeni                    GGACTTAAAGTAGATGCTGGTGTCATTGATAAAACCGATGATATGATCCTTGATGGTATT 180
L.sakei                   GGAATTAAAGTAGATGCTGGTGTCGTTGATAAAACCGATGATATGATCCTTGATGGTATT 150
L.buchneri                --------AGTCGATGCAGGTGTTGTTGATAAAACTGATGATATGATCCTTGATGGTATT 52
Lactobacillus30A          GGTTTGAAAGTTGATGCTGGTGTTCGTGACAAGTCTGATGACGATGTTTTAGATGGTATT 180
Strep.thermophilus        GGTTTAAAGGTCGATGCAGGTGTTCGCGATAAAACTGATGATACGGTTCTTGATGGGATC 177
KS87-14                   --------------------------------------------------ATGGTATT 1
Stap.capitis              GGATTAAAAGTAGATGCAGGTACACGTAAAAAAACGGATGATAATGTACTAGATAATATT 180
Stap.epidermis            GGATTAAAAGTAGATGCGGGTACTCGAAAAAAAACTGATGACAATGTATTAGATAATATC 180

T.muriaticus JCM 10006T   GTTTCTTATGACCGTGCGGAAACAAAGAATGCCTATATTGGACAAATCAACATGACTACT 71
O.oeni                    GTTTCGTATGACCGTGCGGAAACAAAGAATGCCTATATTGGACAAATCAACATGACTACT 240
L.sakei                   GTTTCGTATGACCGTGCGGAAACAAAGAATGCCTATATTGGACAAATCAACATGACTACT 210
L.buchneri                GTTTCGTATGACCGTGCGGAAACTAAAAATGCCTATATTGGACAAATTAACATGACTACC 112
Lactobacillus30A          GTTTCTTATGACCGTGCAGAAACAAAGAATGCTTACATTGGTCAAATTAATATGACCACT 240
Strep.thermophilus        GTTTCTTACGACCGGGCTGAAGCAAAAAATGCCTATATCGGTCAAATCAATATGGAAACT 237
KS87-14                   GTTTCTTATGATCGTGCTGAAGCTAAAAATGCATACATGGGACAAATTAACATGATAACT 61
Stap.capitis              GTTTCTTACGATCGTGCTGAAGCTAAAAATGCATACATGGGACAAATTAACATGATAACT 240
Stap.epidermis            GTATCTTATGATCGTGCTGAAGCAAAAAATGCATATATGGGTCAAATTAATATGATAACT 240
                          ** ** ** ** ** ** *** * ** ***** ** ** ** ***** ** ***   **

T.muriaticus JCM 10006T   GCATCAAGTTTCTCTGGTGTTGGTGGAACTGTTCTTGGTTATGACATCCTTCGTAACCCT 131
O.oeni                    GCATCAAGTTTCTCTGGTGTTGGTGGAACTGTTCTTGGTTATGACATCCTTCGTAACCCT 300
L.sakei                   GCATCAAGTTTCTCTGGTGTTGGTGGAACTGTTCTTGGTTATGACATCCTTCGTAACCCT 270
L.buchneri                GCATCAAGTTTCTCTGGTGTAGGAGGAACAGTTCTTGGATATGACATTCTTCGTAACCCA 172
Lactobacillus30A          GCTTCATCATTTACTGGTGTACAAGGTCGTGTGATTGGTTATGATATTTTGCGGAGCCCA 300
Strep.thermophilus        GCCTCTTCTTTCACTGGTCTTCAAGGGGTGACGTTAGGTTATGATATCCTCCGTAATCCT 297
KS87-14                   GCATCATCTTTCACTGGCTTGCAGGGTTCTACATTGGGATATGATATTTTAAGAAATCCG 121
Stap.capitis              GCATCATCTTTCACTGGCTTGCAGGGTTCTACATTGGGATATGATATTTTAAGAAATCCG 300
Stap.epidermis            GCTTCATCTTTTACTGGCTTACAAGGCTCTACGTTGGGATATGATATTTTAAGAAATCCT 300
                          ** **    **  **** *    **        * ** ***** **  *  * *  **

T.muriaticus JCM 10006T   GAAGTTGACAAAGCTAAGCCATTATTTACTGAAAAACAATGGGACGGTAGTGAACTTCCA 191
O.oeni                    GAAGTTGACAAAGCTAAGCCATTATTTACTGAAAAACAATGGGACGGTAGTGAACTTCCA 360
L.sakei                   GAAGTTGACAAAGCTAAGCCATTATTTACTGAAAAACAATGGGACGGTAGTGAACTTCCA 330
L.buchneri                GAAGTTGATAAGGCTAAGCCTTTATTTACTGAAAAGCAATGGGACGGTAGCGAGTTACCA 232
Lactobacillus30A          GAAGTTGACAAGGCAAAGCCTTTGTTTACTGAAACACAATGGGATGGCAGTGAATTACCA 360
Strep.thermophilus        GCCGTTGATGAAGAGAAACCTTTGTTCGTGGAACCCCAATGGGATGGATCAGAATTACCG 357
KS87-14                   GAGGTAGATGAAGCTAGTTCACTGTTCTCTGTAAAACAATGGGATGGCAGTGAATTACCA 181
Stap.capitis              GAGGTAGATGAAGCTAGTTCATTGTTCTCTGTAAAACAATGGGATGGCAGTGAATTACCA 360
Stap.epidermis            GAAGTCGATGAATCTAATCCATTATTTTCTGTAAAACAATGGGATGGCAGTGAATTACCA 360
                          *  ** **  *    *   *  * **    * *   ******** **    **  * **

T.muriaticus JCM 10006T   ATTTACGATGCAAAGCCTTTACAAGATAATTTAGTTGAATACTTTGGTACTAAAGACGAT 251
O.oeni                    ATTTACGATGCAAAGCCTTTACAAGATACTTTAGTTGAATACTTTGGTACTAAAGACGAT 420
L.sakei                   ATTTACGATGCAAAGCCTTTACAAGATACTTTAGTTGAATACTTTGGTACTAAAGACGAT 390
L.buchneri                ATTTACGACGCAAAGCCTCTGCAAGATACATTGGTTGAATACTTTGGTACCAAAGACGAT 292
Lactobacillus30A          ATTTATGATGCAAAGCCATTGCAAGATGCTTTAGTTGAATACTTTGGTACTGAACAAGAT 420
Strep.thermophilus        ATCTATGATGCCAAACCATTACAAGATGCCTTAGTAGAATATTTTGGCACCGAGTCAGAT 417
KS87-14                   ATTTATGATTCAAAACCATTGCAAAATGCTCTTGTTGAATATTTCGGAACTGAACAAGAG 241
Stap.capitis              ATTTATGATTCAAAACCATTGCAAAATGCTCTTGTTGAATATTTCGGAACTGAACAAGAG 420
Stap.epidermis            ATTTATGATTCAAAACCACTTCAAAATGCACTTGTTGAATATTTTGGTACTGAACAAGAG 420
                          ** ** **  * ** **  * *** **    * ** ***** ** ** **  *    **

T.muriaticus JCM 10006T   ATGCGTCACTATCCTGCTCCAGGTGCATTTGTATGCTGTGCCAACAAAGGTGTAACTGCA 311
O.oeni                    ATGCGTCACTATCCTGCTCCAGGTGCATTTGTATGCTGTGCCAACAAAGGTGTAACTGCA 480
L.sakei                   ATGCGTCACTATCCTGCTCCAGGTGCATTTGTATGCTGTGCCAACAAAGGTGTAACTGCA 450
L.buchneri                ATGCGTCATTATCCAGCTCCTGGTGCTTTTGTATGCTGTGCTAACAAAGGTGTCACTGCA 352
Lactobacillus30A          CGTCGTCACTACCCAGCTCCAGGTTCATTCATTGTTTGTGCTAACAAGGGTGTTACTGCT 480
Strep.thermophilus        CGTCGTCACAATTTAGTTCCTGGCGCCATGGTGGTTTGTGCTAACAAAGGTGTGACTGCA 477
KS87-14                   AGACGACATCCTTTAACTCCAGGAGCAATGTCAATCTGTGCCAATAAAGGAGTTATAGCG 301
Stap.capitis              AGACGACATCCTTTAACTCCAGGAGCAATGTCAATCTGTGCCAATAAAGGAGTTATAGCG 480
Stap.epidermis            AGACGACATCCTTTAACTCCAGGAGCAATGTCAATTTGTGCTAATAAAGGTGTTGTGGCA 480
                             ** **         *** **  *  *       ***** ** ** ** **    **

T.muriaticus JCM 10006T   GAACGTCCAAAGAACGATGCAGATATGAAGCCTGGTCAAGGTTATGGTGTATGGT----- 366
O.oeni                    GAACGTCCAAAGAACGATGCAGATATGAAGCCTGGTCAAGGATATGGTGTTTGGTCAGCA 540
L.sakei                   GAACGTCCAAAGAACGATGCAGATATGAAGCCTGGTCAAGGATATGGTGTTTGGTCAGCA 510
L.buchneri                GAACGTCCAAAGAATGATGAAGACATGAAG------------------------------ 382
Lactobacillus30A          GAACGTCCAAAGAACGATGCTGACATGAAGCCAGGTCAAGGTTATGGTGTATGGTCTGCA 540
Strep.thermophilus        TATCGCCCCCAAGAGGATCGACCCTTCCAAGCCGGTGAAGGCTATGGTGTCTGGTCAGCG 537
KS87-14                   TCAAGACCGAAAGAAAATAGAGAACTAAATGAAGATGAAGGTTATGGTGTATG------- 350
Stap.capitis              TCAAGACCGAAAGAAAATAGAGAACTAAATGAAGATGAAGGTTATGGTGTTTGGTCAGCC 540
Stap.epidermis            TCAAGACCAAAAGAAAATAGAGAATTAAATGAAGATGAAGGATATGGCGTTTGGTCAGCT 540
                              * **  *  *  **       *  *
```

**Figure 4.4** Partially alignment of *hdc*A gene sequences of KS87-14, *T. muriaticus* JCM 10006$^{T}$, *Lactobacillus* 30a ATCC 33222, *L. buchneri* DSM 5987, *L. sakei* LTH 2076$^{T}$, *Oenococcus oeni* IOEB 9204, *Staphylococcus capitis* A9IZZ1, *S. epidermidis* B3Y006 and *Streptococcus thermophilus* CBX24604 using ClustalW2 program. Single, fully conserved residues are indicated by an asterisk.

## 4.2 ALP producing moderate halophilic bacteria

### 4.2.1 Moderate halophillic bacteria isolates and sources of samples

The 76 halophilic bacteria were isolated from 33 samples of traditional fermented fish and salted fish collected from markets and homemade (Table 4.4).

**Table 4.4** Location, sample source, isolate number and number of isolate

| Location/Source of samples | Isolate no. | No. of isolate |
|---|---|---|
| Kuchi narai, Kalasil (Pla-ra, homemade 9 samples) | KL1-1, 1-2, 1-3, 1-4, 2-1, 2-2, 2-3, 2-4, 3-1,3-2,3-3,3-4,4-1,4-2,5-1,5-2, 5-3, 6-1 6-2, 6-3, 7-1, 7-2, 8-1, 8-2, 8-3, 9-1,9-2 | 27 |
| Mahasalakram (Pla-ra, market 3 samples) | MK10-1,10-2,11-1, 11-2, 12-1, 12-2, 12-3 | 7 |
| Nakornsawan (Pla-ra, market 3 samples) | NSW13-1, 13-2, 13-3, 13-4, 13-5, 14-1,14-2,14-3, 25-1,25-2 | 10 |
| Pitsanuloke (Pla-ra, market 1 samples) | PNL15-1,15-2,15-3 | 3 |
| Kuchi narai, Kalasil (Pla-ra, market 5 samples) | KL16-1, 17-1, 17-2, 18-1, 18-2 19-1, 19-2, 27-1, 27-2, 27-3 | 10 |
| Sukhothai (Pla-ra, Pla-som, market 3 samples) | SK20-1, 20-2, 21-1, 21-2, 21-3 22-1, 22-2, 23-1, 23-2, 23-3, 23-4 | 11 |
| Samutsongklam (Pla-ra, Hoi-dong, market 3 samples) | SMK24-1, 24-2, 24-3 | 3 |
| Rayong (Pla-chem, 5 samples) | RY30-1, 30-2, 31-1, 31-2 | 4 |
| Nakornnayok (Pla-ra, market 1 samples) | TPS4-2 | 1 |
| **Total** | | **76** |

*Pla-ra*, fermented fish; *Pla-som,* fermented fish; Hoi-dong , fermented mollusces

### 4.2.2 Screening of ALP-producing halophilic bacteria

ALP producing activity of 76 halophilic bacteria were investgated by growing on Heart Infusion agar (Difco) containing 0.01% (w/v) phenolphthalein bisphosphate tetrasodium salt (Sigma) and 10% NaCl (w/v). Of these isolates, seventeen isolates showed pink color on the tested medium indicating that they could produce ALP.

**Table 4.5** Isolate number, closely relatives, sequence identity (%) and ALP activity.

| Isolate no. | Closely relatives | Identity (%) | ALP Activity* (U/ml) |
|---|---|---|---|
| | **Group I *Staphylococcus saprophyticus*** | | |
| NSW25-1 | *S. saprophyticus* subsp.*saprophyticus* ATCC 15305$^T$ | 99.3 | 12.52±0.03 |
| SK22-1 | *S. saprophyticus* subsp.*saprophyticus* ATCC 15305$^T$ | 99.2 | 12.79±0.07 |
| SK23-2 | *S. saprophyticus* subsp.*bovis* GTC 843$^T$ | 99.2 | 15.12±0.11 |
| | **Group II *Staphylococcus napalensis*** | | |
| RY30-1 | *S. napalensis* CW1$^T$ | 99.6 | 26.16±0.01 |
| RY30-3 | *S. napalensis* CW1$^T$ | 99.5 | 26.05±0.01 |
| RY31-2 | *S. napalensis* CW1$^T$ | 99.7 | 25.58±0.02 |
| | **Group III *Staphylococcus sciuri*** | | |
| KL6-2 | *S. sciuri* subsp. *sciuri* DSM2 0345 $^T$ | 99.8 | 10.78±0.1 |
| | **Group IV *Bacillus vietnamensis*** | | |
| KL2-3 | *B. vietnamensis* 15-1$^T$ | 99.2 | 10.08±0 |
| | **Group V *B. safensis*** | | |
| MK10-2 | *B. pumilus* ATCC 7061$^T$ | 99.8 | 12.29±0.02 |
| | **Group VI *Virgibacillus halodentrifican*** | | |
| KL1-1 | *V. halodentrifican* DSM 10037$^T$ | 99.1 | 49.71±0.02 |
| KL2-4 | *V. halodentrifican* DSM 10037$^T$ | 98.6 | 25.39±0.01 |
| NSW13-2 | *V. halodentrifican* DSM 10037$^T$ | 99.1 | 70.96±0.01 |
| | **Group VII *Oceanobacillus iheyensis*** | | |
| NSW13-4 | *O. iheyensis* JCM11309$^T$ | 99.3 | 13.14±0.06 |
| | **Group VIII *Halobacillus mangrove*** | | |
| NSW13-3 | *H. mangrove* MS10$^T$ | 97.9 | 13.22±0.04 |
| | **Group IX *Halobacillus dabanensis*** | | |
| NSW13-5 | *H. dabanensis* JCM 12772$^T$ | 98.6 | 12.13±0.1 |
| SMK24-1 | *H. dabanensis* JCM 12772$^T$ | 98.5 | 14.77±0.04 |
| | **Group X *Idiomarina zobellii*** | | |
| TPS4-2 | *I. zobellii* KMM231$^T$ | 98.4 | 81.50±0.15 |

* One unit of ALP was defined as the amount of the enzyme yielding one micromole of *p*-nitrophenol within one minute under the assay conditions.

*B. pumilus* ATCC 7061$^T$

To determine ALP 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. Amount of ALP activity in cell-free supernatant of the cultures was analyzed as shown in Table 4.5. The isolate TPS4-2 showed the highest ALP activity (81.50 U/ml), followed by isolate NSW 13-2 (70.96 U/ml), KL1-1 (49.71 U/ml), RY30-1 (26.16 U/ml), RY30-3 (26.05 U/ml), RY31-2 (25.58 U/ml), KL2-4 (25.39 U/ml) and the remained were 10.08-15.12 U/ml respectively. (Table 4.5) Cell and cell-free supernatant of isolate TPS4-2 were confirmed enzymy producing using API-ZYME kit. The isolate TPS4-2 showed positive activity of ALP on API-ZYME testing.

In previously study, the moderately halophilic strains were isolated and identified as *Fillobacillus* sp., *Halobacillus* sp., *Vigibacillus* sp., *Piscibacillus salipiscarius*, *V. dokdonensis*, *V. halodenitrificans*, *V. marismortui*, *V. siamensis*, *B. vietnamnensis*, *Chromohalobacter salexigens*, *Salinivibrio siamensis, Gricilibacillus thailandensis* based on 16S rRNA gene sequences and phylogenetic analysis were distributed in fermented fish (*pla-ra*) and salted fishes (Chamroensaksri *et al*., 2009, 2010; Tanasupawat *et al*., 2007, 2010). Several ALPs have been investigated from variety bacteria such as *Thermotoga neapolitana* (Dong & Zeikus, 1997), *Thermus thermophilus* (Pantazaki *et al*. 1998), the hyperthermophilic archaeon *Pyrococcus abyssi* (Zappa et al., 2001), *Aeropyrum pernix* showed (Helianti *et al*. 2007). However, it is still limited to study ALP from moderately halophilic bacteria with *Vibrio sp*. (Hauksson *et al*., 2000) and *Halomonas sp.* (Ishibashi *et al*., 2005, 2011)

### 4.2.3 Identification and characterization of ALP-producing isolates

Seventeen isolates of ALP-producing bacteria were divided into 10 groups based on their 16S rRNA gene sequence analyses and phenotypic characteristics (Table 4.5). Seven isolates (group I, II and III) belonged to the genus *Staphylococcus* were Gram-positive non-motile and non-spore forming cocci. They were catalase-positive but oxidase-negative. Two isolates in group IV and V belonged to the genus *Bacillus* were Gram-positive spore forming rods. The moderately halophilic bacteria in group VI (3 isolates belonged to the genus *Virgibacillus*), group VII (1 isolate belonged to the genus *Oceanobacillus*), group VIII (1isolate) and group IX (2

isolates) belonged to the genus *Halobacillus*, were Gram-positive, spore forming rods. They produced catalase and most of them were negative with oxidase. All isolates contained *meso*-diaminopimelic acid in the cell wall. They showed the difference in the colonial pigmentation and the growth in high concentration of NaCl (w/v). One isolate of moderately halophilic bacterium in group X belonged to genus *Idiomarina* was a Gram-negative non-spore forming rod shaped bacterium. All the isolates in each group were negative reaction for citrate utilization, indole and tyrosine hydrolysis. The details of their characterizations were described below in each group and shown in Table 4.6 - 4.13.

#### 4.2.3.1 Group I isolates

Group I contained three isolates, NSW25-1, SK22-1 and SK23-2. They were Gram-positive non-motile and non-spore forming cocci. Colonies were circular, low convex, glistening surface, and grey white with yellowish (4-5 mm in diameter). They grew under aerobic and anaerobic condition in 0-15% NaCl (w/v) for SK22-1 and SK23-2, and 0-20% NaCl (w/v) for NSW25-1 and at 25-45$^{o}$C (optimum at 30-37$^{o}$C), at variable optimum pH as shown in Table 2. They were positive for catalase and methyl red but negative for oxidase, nitrate reduction, hydrolysis of aesculin and starch. They produced acid from D-fructose, D-glucose, glycerol, maltose, mannitol and trehalose, but did not produce acid from L-arabinose, D-cellobiose, D-raffinose, galactose, melibiose, rhamnose, salicin and sorbitol. Isolates SK22-1 and SK23-2 were different from NSW25-1 in acid acid production from sucrose. The other variable characteristics were summarized in Table 4.6. These three isolates could tolerate to 15% of NaCl as previously reported (Schleifer & Kloos, 1975; Hajek *et al*., 1996; Trülzsch *et al*., 2007). On the basis of 16S rRNA gene sequence analyses (Figure 4.5), these three isolates were placed in the genus *Staphylococcus*. The isolates NSW25-1 (1459 bp) and SK22-1 (1452 bp) were closely related to *S. saprophyticus* subsp. *saprophyticus* ATCC15305$^{T}$ with 99.1-99.3% similarity (Trülzsch *et al*., 2007) and the isolate SK23-2 (1460 bp) was closely related to *S. saprophyticus* subsp. *bovis* GTC843$^{T}$ with 99.2% similarity (Hajek *et al*., 1996). Therefore, based on the results mentioned above and phenotypic properties indicated that isolates NSW25-1, SK22-1 and SK23-2 were identified as *S. saprophyticus*.

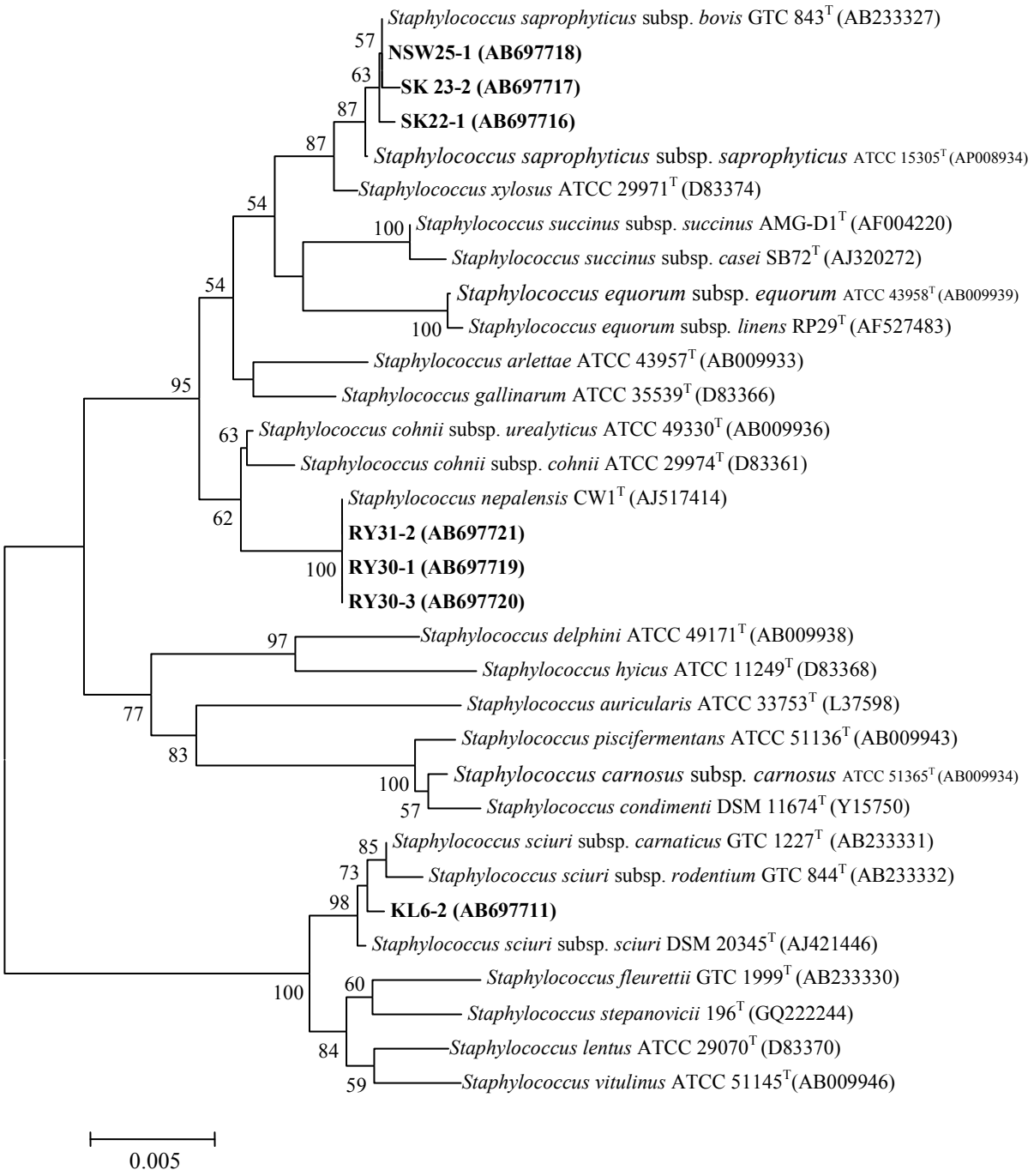


**Figure 4.5** Phylogenetic relationships of isolates in group I (NSW25-1, SK22-1 and SK23-2), group II (RY31-2, RY30-1 and RY30-3), group III (KL6-2) and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages values >50%, base on 1000 replications are shown at the nodes. Bar, 0.005 substitutions per 100 nucleotide position.

**Table 4.6** Differential phenotypic characteristics of group I (NSW25-1, SK22-1, SK23-2) and closely related *Staphylococcus* type strains.

| Characteristics | Group I | | | S1† | S2†† |
|---|---|---|---|---|---|
| | NSW25-1 | SK22-1 | SK23-2 | | |
| Cell shape | Cocci | | | | Spherical cocci |
| Cell form | Single, pair and cluster | | | | |
| Colony color | Yellow | | | | Creamy to pale orange |
| NaCl range (%, w/v) | 0-20 | 0-15 | 0-15 | nd | nd |
| Temperature range (°C) | 30-45 | 30-45 | 30-45 | nd | 10-40 |
| Optimum pH | 7-8 | 6-7 | 7 | nd | nd |
| Oxidase | - | - | - | - | - |
| Catalase | + | + | + | + | + |
| Alkaline phosphatase | + | + | + | - | - |
| Acid from | | | | | |
| L-Arabinose | - | - | - | - | - |
| D-Cellobiose | - | - | - | - | - |
| D-Fructose | + | + | w | + | + |
| Galactose | - | - | - | - | + |
| D-Glucose | + | + | + | nd | + |
| Glycerol | + | + | w | nd | + |
| Lactose | - | - | w | + | - |
| Maltose | + | + | w | + | + |
| Mannitol | + | + | w | + | + |
| D-Mannose | w | - | w | - | - |
| Melibiose | - | - | - | nd | nd |
| D-Raffinose | - | - | - | - | - |
| Rhamnose | - | - | - | nd | + |
| D-Ribose | w | w | w | - | + |
| Salicin | - | - | - | - | - |
| Sorbitol | - | - | - | nd | nd |
| Sucrose | - | + | + | + | + |
| Trehalose | + | + | + | + | + |
| D-Xylose | w | - | - | - | - |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data; *data from Spergser *et al*., 2003. S1; *S. saprophyticus sbsp. Saprophyticus* ATCC15305$^T$, S2; *S. saprophyticus sbsp. bovis* GTC 843$^T$, †Data from Trülzsch *et al*., 2007**.** ††Data from Hajek *et al*., 1996.

#### 4.2.3.2 Group II isolates

Group II contained three isolates, RY30-1, RY30-3 and RY31-2. Colonies were circular, low convex, glistening surface, gray white with yellowish (RY30-1, RY30-3), white (RY31-2) and formed 2-5 mm in diameter after 2 days incubation. Growth occurred aerobically and anaerobically in the presence of 0-20% NaCl (w/v), at 25-45$^{o}$C (optimum at 30-37$^{o}$C), at optimum pH 7 (RY30-1, RY30-3) and at pH 7-8 for strain RY31-2. They grew well in 10% NaCl (w/v). They were positive for methyl red and nitrate reduction, but negative for Voges-Proskauer, gelatin and starch hydrolysis. The isolate RY30-1 produced acid from D-fructose, salicin and trehalose. The isolate RY30-3 produced acid from D-xylose, D-glucose, galactose, L-arabinose, sucrose and trehalose while the isolate RY31-2 produced acid from D-glucose, D-mannose, lactose, L-arabinose and trehalose. The ability to produce acid from sugars of these three isolates was variable and was different from *S. napalensis* CW$^{1}$ (Spergser *et al*., 2003) as shown in Table 4.7. On the basis of 16S rRNA gene sequence analyses (Figure 4.5), these three isolates were placed in the genus *Staphylococcus*. The isolates RY30-1 (1454bp), RY30-3 (1457 bp) and RY31-2 (1456 bp) were closely related to *S.napalensis* CW1$^{T}$ with 99.6, 99.5 and 99.7% 16S rRNA sequence similarity respectively. However, they were identified as *S. napalensis* (Spergser *et al*., 2003).

#### 4.2.3.3 Group III isolates

Group III contained one isolate, KL6-2. Colonies were circular, low convex, glistening surface and gray white with yellowish (less than 3 mm in diameter). Growth occurred aerobically and anaerobically in the presence of 0-15% NaCl (w/v), at 25-45$^{o}$C (optimum at 30-37$^{o}$C), at optimum pH 7-8. The isolate was positive for methyl red, nitrate reduction and hydrolysis of aesculin and gelatin but negative for Voges-Proskauer and starch hydrolysis. Acid was produced from D-mannose, D-raffinose, D-ribose, salicin, sucrose and trehalose, but was not from many sugars. Some of its phenotypic characteristics were different from *S. sciuri* subsp. *scuiri* DSM 20345$^{T}$ (Kloos *et al*., 1976) as shown in Table 4.8. The isolate KL6-2 showed 99.8% sequence (1457 bp) similarity to *S. sciuri subsp. scuiri* DSM 20345$^{T}$ based on 16S rRNA gene (Figure 4.5). Therefore, it was identified as *S. sciuri*.

**Table 4.7** Differential phenotypic characteristics of group II (RY30-1, RY30-3, RY31-2) and closely related *Staphylococcus napalensis* CW1$^T$.

| **Characteristics** | **Group II** | | | ***S. napalensis* CW1$^T$*** |
|---|---|---|---|---|
| | **RY30-1** | **RY30-3** | **RY31-2** | |
| Cell shape | Cocci | | | |
| Cell form | singly, pair & cluster | | | |
| Colony color | Cream-yellow | | White | Opaque white |
| NaCl range (%, w/v) | 0-20 | 0-20 | 0-20 | 0-10 |
| Temperature range (°C) | 30-45 | 30-45 | 30-45 | 20-40 (30) |
| Optimum pH | 7 | 7 | 7-8 | nd |
| Oxidase | - | - | - | - |
| Catalase | + | + | + | + |
| Alkaline phosphatase | + | + | + | + |
| Acid from | | | | |
| L-Arabinose | - | + | + | + |
| D-Cellobiose | - | - | - | - |
| D-Fructose | + | - | - | + |
| Galactose | - | w | - | + |
| D-Glucose | - | w | + | + |
| Glycerol | - | - | - | + |
| Lactose | - | - | w | + |
| Maltose | - | - | w | + |
| Mannitol | - | - | - | + |
| D-Mannose | - | - | + | + |
| Melibiose | - | - | - | - |
| D-Raffinose | - | - | - | - |
| Rhamnose | - | - | - | - |
| D-Ribose | - | - | - | - |
| Salicin | + | + | - | + |
| Sorbitol | - | - | - | nd |
| Sucrose | - | + | w | + |
| Trehalose | + | + | + | + |
| D-Xylose | - | + | - | + |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data, *data from Spergser *et al*., 2003.

**Table 4.8** Differential phenotypic characteristics of group III (KL6-2) and closely related *Staphylococcus scuiuri subsp. sciuri* DSM 20345$^T$

| Characteristics | Group III | *S. scuiuri subsp. sciuri* |
|---|---|---|
| | KL6-2 | DSM 20345$^T$* |
| Cell shape | Cocci | Cocci |
| Cell form | singly, pair & cluster | singly, pair & cluster |
| Colony color | clear colored & small colony | gray-white with yellowish |
| NaCl range (%, w/v) | 0-15 | 0-10 |
| Temperature range (°C) | 30-45 | 25-35 |
| Optimum pH | 7-8 | nd |
| Oxidase | - | + |
| Catalase | + | nd |
| Alkaline phosphatase | + | + |
| Acid from: | | |
| L-Arabinose | - | vary |
| D-Cellobiose | - | + |
| D-Fructose | - | + |
| Galactose | - | + |
| D-Glucose | - | + |
| Glycerol | - | + |
| Lactose | - | - |
| Maltose | - | + |
| Mannitol | - | + |
| D-Mannose | + | + |
| Melibiose | - | nd |
| D-Raffinose | w | + |
| Rhamnose | - | vary |
| D-Ribose | + | + |
| Salicin | + | + |
| Sorbitol | - | vary |
| Sucrose | + | + |
| Trehalose | + | + |
| D-Xylose | - | - |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data, *data from Kloos *et al*.,1976.

#### 4.2.3.4 Group IV isolates

Group IV contained one isolate, KL2-3. They were Gram-positive spore forming rods. Colonies were irregular, raised and orange to peach (0.5-3.5 mm diameter). Growth occurred under aerobic and anaerobic conditions, in 0-15% NaCl (w/v), at 25-45$^{o}$C (optimum at 30-37$^{o}$C), at pH 7-8. This isolate was positive for hydrolysis of aesculin, gelatin, Tween 80 and starch but negative for methyl red, voges-proskauer, arginine hydrolysis and nitrate reduction. Acid was produced from D-fructose, D-glucose, D-ribose, D-trehalose, sucrose, glycerol and maltose. No acid was produced from L-arabinose, D-cellobiose, D-galactose, inositol, inulin, lactose, D-mannitol, D-mannose, D-melezitose, D-melibiose, raffinose, rhamnose, salicin and D-xylose. On the basis of 16S rRNA gene sequence analyses, isolate KL2-3 was placed in the genus *Bacillus* and showed 99.2% sequence (1458 bp) similarity to *B. vietnamensis* 15-1$^{T}$ (Figure 4.6). The isolate KL2-3 showed almost the same phenotypic characteristics as *B. vietnamensis* 15-1$^{T}$ as shown in Table 4.9. Therefore, it was identified as *B. vietnamensis* (Noguchi *et al*., 2004).

#### 4.2.3.5 Group V isolates

Group V contained one isolate, MK10-2. They were Gram-positive spore forming rods. Colonies were round, opaque, creamy white and irregular margins. This isolate was positive for Methyl Red-Voges-Proskauer, hydrolysis of aesculin and gelatin but negative for nitrate reduction, hydrolysis of Tween 80 and starch. Acid was produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and D-trehalose. No acid was produced from inositol, inulin, lactose, D-melezitose, D-melibiose, raffinose, rhamnose andD-xylose. The isolate MK10-2 produced no acid from inositol and D-xylose. This isolate was closely related to *B. pumilus* ATCC 7061$^{T}$ and *B. safensis* FO36$^{T}$ with 99.8 and 99.7% sequence similarity, respectively (Figure 4.6). Their phenotype characteristics were listed in Table 4.9.

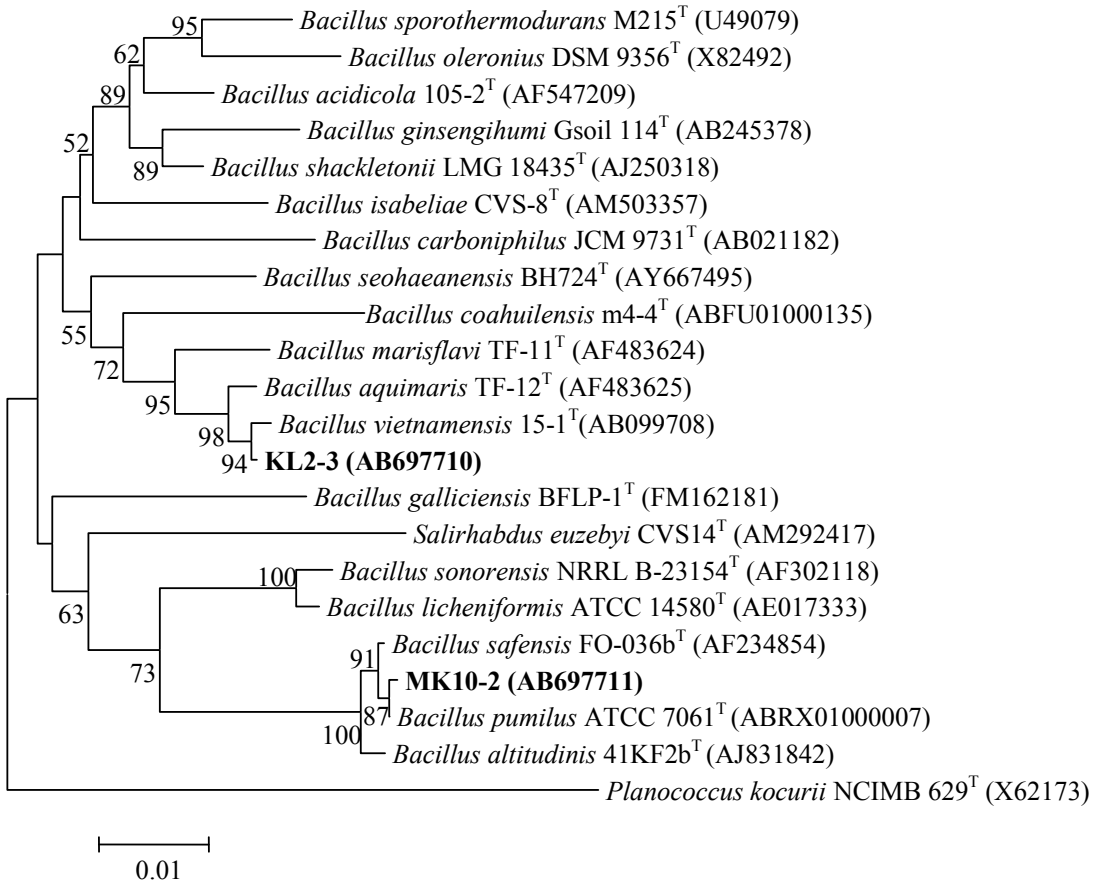


**Figure 4.6** Phylogenetic relationships of isolates in group IV (KL2-3), group V (MK10-2) and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages over 50%, base on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per 100 nucleotide position.

**Table 4.9** Differential phenotypic characteristics of group IV (KL2-3), group V (MK10-2) and closely related *Bacillus* type strains.

| Characteristics | Group IV KL2-3 | *B. vietnamensis* JCM 11124$^{T}$ † | Group V MK10-2 | *B. safensis* FO-36$^{T}$ †† | *B. pumilus* ATCC 7061$^{T}$ †† |
|---|---|---|---|---|---|
| Cell shape | Rod(oval) | Rods | Rods | Rods | Rods |
| Colony colour | Orange&peach | Cream to orange | Creamy white | Dull white | white |
| Tween 80 hydrolysis | + | nd | - | - | + |
| Arginine hydrolysis | - | - | - | - | nd |
| NaCl range (%w/v) | 0-15 | 0-15 | 0-15 | 0-10 | 0-10 |
| pH range | 7-8 | 6.5-10 | 7-8 | 6.5-10 | 5-10 |
| Temperature range ($^{o}$C) | 30-45 | 10-40 | 30-45 | 10-50 | 10-50 |
| Acid form | | | | | |
| L-Arabinose | - | - | w | + | + |
| D-Cellobiose | - | - | + | + | + |
| D-Fructose | + | + | + | + | + |
| D-Galactose | - | - | + | + | + |
| D-Glucose | + | + | + | + | + |
| Glycerol | w | + | w | + | + |
| Inositol | - | - | - | + | - |
| Inulin | - | + | - | - | nd |
| Lactose | - | - | - | - | + |
| Maltose | w | + | w | + | - |
| D-Mannitol | - | + | w | + | + |
| D-Mannose | - | - | + | + | nd |
| D-Melezitose | - | - | - | - | - |
| D-Melibiose | - | - | - | Vary(+) | + |
| Raffinose | - | - | - | - | - |
| Rhamnose | - | - | - | - | - |
| D-Ribose | + | + | + | + | + |
| Salicin | - | - | + | + | + |
| D-Sorbitol | - | - | - | - | - |
| Sucrose | + | + | + | + | nd |
| D-Trehalose | + | + | + | + | _ |
| D-Xylose | - | - | - | + | + |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data, †Data from Noguchi *et al*., 2004. ††Data from Satomi *et al*., 2006.

#### 4.2.3.6 Group VI isolates

Group VI contained three isolates KL1-1, KL2-4 and NSW 13-2. They were Gram-positive, spore forming rods. Colonies were circular to slightly irregular, raised, opaque cream yellow for KL1-1 and NSW 13-2 and cream yellow colour for KL 2-4, (1-2 mm diameter). They grew under aerobic and anaerobic conditions, in NaCl up to 20% (w/v), at 25-45 $^{o}$C, at pH 7-8 (KL1-1, NSW13-2) and pH 6-8 for KL2-4. They were positive for catalase, nitrate reduction and hydrolysis of gelatin but negative for oxidase, Methyl Red-Voges-Proskauer, hydrolysis of aesculin and starch. The isolate KL2-4 hydrolyzed Tween 80 but KL1-1 and NSW13-2 did not. Acid was produced from D-glucose, D-ribose, maltose and sucrose. No acid was produced from glycerol, inositol, inulin, D-mannose, D-melezitose, D-melibiose, raffinose, rhamnose, salicin and D-xylose. The variable of acid productions from sugars between these three isolates and the closely type strains were shown in Table 4.10. The isolates KL1-1(1468 bp), KL2-4 (1477 bp) and NSW13-2 (1474 bp) were closely related to *V. halodenitrificans* DSM 10037$^{T}$ with 99.1, 98.6 and 99.1% sequence similarity, respectively (Figure 4.7). On the basis of 16S rRNA gene sequence analyses, these three isolates were placed in the genus *Virgibacillus*. Their phenotypic characteristics were closely related to *V. halodenitrificans* DSM 10037$^{T}$ as shown in Table 4.10. Therefore, they were identified as *V. halodenitrificans* (Yoon *et al*., 2004).

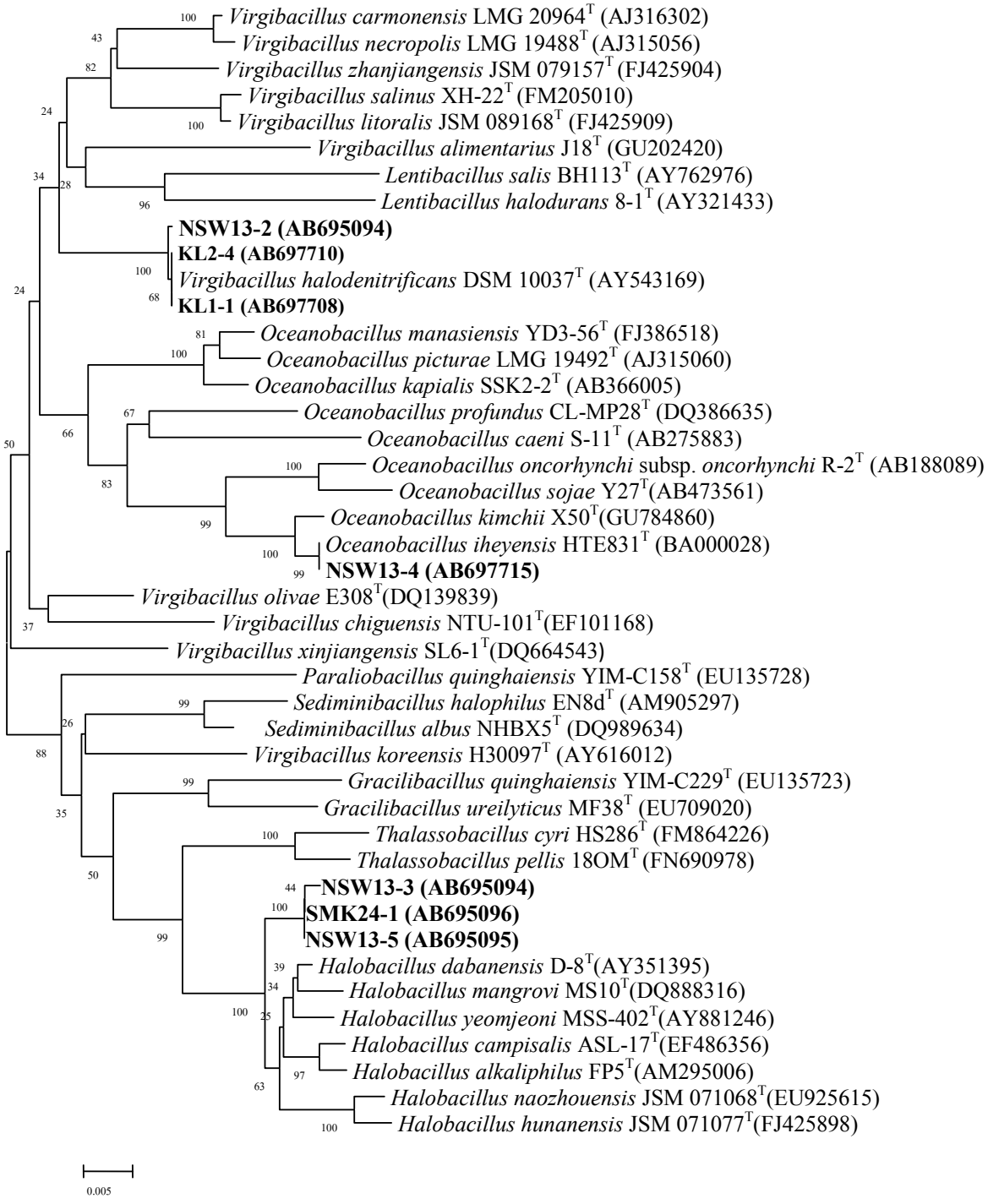


**Figure 4.7** Phylogenetic relationships of isolates group VI (KL1-1, KL2-4, NSW13-2), Group VII (NSW13-4), group VIII (NSW13-3), group IX (NSW13-5, SMK24-1) and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap percentage values base on 1000 replications are shown at the nodes. Bar, 0.005 substitutions per 100 nucleotide position.

**Table 4.10** Differential phenotypic characteristics of group VI (KL1-1, KL2-4, NSW13-2) and closely related *V. halodenitrificans* DSM10037$^{T}$.

| Characteristics | Group VI | | | *V. halodenitrificans* DSM 10037$^{T*}$ |
|---|---|---|---|---|
| | KL1-1 | KL2-4 | NSW13-2 | |
| Cell shape | Rods | Rods | Rods | Rods |
| Colony colour | cream | cream | cream | cream |
| Gram stain | + | + | + | vary |
| Tween 80 hydrolysis | - | + | - | - |
| Arginine hydrolysis | - | - | - | nd |
| NaCl range (%w/v) | 0-15 | 0-20 | 0-15 | 0-23 |
| pH range | 7-8 | 6-8 | 7-8 | 5.8-9.6 |
| Temperature range ($^{o}$C) | 30-45 | 30-40 | 30-45 | 10-45 |
| Acid from | | | | |
| L-Arabinose | - | - | - | - |
| D-Cellobiose | w | - | w | - |
| D-Fructose | - | + | + | + |
| D-Galactose | w | w | - | + |
| D-Glucose | w | w | + | + |
| Glycerol | - | - | - | - |
| Inositol | - | - | - | - |
| Inulin | - | - | - | nd |
| Lactose | - | - | w | vary |
| Maltose | + | w | w | + |
| D-Mannitol | + | - | w | + |
| D-Mannose | - | - | - | + |
| D-Melezitose | - | - | - | - |
| D-Melibiose | - | - | - | - |
| Raffinose | - | - | - | - |
| Rhamnose | - | - | - | - |
| D-Ribose | + | + | + | + |
| Salicin | - | - | - | nd |
| D-Sorbitol | - | - | - | - |
| Sucrose | + | + | + | + |
| D-Trehalose | - | + | + | + |
| D-Xylose | - | - | - | - |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data, *data from Yoon *et al*., 2004

#### 4.2.3.7 Group VII isolates

Group VII contained one isolate, NSW13-4 was Gram-positive, spore forming rods. Colonies were circular, raised and creamy white (2-3 mm in diameter). Growth occurred in the presence of NaCl range 0-15% (w/v), at 25-45$^{o}$C (optimum at 30-37$^{o}$C) and at pH 7-9. This isolate was positive for catalase, hydrolysis of gelatin but negative for oxidase, MR-Voges-Proskauer, nitrate reduction, hydrolysis of aesculin, arginine, Tween 80 and starch. Acid was produced from L-arabinose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, rhamnose, D-ribose, sucrose and D-xylose. No acid was produced from D-cellobiose, D-frutose, D-galactose, inositol, inulin, lactose, D-melezitose, D-melibiose, raffinose, salicin and D-trehalose. On the basis of 16S rRNA gene sequence analyses, this isolate was placed in the genus *Oceanobacillus*. The isolate NSW13-4 (1467 bp) was closely related to *O. iheyensis* JCM11309$^{T}$ with 99.3% sequence similarity (Figure 4.7). The ability to produce acid from sugars of isolate NSW13-4 was variable and was different from *Oceanobacillus iheyensis* JCM11309$^{T}$ (Lu *et al*., 2001) as shown in Table 4.11. However, this isolate was identified as *O. iheyensis*.

**Table 4.11** Differential phenotypic characteristics of group VII (NSW13-4) and closely related *Oceanobacillus iheyensis* JCM 11309$^T$

| **Characteristics** | **Group VII** | ***O. iheyensis* JCM 11309$^T$*** |
|---|---|---|
| | **NSW13-4** | |
| Cell shape | Rods | Rods |
| Colony colour | Creamy white | Creamy white |
| Tween 80 hydrolysis | - | nd |
| Arginine hydrolysis | - | nd |
| NaCl range (%w/v) | 0-15 | 0-21 |
| pH range | 7-9 | 6.5-10 |
| Temperature range ($^o$C) | 30-45 | 15-42 |
| Acid form | | |
| L-Arabinose | + | - |
| D-Cellobiose | + | - |
| D-Fructose | + | - |
| D-Galactose | - | - |
| D-Glucose | + | -(u) |
| Glycerol | + | - |
| Inositol | - | - |
| Inulin | - | - |
| Lactose | - | - |
| Maltose | + | -(u) |
| D-Mannitol | + | - |
| D-Mannose | + | -(u) |
| D-Melezitose | - | - |
| D-Melibiose | - | - |
| Raffinose | - | - |
| Rhamnose | w | - |
| D-Ribose | + | - |
| Salicin | - | - |
| D-Sorbitol | - | - |
| Sucrose | + | - |
| D-Trehalose | - | - |
| D-Xylose | + | - |

+; positive reaction, -; negative reaction, w; weak reaction, u; utilized, nd; no data, *Data from Lu *et al*., 2001

#### 4.2.3.8 Group VIII isolates

Group VIII contained one isolate, NSW13-3 was Gram-positive, spore forming rods. Colonies of isolate NSW13-3 were entire, circular, low convex and pale yellow colour. Growth occurred aerobically in the presence of 5-20% (w/v) NaCl, at 25-45$^{o}$C (optimum at 30-37$^{o}$C) and at pH 7-8. It was positive for catalase, methyl red, hydrolysis of gelatin, starch and Tween 80 but negative for oxidase, Voges-Proskauer, nitrate reduction and hydrolysis of aesculin and arginine. Not able to produced acid from L-arabinose, inulin, D-melezitose, D-melibiose, rhamnose and D-xylose. On the basis of 16S rRNA gene sequence analyses, this isolate was placed in the genus *Halobacillus*. The isolate NSW13-3 (1472 bp) was closely related to *H. mangrovi* MS10$^{T}$ with 97.9% sequence similarity (Figure 4.7) (Soto-Ramírez *et al*., 2008). The isolate showed different phenotypic characteristics from *Halobacillus mangrovi* MS10$^{T}$ (Table 4.12) and contained *meso*-diaminopimelic acid in the cell wall which was absent in *Halobacillus mangrovi* MS10$^{T}$ (Soto-Ramírez *et al*., 2008). Therefore, the isolate NSW13-3 was kept unidentified and should be confirmed for DNA-DNA hybridization for the proposal of the new species.

#### 4.2.3.9 Group IX isolates

Group IX contained two isolates, NSW13-5 and SMK24-1. Colonies of both isolates were circular, smooth and raised, creamy yellow to yellow (3-4 mm in diameter). Growth occurred aerobically in the presence of 1-20% (w/v) NaCl, at 25-45$^{o}$C (optimum at 30-37 $^{o}$C) and at pH 7-8. They were positive for MR, hydrolysis of gelatin, starch and Tween 80 but negative for Voges-Proskauer, nitrate reduction and hydrolysis of aesculin and arginine. Both isolates produced acid from D-glucose, glycerol, inositol, maltose, D-mannitol, raffinose, D-ribose, sucrose and D-trehalose. They did not produced acid from L-arabinose, D-melezitose, D-melibiose, rhamnose and D-xylose. The isolate NSW13-5 produced acid from D-cellobiose, D-fructose, D-galactose, lactose, D-mannose and salicin while the isolate SMK24-1 did not as listed in Table 4.12. Base on 16S rRNA gene sequence analyses, both isolates were placed in the genus *Halobacillus*, the isolate NSW13-5 (1467 bp) and SMK24-1 (1468 bp) were closely related to *H. dabanensis* JCM 12772$^{T}$ with 98.6 and 98.5% similarity respectively (Figure 4.7). They showed different phenotypic characteristics from *H. dabanensis* JCM 12772$^{T}$ (Table 4.12) and contained *meso*-diaminopimelic

acid which was absent in *H. dabanensis* JCM 12772$^{T}$ (Liu *et al*., 2005). Therefore, these isolates were kept unidentified.

**Table 4.12** Differential phenotypic characteristics of group VIII (NSW13-3), group IX (NSW13-5, SMK24-1) and closely related *Halobacillus* species.

| Characteristics | Group VIII | *H. mangrovi* | Group IX | | *H. dabanensis* |
|---|---|---|---|---|---|
| | NSW13-3 | MS10$^{T\dagger}$ | NSW13-5 | SMK24-1 | JCM12772$^{T\dagger\dagger}$ |
| Cell shape | Rods | Short rods | Rods | Rods | Rods |
| Colony colour | Pale yellow | Cream&brown | Creamy yellow | Yellow mucoid | Cream to orange |
| Gram stain | + | + | + | + | + |
| Tween 80 hydrolysis | + | - | + | + | - |
| Arginine hydrolysis | - | | - | - | |
| NaCl range (%w/v) | 5-20 | 5-20 | 1-20 | 1-20 | 0.5-25 |
| pH range | 7-8 | 6-9 | 7-8 | 7-8 | 5-11 |
| Temperature range ($^{o}$C) | 30-45 | 10-50 | 30-45 | 30-45 | 15-50 |
| Acid from | | | | | |
| L-Arabinose | - | - | - | - | nd |
| D-Cellobiose | + | - | + | - | nd |
| D-Fructose | + | - | + | - | + |
| D-Galactose | + | - | + | - | - |
| D-Glucose | + | - | + | + | + |
| Glycerol | + | | + | + | nd |
| Inositol | + | nd | + | + | nd |
| Inulin | - | nd | - | + | nd |
| Lactose | + | nd | + | - | nd |
| Maltose | + | - | + | + | + |
| D-Mannitol | + | - | + | w | + |
| D-Mannose | + | - | + | - | nd |
| D-Melezitose | - | nd | - | - | nd |
| D-Melibiose | - | - | - | - | nd |
| Raffinose | + | nd | + | + | nd |
| Rhamnose | - | - | - | - | nd |
| D-Ribose | + | nd | + | + | nd |
| Salicin | + | nd | + | - | nd |
| D-Sorbitol | - | - | - | - | |
| Sucrose | + | - | + | + | + |
| D-Trehalose | + | - | + | + | + |
| D-Xylose | - | - | - | - | + |

+; positive reaction, -; negative reaction, w; weak reaction, nd; no data, sp; spore $^{\dagger}$Data from Soto-Ramírez *et al*., 2008. $^{\dagger\dagger}$Data from Liu *et al*., 2005

#### 4.2.3.10 Group X isolates

Group X contained one isolate, TPS4-2 which was Gram-negative, non-endospore-forming, slightly curved rods, approximately 0.3-0.4 µm in width and 1.2-2 µm in length. after 48 h of incubation at 37 °C on JCM medium no. 377 agar. Motile by means of one polar flagellum (Fig.1). Colonies were circular with entire edges, smooth, convex, opaque and pale yellow colour, diameter reached 0.9-2.5 mm

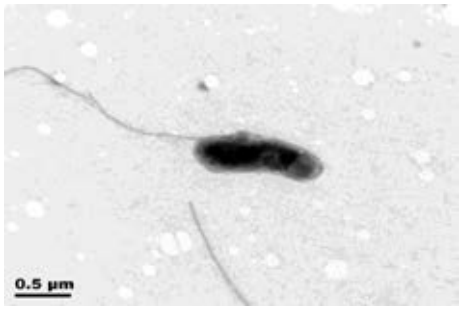


**Figure 4.8** Transmission electron micrograph of strain TPS4-2$^T$ with the single polar flagellum, grown on JCM medium no. 377. Bar 0.5 µm.

On the basis of 16S rRNA gene sequence and phylogenetic analyses, the strain TPS4-2$^T$ was placed in a monophyletic cluster consisting of the genus *Idiomarina* and closely related to the species as shown in Figure. The TPS4-2$^T$ (1375 bp) was closely related to *Idiomarina zobelli* DSM 15924$^T$, *I. baltica* DSM 15154$^T$, *I. fontislapidosi* DSM 16139$^T$ and *I. seosinensis* KTCT 12296$^T$ with 98.7, 96.9, 96.6 and 96.4 % 16S rRNA gene sequence similarity, respectively (Figure 4.9).

DNA–DNA hybridization was performed to determine the genetic relatedness between strain TPS4-2$^T$ with its closest phylogenetic neighbours (Table 4.13). The strain TPS4-2$^T$ exhibited low level DNA-DNA relatedness with *I. zobellii* DSM 15924$^T$ (47.7%), *I. baltica* DSM 15154 $^T$ (24.7%) and *I. fontislapidosi* DSM 16139$^T$ (7.4%) and *I. seosinensis* KTCT 12296$^T$ (14.1%). *I. zobellii* DSM 15924$^T$ exhibited reciprocally low DNA-DNA relatedness (51.4%) to strain TPS4-2$^T$. The DNA–DNA relatedness of strain TPS4-2$^T$ with its closest phylogenetic neighbours was well below the 70% cut-off point recommended for the assignment of the strains to the same genomic species. (Wayne *et al*., 1987) Based on the above DNA–DNA relatedness data, strain TPS4-2$^T$ warrants separate species of the genus *Idiomarina.*

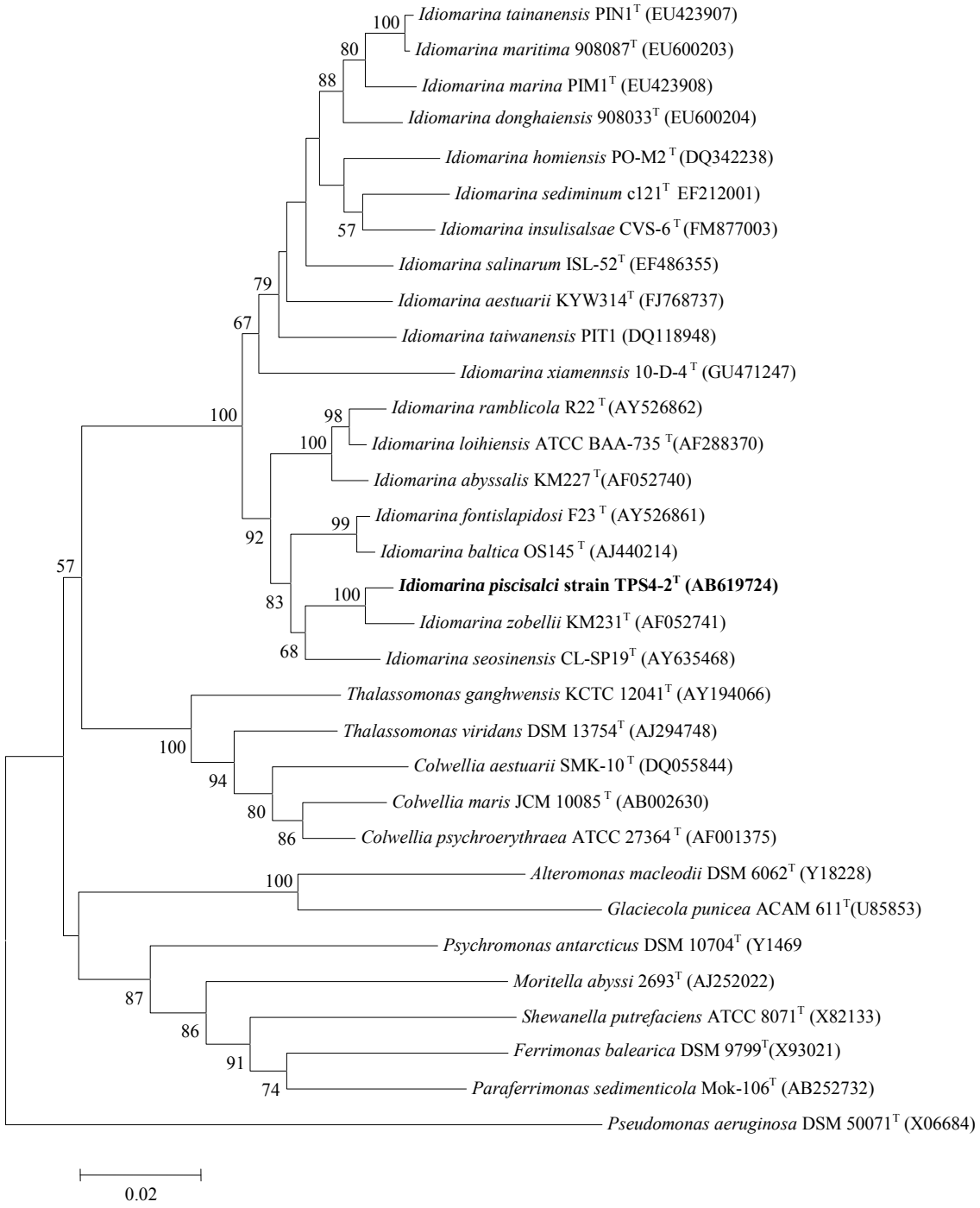


Figure 4.9 Phylogenetic tree showing the relationships between strain TPS4-2$^T$, *Idiomarina sp*. and related taxa based on 16S rRNA gene sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.02 substitutions per nucleotide position

**Table 4.13** DNA-DNA relatedness of TPS4-2$^T$ and related *Idiomarina* species.

| Strain | DNA-DNA relatedness (%) with labelled strains[a] | |
|---|---|---|
| | TPS4-2$^T$ | DSM 15924$^T$ |
| TPS4-2$^T$ | 100 ±0.02 | 51.4 ±0.01 |
| *I. zobellii* DSM 15924$^T$ | 47.7 ±0.01 | 100 ±0.04 |
| *I. fontislapidosi* DSM 16139$^T$ | 7.4 ±0.02 | 11.6 ±0.01 |
| *I. baltica* DSM 15154$^T$ | 24.7 ±0.01 | 27.2 ±0.03 |
| *I. seosinensis* KCTC 12296$^T$ | 14.1±0.02 | 16.8 ±0.06 |

[a]Values are expressed as the means of triplicate determinations.

The relationship among strain TPS4-2$^T$ with its closest phylogenetic neighbours (*I. zobellii* DSM 15924$^T$, *I. baltica* DSM 15154$^T$, *I. fontislapidosi* DSM 16139$^T$ and *I. seosinensis* KTCT12296$^T$) was determined by the $(GTG)_5$ rep-PCR fingerprinting method, a useful technique for determining inter- and intra-species relatedness (Versalovic *et al.,* 1994). The similarity of the fingerprint patterns resulted in the delineation of two $(GTG)_5$-PCR clusters at 53.5% Dice coefficient and UPGMA in Fig. 4.10. Within the first cluster comprises of three strains as TPS4-2$^T$, *I. seosinensis* KTCT12296$^T$ and *I. baltica* DSM 15154$^T$, the $(GTG)_5$-PCR patterns resulted in the delineation at 57.3 and 68.6% Dice coefficient. While the second cluster comprises of two strains as *I. fontislapidosi* DSM 16139$^T$ and *I. zobellii* DSM 15924$^T$, the $(GTG)_5$-PCR patterns resulted in the delineation at 70.6% Dice coefficient and UPGMA.

The result of cluster analysis of the generated $(GTG)_5$-PCR banding patterns of the TPS4-2$^T$ and 4 reference strains are shown in a Fig. 4.10. All strains clearly grouped in separate single-strain clusters. The $(GTG)_5$-PCR genomic fingerprint displayed a high heterogeneity for all strains. It was found that the TPS4-2$^T$ generated unique pattern and different genomic fingerprints from all reference strains, indicated that the strain TPS4-2$^T$ was genotypically different species.

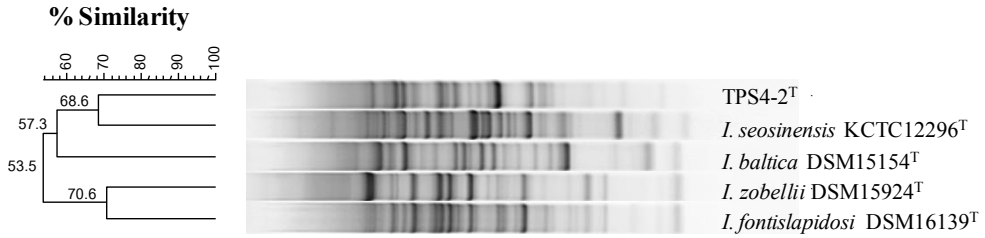


**Figure 4.10** Dendrogram and agarose gel electrophoresis of $(GTG)_5$ -PCR genomic fingerprint of strain TPS4-2$^T$ and 4 type strians of closely related *Idiomarina* species.

The result of phenotypic and chemotaxonomic analyses is shown in Table 4.14. Growth of strain TPS4-2$^T$ occurred within the pH range 6-9 (optimally at pH 8), at 25-45 °C (optimally at 30-37°C) and in 3-25% (w/v) NaCl (optimally in 10-15%, w/v NaCl) but no growth in the absence of NaCl. No growth was observed at 4, 10°C and above 45°C. Positive for catalase, oxidase, hydrolysis of arginine, casein, gelatin and Tween80 but negative for L-tyrosine hydrolysis. In API ZYM test, the strain is positive for acid phosphatase and alkaline phosphatase, esterase(C4), esterase lipase (C8), α-chymotrypsin, napthol-AS-BI-phosphohydrolase, leucine arylamidase, valine arylamidase and cystein arylamidase but negative for nitrate reduction, hydrolysis of aesculin, starch and trypsin, lipase (C14), α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, urease, $H_2S$ production, methyl-red and indole formation. Facultative anaerobe. This strain grew under anaerobic conditions even if $KNO_3$ was omitted. The major respiratory lipoquinone was Q-8.

**Table 4.14** Differential characteristics of TPS4-2$^T$ and related *Idiomarina* species

| **Characteristic** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| Cell shape | SCR | Rods | Curved Rods | SCR | SCR |
| Pigmentation | Opague/ Pale yellow | Clear/ Pale yellow | Cream | Cream | Cream |
| NaCl range (%, w/v) | 3-20 | 1-10 | 0-25 | 3-20 | 1-20 |
| Optimum NaCl (%) | 10-15 | 3-5 | 5 | 5 | 10 |
| Maximum Temperature (°C) | 45 | 30 | 45 | 45 | 40 |
| pH range | 6-9 | 6-9 | 5-9 | 5-9 | 6-10 |
| Arginine hydrolysis | + | - | + | - | + |
| Esculin hydrolysis | - | - | + | + | + |
| Gelatin hydrolysis | + | - | + | + | + |
| Tween 80 hydrolysis | + | - | + | + | + |
| Utilization of (Biolog GN2) | | | | | |
| L-Glutamic acid | - | + | + | - | + |
| L-Alaninamide | + | + | - | + | - |
| L-Arabinose | + | - | + | + | + |
| L-Asparagine | - | + | - | + | + |
| L-Aspartic acid | - | - | - | - | + |
| β-Hydroxybutyric acid | + | - | - | - | - |
| γ-Hydroxybutyric acid | - | + | - | - | - |
| α-Keto Butyric acid | + | + | + | + | - |
| α-Keto Valeric acid | + | - | - | + | - |
| L-Leucine | + | - | - | - | - |
| L-Proline | + | - | - | - | - |
| Propionic acid | + | + | + | - | - |
| Pyruvic acid methyl ester | + | - | - | - | - |
| L-Serine | + | + | - | + | - |
| Succinic acid | - | - | + | - | - |
| Susceptibility to | | | | | |
| Kanamycin | - | + | - | - | - |
| Penicillin G | + | - | - | + | + |
| Sulphonamide | - | - | + | - | - |
| Vancomycin | - | - | - | + | + |
| G+C content (mol%) | 47.0 | 48.6 | 48.0 | 48.2 | 45 |
| Source of isolation | Fermented fish | Sea water* | Baltic sea† | Hypersaline†† | Hypersaline††† |

Strains: 1, TPS4-2$^T$; 2, *I. zobellii* DSM 15924$^T$; 3, *I. baltica* DSM 15154 $^T$; 4, *I. fontislapidosi* DSM 16139$^T$; 5, *I. seosinensis* KCTC 12296$^T$;. +, Positive reaction/growth; w, weakly positive; -, negative reaction /no growth; SCR, slightly curved rods; *Data from Ivanova *et al*., 2000. †Data from Brettar *et al*., 2003. ††Data from Martínez-Cánovas *et al*., 2004. †††Data from Choi *et al*., 2005.

The TPS4-2$^T$ did not produce acid from D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, ribose, sucrose, trehalose and D-xylose. Utilizes L-arabinose, pyruvic acid methyl ester, acetic acid, β-hydroxybutyric acid, α-keto butyric acid, α-keto valeric acid, propionic acid, L-alaninamide, L-alanyl-glycine, Glycyl-L-glutamic acid, L-leucine, L-proline and L-serine but not D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, ribose, sucrose, trehalose and D-xylose. Susceptible to carbenicillin (100 μg), penicillin G (20 units), sulphonamide (300 μg), imipenem (10 μg), erythromycin (15 μg), ampicilllin (10 μg), cephalothin (30 μg) and novobiocin (5 μg) but resistant to kanamycin (30 μg), tobramycin (10 μg), vancomycin (30 μg), streptomycin (10 μg) and gentamicin (10 μg). DNA G+C content of the strain TPS4-2$^T$ was 47.0 mol% which was close to the values observed for other members of the genus *Idiomarina*.

The predominant cellular fatty acids of TPS4-2$^T$ were iso-$C_{15:0}$ (39.8%) and iso-$C_{17:0}$ (14.16%) similar to the closely type strains. Strain TPS4-2$^T$, *I. zobellii* DSM 15924$^T$, *I. baltica* DSM 15154$^T$, *I. fontislapidosi* DSM 16139$^T$ and *I. seosinensis* KTCT 12296$^T$ showed the same cellular fatty acid profiles of iso-$C_{11:0}$, iso-$C_{11:0}$3-OH, iso-$C_{13:0}$, iso-$C_{13:0}$3-OH, $C_{14:0}$, $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$ F, $C_{16:0}$, iso-$C_{16:0}$, $C_{17:0}$, iso-$C_{17:0}$, $C_{17:0\ Cyclo}$, anteiso-$C_{17:0}$, $C_{17:1}\omega8c$, iso-$C_{17:1}\omega9c$, $C_{18:1}\omega7c$, $C_{18:1}\omega9c$, $C_{18:0}$, $C_{18:1}\omega7c$ 11 methyl and $C_{19:0\ Cyclo}\omega8c$ but the significant quantitative amounts were different as shown in Table 4.15.

The strain TPS4-2$^T$ was clearly differentiated from *I. zobellii* DSM 15924$^T$, *I. baltica* DSM 15154$^T$, *I. fontislapidosi* DSM 16139$^T$ and *I. seosinensis* KTCT 12296$^T$ by the growth at a maximum 20% NaCl and at 45 °C, hydrolysis of arginine, gelatin and Tween 80, utilization of carbon sources and susceptible to antibiotics . On the basis of its phenotypic characteristics as well as its phylogenetic position, DNA G+C content, the differential of $(GTG)_5$ rep-PCR fingerprinting and the level of DNA-DNA relatedness less than 70%, the strain represents a novel species of the genus *Idiomarina*, for which the name *Idiomarina piscisalsi* sp. nov. was proposed.

**Table 4.15** Cellular fatty acid composition of strain TPS4-2$^T$ and related *Idiomarina* species. Strains: 1,TPS4-2$^T$; 2, *I. zobellii* DSM 15924$^T$; 3, *I. baltica* DSM 15154$^T$; 4, *I. fontislapidosi* DSM 16139$^T$; 5, *I. seosinensis* KCTC 12296$^T$. -, not detected.

| | Percentage of total | | | | |
|---|---|---|---|---|---|
| **Fatty acid**[a] | **1**[b] | **2**[b] | **3**[b] | **4**[b] | **5**[c] |
| C10:0 3-OH | tr | tr | 1.0 | 1.6 | 1.3 |
| C11:0 ISO | 1.2 | 3.2 | 2.5 | 2.7 | 3.2 |
| C11:0 ISO 3-OH | 3.1 | 3.5 | 2.7 | 2.5 | 5.0 |
| C13:0 ISO | 3.1 | 1.9 | 1.2 | 1.1 | - |
| C13:0 ISO 3-OH | 2.5 | 5.6 | 3.0 | 3.4 | 4.2 |
| C15:0 | tr | tr | 1.0 | tr | - |
| C15:0 ISO | 39.8 | 42.1 | 45.4 | 32.7 | 17.1 |
| C15:1 ISO F | 1.9 | tr | 1.6 | 1.3 | tr |
| C16:0 | 4.5 | 6.7 | 8.3 | 7.2 | 8.9 |
| C16:0 ISO | 1.0 | tr | 1.1 | tr | - |
| C17:0 | 1.2 | 1.3 | 1.2 | 1.6 | 0.8 |
| C17:0 ISO | 14.2 | 15.5 | 10.6 | 11.6 | 15.2 |
| C17:0 CYCLO | 1.5 | 2.5 | 3.2 | 2.3 | 4.5 |
| C17:1 ω8c | tr | tr | tr | 1.2 | - |
| C17:1 ω9c ISO | 8.9 | 4.4 | 4.3 | 4.9 | 8.8 |
| C18:1 ω7c | tr | tr | tr | 1.0 | 8.7 |
| C18:1 ω7c 11 methyl | tr | - | 2.9 | 2.1 | - |
| C18:1 ω9c | 3.2 | 2.7 | 1.2 | 6.5 | tr |
| C18:0 | 1.2 | 1.3 | 1.1 | 2.2 | 3.9 |
| C19:0 CYCLO ω8c | tr | tr | tr | tr | 3.5 |
| Summed feature 3[e] | 3.3 | 1.4 | 3.0 | 7.8 | 2.5 |

[a]Values are percentage of total cellular fatty acids. [b]Data were obtained in this study under the same method. [c]Data from Choi and Cho (2005). [e]Summed feature 3 contained $C_{16:1}$ω7c and /or iso-$C_{15:0}$ 2OH. tr; trace (< 1%)

From above results, all seventeen ALP-producing isolates were identified as follow this. Three isolates in group I, NSW25-1 and SK23-2 from *pla-ra*, and SK22-1 from *pla-som* were identified as *S. saprophyticus* and three isolates in group II, RY30-1, RY30-3 and RY31-2 were identified as *S. napalensis* were isolated from fermented fish with high salt that are different source previously reported (Schleifer & Kloos, 1975; Spergser *et al*., 2003). One isolate, KL6-2 (group III) from *pla-ra* was identified as *S. sciuri*. One isolate, KL2-3 (group IV) from *pla-ra* was identified as *B. vietnamensis*. One isolate, MK10-2 (group V) from *pla-ra* was identified as *B. safensis* was found. Three isolates, KL1-1, KL2-4 and NSW 13-2 (group VI) from *pla-ra* were identified as *V. halodenitrificans*, isolated as mentioned above (Tanasupawat *et al*., 2010). One isolate, NSW13-4 (group VII) from *pla-ra* identified as *O. iheyensis* was found. Three novel species of the isolates in group VIII (NSW13-3 from *pla-ra*) and group IX (NSW13-5 from *pla-ra* and SMK24-1 from *hoi-dong*, fermented mollusces) were closely related to *H. mangrovi* MS10$^T$ and *H. dabanensis* JCM12772$^T$, respectively. One isolate, TPS4-2$^T$ (group X) from *pla-ra* that related to *I. zobellii* KMM231$^T$ was identified as novel specie name *Idiomarina piscisalsi.*

In previously reported, ALPs have been widely studied in extremely halophile bacteria such as *Halobacterium halobium, Halobacterium cutirubrum*, *Haloarcula morismortui* (Goldman *et al*., 1990), the hyperthermophilic archaeon *Pyrococcus abyssi* (Zappa *et al*., 2001) and the hyperthermophilic archaea, *Aeropyrum pernix* (Helianti *et al*. 2007). However, publications of ALPs from the moderately halophile are still limited to *Vibrio* sp. and *Halomonas* sp.593.

The moderately halophilic bacteria isolates from thai fermented fish that produced ALP, were identify as spiecies of genus *Virgibacillus, Halobacillus, Oceanobacillus and Idiomarina*. The isolate TPS4-2$^T$ was identified as novel species name *Idiomarina piscisalsi,* produced highest extracellular ALP activity at 81.5 U/ml. It was provided as potential ALP-producing strains. Because of its novelty strain and high ALP production, the moderately halophile bacteria, *I. piscisalsi* TPS4-2$^T$ was selected to further study ALP purification and characterization.

### 4.2.4 Time course of growth and ALP production.

The growth and ALP production of strain TPS4-2$^T$ were investigated in JCM no.377 medium as shown in Figure 4.11. The lag and exponential phase of bacterial growth was 2 and 22 h respectively and after 24 h the bacterial growth reached to the stationary phase. The extracellular and intracellular ALP began to display at the beginning of exponential phase. ALP production slightly decreased during the middle of cultivation. The highest level of extra- and intracellular ALPs were produced in the stationary phase at 54 and 36 h incubation time, respectively. Both of ALPs virtually did not differ when they were determined by activity staining. Due to a higher activity, intracellular ALP was selected for further studies. The results revealed low level of extracellular production and increased in the late of stationary phase. It should be mentioned that the amount of extracellular ALP might be due to cell autolysis. However, in general of Gram-negative bacteria have periplasmic enzymes, these may be released through the outer membrane under special conditions such as ALP of *Lysobacter enzymogenes* (von Tigerstrom, 1984).

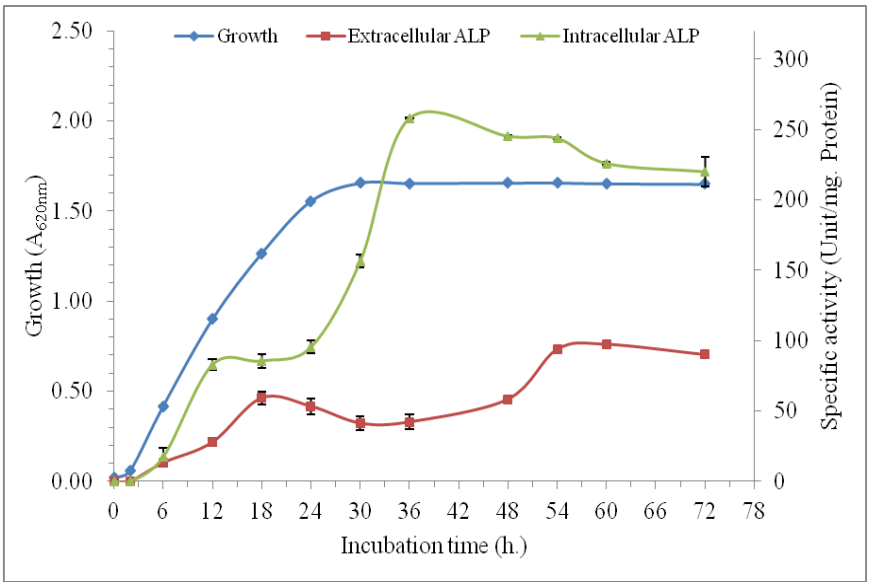


**Figure 4.11** Time course of ALP production by TPS4-2$^T$ in JCM no. 377 medium at 37$^o$C 200 rpm. Means of triplicate determinations are presented, and bars indicate standard deviation. Specific ALP activity is defined as the amount of enzyme that liberates µmol of *p*-nitrophenol per min per mg protein in 0.2 M Tris HCl buffer pH 10.0 at 37 $^o$C 15 min.

### 4.2.5 Characterization of crude intracellular ALP

#### 4.2.5.1 Effect of temperature on crude intracellular ALP activity and stability

The effect of temperature on crude intracellular ALP activity and stability was determined in a range 37-75°C with 0.2 Tris HCl buffer pH 10 in final reaction. As shown in Figure 4.12A and 4.12B, ALP exhibited at least 80% of its optimum activity over a range of temperature from 37 °C to 60°C, and decreased at above 60°C. Therefore, the optimum temperature for crude intracellular ALP activity of TPS4-2$^T$ was 60 °C. This optimum temperature was higher than the previously reported for moderately halophilic bacteria such as *Halomonas sp.* 593 (37-50°C), *Vibrio* sp. (25°C) (Hauksson *et al*., 2000) and halophilic archaeon such as *Haloarcula merismortui* (25°C) (Goldman *et al*., 1990). Thermal stability was determined from ALP incubation at each temperature for 1 h**,** the crude intracellular ALP activity of TPS4-2$^T$ was fully stabled at 37-55°C. The remaining activity was 100% at 37-55°C and decreased gradually at temperature higher than 55°C.

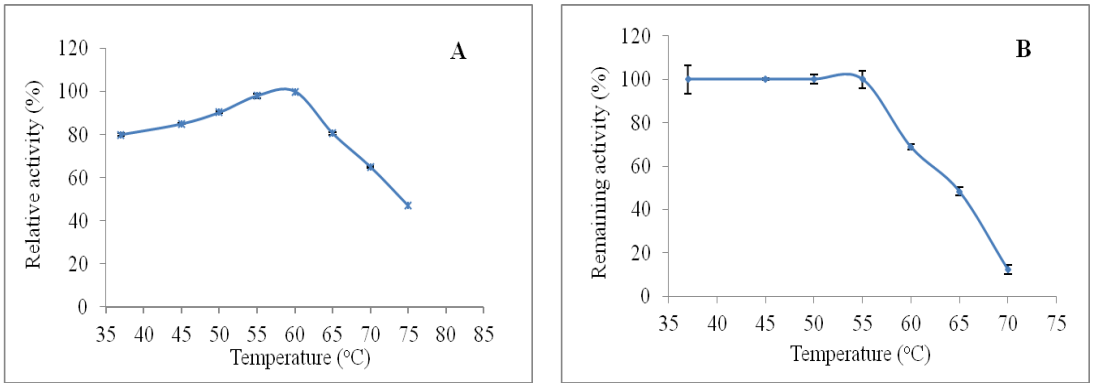


**Figure 4.12** Effect of temperature on crude intracellular ALP activity (A) and stability (B). Means of triplicate determinations are presented, and bars indicate standard deviation. For stability test, the remaining activity were analyzed in 0.2 M Tris HCl buffer pH 10.0 at 60°C for 15 min after incubating the enzyme at various indicated temperature in 50 mM Tris HCl buffer pH 8 for 1 h.

#### 4.2.5.2 Effect of pH on crude intracellular ALP activity and stability

The effect of pH on crude intracellular ALP was determined in 0.2 M Tris HCl buffer pH 7-11 and 0.2 M NaOH-glycine buffer at pH 11-12. As shown in Figure 4.13A, the crude intracellular ALP was active in pH range 7-11 and showed maximum activity at pH 10. The pH stability of ALP activity is shown in Figure 4.13B. The ALP activity was fully stable at pH range of 7-8.8, and the remaining activity slightly decreased to 80% at pH 11 after 1 h incubation at 25°C.

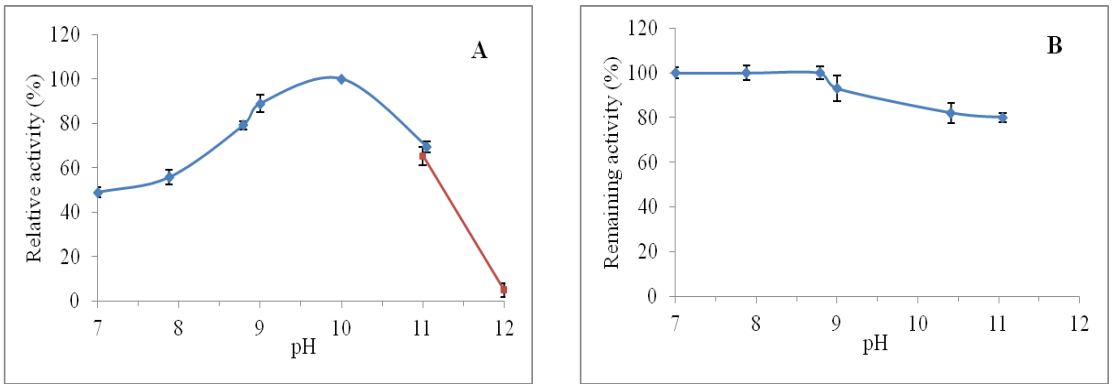


**Figure 4.13** Effect of pH on crude intracellular ALP activity (A) and stability (B). Specific ALP activity was determined the amount of *p*-nitrophenol per min in 0.2 M Tris HCl buffer pH 7-11 and 0.2 M NaOH-glycine buffer pH 11-12 at 60 °C 15 min. For stability test (B), the remaining activity was analyzed after incubating the enzyme at in various indicated pH buffer from 7-11 for 1 h at 25°C. Means of triplicate determinations are presented, and bars indicate standard deviation.

#### 4.2.5.3 Effect of inhibitors on crude intracellular ALP activity

The crude intracellular ALP of TPS4-2$^T$ was completely inhibited by EDTA and EGTA, while inhibitor of acid proteinase, serine protease, chymotrysin & cysteine proteinase, trysin-like proteinase had no significant affect this enzyme.(Table 4.16) Therefore, the ALP from TPS4-2$^T$ was identified to be a metalloenzyme, base on its inhibition profile. Due to the complete inhibition by EGTA, this ALP required calcium for its activity

**Table 4.16** Effect of various inhibitors on crude intracellular ALP activity.

| Inhibitors | Targeted enzyme | Final concentation | Inhibition (%) |
|---|---|---|---|
| Leupeptin | Trypsin-like and some cysteine protease | 10 μM | 0.15±0.01 |
| Soybean trypsin inhibitor | Trypsin-like protease | 0.1 mg/ml | 0 |
| PMSF | Serine protease | 1 mM | 6.21±0.05 |
| Chymostatin | Chymotrypsin-like serine protease | 10 μM | 3.9±0.05 |
| TLCK | Trypsin-like serine protease | 10 μM | 3.9±0.01 |
| TPCK | Chymotrypsin & Cysteine protease | 10 μM | 0 |
| E64 | Cysteine protease | 1 μM | 0 |
| Pepstatin A | Acid proteinase | 2 μM | 4.7±0.01 |
| EDTA | Metalloprotease | 1 mM | 100 |
| EGTA | Metalloprotease : high affinity for Calcium ion | 1 mM | 100 |

#### 4.2.5.4 Effect of NaCl concentration on crude intracellular ALP activity

The effect of NaCl on ALP activity was determined in 0.2 Tris HCl buffer with the presence of NaCl at various concentrations ranging from 0 to 3.5 M under the optimum condition (at 60ºC pH 10.0). The result was shown in Figure 4.14. ALP exhibited the maximum activity in the presence of final concentration of NaCl at 2.5 M or 14.5% NaCl (w/v) in reaction. ALP activity was obtained 80% relative activity in the reaction containing 1.5-3.5 M NaCl. The strain TPS4-2$^T$ grew well in medium with concentration of NaCl 1-2.5 M and was unable to in the absence of NaCl, indicated that TPS4-2$^T$ was a moderate halophilic bacterium according to the definition of Kushner (1985).

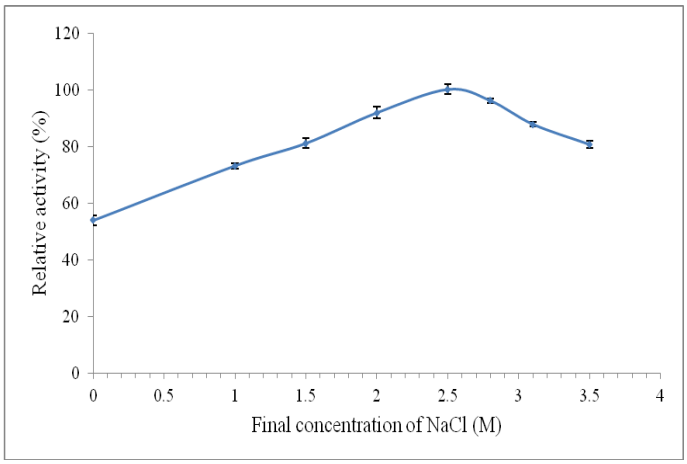


**Figure 4.14** Effect of NaCl concentration on crude intracellular ALP activity.

#### 4.2.5.5 Effect of metal ion on crude intracellular ALP activity

The effect of divalent cation were examined for their influence catalysis on ALP activity (Table 4.17). $Ca^{2+}$ and $Mg^{2+}$ ions enhanced ALP activity. The enzyme exhibited a twofold activation in the presence of 1mM $Ca^{2+}$ in reaction. Such result confirms a strong dependency on $Ca^{2+}$ and the metalloenzymatic nature of ALP from TPS4-$2^{T}$. This ALP activity was inhibited by $Zn^{2+}$ and $Mn^{2+}$ions. This result was similar to ALPs produced from Psychrophilic *Arthrobacter* sp. (De Prada *et al*., 1996), *Thermus caldophilus* GK24 (Park *et al*., 1999) and *B. intermedius* S3-19 (Sharipova *et al*., 2000), their ALPs were inactivated by $Zn^{2+}$ ion. However, many researchers have previously reported that $Zn^{2+}$ and $Mg^{2+}$ ions are important for the structure and activity of ALPs from human, shrimp, *E. coli*, *Vibrio* sp., *Halobacterium salinarum* and other bacteria (Wende *et al*., 2010).

**Table 4.17** Effect of divalent metal ions on activity of crude intracellular ALP

| Metal ion | Final concentration | Relative activity (%) |
|---|---|---|
| None | - | 100±0.08 |
| $Ca^{2+}$ | 1 mM | 232.4±0.20 |
| $Cu^{2+}$ | 0.1 mM | 100±0.11 |
| $Mg^{2+}$ | 1 mM | 151.4±0.09 |
| $Mn^{2+}$ | 0.1 mM | 77.9±0.99 |
| $Zn^{2+}$ | 1 mM | 54.6±0.22 |

*ALP activity in the absence of chemical mentioned above was set as 100%. Relative activity are presented in the means value of triplicate determinations with standard deviation.

### 4.2.6 Purification of *Idiomarina piscisalsi* TPS4-2$^T$ ALP

Purification step of ALP is summarized in Table 4.18. After the filtrate was concentrated by ultrafiltration with 10,000 MWCO, total activity (24604.7 units) of approximately 91.4% remained, while 8.6% of total protein was removed. From this result, purity of 1.3 fold was achieved after ultrafiltration. After loading the concentrated filtrate onto anion exchanger, HiTrap DEAE FF column, the column was eluted by a 0-1.0 M NaCl gradient. Five protein peaks ($A_{280}$) was separated which an activity peak was eluted at 0.2-0.4M NaCl (Figure 4.15). ALP activity was detected at 0.26 M NaCl in buffer elution or fraction number 37-38. Large amount of proteins was removed after elution with 0-0.2 M NaCl, leading to an increase in purity fold of 11. Fractions number 37-38 with ALP activity were pooled. Specific activity of the pooled fractions increased to 1572.2 units/mg protein.

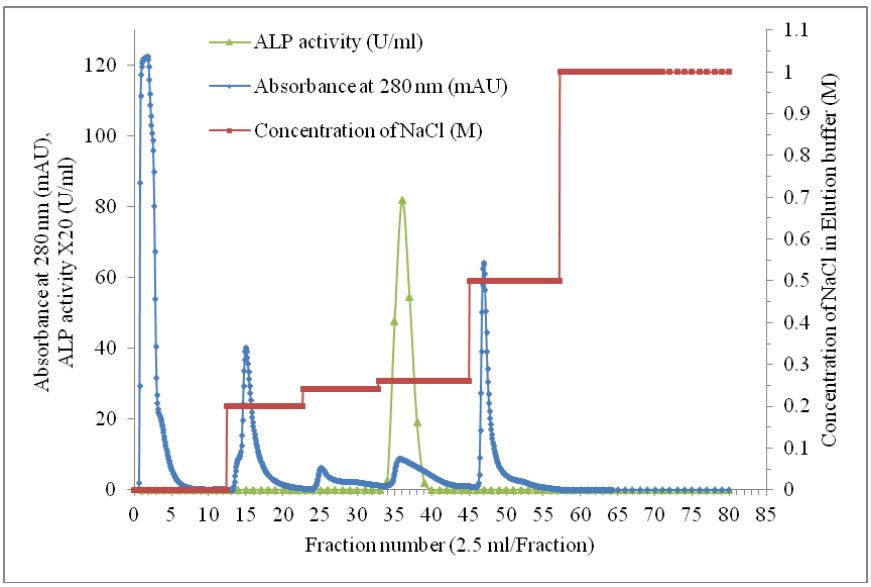


**Figure 4.15** Purification of TPS4-2$^T$ ALP using FPLC on HiTrap DEAE FF column equilibrated with 50 mM Tris HCl pH8.0, then eluted at a flow rate of 2.5 ml/min with step-wise gradient of 0 to 1.0 M and 2.5 ml per fraction were collected. The elution profile was monitored by spectrophotometry at 280 nm.

The pooled active HiTrap DEAE FF fractions were further purified by a size exclusion chromatography using Superose 12 10/300. The column separated ALP enzyme from other proteins by molecular size (Figure 4.16). After loading the concentrated filtrate onto a Superose 12 10/300 column, the column was eluted by a 0.15 M NaCl in 50 mM Tris HCl pH 8.0. Four protein peaks ($A_{280}$) was separated which an activity peak was eluted at fraction number 23-24 as shown in Figure 4.16. Fractions with activity were pooled. Specific activity of the pooled fractions increased to 1727.8 units/mg protein. Purity of ALP increased to 12.1 fold with a yield of 12.1 % in this step.

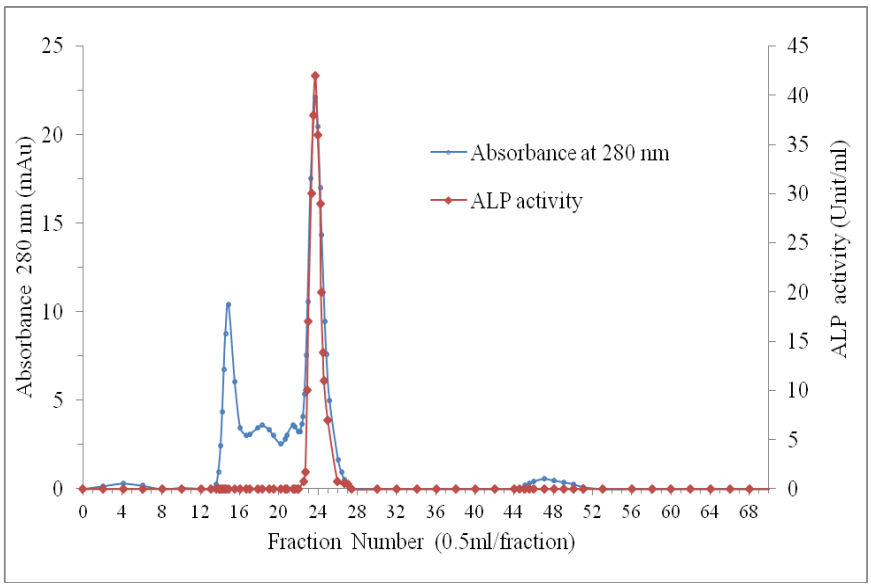


**Figure 4.16** Size-exclusion of patially purified ALP from TPS4-2$^T$ using FPLC on Superose 12 10/300 GL column. The standard marker was eluted through a Superose 12 10/300 GL column equilibrated with 50 mM Tris HCl pH8.0 containing 0.15 M NaCl pH 8.0 at a flow rate of 0.1 ml/min and 0.5 ml per fraction were collected. The elution profile was monitored by spectrophotometry at 280 nm.

**Table 4.18** Preparation of partially purified enzyme for characterization

| **Purification steps** | **Total activity (Units*)** | **Total protein (mg**)** | **Specific activity (U/mg)** | **Purity (Fold)** | **Yield ( %)** |
|---|---|---|---|---|---|
| Crude extract | 26930.6 | 189.0 | 142.5 | 1.0 | 100 |
| Amicon 10K | 24604.7 | 138.6 | 177.5 | 1.3 | 91.4 |
| Hitrap DEAE FF | 6296.5 | 4.0 | 1572.2 | 11.0 | 23.4 |
| Superose 12 | 3265.4 | 1.9 | 1727.8 | 12.1 | 12.1 |

The unit of enzyme activity is expressed as the µmol of *p*-nitrophenol released per min under standard condition assay. **Protein concentration was measured by Lowry method.

The purity of the purified ALP enzyme from TPS4-2$^T$ was evaluated by native gel electrophoresis, followed by activity staining (Figure 4.17A and 4.17B). Protein pattern of concentrated ALP during purification process is also shown in Figure 4.17A. The concentrated filtrate (crude enzyme) contained variety of proteins with different molecular weights (lane 1 and 2). After subjected to Hitrap DEAE FF column (lane 3-7), some proteins bands disappeared due to gradient elution below 0.26 M NaCl and above 0.4 M NaCl in 50 mM Tris HCl buffer pH 8.0. However, further purification on Superose 12 10/300 size exclusion chromatography did not result in a significant protein removal and the dominated protein band position was agreed well with the activity band observed on activity staining gel (Figure 4.17B)

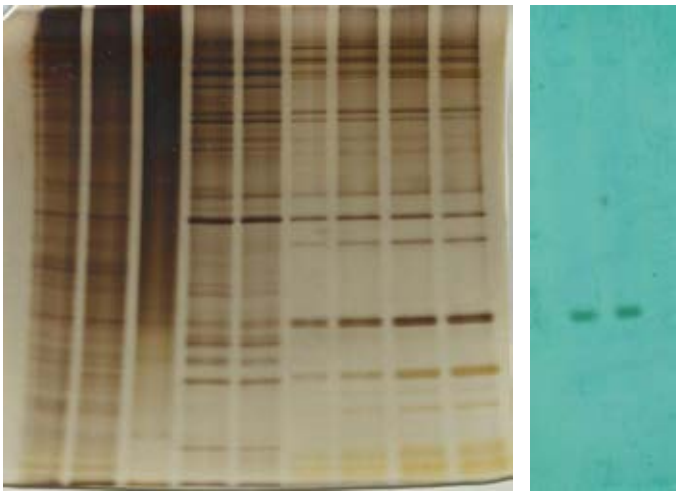


**Figure 4.17** Protein pattern (A) and activity staining (B) of the partially purified ALP from TPS4-2$^T$ on native gel electrophoresis and ALP activity staining at pH 10.0, 37°C. Lane 1-2 crude enzyme extract; lane 3, Unbound in fraction 2 pass HiTrap DEAE FF; lane 4-5, HiTrap DEAE FF (fraction no.25 and 26), lane 6-7, HiTrap DEAE FF (fraction no.37 and 38), lane 8-9, Superose 12 10/300 (fraction no.23 and 24). Approximately same amount (0.85 μg) of protein on each lane was applied to 12.5% polyacrylamide gel and electrophoresis was carried out for 85 minute at 20 mA per gel. After electrophoresis, the left-hand gel (A) was protein stained with silver nitrate and the right-hand gel (B) was activity stained by the method described in materials and methods (5 Units/well).

### 4.2.7 Characteristics of the partially purified TPS4-2$^T$ ALP

#### 4.2.7.1 Molecular mass of partially purified TPS4-2$^T$ ALP

After loading the concentrated sample onto size exclusion chromatography using Superose 12 10/300 column, the column was eluted by a 0.15 M NaCl in 50 mM Tris HCl pH 8.0. The elution chromatrogram was shown in Figure 4.16, five protein peaks ($A_{280}$) was separated which the ALP activity was deteced in the fouth peak at elution fraction number 23 and 24. The molecular mass of the partially purified ALP calculated by buffer elution volume that had ALP activity, it was 53±0.2 kDa by gel filtration (Figure 4.18). The molecular mass was closest to ALP of the euryarchaeon *Pyrococcus abyssi* (54kDa)(Zappa *et al.,* 2001).

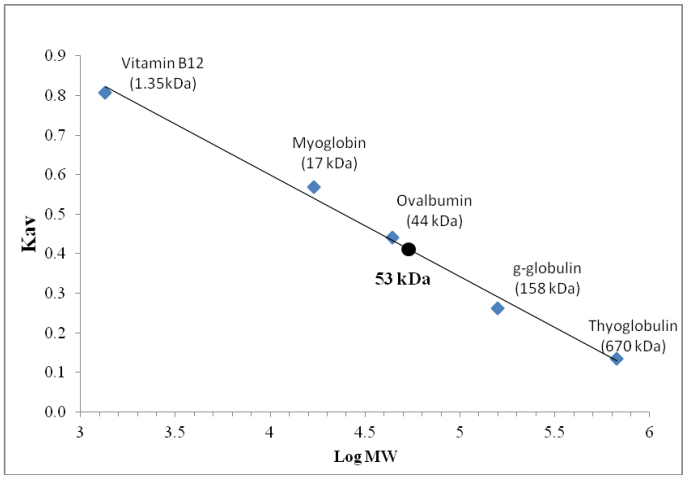


**Figure 4.18** Calibration curve for the molecular weight determination on Superose 12 10/300 chromatography. ●; partially purified ALP from TPS4 -2$^T$.

#### 4.2.7.2 Effect of temperature on activity and thermal stability of the partially purified ALP

The temperature profile of the partially purified ALP activity and stability was shown in Figure 4.19. The result revealed that the ALP of TPS4-2$^T$ was moderately thermoactive ALP, that the temperature increased the activity of enzyme also increased. Its optimum activity showed et 60$^o$C and becamed inactivated at 65$^o$C. This optimum temperature was higher than the optimum growth temperature

(30-37°C). However, this strain can grow at 45°C. The temperature range of ALP activity was 50-70°C with 70% of maximum activity (Figure 4.19A).

The activity decreased when beyond this range temperature. However its activity remained 40% of maximum activity at 37°C and 75°C. The optimum temperature was higher than the value reported for moderately halophile *Halomonas* sp. 593 (37-50°C) (Ishibashi *et al*., 2005) and *Vibrio* sp. (25°C) (Hauksson *et al*., 2000). As known the ability of moderately halophilic bacteria to grow over wide range of salinities make all of their biosynthetic inventory such as compatible solutes including enzymes showing optimal activities at different values of pH, salt concentration and temperature. In addition, their compounds or enzymes were accumulated in periplasmic space, must function under more fluctuation or severe conditions than cytoplasmic enzymes, because they are exposed directly to varities environment. Therfore, ALP of the moderately halophilic TPS4-2$^T$ could active at higher temperature than optimum growth temperature (Ishibashi *et al*., 2005).

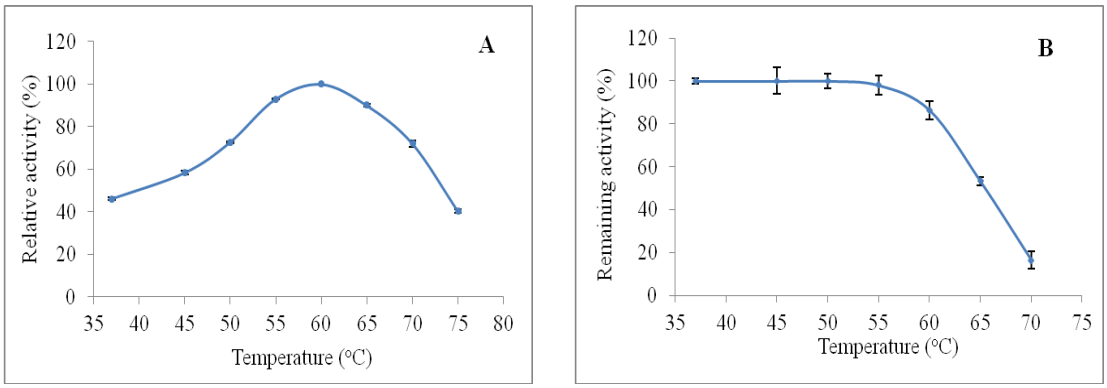


**Figure 4.19** The effect of temperature on ALP activity (A) and stability (B) of the partially purified ALP from TPS4-2$^T$. Means of triplicate determinations are presented, and bars indicate standard deviation. Optimum activity (A) was analyzed using pNPP as a substrate for 15 min, pH 10.0 at various indicated temperature. For the stability test (B), the enzyme was incubated at various indicated temperature for 1 h in 0.2 M Tris-HCl buffer (pH 8) and then cooled on ice. The remaining activity was analyzed using *p*-NPP as a substrate pH 10.0 at 60°C for 15 min.

Thermal stability profile was shown in Figure 4.19B. ALP activity of TPS4-2$^T$ was fully stable at 37-55°C with 100% of its remaining activity after incubating at each temperature for 1 h. It was not stable under its optimum temperature. The decrease of stability at higher temperature or above 55$^o$C, was possibly due to the partially unfolding of the enzyme molecule. The high temperature increases the kinetic energy of molecules which in turn breaks the bond that holding the active amino groups and enzyme is finally denatured and losses enzyme activity (Bryan and Keith, 1981). The activity of TPS4-2$^T$ remained 80% and 50% of maximum activity after incubating for 1 h at 60$^o$C and 65$^o$C, respectively. Comparison on other moderately halophile bacteria, It was more stable than reported ALP of *Halomonas* sp. 593, that lost 80% activity with heat at 60$^o$C for 5 min. and ALP of *Vibrio* sp., that was heat-labile with half life of only six minute at 40$^o$C. For mesophillic such as *Bacillus substlis* had a half-life at 65$^o$C for 28 min. and *Rhizopus microsporus* var. *rhizopodiformis* (Junior *et al*., 2008) was stable up to 50 °C for at least 15 min. The thermostability of ALP of TPS4-2$^T$ at optimum temperature 60$^o$C pH 10.0 may be convenient for biotechnology applications.

#### 4.2.7.3 Effect of pH on partially purified ALP activity and stability

The effect of pH on the activity of partially purified ALP from TPS4-2$^T$ was determined in different pH between 7-12 with 0.2 M Tris HCl buffer pH 7-11 and 0.2 M NaOH-glycine buffer pH 11-12. As shown in Figure 4.20A, the partially purified enzyme was active at pH 9-10.5, and exhibited maximum activity at pH 10. The activity was found 80% of the maximum activity pH range 9-10.5. The pH range was very narrow. Only 60% of the maximum activity was detected at pH 11. It was considerable loss of activity at pH ≤8.0 and at pH ≥11.5. The pH has effect to the charges on the amino acids in the active site such that the enzyme is unable to form enzyme substrate complex. Thus, enzyme will decrease or lost its activity. (Hulett *et al.*, 1971; Mahesh *et al*., 2010)

An effect of pH on ALP stability was shown in Figure 4.20B. Only 70% of remaining activity was stable in narrow pH range value 7.0-8.0 after incubation for 1 h at 25$^o$C. The remaining activity after incubation for 1 h at pH 9, 10 and 11 was decrease to 50, 45 and 40% respectively. The optimal pH of the enzyme activity was

10.0, similar to the values reported for moderately halophilic bacteria such as *Vibrio sp.,* (Hauksson *et al*., 2000), *Halomonas sp.* (Ishibashi *et al*., 2005) and archaeon, *Aeropyrum pernix* K1 (Helianti *et al*.,2007)

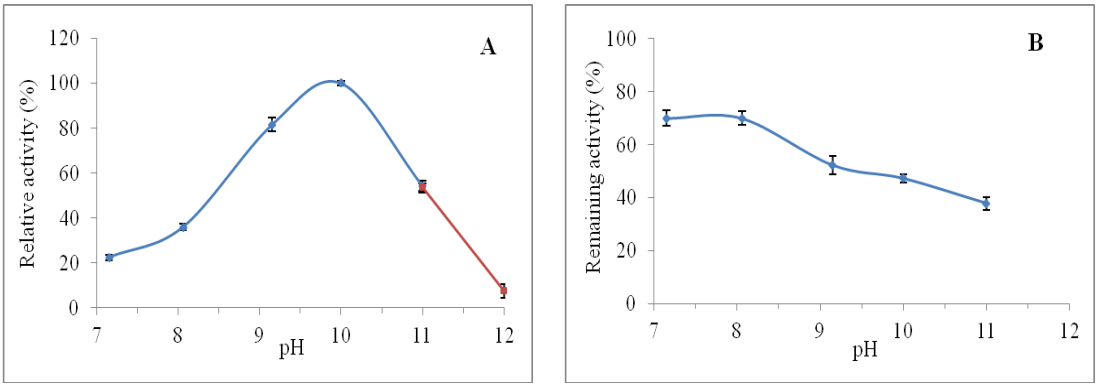


**Figure 4.20** The effect of pH on ALP activity (A) and stability (B) of the partially purified ALP from TPS4-2$^{T}$. Means of triplicate determinations are presented, and bars indicate standard deviation. An activity (A) was analyzed at various indicated pH by using 0.2 M Tris HCl buffer pH 7-11 and NaOH-glycine buffer pH 11-12. For the stability test (B), The remaining activity was analyzed after incubating the enzyme at various indicated pH buffer for 1 h at 25°C. Activity analyzed by using pNPP as substrate, pH 10.0 at 60°C for 15 min.

#### 4.2.7.4 Effect of salt concentration on partially purified ALP

ALP activity was measured in the presence of various NaCl concentrations (0-4 M NaCl), the maximal activity was obtained in the presence of 0.3 M NaCl and the activity more than 70% of the maximal activity was detected in the presence of 0.1-0.5 M NaCl. It was 50-57% in the presence of NaCl lower than 0.1 M. The activity decreased when concentration of NaCl increased higher than 0.3 M. It decreased to 38-40% of the maximal activity if concentration of NaCl reached to 1-4 M. The result was slightly similar to purified ALP of moderately halophillic bacteria *Halomonas* sp. 593 that its activity was maximum at 0 M NaCl concentration. And the level of activity gradually decreased when the concentration of NaCl increased. However, it required at least 0.3 M for its stability. ALP activity of *Vibrio cholerae*

was severely inhibited in the presence of NaCl, its activity decreased to 21-47% in the presence of 0.2 M NaCl. (Ishibashi *et al*., 2005)

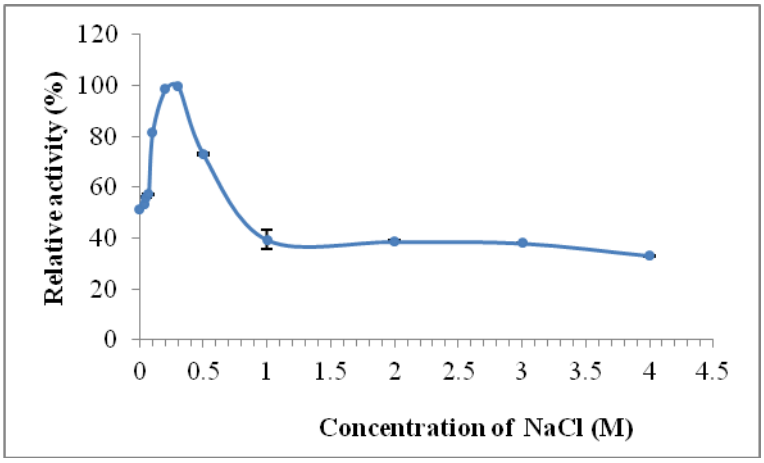


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**Figure 4.21** The effect of NaCl on ALP activity of the partially purified ALP from TPS4-2$^T$. Means of triplicate determinations are presented, and bars indicate standard deviation. Activity was analyzed in various NaCl concentration using *p*-nitrophenylphosphate as a substrate at 60°C, pH 10.0 for 15 min.

Results from purification process revealed that ALP from TPS4-2$^T$ might required low salt concentration for their activity. It was shown that the relative activity (50-57%) was detected in the absence of salt (0-0.1M) but higher activity was observe when salt increased and reached the maximum activity at 0.3 M but it seemed to be higher in the presence of salt and reached the maximum at 0.3 M NaCl (Figure 4.21). Nevertheless, the ALP activity declined at 0.5-4 M NaCl. In general, the solubility of each protein depends on the level of hydrophobic or hydrophilic properties of their surface (Lanyi, 1974). The addition of ions such as sodium chloride creates an electron shielding between water molecules and the protein which in turn reducing protein solubility resulting in aggregation of protein. In the case of halophilic or salt requirement enzymes, they have large excess of acidic amino acids such as glutamic acid and aspartic acid shielding on their surface. These amino acids bind with hydrate ions which reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentrations (Boutaiba *et al*., 2006).

However, the activity of enzyme will subsequently decline when reaching the maximum because the salt bridge of acidic amino acid is occurred which affects straightening of an alpha helix that promotes unfolding of proteins (Ophardt, 2003). This changes the shape of enzyme and decreases the rate of reaction which known as denaturation. The results obtained here showed that crude ALP from TPS4-2$^T$ tended to be halophilic enzyme as the relative activity was found to increase when sodium chloride was added (2.5 M). In addition, the relative activity of partial purify ALP from TPS4-2$^T$ tended to decrease when high concentration of sodium chloride (>0.3M) was added. These was suggested that some acidic amino acids (i.e. glutamic acid and aspartic acid) shielding on crude enzyme surface may be eluded during purification process especially in HiTrap DEAE FF column, therefore, surface of partial purified protein became more hydrophobic allowing the proteins bind with each others and begin to aggregate at high salt concentration. For that reason, its activity was decreased when high sodium chloride concentration was added in the assay reaction.

**Table 4.19** Effect of various inhibitors on activity of the partially purified ALP.

| Inhibitors | Targeted enzyme | Final concentation | Inhibition (%) |
|---|---|---|---|
| None | Without protease inhibitor | | 9.3±0.55 |
| Leupeptin | Trypsin-like and some cysteine protease | 10 μM | 0 |
| Soybean trypsin inhibitor | Trypsin-like protease | 0.1 mg/ml | 29.1±2.08 |
| PMSF | Serine ALP | 1 mM | 14.1±2.08 |
| Chymostatin | Chymotrypsin-like serine protease | 10 μM | 38.4±1.32 |
| TLCK | Trypsin-like serine protease | 10 μM | 11.2±1.32 |
| TPCK | Chymotrypsin & Cysteine protease | 10 μM | 4.72±3.18 |
| E64 | Cysteine protease | 1 μM | 7.12±2.3 |
| Pepstatin A | Acid proteinase | 2 μM | 9.1 ±2.5 |
| EDTA | Metalloprotease | 1 mM | 0 |
| EGTA | Metalloprotease : high affinity for $Ca^{2+}$ion | 1 mM | 0 |

**4.2.7.5 Effect of inhibitors on the partially purified ALP activity**

Activity of the partially purified ALP of TPS4-2$^T$ was completely inhibited by metal ion chelators, EDTA and EGTA. There was no significant effect of inhibitors of acid proteinase, serine protease, cysteine proteinase and trypsin-like proteinase (Table 4.19). The result suggested that the ALP from TPS4-2$^T$ was a metalloenzyme and it was related to calcium metalloenzyme because it was blocked with EGTA that was high affinity for $Ca^{2+}$ion. In addition, trypsin-like protease and chymotrypsin-like serine protease could decrease this enzyme around 29-38% of maximum activity because the enzymatic reaction proceeds through a covalent serine-phosphate intermediate to produce inorganic phosphate and an alcohol (Schwartz & Lipmann, 1961).

**4.2.7.6 Effect of metal ion on the partially purified ALP activity**

Effect of divalent metal ions on activity of the partially purified ALP from TPS4-2$^T$ was examined at 1mM of $CaCl_2$, $MgCl_2$ and $ZnCl_2$ and at various. $Ca^{2+}$ and $Mg^{2+}$ ions enhanced the activity, whereas $Zn^{2+}$ had affected on the activity (Table 4.20). $Zn^{2+}$ concentration higher than 0.1 mM inhibited metalloenzyme (Zhang *et al*., 2003 and Karbalaei-Heidari *et al*., 2006). The inhibition was probably due to the formation of zinc monohydroxide that bridged the catalytic zinc ion to side chain of an active site (Larsen and Auld. 1991). It was similar to the membrane-bound and extracellular ALP form *B. intermedius* S3-19 that was activated by 1-3 M $Ca^{2+}$and $Mg^{2+}$ion but was inactivated by $Zn^{2+}$ion. (Sharipova *et al*., 2000) and ALP from *Aeropyrum premix* K1 was stimulate by $Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$ and $Co^{2+}$ion (Helianti *et al*., 2007). Previously, ALPs from many bacteria were characterized and reported that they were stimulated by $Zn^{2+}$, and $Mg^{2+}$ion such as *E. coli*, psychrophilic *Vibrio* sp., *Lyzobacter enzymogen* and *Bacillus* sp.(Dhaked *et al*., 2005).

Therefore, $Ca^{2+}$ and $Mg^{2+}$ ion are more necessary for partially purified ALP activity from TPS4-2$^T$ than $Zn^{2+}$ with the optimal concentration at 1 mM (Table 4.20). These can be explained that kinetic rate constant of ALP from TPS4-2$^T$ can increased via $Ca^{2+}$ and $Mg^{2+}$ ion by binding with the active enzyme [$E_a$] to make enzyme in more active form, thus it make enzyme as suitable conformation (native form) to bind with substrate (Goldman *et al*., 1990), therefore, the rate of reaction was increased when $Ca^{2+}$ and $Mg^{2+}$ ion was added in the assay condition. ALP from

TPS4-2$^T$ exhibited low activity when $Zn^{2+}$ added. In typical, the conserved active site of ALP having three divalent metal ions (two $Zn^{2+}$and one $Mg^{2+}$) as the essential catalytic mechanism of ALP (Bobyr *et al*., 2012). Hoever, addition of $Zn^{2+}$ could decrease ALP activity as reported in many previous ALPs (Bosron *et al*., 1997; Stec *et al*., 1998; Helland *et al*., 2009;) because $Zn^{2+}$ bind hydroxide ion in the initial nucleophilic serine that $Mg^{2+}$ usually bind for catalysis and make enzyme more stable. Therefore, adding $Zn^{2+}$ may inhibit ALP activity by repressing $Mg^{2+}$ to bind hydroxide ion of nucleophilic serine. This result was as same as ALP from the archaebacterium *Haloarcula marismortui* that requires $Ca^{2+}$ and $Mg^{2+}$ but not $Zn^{2+}$ ions for its activity (Glodman *et al*., 1990).

**Table 4.20** Effect of divalent metal ions on activity of the partially purified ALP

| Metal ion | Final concentration | Relative activity (%) |
|---|---|---|
| None | - | 100* |
| $Ca^{2+}$ | 1 mM | 147.8±0.01 |
| $Cu^{2+}$ | 0.1 mM | 100±0.02 |
| $Mg^{2+}$ | 1 mM | 142.6±0.02 |
| $Mn^{2+}$ | 0.1 mM | 90±0.01 |
| $Zn^{2+}$ | 1 mM | 87.4±0.02 |

*ALP activity in the absence of chemical mentioned above was set as 100%. Relative activity are presented in the means value of triplicate determinations with standard deviation

#### 4.2.7.7 Kinetic studies

The kinetic parameters of the partially purified ALP was studied using *p*-nitrophenylphosphate as substrate under the optimum conditions (60$^o$C, pH 10.0 for 15 min). The following kinetic constant was calculated from the double reciprocal plots of the initial reaction rates and substrate concentration between 0.1-4 mM. The partially purified ALP has $V_{max}$ of 28.01 µmol/min which was higher than purified ALP from euryarchaeon *Pyrococcus abyssi* that have $V_{max}$ at 8.31 µmol/min (Zappa *et al*., 2001). The $K_m$ value for *p*-NPP of partially purified ALP from TPS4-2$^T$ was 0.09 mM. This value was closely to the $K_m$ values of *E. coli* ($K_m$ = 0.07 mM) that assay at pH 10, 15°C. This value was compared to the $K_m$ values of ALP from *Vibrio* sp. and *E. coli* as reported by Hauksson *et al*., (2000) at various conditions assay was shown in Table 4.21. At pH 10 and optimum temperature, the $K_m$ of TPS4-2$^T$ was lower than the $K_m$ values from *Vibrio* sp. G15-21 ($K_m$ =2 mM) and *E.coli* ($K_m$ =0.22

mM). In addition, It also lower than others previously reported $K_m$ values such as the purified ALP from *Pyroccocus abyssi* (0.167 mM) (Zappa *et al*., 2001), *Cenococcum graniforme* ($2.1x10^{-4}$M) (Bae and Barton, 1989) and calf intestine ($9.6x10^{-4}$M). The turnover number ($k_{cat}$) and catalytic efficientcy ($k_{cat}/K_m$) were 29.1 $s^{-1}$ and 3.3 x $10^5$ $M^{-1}s^{-1}$, respectively. The $k_{cat}$ and $k$cat/$K_m$ values were compared to the values of purified ALPs from *Vibrio* sp. G15-21 *and E.coli* in Table 4.21. The $k_{cat}$ of ALP from TPS4-$2^T$ was less than but the $k_{cat}/K_{m\ value}$ was in the same range.

**Table 4.21** Kinetic analysis of partially ALP from TPS4-$2^T$ compared to the previously reported ALP from *Vibrio sp. G15-21* and *E. coli.*

| Microorganism | Temperature ($^oC$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|---|
| TPS 4-2 | 60 | 0.09 | 29.1 | 3.3 x $10^5$ |
| *Vibrio sp.* G15-21* | 37 | 2.00 | 1024 | 5.1 x $10^5$ |
| *E. coli** | 37 | 0.22 | 104 | 4.9 x $10^5$ |
| *Vibrio sp.* G15-21* | 25 | 0.96 | 350 | 3.7 x $10^5$ |
| *E. coli** | 25 | 0.14 | 38 | 2.8 x $10^5$ |

*All data at pH 10.0 from Hauksson *et al*., 2000

The moderately halophilic bacterium, strain TPS4-$2^T$ was isolated and identified to propose as a novel species named *Idiomarina piscisalsi* TPS4-$2^T$. This novel, TPS4-$2^T$ species produced intracellular alkaline phosphatase (ALP) higher than extracellular ALP. An intracellular ALP was purified 11-fold with the specific activity 1572.2 U/mg using Hitrap DEAE FF column. The partially purified ALP had a molecular mass of about 53 kDa (calculated by gel filtration). The optimal pH and temperature for its activities was pH 10.0 and 60$^o$C. The enzyme had a maximal activity in the presence of 1 mM $CaCl_2$ or 0.3 M NaCl, pH 10.0 and at 60°C. Its activity remained about 40% in the presence of NaCl ranging 1-4 M, which distinguished from crude intracellular ALP activity that increased when NaCl was increased to 2.5 M in reaction under the same other condition. The partially purified ALP was stabled at pH 7.0-8.0 and at 37–55°C for 1 h. The ALP of TPS4-$2^T$ was identified to be metalloenzyme that was stimulated by $Ca^{2+}$ and $Mg^{2+}$.

# CHAPTER V

# CONCLUSION

In the course of investigation of halophilic lactic acid bacteria presented in fermented fish, forty-one bacterial isolates were characterized taxonomically. Based on their phenotypic characteristics, 16S rRNA PCR products with restriction endonucleases digestions and 16S rRNA gene sequence analyses of the representative isolates. They were classified as the genus *Tetragenococcus* and could be identified into two species namely *T. halophilus* (22 isolates in group A) and *T. muriaticus* (19 isolates in group B). All strains were Gram-positive moderately halophilic bacteria, facultatively anaerobic, non-spore forming cocci, non-motile, arranged in pairs or tetrads. Colonies on MRS agar plate supplemented with 10% (w/v) NaCl were circular low convex with entire margin and nonpigmented. All produced L-lactic acid homofermentatively from glucose. Most of the strains were able to grow at 40$^{o}$C (but not at 45$^{o}$C), in the presence of 5-25% (w/v) NaCl at pH ranging from 6-9 and produced acid from glucose, D-galactose, D-maltose, D-mannose and D-ribose.

Of the identified isolates, *T. muriaticus* strain KS87-14 could produce the highest level of histamine at 626 mg/l when compared with *T. muriaticus* JCM 10006$^{T}$ (78 mg/l) and *T. muriaticus* JCM 10007 (45 mg/l) that were used as reference strains and cultured under the same condition for identification and histamine formation, respectively. This strain produced histamine 10 times (626 mg/ml) higher than the reported *T. muriaticus* strains did. The positive detection of KS87-14 was achieved by using primers JV16HC and JV17HC specific for the *hdc*A, a structural gene of pyruvoyl dependent histidine decarboxylase of various histamine-producing Gram-positive bacteria. Compared with previously published partial nucleotide sequences for the *hdc*A, the amplified fragment sequence of KS 87-14 appeared to be 100% similar to the staphylococcal *hdc*A gene.

In the part of screening of ALP-producing moderately halophillic acid bacteria isolation. The seventy-six isolates moderately halophillic acid from thirty-three fermented fish were screened. Only seventeen isolated exhibited ALP activity and they were characterized taxonomically. Seven isolates (group I, II and III) were

Gram-positive non-motile and non-spore forming cocci. They were catalase-positive but oxidase-negative. They were identified as *S. saprophyticus*, *S. napalensis* and *S. sciuri*, respectively. Two isolates in group IV and V belonged to the genus *Bacillus* were Gram-positive spore forming rods and identified as *B. vietnamensis* and *B*. *pumilus*, respectively.

Three isolates in group VI (KL1-1, KL2-4 and NSW13-2) were Gram-positive, spore forming rods and identified as *V. halodenitrificans*, group VII (1 isolate) was identified as *O. iheyensis*. Group VIII (1 isolate, NSW13-3) was Gram-positive, spore forming rods. On the basis of 16S rRNA gene sequence analyses, this isolates was closely related to *H. mangrovi* MS10$^T$ with 97.9% sequence similarity. The isolate showed different phenotypic characteristics from *Halobacillus mangrovi* MS10$^T$ and contained *meso*-diaminopimelic acid in the cell wall which was absent in *Halobacillus mangrovi* MS10$^T$. Therefore, the isolate NSW13-3 should be confirmed for DNA-DNA hybridization with the related *Halobacillus* species for the proposal of the new species. Group IX (2 isolates, NSW13-5 and SMK24-1) belonged to the genus *Halobacillus*, were Gram-positive, spore forming rods. They produced catalase and most of them were negative with oxidase. All isolates contained *meso*-diaminopimelic acid in the cell wall. They showed the difference in the colonial pigmentation and the growth in high concentration of NaCl. Their phenotypic characteristics and DNA-DNA hybridization are required for further studies with the related *Halobacillus* species. One isolate in group X (TPS4-2$^T$) belonged to genus *Idiomarina* was a Gram-negative non-spore forming rod shaped bacterium. All the isolates in each group were negative reaction for citrate utilization, indole and tyrosine hydrolysis. Group I, II, III, IV, V and VII isolates were halotolerant and group VI, VIII, IX and X isolates were moderately halophilic bacteria.

A Gram-negative, aerobic, moderately halophilic bacterium, strain TPS4-2$^T$, was isolated from fermented fish (*pla-ra*) in Thailand. The cells were slightly curved rods, motile and non-endospore-forming. This strain grew optimally at 30-37 $^oC$, pH 8 and in the presence of 10-15% (w/v) NaCl. The predominant ubiquinone was Q-8. The major cellular fatty acids were iso-$C_{15:0}$ and iso-$C_{17:0}$. DNA G+C content was 47.0 mol%. Comparative 16S rRNA gene sequence analyses indicated that strain TPS4-2$^T$ was placed in genus *Idomarina* and closely related to the species *I. zobellii*

DSM 15924$^T$ (98.7%), *I. baltica* DSM 15154$^T$ (96.9%), *I. fontislapidosi* DSM 16139$^T$ (96.6%) and *I. seosinensis* KTCT12296$^T$ (96.4%). Strain TPS4-2$^T$ showed a low degree similarity of rep-PCR fingerprinting and low DNA-DNA relatedness with the above-mentioned species. The phenotypic, chemotaxonomic and phylogenetic data supported that the strain TPS4-2$^T$ should be classified as a novel species of genus *Idomarina* for which the name *Idiomarina piscisalsi* TPS4-2$^T$ is proposed.

The TPS4-2$^T$ was selected for further studies due to its novel species and high alkaline phosphatase (ALP) production. Intracellular ALP was produced at the beginning of exponential phase and the highest ALP was observed at the beginning of stationary phase. Intracellular ALP was detected higher than extracellular ALP in JCM no.377 broth pH 7.0-7.2 at 37°C on rotary shaking at 200 rpm for 36 h. Therefore, intracellular ALP was further purified by Hitap DEAE FF and Superose 12 10/300 chromatography. The partially purified ALP presented a molecular mass of 53 kDa, calculated by gel filtration. It had maximal activity in the presence of 1 mM of $CaCl_2$, pH 10.0, at 60°C. Stability was fully at pH 7.0-8.8 and 37–55°C. The $K_m$ and $V_{max}$ values of the partially purified ALP were 0.09 mM and $V_{max}$ of 28.01 μmol/min. It was stimulated by $Mg^{2+}$ and $Ca^{2+}$. The activity increased when concentration of NaCl increased from 0 to 0.3 M. Beyond this concentration, its activity decreased and remained 40% when increased NaCl to 1-4 M in reaction. Because of EGTA, specific $Ca^{2+}$ chelating agent, could completely inhibit ALP activity. This study indicated that the TPS4-2$^T$ ALP was metalloenzyme that can be activated by $Ca^{2+}$ and $Mg^{2+}$ ions.

From the results, the moderately halophilic bacteria, *Idiomarina*, *Virgibacillus, Halobacillus* and *Tetragenococcus* strains including the halotolerant *Bacillus* and *Staphylococcus* strains were distributed in many samples of fermented fishery. They are the most likely source of enzymes and constitute a heterogeneous group of halophiles belonging to different genera. The isolation of halophiles able to produce enzymes will provide the possibility to have optimal activities at different salt concentrations. TPS4-2$^T$ produced a substantial level of intracellular ALP activity that was active in the extreme conditions. The applications of this strain and the roles of the remained strains should be further studied.

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# APPENDICES

# APPENDIX A

## CULTURE MEDIA AND REAGENT FOR IDENTIFICATION

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

**1. JCM medium no. 377**

| | | |
|---|---|---|
| Yeast extract | 5 | g |
| Casamino acid | 5 | g |
| Sodium glutamate | 1 | g |
| Tri-sodium citrate | 3 | g |
| $MgSO_4.7H_2O$ | 20 | g |
| KCl | 2 | g |
| NaCl | 100 | g |
| $FeCl_2.4H_2O$ | 0.362 | g |
| $MnCl_2.4H_2O$ | 0.0362 | g |
| Agar | 20 | g |
| Distilled water | 1 | L |
| Adjust pH 7.0-7.2 with NaOH | | |

**2. Nitrate broth**

| | | |
|---|---|---|
| Beef extract | 10 | g |
| Peptone | 10 | g |
| NaCl | 5 | g |
| Distilled water | 1 | L |
| Dissolve and adjusted pH to 7.2. | | |

**3. Nitrate test reagent**

**Solution A:** 0.33% sulphanilic acid in 5 N- acetic acid was dissolved by gentle heating.

**Solution B:** 0.6% dimethyl-α-napthylaminein 5 N-acetic acid was dissolved by gentle heating.

**4. 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 hydrochloride | 10.0 | g |
| Agar | 3.0 | g |
| Distilled water | 1 | L |

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

**5. Casein agar**

| | | |
|---|---|---|
| MRS agar with 10% (w/v) NaCl | 990 | ml |
| 10% Skim milk solution | 10 | ml |

10% Skim milk solution in distilled water was sterile separately at 110$^{o}$C 10 min, after that poured into MRS agar with 10% (w/v) NaCl, that was seperated to sterile at 121$^{o}$C 15 min, then mixed and pour plate.

**6. Gelatin agar**

| | | |
|---|---|---|
| Base agar medium (MRS or JCM no.377 with 10%(w/v) NaCl (omitted casamino acid ) | 100 | ml |
| Gelatin | 1% | (w/v) |

Dissolve and adjust pH 7-7.2.

**7. Base medium for acid formation from carbohydrate**

| | | |
|---|---|---|
| Yeast extract | 5 | g |
| Peptone | 5 | g |
| Beef extract | 2 | g |
| Sodium acetate | 2 | g |
| Tween 80 | 0.25 | g |
| $MgSO_4.7H_2O$ | 200 | mg |
| $MnSO_4.4H_2O$ | 10 | mg |
| $FeSO_4.7H_2O$ | 10 | mg |

| | | |
|---|---|---|
| NaCl | 100 | g |

Supplement with carbon sousce to 1% (w/v), use bromcresol purple as indicator and adjust pH to 6.8 , then make volume to 1000 ml by distilled water.

**8. Histidine broth**

| | | |
|---|---|---|
| Glucose | 1 | g |
| Peptone | 5 | g |
| Yeast extract | 3 | g |
| L-histidine monohydrochloride | 5 | g |
| NaCl | 100 | g |

Adjust pH to 6.5 make volume to 1000 ml by distilled water.

**9. Flagella staining**

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

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

**10. Aesculin broth**

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

Dissolve the aesculin and iron salt in the peptone water, then adjust pH 7.4 and sterilized at 115 ºC for 10 min.

**11. Starch agar**

| | | |
|---|---|---|
| JCM no.377 agar medium | 100 | ml |
| Starch | 1% | (w/v) |

Dissolve and adjust pH 7-7.2.

**12. Tyrosine agar**

| | | |
|---|---|---|
| JCM no.377 agar medium (omitted casamino acid) | 100 | ml |
| L-tyrosine | 0.5% | (w/v) |

Dissolve and adjust pH 7-7.2.

**13. Tween 80 agar medium**

| | | |
|---|---|---|
| JCM no. 377 agar medium (omitted casamino acid) | 100 | ml |
| Tween 80 | 2 | ml |

Dissolve and adjust pH 7-7.2.

**14. Tryptone broth**

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

Adjust pH 7-7.2.

**15. Kovacs'reagent**

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

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50-55 ºC). Cool and the acid with care. Protect from light and storere at $4^{o}C°$

**16. Cellular fatty acid analysis**

**16.1 Reagent 1** (Saponification reagent)

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

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

**16.2 Reagent 2** (Methylation reagent)

| | | |
|---|---|---|
| 6 N HCl | 65 | ml |
| MeOH (HPLC grade) | 55 | ml |

pH must be below 1.5.

**16.3 Reagent 3** (Extraction solvent)

| | | |
|---|---|---|
| *n*-Hexane (HPLC grade or n-Hexane 1000) | 50 | ml |
| Methyl-tert-Butyl Ether (HPLC grade) | 50 | ml |

**16.4 Reagent 4** (Base wash reagent)

| | | |
|---|---|---|
| Sodium hydroxide | 1.2 | g |
| Mili-Q water | 100 | ml |

**16.5 Reagent 5** (Saturated sodium chloride)

# APPENDIX B

## REAGENT FOR DNA EXTRACTION AND PURIFICATION DNA BASE COMPOSITION, DNA-DNA HYBRIDIZATION AND 16S rRNA SEQUENCING

### 1. DNA extraction and DNA base composition

#### 1.1 Nuclease P1 solution

| | | |
|---|---|---|
| Nuclease P1 | 0.1 | mg |
| 40 mM $CH_3COONa$ + 12 mM $ZnSO_4$ (pH 5.3) | 1 | ml |

Dissolved and stored at 4°C. Made to 100 ml with distilled water.

#### 1.2 Alkaline phosphatase solution

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

#### 1.3 Saline –EDTA buffer pH 8.0 (0.15m NaCl + 0.1 M EDTA)

| | | |
|---|---|---|
| NaCl | 8.76 | g |
| EDTA | 37.22 | g |

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

#### 1.4 0.1 M Tris-buffer pH 9.0

| | | |
|---|---|---|
| Tris HCl | 1.21 | g |
| Distilled water | 90 | ml |

Dissolved and adjusted to pH 9.0 then made volume up 1000 ml with distilled water

#### 1.5 10% (W/V) SDS

| | | |
|---|---|---|
| Sodium dodecyl sulphate | 10 | g |
| Distilled water | 90 | ml |

Dissolved and made up to 100 ml with distilled water.

**1.6 Phenol: Chloroform (1:1, v/v)**

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

**1.7 20 x SSC (20 x standard saline citrate)**

| | | |
|---|---|---|
| NaCl | 17.5 | g |
| Sodium citrate | 8.8 | g |
| Distilled water | 900 | ml |

Adjusted pH to 7.0 and made volume up 1 L , then steriled at 121 ºC for 15 minutes. Note: To prepare 0.1× SSC and 0.2× SSC, the 20× SSC were diluted at 200 and 100 times, respectively before used.

**1.8 RNase A solution**

| | | |
|---|---|---|
| RNase A | 20 | mg |
| 0.15MNaCl | 10 | ml |

Dissolved 20 mg of RNase A in 10 ml 0.15 M NaCl and heated at 95°C for 5-10 min. Kept in -20°C.

**2. DNA-DNA hybridization**

**2.1 Hybridization solution**

| | | |
|---|---|---|
| Prehybridization | 100 | ml |
| Dextran sulfate | 5 | g |

Dissolved dextran sulfate in Prehybridization solution and kept at 4 ºC.

**2.2 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 |

All were dissolved in nanopure water steriled and kept at 4 ºC.

**2.3 Solution I**

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

All of ingredients were mixed and keep at 4 ºC.

**2.4 Solution II**

| | | |
|---|---|---|
| Strepavidin –POD conjugate | 1 | µl |
| Solution1 | 4 | ml |

DissolvednStrepavidin- POD conjugate in solution 1 before used. The solution 2 was freshly prepared.

**2.5 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 |

Steriled by autoclaveing at 121 ºC, 15pounds/inch 2 pressure, for 15 minutes

**2.6 Solution III**

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

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

All of ingredients were mixed and used. The solution 3 was freshly prepared.

**3. 1 Primers for 16S rRNA amplification and Sequencing**

| | |
|---|---|
| Forward primers | 27F 5'-AGAGTTTGATCCTTGGCTCAG-3’ |
| | 339F 5'-CTCCTACGGGAGGCAGCAG-3’ |
| Reverse primers | 1530R 5'-AAGGAGGTGATCCAGCC-3’ |
| | 520R 5'-ACCGCGGCTGCTGGC-3’ |
| | 802R 5’-TACCAGGGTATCTAATCCC-3’ |

# APPENDIX C

## STANDARD ASSAY METHODS

### 1. Determination of histamine formation

#### 1.1 Reagents

1.1.1 0.4 N Perchloric acid **(**$HClO_4$)

1.1.2 1,7- Diaminoheptane (1,7-Dh) 2 mg/ml

1.1.3 Mobile phase: Acetonitrile + 0.1% acetic acid

#### 1.2 Procedure

1.2.1 Sample extraction: Supernatant 480 µl was added 1,7-Diaminoheptane (1,7-Dh) 2 mg/ml and 0.4 N Perchloric acid ($HClO_4$) 500 µl, then mixed by vortex for 5 min and centrifuged at 12,000 rpm for 5 min, after that took the supernatant 300 µl for derivatization.

1.2.2 Derivatization: The histamine in the extracted sample was derivatized by the method modified from Eerola *et al*., 1993. Dansyl chloride was used as derivatizing agent and freshly prepared by dissolving with acetone to concentration of 10 mg/ml and protected from light. Each 300 µl of a supernatant or standard solution was pipetted into microcentrifuge tube. A 60 µl of sodium hydroxide and a 90 µl of a saturated solution of sodium hydrogencarbonate were added to supernatant or standard solution and mixed together with vortex. A 600 µl of dansyl chloride solution was added and introduced to the incubator temperature 40 °C for 45 min. After the reaction finished, 30 µl of ammonia solution was added for terminating the reaction. Sample solution was placed for 30 min at room temperature and then centrifuged at 2,500 rpm for 5 min. The supernatant was filtered through Minisart RC4 0.45 micron (Sartorius, Germany) and the filtrate was introduced to HPLC analysis.

1.2.3 HPLC analysis : The histamine derivatives analysis was performed with Alliance HPLC system (WATERS, USA) model 2690 equipped with Photodiode Diode Array (WATERS, USA) model 996 for the detection. A 20 µl of standard or sample derivatives was injected. Histamine was separated on Hypersil BDS C18 column (Thermo, USA) size 4.6x200 mm, temperature column at 40 °C. A gradient

mobile phase composed of 0.1% acetic acid as solvent A and the mixture of acetonitrile with 0.1% acetic acid as solvent B. The flow rate was 1 ml/min. A gradient program began at 50%A and 50%B and then solvent B was raised to 90% within 25 min, after that the gradient was switched to 50%A and 50%B within 10 min and hold this gradient for 5 min before next running started. The absorbance of histamine was detected at wavelength 254 nm. The peak area calculation was accomplished by Empower software (WATERS, USA). The histamine content in sample could be calculated by comparing with the authentic standard by internal standard quantitation method.

**2. Determination of protein and soluble peptide**

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

**2.1 Reagents**

**A**: 2% sodium carbonate in 0.1N NaOH

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

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

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

**2.2 Procedure**

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

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

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

**2.3 Preparation of standard curve of Bovine serum albumin (BSA)**

Standards solution of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg/ml were prepared from BSA.

**3. SDS-PAGE and zymogram**

**3.1 Polyacrylamide gel electrophoresis (PAGE) reagents**

**Monomer solution**

| | | |
|---|---|---|
| Acrylamide | 30% | (w/v) |
| Bisacrylamide | 0.8% | (w/v) |

Made up to 100 ml with deionized water.

Note: Acrylamide is a neurotoxin observes extreme caution to minimize skin contact and inhalation. The solution can be store up to 3 months at 4°C in the dark.

**3.2 4X Resolving gel buffer**

| | | |
|---|---|---|
| Tris(hydroxymethyl)aminomethane | 18.15 | g |
| Deionized water | 90 | ml |

Dissolved and adjusted the pH to 8.8 by using 0.1 N HCl. Made up to 100 ml with deionized water. Note: The solution can be store up to 3 months at 4°C in the dark.

**3.3 4X Stacking gel buffer**

| | | |
|---|---|---|
| Tris(hydroxymethyl)aminomethane | 6 | g |
| Deionized water | 90 | ml |

Dissolved and adjusted the pH to 6.8 by using 0.1 N HCl. Made up to 100 ml with deionized water. Note: The solution can be store up to 3 months at 4°C in the dark.

**3.4 10X Tank buffer for SDS-PAGE**

| | | |
|---|---|---|
| Tris(hydroxymethyl)aminomethane | 30.28 | g |
| Glycine | 144.13 | g |
| Sodium dodecyl sulfate | 10 | g |

| | | |
|---|---|---|
| Distilled water | 900 | ml |

Dissolved and made up to 1 litter with distilled water.

Note: Diluted 10 times before use. The solution can be store up to 1 month at room temperature. 10× Tank buffer for Native-PAGE is prepared in a similar manner, except that the addition of SDS was omitted.

**3.5 10% Sodium dodecyl sulphate (SDS)**

| | | |
|---|---|---|
| Sodium dodecyl sulfate | 10 | g |
| Deionized water | 90 | ml |

Dissolved and made to 100 ml with deionized water.

**3.6 2X Sample buffer for SDS-PAGE**

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

Dissolved and made up to 10 ml with deionized water.

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

**3.7 4% Stacking gel for SDS-PAGE**

| | | |
|---|---|---|
| Deionized water | 3.053 | ml |
| 4× Stacking gel buffer | 1.25 | ml |
| Monomer solution | 667 | µl |
| 10% (w/v) SDS | 50 | µl |
| 10% (w/v) Ammonium persulfate | 25 | µl |
| TEMED | 5 | µl |

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

**3.8 12.5% Running gel for SDS-PAGE**

| | | |
|---|---|---|
| Deionized water | 4.1314 | ml |
| 4× Running gel buffer | 3.25 | ml |
| Monomer solution | 5.4171 | ml |
| 10% (w/v) SDS | 130 | µl |
| 10% (w/v) Ammonium persulfate | 65 | µl |
| TEMED | 6.5 | µl |

Note: 12.5% running gel for Native-PAGE is prepared in a similar manner, except that the addition of SDS was omitted.

**3.9 Staining solution**

| | | |
|---|---|---|
| Coomassie brilliant blue (R-250) | 1.25 | g |
| Ethanol | 450 | ml |
| Acetic acid | 100 | ml |

Dissolved and made up to 1 litter with distilled water.

Note: The reagent should be filtered before use. Store the solution in the dark.

**3.10 Gel coloration** for ALP activity staining

**A**: 7.5 %(w/v) ammonium molybdate in distilled water.

**B**: 11% (v/v) Tween 20 in distilled water.

**C**: 60 ml $H_2SO_4$ in 300 ml distilled water.

**D**: After reagent C cooled, then added 440 mg malachite green. This solution could be stored up to 3 months at room temperature in the dark. Dilution was freshly prepared two times by reagent C before using.

**Gel coloration**: A 2.5 ml of solution A and 0.2 ml solution B were added into a 10 ml of solution D. This mixture solution was used to stain native PAGE. A 1.5 ml of mixture solution was added in to 10 ml of optimum buffer that the native gel was immersed to check activity of ALP.

# APPENDIX D

## STANDARD CURVE

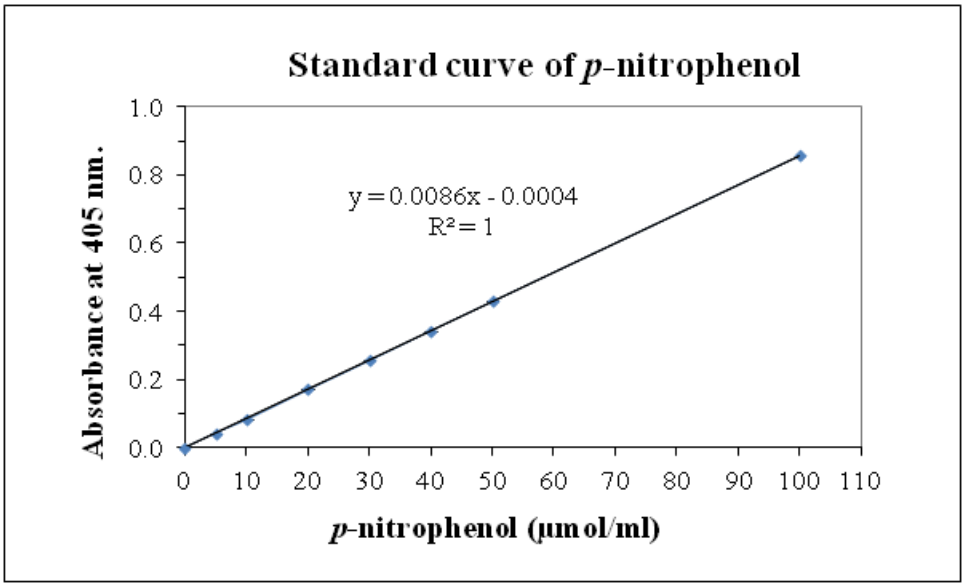


**Figure** 4.22 Standard curve of *p*-nitrophenol

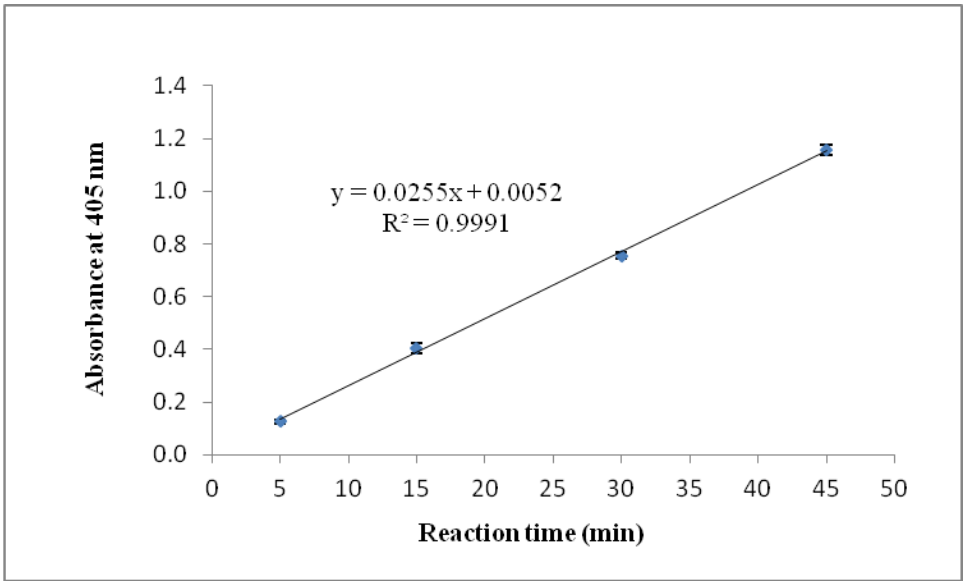


**Figure** 4.23 Relation on ALP activity and reaction time.

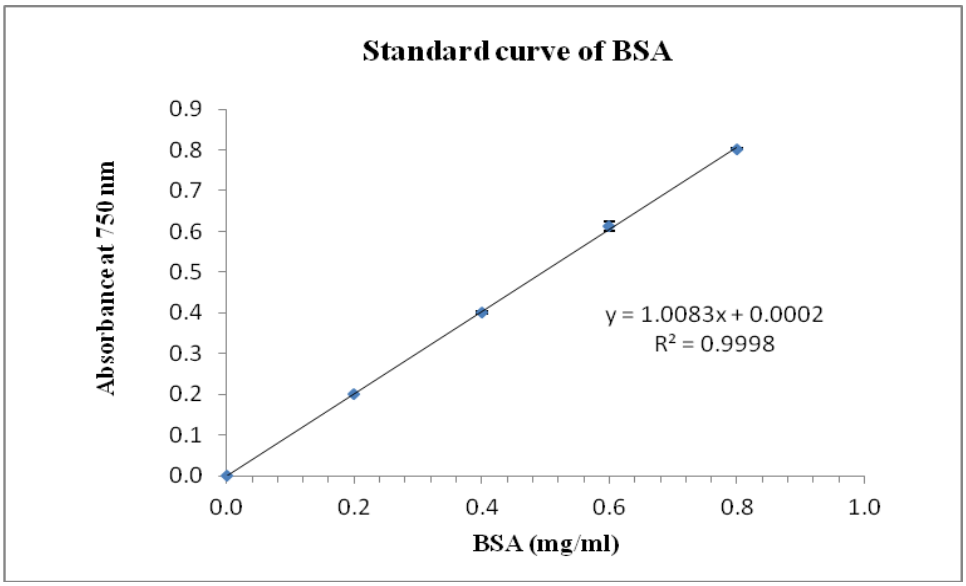


**Figure** 4.24 Standard curve of bovine serum albumin (BSA)

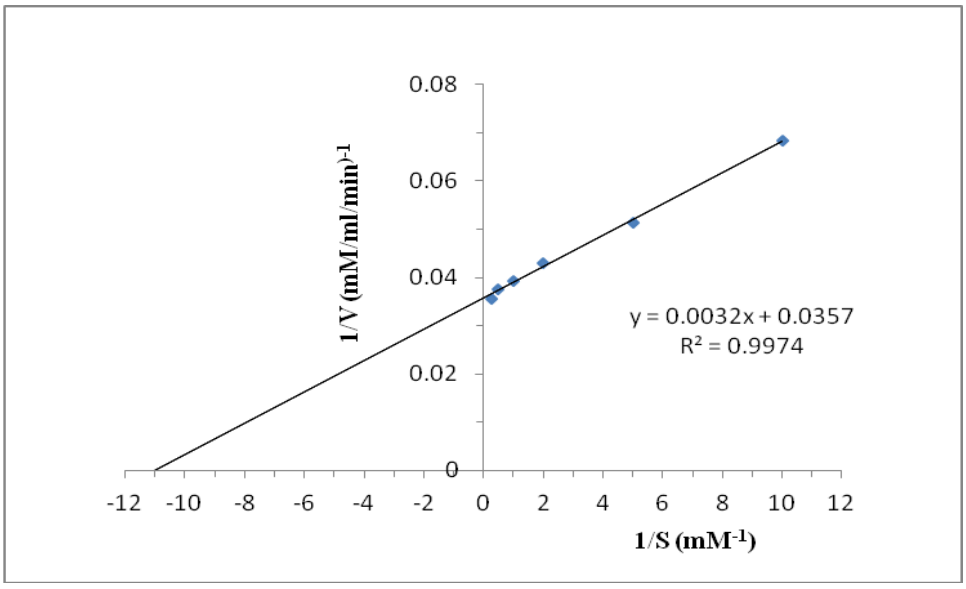


**Figure** 4.25 Lineweaver-Burk plot generated from the initial reaction rates using *p*-nitrophenolphosphate as substrate in the range of 0.1-4 mM and 0.85 µg of partially purified ALP per assay.

# APPENDIX E

## TAXONOMICAL CHARACTERISTICS

**Table 4.22** Cellular fatty acid composition of strain NSW13-3 and SMK 24-1.

| Fatty acid | Percentage of total | |
|---|---|---|
| | NSW 13-3 | SMK 24-1 |
| C12:0 | 0.2 | 0.35 |
| C13:0 iso 3OH | 0.72 | 0.31 |
| C14:0 iso | 6.225 | 8.58 |
| C14:0 | 0.27 | 0.26 |
| C15:0 iso | 17.315 | 20.38 |
| C15:0 anteiso | 36.415 | 34.73 |
| C15:0 | 0.35 | 0.20 |
| C16:1 ω7c alcohol | 3.08 | 4.46 |
| C16:0 iso | 14.46 | 14.12 |
| C16:1 ω11c | 0.39 | 0.37 |
| C16:0 | 1.455 | 1.31 |
| C17:1 iso ω10c | 0.26 | 0.38 |
| C17:0 iso | 4.05 | 3.47 |
| C17:0 anteiso | 12.785 | 9.58 |
| C18:1 ω9c | 0.195 | 0.14 |
| C18:0 | 0.435 | 0.34 |
| Summed Feature 4 | 0.97 | 1.09 |

**Table 4.23** Physiological and biochemical characteristics of the isolates.

| Parameters | KM1-1 | KM1-2 | KM1-3 | KM1-4 | KM1-5 | KM1-6 | KM2-2 | KM3-1 | KM3-2 | KM3-3 | KM3-4 | KM4-1 | KM6-1 | KM6-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arginine hydrolysis | - | - | - | - | - | - | - | - | - | - | - | - | w | - |
| Casein hydrolysis | - | - | + | + | + | - | + | + | + | + | + | - | + | + |
| NaCl (% w/v) | | | | | | | | | | | | | | |
| 0 | - | - | - | - | - | - | - | + | + | - | + | - | - | - |
| 5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 15 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 20 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 25 | - | - | + | - | + | - | + | + | + | + | + | - | - | + |
| pH | | | | | | | | | | | | | | |
| 4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Temperature ( $^{o}$C) | | | | | | | | | | | | | | |
| 37 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 40 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 45 | w | w | - | - | - | - | - | - | - | - | - | - | + | - |

**Table 4.23** (Cont.1) Physiological and biochemical characteristics of the moderate halophile isolates.

| Parameters | KM6-3 | KS54-9 | S25-12 | A80 | KS87-14 | IB10-2 | IB20-7 | IS40-7 | IS60-1 | KS11-1 | KS35-4 | KS54-11 | KS87-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arginine hydrolysis | - | - | - | - | - | + | + | + | + | + | + | + | + |
| Casein hydrolysis | + | + | + | - | + | - | - | - | - | + | - | - | - |
| NaCl (% w/v) | | | | | | | | | | | | | |
| 0 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 15 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 20 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 25 | + | + | + | w | + | - | - | + | + | - | - | - | - |
| pH | | | | | | | | | | | | | |
| 4 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | + | + | - | - | - | - |
| 6 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Temperature ( $^{o}$C) | | | | | | | | | | | | | |
| 37 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 40 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 45 | - | - | - | w | - | - | - | - | - | - | w | w | + |

**Table 4.23** (Cont.2) Physiological and biochemical characteristics of the moderate halophile isolates.

| Parameter | Ph27 | Ph65 | Ph88 | Ph120 | Ph136 | Ph155 | SO25-6 | SO45-1 | SP34-3 | SP36-1 | SP37-2 | SP39-3 | SP40-1 | SP43-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arginine hydrolysis | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Casein hydrolysis | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| NaCl (% w/v) | | | | | | | | | | | | | | |
| 0 | - | - | - | - | - | + | - | + | + | - | - | - | - | - |
| 5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 15 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 20 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 25 | - | - | - | - | - | + | - | + | + | - | - | - | - | - |
| pH | | | | | | | | | | | | | | |
| 4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| 6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Temperature ( $^{o}$C) | | | | | | | | | | | | | | |
| 37 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 40 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 45 | - | - | - | - | - | - | - | + | - | - | + | + | + | w |

**Table 4.24** Acid from carbohydrate of the moderate halophile isolates.

| Carbon source | KM1-1 | KM1-2 | KM1-3 | KM1-4 | KM1-5 | KM1-6 | KM2-2 | KM3-1 | KM3-2 | KM3-3 | KM3-4 | KM4-1 | KM6-1 | KM6-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amygdalin | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| L-Arabinose | w | w | - | - | - | - | - | - | - | + | - | - | - | + |
| D-cellobiose | - | - | - | - | + | - | + | w | w | - | w | - | - | - |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Galactose | + | + | + | + | + | - | + | + | + | + | + | - | w | + |
| Glycerol | - | - | - | - | - | - | - | - | - | - | - | - | w | - |
| Lactose | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Maltose | + | + | + | + | + | + | + | + | + | + | + | + | - | + |
| D-Mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Mannose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Melibiose | + | + | w | w | w | - | w | - | - | - | - | - | + | + |
| D-Melezitose | + | + | + | + | + | - | + | + | + | + | + | - | - | + |
| Methyl-D-glucoside | + | + | - | - | - | - | - | - | - | - | - | - | - | w |
| Raffinose | w | w | - | - | - | - | - | - | - | - | - | - | - | w |
| Rhamnose | - | - | - | - | - | - | - | - | - | - | - | - | - | w |
| D-Ribose | + | + | w | w | w | + | w | w | w | - | w | + | + | - |
| Salicin | - | - | w | w | w | + | w | w | w | w | w | + | + | w |
| D-sorbitol | - | - | - | - | w | w | w | - | - | - | - | w | - | - |
| L-Sorbose | w | w | - | - | - | - | - | - | - | - | - | - | - | - |
| Sucrose | + | + | + | + | + | w | + | + | + | w | + | w | w | + |
| D-Trehalose | w | w | - | - | - | - | - | - | - | - | - | - | - | + |
| D-Xylose | w | w | - | - | - | w | - | - | - | - | - | w | w | - |

**Table 4.24** (Cont.1) Acid from carbohydrate of the moderate halophile isolates.

| Carbon source | KM6-3 | A80 | KS54-9 | KS87-14 | KS87-1 | S25-12 | IB10-2 | IB20-7 | IS40-7 | IS60-1 | KS11-1 | KS35-4 | KS54-11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amygdalin | - | - | - | - | + | - | + | + | + | + | + | + | + |
| L-Arabinose | + | - | - | - | + | - | + | - | + | + | - | + | + |
| D-cellobiose | - | - | w | w | + | w | + | + | + | + | + | + | + |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Galactose | + | w | w | w | + | w | + | + | + | + | + | + | + |
| Glycerol | - | w | - | - | + | - | + | + | w | w | + | w | w |
| Lactose | - | - | - | - | - | - | - | - | - | - | - | w | w |
| Maltose | + | + | w | w | + | w | + | + | + | + | + | + | + |
| D-Mannitol | + | + | + | + | + | + | - | + | + | + | + | - | - |
| D-Mannose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Melibiose | + | - | - | - | - | - | - | w | w | w | w | w | w |
| D-Melezitose | + | - | - | - | - | - | + | w | w | w | + | + | + |
| Methyl-D-glucoside | w | - | - | - | w | - | w | - | + | + | w | w | w |
| Raffinose | w | - | + | + | - | + | - | - | + | + | w | w | w |
| Rhamnose | w | + | w | w | - | w | - | - | + | + | w | w | w |
| D-Ribose | - | + | - | - | + | - | + | + | + | + | + | + | + |
| Salicin | w | w | - | - | + | - | + | + | + | + | + | + | + |
| D-sorbitol | - | + | - | - | - | - | w | + | w | w | w | - | - |
| L-Sorbose | - | - | - | - | - | - | w | - | - | - | w | - | - |
| Sucrose | + | - | - | - | + | - | + | + | + | + | + | + | + |
| D-Trehalose | + | - | - | - | + | - | + | + | + | + | + | + | + |
| D-Xylose | - | - | - | - | - | - | + | - | + | + | + | + | + |

**Table 4.24** (Cont.2) Acid from carbohydrate of the moderate halophile isolates.

| Carbon source | Ph27 | Ph65 | Ph88 | Ph120 | Ph136 | Ph155 | SO25-6 | SO45-1 | SP34-3 | SP36-1 | SP37-2 | SP39-3 | SP40-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amygdalin | + | w | w | w | + | - | + | + | + | + | + | + | + |
| L-Arabinose | + | - | - | - | + | + | + | + | + | + | + | + | + |
| D-cellobiose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Galactose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Glycerol | w | + | + | + | w | + | w | - | + | w | + | + | + |
| Lactose | w | - | - | - | w | - | w | + | w | w | - | - | - |
| Maltose | + | w | w | w | + | + | + | + | + | + | + | + | + |
| D-Mannitol | - | - | - | - | - | + | - | + | + | - | + | + | + |
| D-Mannose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Melibiose | w | w | w | w | w | + | w | - | - | w | - | - | - |
| D-Melezitose | + | - | - | - | + | + | + | + | + | + | - | - | - |
| Methyl-D-glucoside | w | - | - | - | w | + | w | + | + | w | w | + | w |
| Raffinose | w | - | - | - | w | - | w | - | - | w | - | - | - |
| Rhamnose | w | - | - | - | w | - | w | - | - | w | - | + | - |
| D-Ribose | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Salicin | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-sorbitol | - | - | - | - | - | + | - | + | + | - | - | - | - |
| L-Sorbose | - | - | - | - | - | - | - | - | + | - | - | + | - |
| Sucrose | + | + | + | + | + | + | + | w | + | + | + | + | + |
| D-Trehalose | + | + | + | + | + | + | + | w | + | + | + | + | + |
| D-Xylose | + | - | - | - | + | + | + | w | + | + | - | + | - |

# APPENDIX F

## 16S rRNA GENE SEQUENCE OF REPRESENTATIVE ISOLATES

### 1. The 16S rRNA gene nucleotide sequence of KL1-1

GGCCAAGGGGCGGCTATACATGCAAGTCGAGCGCGGGAAGCAAGCTGATCCTCTTCGGAGGTGACG
CTTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACC
CCGGGAAACCGGGGCTAATACCGGATAATACTTTTCATCACCTGATGAGAAGTTGAAAGGTGGCTTT
TAGCTACCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA
ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGCA
CCTTGACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGT
CTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGCAATC
GGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCAAAACCGCGAGGTCAA
GCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC
ACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTGGAGCCAGCCGCCGAAAGGGGAC
CCCA

### 2. The 16S rRNA gene nucleotide sequence of KL2-4

CCCAAAGGGACGCCAGTACATGGCAAGTCGAGCGCGGGAAGCAAGCTGATCCTCTTCGGAGGTGAC
GCTTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAAC
CCCGGGAAACCGGGGCTAATACCGGATAATACTTTTCATCACCTGATGAGAAGTTGAAAGGTGGCTT
TTAGCTACCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGC
ACCTTGACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAA
GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGA
ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGCAA
TCGGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCAAAACCGCGAGGTCA
AGCAAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAAT
CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
CACCACGAGAGTTGGTAACACCCGAAGTCGGTGAGGTAACCTTTGGAGCCAGGCCCGCCCGAAGGG
GGTCCCCCCCCCG

## 3. The 16S rRNA gene nucleotide sequence of NSW13-2

GGGGGGGGGGGGGGGCTATACATGCAAGTCGAGCGCGGGAAGCAAGCTGATCCTCTTCGGAGGTGACGCTTGTG
GAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACCCCGGGAAACCGGGG
CTAATACCGGATAATACTTTTCATCACCTGATGGGAAGTTGAAAGGTGGCTTTTAGCTACCACTTACAGATGGGCC
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTG
CCGTTCGAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAGAGTGGAATTCCA
CGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGC
TGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
AAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTCTGCAATCGGTAGAGATACCGAGTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCA
TTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCAAAACCGCGAGGTCAAGCAAATCCC
ATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAG
TCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGGGGACCCAAGGG

## 4. The 16S rRNA gene nucleotide sequence of NSW13-3

GGGGGGGGCCGCCTAGTAGATGTCTAGTCGAGCGCGGGAGCGAGCTGATCCCTTCGGGGTGACGCTCGTGGAACG
AGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGGTAATACTTTCTTTCGCATGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA
CGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTGC
GAATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGGT
GCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGG
GGGCTTCCACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCCTTGGACCTCCCTAGAGATAGGGATTTCCCTTCGGGGACCAAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTCAG
TTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTAT
GACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTGTAGCAAATCCCATAA
AACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACAGGGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGG
TGAGGTAACCTTTGGAGCCCAGCCCCCCGAAGGGGTTCCAGGGGGG

## 5. The 16S rRNA gene nucleotide sequence of NSW13-4

GGGGGGGGGGGGGGGCTATACATGCAGTCGAGCGCATGAAATTATTTGATTCTCTTCGGAGTTGACGATAATGGAA
TGAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTAAGACTGGGATAACTCGTGGAAACGCGAGCTA
ATACCGGATAACACTTTTCATCTCCTGATGAGAAGTTGAAAGGCGGCTTTTGCTGTCACTTACAGATGGGCCTGCG
GCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACTATA
GTAACTGATAGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCTCGCAGGCGGTTCTTTAAGTCTGATGTGAAATCTTA
CGGCTCAACCGTAAACGTGCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGG
GTTTCCGCCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAA
AAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATCCTCTGAACACTCTAGAGATAGAGTTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTAAGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAC
CTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGAAGCGAACCCGCGAGGTCAAGCAAATCCCACAAAAC
CATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGGTGA
GGTAACCGTAAGGAGCCAGCCGCCGAAGGGGGACCAAA

## 6. The 16S rRNA gene nucleotide sequence of NSW13-5

GGGGGGGGGGGGGGCTATACATGCAGTCGAGCGCGGGAAGCGAGCTGATCCCTTCGGGGTGACGCTCGTGGAAC
GAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGGTAATACTTTCTTTCGCATGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTG
CGAATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGG
TGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAG
GGGGCTTCCACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
AGGTCTTGACATCCTTGGACCTCCCTAGAGATAGGGATTTCCCTTCGGGGACCAAGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTCA
GTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTGTAGCAAATCCCATAA
AACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGG
TGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGGGAACCCAA

## 7. The 16S rRNA gene nucleotide sequence of NSW25-1

GGGGGGGGGGGGGGCTAATACATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACAT
TTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTT
GGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCACG
TCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGC
AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGG
ATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTT
TGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAG
GTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACA
CACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTT
CGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATAC
GTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTA
TGGAGCTAGCCGTCGAAGGGGGGACCAAAAA

## 8. The 16S rRNA gene nucleotide sequence of SK22-1

GGGGGGGGGGGGGCTATACATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTG
GAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGT
AAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCACGTCTT
GACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGA
GATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGA
AAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTT
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACAC
GTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGG
ATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTT
CCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGG
AGCTAGCCGTCGAAGTGACAAAG

## 9. The 16S rRNA gene nucleotide sequence of SK23-2

GGGGGGGGCGGGCGGCTGATACATGGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAA
CATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTA
GTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCA
CGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGC
GCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGG
GGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCC
CTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATC
CTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTC
TAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCT
ACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCA
GTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAA
TACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCAT
TTATGGGAGCTAGCCGTCGAAGGGGGCCCAA

## 10. The 16S rRNA gene nucleotide sequence of RY30-1

GGGGGGGGGGGGCTATACATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAATATTTA
GAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGT
GGGGTAATGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACGTCTTG
ACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGAC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAC
AACTCTAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAAGTTGGGCACTCTAAGTTG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACG
TGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGA
TTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTC
CCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTCTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGG
AGCTAGCCGTCGAAGTGACAAGAGG

## 11. The 16S rRNA gene nucleotide sequence of RY30-3

GGGGGGGGGGGGGGCTATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAATA
TTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGT
TGGTGGGGTAATGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACG
TCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGC
AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGG
ATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTT
TGACAACTCTAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAAGTTGGGCACTCTAA
GTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACA
CACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTT
CGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATAC
GTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTCTGTAACACCCGAAGCCGGTGGAGTAACCATTTA
TGGAGCTAGCCGTCGAAGTGACAATAGG

## 12. The 16S rRNA gene nucleotide sequence of RY31-2

GGGGGGGGGGGGGGCTATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAATA
TTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGT
TGGTGGGGTAATGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACG
TCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGC
AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGG
ATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTT
TGACAACTCTAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAAGTTGGGCACTCTAA
GTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACA
CACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTT
CGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATAC
GTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTCTGTAACACCCGAAGCCGGTGGAGTAACCATTTA
TGGAGCTAGCCGTCGAAGTGACAATAG

## 13. The 16S rRNA gene nucleotide sequence of KL6-2

GGGGGGGGGGGGGGCTATACATGCAAGTCGAGCGAACAGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATA
TTTTGAACCGCATGGTTCGATAGTGAAAGACGGTTTCGGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAG
TTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTGAACAA
GTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCG
CAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGG
GATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCT
TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACG
GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCT
TTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTA
GGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTAC
ACACGTGCTACAATGGATAATACAAAGGGCAGCGAATCCGCGAGGCCAAGCAAATCCCATAAAATTATTCTCAGT
TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA
CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCTTTT
AGGAGCTAGCCGTCGAAGGGACAAAGGG

## 14. The 16S rRNA gene nucleotide sequence of KL2-3

GGGGGGGGGGGGGGCTATACATGCAAGTCGAGCGGATTGATGGGAGCTTGCTCCCTGATATCAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACTCATT
TCCTCGCATGAGGAAATGTTGAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGCA
CCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGT
AGATATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC
TGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAA
GATGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACGGTACAAAGGGCAGCGAGACCGCGAGGTTTAGCCAATCCCATAAAACCGTTCTCAGTT
CGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTT
GGAGCCAGCCGCCTAAGGGGACAGAAGGGG

## 15. The 16S rRNA gene nucleotide sequence of MK10-2

GGGGGGGGGGGCGGCTAATACATGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTC
CTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGGGGTAATGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCA
CCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGG
GAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG
GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCC
TTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC
TCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCGAATCCCATAAATCTGTTCTCAGTT
CGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCTTTA
TGGAGCCAGCCGCCGAAGGGGGGCCCAAA

## 16. The 16S rRNA gene nucleotide sequence of SMK24-1

GGGGGGGGGGGGGGGCTATACATGCAGTCGAGCGCGGGAAGCGAGCTGATCCCTTCGGGGTGACGCTCGTGGAAC
GAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGGTAACACTTTCTTTCGCATGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTG
CGAATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGG
TGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAG
GGGGCTTCCACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
AGGTCTTGACATCCTTGGACCTCCCTAGAGATAGGGATTTCCCTTCGGGGACCAAGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTCA
GTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCCGCGAGGTGTAGCAAATCCCATAA
AACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTAACACCCGAAGTCGG
TGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGGGACCCAAGG

## 17. The 16S rRNA gene nucleotide sequence of TPS4-2$^{T}$

CCTAACACATGCAAGTCGAGCTAACGAGAAGCTTGTTCGCTCGAGCGGCGGACGGGTGAGTAAGACTTGGGAATT
TGCCTTTAGGCGGGGGACAACCACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGTGGGGTTCG
GCTCACACCTAAAGATGAGCCCAAGCGGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCTTA
GCTGTTCGAGAGGATGATCAGCCACACTGGGACTGAAGCACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGGGAAACCCTGATGCAACCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTT
TCAGTGGGGAGGAAAGGGTAATCGTTAATACGACTCAGTTGACGTTAGCCACAGAAGAAGCACCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTACGTAGGCGGTG
TGTTAAGCTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATAGCATTTAGAACTGGCACGCTAGAGTCCTGAAGA
GGTGGTAGAATTTCCAGTGTAGCGGTGAAATGCGTAGATATTGGAAGGAATACCGGTGGCGAAGGCGGCCACCTG
GTCAGGACTGACGCTGAGGTACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCAACTAGTTGTTCGTGTCATTAAGACGTGAGTAACGCAGCTAACGCACTAAGTTGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAGGTGGTTTAATTCGATGCAA
CGCGAAGAACCTTACCATCCCTTGACATCCAGTGAATTTTCCAGAGATGGAATAGTGCCTTCGGGAACACTAAAA
AAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTAGTTGCCAGCGTTCGGCCGGGAACTCTGGGGAGATTGCCGATGGAAAACCGGAGGAAAGTGGGGACGACGTCA
AGTCATCATGGCCCTTACGGGATGGGCTACACAAGTGCTACAAAGCGTACAAAAAGAGCAACCAACCCTGAGGGT
AAGCGAATCTCATAAAGCGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT
AATCGTGGATCAAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGT
GCACCAGAAGTGGTTAGTTTAACT

## 18. The 16S rRNA gene nucleotide sequence of KS87-14

TAGGCTCCTTGCGGTTACCTCACCGACTTCGGGTGTTACGAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCC
CGGGAACGGATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTG
CAATCCGAACTGAGAGAAGCTTTCAGAGATTCGCTGGGCCTTGCGGCTTCGCGCGCTCGTTGTACTCCCCATTGTA
GCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCA
GTCTGACTAGAGTGCCCAACTAAATGCTGGCAACTAGCCATAAGGGTTGCGCTCGTTACGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCTTTGCCCCCGAAGGGGAAACCCTATCTCTAGGGCG
GTCAAAGGATGTCAAGACCTGGTAAGGGTCTTCGCGTTGCTTCCAATTAAACCACATGCTCCACCGCTTGGGCGGG
CCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTGCAGCAC
TGAAGGGCGGAAACCCTCCAACACTTAACACTCAGCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TACCCACGCTTTCGAGCCTCAGCGTCAGTCACAGACCAGAAAGTCGCCTTCGCCACCGGTGTTCCTCCATATATCT
ACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGTACTCAAGTTCGCCAGTTTCCAATGCCCCTCCTC
GGTTGAGCCGAGGGCTTTCACATCAGACTTAACGAACCGCCTGCGCTCCCTTTACCCCCAATAAATCCGGACAACG
CTTGGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGCTACCGTCATAGAAA
AAGCATTCCCTCTTTTTCCTGTTCTTGGCTGACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGT
TGCTCGGTCAGACTTGCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCTTTGGTGAGCCGTTACCTCACCAACTGGCTAA
TGCACCGCGGGCCCCTCCAGCAGTGACGCTTAAAGCGCCTTTTCCCTTTCTTCCAGGCGAAAAAAAGGCCTATGCG
GTATTAGCACCTGTTTCCAAGTGTTATCCCCCGCTGCTGGGTAGGTTCCCCACGTGTTACTCACCCGTCCGCCACTC
TGCCACCTCTTCCCCCGAAGGGGAACAGGGACAGCGTTCGACTTGCAT

## 19. The 16S rRNA gene nucleotide sequence of KM 1-5

CGGTAGGCTCCTTGCGGTTACCTCACCGACTTCGGGTGTTACGAACTCTCGTGGTGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGGATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAG
CCTGCAATCCGAACTGAGAGAAGCTTTCAGAGATTCGCTGGGCCTTGCGGCTTCGCGCGCTCGTTGTACTCCCCAT
TGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCTGACTAGAGTGCCCAACTGAATGCTGGCAACTAGCCATAAGGGTTGCGCTCGTTACGGGACTTAACCC
AACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCTTTGCCCCCGAAGGGGAAACCCTATCTCTA
GGGCGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTG
CAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCAGCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTTGCTACCCACGCTTTCGAGCCTCAGCGTCAGTCACAGACCAGAGAGTCGCCTTCGCCACCGGTGTTCCTCCA
TATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGTACTCAAGTTCGCCAGTTTCCAATGCC
CCTCCTCGGTTGAGCCGAGGGCTTTCACATCAGACTTAACGAACCGCCTGCGCTCCCTTTACGCCCAATAAATCCG
GACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGCTACCGTCA
TAGAAAAAGCATTCCCTCTTCTTCCTGTTCTTGGCTGACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACG
CGGCGTTGCTCGGTCAGACTTGCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT
CTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCTTTGGTGAGCCGTTACCTCACCAACT
GGCTAATGCACCGCGGGCCCCTCCAGCAGTGACGCTTAAAGCGCCTTTTCCCTTTCTTCCAGGCGAAAAAAAGGCC
TATGCGGTATTAGCACCTGTTTCCAAGTGTTATCCCCCGCTGCTGGGTAGGTTCCCCACGTGTTACTCACCCGTCCG
CCACTCTGCCACCTCTTCCCCCGAAGGGGAACAGGGGCAGCGTTCGACTGCATGTATAGA

## 20. The 16S rRNA gene nucleotide sequence of KS87-1

GGCGGCTGGCTCCCCTAGGGGTTGCCGCACCGACTTTGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGGATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTT
GCAGCCTGCAATCCGAACTGAGAGAAGCTTTCAGAGATTCGCTCGGCCTTGCGGCTTGGCGCGCTCGTTGTACTTC
CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGGTTTGT
CACCGGCAGTCTTGCTAGAGTGCCCAACGCAATGCTGGCAACTAACAATAAGGGTTGCGCTCGTTACGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAACCCTAT
CTCTAGGGCGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTA
ACTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTA
ATCCTGTTTGCTACCCACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCT
CCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTGATCCAGTTTCCAAT
GACCCTCCCCAGTTGAGCTGGGGGCTTTCACATCAGACTTAAATCACCGCCTGCGCTCGCTTTACGCCCAATAAAT
CCGGAAAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGCTACCG
TCATAGAAAAAGCATTGCCTCTTTCTCCTGTTCTTTGCTGACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTC
ACGCGGCGTTGCTCGGTCAGACTTGCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG
TGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCTTTGGTGAGCCGTTACCTCACCA
ACTGGCTAATGCACCGCGGGACCAGCCATCAGTGACGCTGTAAAGCGCCTTTGAGCTTTCTTTCAGGTGAAAAAA
AGCCGTATGCGGTATTAGCACCTGTTTCCAAGTGTTATCCCCCGCTGATGGATAGGTTCCCCACGTGTTACTCACCC
GTCCGCCACTCCGCTTAAGAAAAAACCGAAGTTTCTTCTTAAGCAGCGTTCGACTTGCATG

## 21. The 16S rRNA gene nucleotide sequence of S25-1

TTCGGCGGTAGGCTCCTTGCGGTTACCTCACCGACTTCGGGTGTTACGAACTCTCGTGGTGTGACGGGCGGTGTGT
ACAAGGCCCGGGAACGGATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGT
TGCAGCCTGCAATCCGAACTGAGAGAAGCTTTCAGAGATTCGCTGGGCCTTGCGGCTTCGCGCGCTCGTTGTACTC
CCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG
TCACCGGCAGTCTGACTAGAGTGCCCAACTGAATGCTGGCAACTAGCCATAAGGGTTGCGCTCGTTACGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCTTTGCCCCCGAAGGGGAAACCCTATC
TCTAGGGCGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAA
CTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCAGCGTTTACGGCGTGGACTACCAGGGTATCTAA
TCCTGTTTGCTACCCACGCTTTCGAGCCTCAGCGTCAGTCACAGACCAGAGAGTCGCCTTCGCCACCGGTGTTCCTC
CATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGTACTCAAGTTCGCCAGTTTCCAATG
CCCCTCCTCGGTTGAGCCGAGGGCTTTCACATCAGACTTAACGAACCGCCTGCGCTCCCTTTACGCCCAATAAATC
CGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGCTACCGT
CATAGAAAAAGCATTCCCTCTTCTTCCTGTTCTTGGCTGACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCA
CGCGGCGTTGCTCGGTCAGACTTGCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGT
GTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCTTTGGTGAGCCGTTACCTCACCAA
CTGGCTAATGCACCGCGGGCCCCTCCAGCAGTGACGCTTAAAGCGCCTTTTCCCTTTCTTCCAGGCGAAAAAAAGG
CCTATGCGGTATTAGCACCTGTTTCCAAGTGTTATCCCCCGCTGCTGGGTAGGTTCCCCACGTGTTACTCACCCGTC
CGCCACTCTGCCACCTCTTCCCCCGAAGGGGAACAGGGGCAGCGTTCGACTTGCATGTATAGA

## 22 The 16S rRNA gene nucleotide sequence of SP37-2

GGCGGCTGGCTCCCCTAGGGGTTGCCGCACCGACTTTGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGGATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTT
GCAGCCTGCAATCCGAACTGAGAGAAGCTTTCAGAGATTCGCTCGGCCTTGCGGCTTGGCGCGCTCGTTGTACTTC
CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGGTTTGT
CACCGGCAGTCTTGCTAGAGTGCCCAACGCAATGCTGGCAACTAACAATAAGGGTTGCGCTCGTTACGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAACCCTAT
CTCTAGGGCGGTCAAAGGATGTCAAGACCTGGTAAGGTTTCTTTCGCGTTGCTTCGAATTAAACCAAATGCTCCAC
CGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGT
TAACTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTACCCACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTC
CTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTGATCCAGTTTCCA
ATGACCCTCCCCAGTTGAGCTGGGGGCTTTCACATCAGACTTAAATCACCGCCTGCGCTCGCTTTACGCCCAATAA
ATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGCTAC
CGTCATAGAAAAAGCATTGCCTCTTTCTCCTGTTCTTTGCTGACAACAGAGCTTTACGATCCGAAAACCTTCTTCAC
TCACGCGGCGTTGCTCGGTCAGACTTGCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGC
CGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCTTTGGTGAGCCGTTACCTCAC
CAACTGGCTAATGCACCGCGGGACCAGCCATCAGTGACGCTGTAAAGCGCCTTTGAGCTTTCTTTCAGGTGAAAAA
AAGCCGTATGCGGTATTAGCACCTGTTTCCAAGTGTTATCCCCCGCTGATGGATAGGTTCCCCACGTGTTACTCACC
CGTCCGCCACTCCGCTTAAGAAAAAACCGAAGTTTCTTCTTAAGCAGCGTTCGACTTGCATGTATAG

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**Proceeding :**

Sitdhipol, J., Yuphan, P., Visessanguan, W., Tosukhowong, A., Tepkasikul, P., Itoh, T., and Tanasupawat, S. Identification of *Tetragenococcus* strains based on 16S rRNA gene restriction fragment pattern and sequence analyses. The 3rd International Conferences on Fermentation Technology for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University. 26-28 August 2009.

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