DIVERSITY OF HALOPHILIC BACTERIA IN SALINE SOIL AT NONG BO RESERVOIR, MAHASARAKHAM PROVINCE, THAILAND

Mrs. Kannika Chookietwattana

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

Academic Year 2003

ISBN 974-283-006-1

ความหลากหลายของแบคทีเรียชอบเจริญในที่เค็มในดินเค็มบริเวณอ่างเก็บน้ำหนองบ่อ จังหวัดมหาสารคาม ประเทศไทย

นางกรรณิการ์ ชูเกียรติวัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2546
ISBN 974-283-006-1

DIVERSITY OF HALOPHILIC BACTERIA IN SALINE SOIL AT NONG BO RESERVOIR, MAHASARAKHAM PROVINCE, THAILAND

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Thesis Examining Committee

.....
(Dr. Nathawut Thanee)
Chairperson

.....
(Asst. Prof. Dr. Sureelak Rodtong)
Member (Thesis Advisor)

.....
(Dr. Shu Chen)
Member

.....
(Dr. Joseph A. Odumeru)
Member

.....
(Asst. Prof. Dr. Panee Wannitikul)
Member

.....
(Assoc. Prof. Dr. Sarawut Sujitjorn)
Vice Rector for Academic Affairs

.....
(Assoc. Prof. Dr. Prasart Suebka)
Dean of the Institute of Science

กรรณิการ์ ชูเกียรติวัฒนา: ความหลากหลายของแบคทีเรียชอบเจริญในที่เค็มในดินเค็มบริเวณอ่างเก็บน้ำหนองบ่อ จังหวัดมหาสารคาม ประเทศไทย (DIVERSITY OF HALOPHILIC BACTERIA IN SALINE SOIL AT NONG BO RESERVOIR, MAHASARAKHAM PROVINCE, THAILAND)
อาจารย์ที่ปรึกษา: ผศ. ดร. สุรีลักษณ์ รอดทอง, 213 หน้า. ISBN 974-283-006-1

การศึกษาความหลากหลายและความหนาแน่นของแบคทีเรียชอบเจริญในที่เค็มในดินเค็มบริเวณอ่างเก็บน้ำหนองบ่อ อำเภอบรบือ จังหวัดมหาสารคาม ประเทศไทย โดยเก็บตัวอย่างดินเค็มทุกเดือนเป็นเวลาหนึ่งปี ตั้งแต่เดือนมิถุนายน พ.ศ. 2544 ถึง พฤษภาคม พ.ศ. 2545 จากห้าแปลงศึกษาที่มีระบบนิเวศที่แตกต่างกัน ซึ่งเมื่อวิเคราะห์ลักษณะทางกายภาพและเคมีของตัวอย่างดินพบว่า เนื้อดินเป็นดินทรายปนดินร่วนถึงดินร่วนปนดินทราย ดินมีความชื้นต่ำและเป็นดินไม่เค็มถึงเค็มน้อย และมีปริมาณอินทรีย์วัตถุต่ำมาก เมื่อตรวจหาความหนาแน่นของแบคทีเรียชอบเจริญในที่เค็มน้อย เค็มปานกลาง และเค็มจัด โดยใช้อาหารเลี้ยงเชื้อ halobacteria ที่เติมเกลือโซเดียมคลอไรด์ 3% 8% และ 25% ตามลำดับ พบว่าแบคทีเรียชอบเจริญในที่เค็มน้อยมีความหนาแน่นมากที่สุด และพบแบคทีเรียชอบเจริญในที่เค็มปานกลางและที่เค็มจัดมีความหนาแน่นน้อยลงตามลำดับ จากการระบุและจัดจำแนกชนิดของแบคทีเรียชอบเจริญในที่เค็มที่แยกและคัดเลือกมาศึกษาจำนวน 152 ไอโซเลท โดยอาศัยลักษณะปรากฏ การวิเคราะห์กรดไขมันของเซลล์ และการวิเคราะห์ลำดับเบสของ 16S rDNA สามารถจัดแบคทีเรียอยู่ใน 16 สกุล และ 38 สายพันธุ์ ซึ่งส่วนใหญ่เป็นแบคทีเรียในสกุล *Bacillus Halobacillus* และ *Halomonas* ผลการจัดจำแนกชนิดของแบคทีเรียชอบเจริญในที่เค็มทั้ง 152 ไอโซเลท นี้ยังให้ข้อมูลที่เป็นประโยชน์ต่อการนำแบคทีเรียเหล่านี้ไปใช้ประโยชน์ทางเทคโนโลยีชีวภาพ

สาขาวิชาชีววิทยา
ปีการศึกษา 2546

ลายมือชื่อนักศึกษา .....กรรณิการ์ ชูเกียรติวัฒนา.....
ลายมือชื่ออาจารย์ที่ปรึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

KANNIKA CHOOKIETWATTANA: DIVERSITY OF HALOPHILIC BACTERIA IN SALINE SOIL AT NONG BO RESERVOIR, MAHASARAKHAM PROVINCE, THAILAND
THESIS ADVISOR: ASSISTANT PROFESSOR SUREELAK RODTONG, Ph.D. 213 PP. ISBN 974-283-006-1

The diversity and density of halophilic bacteria in saline soil at Nong Bo Reservoir, Borabu District, Mahasarakham Province, Thailand, were studied monthly from June 2001 to May 2002 on five study plots with different ecological systems. The physical and chemical properties of soil samples were analyzed. The soil was loamy sand to sandy loam texture with low moisture content, non-saline to slight saline, and extremely low organic matter. The densities of slightly-, moderately-, and extremely halophilic bacteria were measured using the halobacteria medium containing 3%, 8%, and 25% sodium chloride, respectively. The density of slightly halophilic bacteria was the highest, followed by the moderately- and extremely halophilic bacteria. One hundred and fifty-two halophilic bacterium isolates were selected for characterization using the combination of phenotypic characteristics, fatty acid analysis, and 16S rDNA sequence analysis. These bacteria could be identified as belonging to sixteen genera and thirty-eight species. The dominant genera were *Bacillus*, *Halobacillus*, and *Halomonas*. The characterization of these 152 halophilic bacterium isolates provides valuable information required for future use of these isolates for biotechnological application.

School of Biology
Academic Year 2003

Student's Signature ...Kannika Chookietwattana...
Advisor's Signature ...Sureelak Rodtong...
Co-advisor's Signature .....
Co-advisor's Signature .....

Acknowledgement

I would like to express the deepest gratefulness to my thesis advisor, Asst. Prof. Dr. Sureelak Rodtong, for kindly accepting me as her graduate student to discover the halophiles' world in saline soil. She kindly provided me the laboratory facilities, chemicals, and microbiological media. All her advice is very valuable for my life and future career. My deepest gratitude also to my two co-advisors, Dr. Joseph A. Odumeru and Dr. Shu Chen, for giving me the opportunity to work in the Laboratory Services Division, University of Guelph, Canada, and for their continuous support, encouragement, and valuable suggestions from which I have greatly profited. My warmest thanks to all Thais in Guelph and all the staff of Guelph Molecular Supercentre and Food Microbiology, the Laboratory Services Division, for their readiness to help and warmth support for me throughout my stayed in Canada. All the experience I gained from the Laboratory Services Division is a great value.

I would like to thank Dr. Usa Klinhom for encouraging me to study microbial diversity in saline soil and the kindly cooperative staff of Walairukhavej Botanical Research Institute, Mahasarakham University, in the soil samples collection. My special thanks to Mahasarakham University for providing funds through the University Development Scholarship to pursue my Ph.D. graduate work at Suranaree University of Technology (SUT) and the Mahasarakham University Staff Development Fund for training at the Laboratory Services Division. I also thank the SUT for partial funding for this work with the thesis grant. My thanks and great appreciation to the

following people in the SUT: Areeya, Jitraporn, Kunthika, Nuttika, Phetchara, Wantanee, and Woottiput.

Lastly, I would like to express my deepest gratitude to my mother, my father, and my husband for their greatest love, encouragement, help and support for me to overcome all the challenges and difficulties in this work.

Kannika Chookietwattana

Contents

Page

Contents (Continued)

Page

Contents (Continued)

Page

Contents (Continued)

Page

Contents (Continued)

Page

Contents (Continued)

Page

Contents (Continued)

Page

List of Tables

Page

Table

List of Tables (Continued)

Page

List of Figures

Page

Figure

List of Figures (Continued)

Page

Lists of Abbreviations

ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American type culture collection
bp	Base pair
CFU	Colony forming unit
CTAB	Cetyltrimethylammonium bromide
°C	Degree Celsius
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DGGE	Denaturing gel gradient electrophoresis
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
dTTP	Deoxythymidine triphosphate
EC	Electrical conductivity
EDTA	Ethylene diamine tetraacetic acid
et al.	et alia (and others)
FAME	Fatty acid methyl ester
(k, c, m, μ, n) m	(kilo, centi, milli, micro, nano) Meter
(m, n) g	(milli, nano) Gram
(m, μ) L	(milli, micro) Liter

Lists of Abbreviations (Continued)

(m) M	(milli) Molar
(m, μ) S/cm	(milli, micro) Siemen per centimeter
%	Percent
PCR	Polymerase Chain Reaction
(p) Moles	(pico) Moles
pp	Pages
ppm	Part per million
psi	Pound per square inch
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Round per minute
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
TE	Tris/EDTA (buffer)
Temp	Temperature
T-RFLP	Terminal restriction fragment length polymorphism
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultraviolet
v/v/v	Volume/volume/volume
w/v	Weight/volume
w/w	Weight/weight

CHAPTER I

INTRODUCTION

1.1 Overview of saline soil problems and significance of halophilic bacterial study

Saline soil causes a serious problem of reduced agricultural production in over 100 countries especially in China, India, Pakistan, the United States, and Thailand. In Thailand, there is a widespread saline soil problem in the northeast, which results in low income and poverty of farmers. Nong Bo Reservoir at Mahasarakham Province is one of the areas having a serious problem with saline soil since 1980. The crisis event occurred in 1989 when 50,000 hectares of rice field were affected by the high salinity of reservoir water. The reservoir had received much attention from the Thai Government and other organizations. The reservoir has a total area of approximately 2,000 hectares, and is the Siew River's headwater that serves as the main catchment for large agricultural areas in the northeast Thailand. The National Center for Genetic Engineering and Biotechnology, Thailand, with the cooperation of Khon Kaen University, King Mongkut's University of Technology Thonburi, Land Development Department, Mahasarakham University, Rajabhat Institute Mahasarakham, Rajapruek Institute Foundation, Royal Forestry Department, and Royal Irrigation Department, have been trying to restore the environment of the reservoir to increase the yield of natural resources.

Both physical and chemical methods are not cost-effective for saline soil reclamation of the reservoir because of the occurrence of rock salt strata within the

drainage basin. During the last five years, the main remedial action for salinity amelioration has been replantation. This strategy is to prevent the uplifting of saline groundwater. Unfortunately, the implementation of this strategy was not very successful because of the complicated problems of the soil salinity itself and the other soil problems.

Halophilic microorganisms are organisms that grow optimally in the presence of NaCl at least 0.2 M. The high potential for biotechnological remediation applications using halophilic bacteria have been reported by several authors (Oren, 2002a; Ramos-Cormenzana, 1990; and Ventosa *et al*., 1998b). The applications of halophilic bacteria include recovery of saline soil by directly supporting the growth of vegetation thus indirectly increasing crop yields in saline soil. The other applications of halophilic bacteria were in food and pharmaceutical industries, production of enzymes, polymers and various cosmetic products. With the possibility of application of halophilic bacteria in saline soil recovery and the importance of microbial diversity in soil, the study of halophilic bacterial diversity in saline soil at Nong Bo Reservoir is important in order to realistically access their future application in the rehabilitation of Nong Bo Reservoir. In addition, the halophilic bacterial isolates obtained can be used for the study of other potential applications. The information obtained from this study would also provide information for taxonomists, ecologists, biotechnologists and others who are interested in halotolerant and halophilic bacteria for the effective management of biodiversity conservation to reach a goal of resource sustainability.

1.2 Research objectives

1.2.1 To determine the diversity and density of halophilic bacteria in saline soil at Nong Bo Reservoir, Borabu District, Mahasarakham Province, Thailand.

1.2.2 To isolate and collect halophilic bacteria from saline soil for future investigations, particularly halophilic bacterium applications regarding the recovery of saline soil and increasing crop yield in saline soil and production of specific bacterium metabolites such as enzymes, antimicrobial compounds, and pharmaceutical compounds.

1.3 Expected results

There are two aspects of expected outcomes. The first aspect will be short-term expected outcomes concerning a reveal of halophilic bacterium diversity in saline soil at Nong Bo Reservoir, Borabu District, Mahasarakham Province, nucleic acid techniques for the investigation of halophilic bacteria, and cultural techniques of halophilic bacteria for application in the future. The second aspect will be long-term expected outcomes in which better understandings of taxonomy, physiology, and genetics of halophilic bacteria lead to novel applications; and data from this study allow scientists to modify and control the biodiversity in hypersaline environments. Then it will certainly yield the beneficial outcome for implementation of the conservation and sustainable utilization of microbial diversity in Thailand.

CHAPTER II

LITERATURE REVIEW

2.1 Saline soil

2.1.1 Characteristics of saline soil

Saline soil is soil that has an excess of soluble salts mostly comprising chlorides, sulfates, and bicarbonates of sodium, calcium, and magnesium. Soil is considered saline when the electrical conductivity of the saturated soil extract exceeds 4,000 microsiemen per centimeter (μS/cm), the exchangeable sodium percentage value is less than 15 and the sodium adsorption ratio is less than 13. In addition, the soil has a pH value of 8.5 or less (Ghassemi *et al.*, 1995), and its salinity is greater than 0.2 % (Kaurichev, 1980).

Electrical conductivity (EC) is the most preferred index to assess soil salinity. It is based on the concept that the amount of electrical current carried by a salt solution under standard condition increases as the salt concentration of the solution increases. The EC is expressed in millimhos per centimeter (mmhos/cm) or millisiemen per centimeter (mS/cm) in SI units. Two different methods for measuring EC are commonly used in laboratories and are expressed in two different EC values; EC_e and EC_w (Brady and Weil, 1999). The EC_e refers to conductivity of solution extracted from a water-saturated soil paste. The EC_w refers to conductivity of solution extracted from a 1:2 soil water mixture.

The EC_e and the EC_w values are correlated with each other so that calculations can be made of soil salinity expressed conventionally in terms of EC_e (Brady and Weil, 1999). The following relationship is used to convert the EC_w to EC_e.

$$EC_e = 1.5 \times EC_w \text{ (Landon, 1991)}$$

There are four categories of soil that are defined based on the level of salinity and crop reaction. The four categories are summarized in Table 1.

Table 1. Interpretation of EC_e and total salt content (Landon, 1991).

USDA soil class	**Designation**	**EC_e (mS/cm)**	**Total salt content (%)**	**Crop reaction**
0	Salt free	0-2	<0.15	Salinity effects are mostly negligible except for the most sensitive plants
1	Slightly saline	4-8	0.15-0.35	Yield of many crops restricted
2	Moderately saline	8-15	0.35-0.65	Only tolerant crops yield satisfactory
3	Strongly saline	> 15	> 0.65	Only very tolerant crops yield satisfactory

2.1.2 Cause of soil salanisation

Salanisation is the process whereby the concentration of total dissolved solids (salts) in water and soil is increased. Soil salanisation can be caused by natural processes, also known as primary salanisation, and human-induced processes that known as secondary salanisation.

Primary salanisation is produced as a result of a gradual accumulation of products from the weathering of native rocks, from fossil salts that are derived from prior deposits or from entrapped solutions found in earlier marine sediments (Spark, 1995). The released salts are then transported away from their source of origin through groundwater streams.

The secondary salanisation is the result of the salts stored in the soil profile and/or groundwater being mobilized by extra water provided by human activities. The extra water raises the watertable elevation or increases the pressure of confined aquifers. This creates an upward leakage to watertable aquifers. When the watertables are close to the soil surface, water is evaporated, leaving salts behind and causing land salanisation. There are five human activities causing soil salanisation. These include deforestation, the construction of reservoirs, salt farming, irrigation using saline water, and the lowering of land levels due to erosion and engineering works (Mitsuchi *et al.*, 1986).

2.1.3 Effects of soil salinity

The multiple effects of saline soil can be described as follows:

2.1.3.1 Effects on soil structure properties

Soil salinity has critical effects on the permeability and infiltration of soil. When soil has high sodium ion concentration and low electrical conductivity then soil permeability, hydraulic conductivity, and the infiltration rate are decreased due to swelling and dispersion of clays and slaking of aggregates. Swelling causes the soil pores to become narrow, and slaking leads to reduction of the number of macropores through which water and solutes can flow, resulting in plugging of pores by the dispersed clay.

2.1.3.2 Effects on plant growth

Saline soil causes both poor growth and poor yield of plants. Soluble ions in saline soil can affect plants by reducing the osmotic potential. Thus, salinity contributes to forces which reduce photosynthesis and transpiration by preventing water from entering plant roots (Waisel, 1972). In addition, salinity may reduce plant growth by the direct chemical effect of salt disrupting the nutritional and metabolic process in the plant and/or the indirect effect of salt altering the structure, permeability, and aeration of the soil. The effect of salinity on plant growth is affected by climate, soil conditions, agronomic practices, irrigation management, crop types and varieties, growth stage, and salt compositions (Sparks, 1995). Salinity does not usually affect crop yield until the electrical conductivity exceeds a certain value for each crop. This is known as the threshold salinity level or the threshold EC_e value and it differs for various crops (Bresler *et al.*, 1982; Maas, 1990). In addition to the reduction in crop yield, salinity may also result in poor trafficability, delayed seeding, and limited choice of crops.

2.1.3.3 Effects on soil microorganisms

Soluble ion concentrations (especially sodium ion) greater than about 0.15 M ions in soil leads to hyperosmotic conditions which forces water to diffuse out of a microbial cell. The cells will then shrink or plasmolyse. In addition, the high sodium ion concentration also causes the water associated with such solutes to become unavailable to microorganisms. Basically, the effect of sodium ion on the growth of microorganisms of different species will differ due to growing water activity of each microorganism. Microorganisms under hypertonic environments (low water activity) either die or remain dormant except halotolerant and halophilic microorganisms that can combat this problem. Generally, high soil salinity can interfere with the growth and activity of soil microbes hence it indirectly affects the nutrient availability to plants. Therefore, the study of interaction between soil microorganisms and plant is needed.

2.1.4 Saline soil in the northeast Thailand

In Thailand, saline soil covers an area of 3.61 million hectares (22.5 million rais) (Arunin, 1992). There are two major areas of saline soil in Thailand: the inland in the northeast and along the coastal areas. The majority of saline soil is in the northeast region covering an area of 2.85 million hectares (17.8 million rais). The coastal saline soil covers a total area of 0.58 million hectares (3.6 million rais) (Ghassemi *et al.*, 1995). Saline soil is one of the major problems with soils in the northeast Thailand (Mitsuchi *et al.*, 1986) as can be seen in Figure 1.

The northeast Thailand is located between latitude 14°18′ to 18°33′ North and longitude 100°56′ to 105°07′ East. It is also known as the Korat plateau. The Korat plateau is composed of two basins separated by Phu Phan range. The Korat basin is in the

south of the plateau while the Sakhon Nakhon basin is in the north. The majority of saline soil in the northeast can be found in the Korat basin because the Korat basin lies on a rock salt dome. In some areas the thickness of the rock salt strata is greater than 1 km. The soil salanisation in the northeast Thailand is the result of two causes. The first cause is the naturally occurring salt in the area originating from rock salt strata called the Mahasarakham

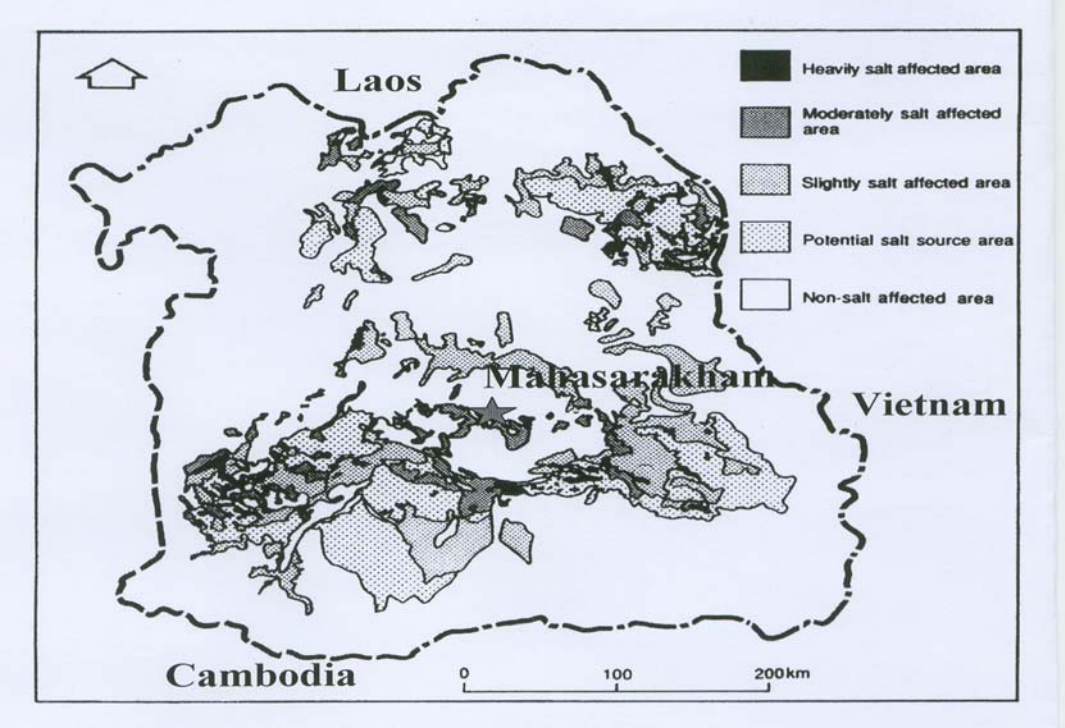


Figure 1 Distribution of salt affected soil in the northeast Thailand (Ghassemi *et al.*, 1995).

formation. The second cause is human activities including deforestation, the construction of reservoirs, the salt farming, and the irrigation of saline water.

Both moderately and heavily salt-affected areas are found mainly in Mahasarakham, Khon Kaen, Nakhon Ratchasima, and Roiet (Rimwanich and Suebsiri, 1984). In Mahasarakham Province, Nong Bo Reservoir is one of the heavily salt-affected areas. Nong Bo Reservoir is located at Borabu District, Mahasarakham Province, Thailand, and was constructed in 1951. It has an area of 320 hectares (2,000 rais), of which about 160 hectares (1,000 rais) hold water while the rest of the area is non-water-holding. The reservoir has the capacity for water holding of up to 2,000,000 cubic meter. It is the Siew River headwater that flow through three provinces in the northeast Thailand: Mahasarakham, Roiet, and Sisaket. The length of the Siew River is approximately 250 km. Nong Bo Reservoir is located on the Mahasarakham formation, a rock salt strata, which is formed as a dome and is only about 10 m depth from the soil surface in the area of the reservoir (นเรศ สัตยารักษ์ และคณะ, 2542). Nong Bo reservoir and the land nearby are good places for salt production. The salt production at Nong Bo Reservoir commenced in 1969 for household consumption and then expanded for the commercial purposes. Severe problems arose in 1980 because the practice of salt farming caused a dramatic increase in water salinity of the reservoir then affected large areas of paddy fields. The salinity of water in the reservoir reached 40.3 mS/cm at the sampling date of August 1986 (Mitsuchi *et al.*, 1986). Tasker (1990) reported that in October 1989 there was an unofficial estimate of 300,000 farmers in 500 villages of Mahasarakham, Roiet, and Sisaket Provinces, covering 50,000 hectares (312,500 rais) of rice field which were affected by the salanised of the Siew River. Therefore, in November 1989, the Thai government banned salt farming practices, especially in the Borabu District. However the

illegal rock salt farming has continued due to the substantial profit and the saline soil problems continue.

According to the 8th National Economic and Social Development Plan of Thailand (1997-2001), there are two strategies for the development of saline soil at Nong Bo Reservoir. The first strategy is the plantation of salt tolerant species and halophytes such as *Acacia mangium* Willd., *Eucalyptus* spp., and *Sporobolus virginicus* in saline soil areas for the environmental rehabilitation and protection of saline soil distribution. The screening of plant species to select those suited to each area problem is being implemented. The plant species chosen to grow in the saline soil area of Nong Bo Reservoir are based on having a fast growth rate, having characteristics to serve as a source of fuel and food, and able to increase soil fertility by fixing nitrogen (ชัยนาม ดิสถาพร, 2542).

The second strategy is to study the application of subsurface drainage system for salt removal from the problematic area (สมศรี อรุณินท์, 2542ข). Since 1994, the National Center for Genetic Engineering and Biotechnology, Thailand, with the cooperation of Khon Kaen University, King Mongkut's University of Technology Thonburi, Land Development Department, Mahasarakham University, Rajabhat Institute Mahasarakham, Rajapruek Institute Foundation, Royal Forestry Department, and Royal Irrigation Department, have tried to recover Nong Bo Reservoir. However, their work is still under way.

2.2 Bacterial diversity in soil

The soil is an important habitat for bacteria. Soil bacteria can be found as single cells or as microcolonies, embedded in a matrix of polysaccharides. Bacteria inhabiting soil play a role in conservation and restoration biology of higher organisms, produce potentially important biotechnology products and are critically important sources of knowledge about the strategies and limit of life. Knowledge of soil bacterial diversity provides a baseline measure against which the impact of environmental changes can be measured. It also leads to better management of bacterial communities, such as improving efficiency of waste treatment systems and developing more effective biological control agents.

2.2.1 Species concept in the study of bacterial diversity

Wiley (1978) defined a species in the evolutionary species concept as "a single lineage of ancestral descendent populations of organisms which maintains its identity from other such lineage and which has its own evolutionary tendencies and historical fate. A key feature of this species concept, relevant to microorganisms, is that it accommodates the capacity to share genetic information between population without the loss of distinct evolutionary roles". Before the availability of molecular techniques, bacterial identification was mainly based on observable phenotypes. The advent of molecular technique together with evolution of the evolutionary species concept has enabled species concepts derived from phenotypic methods to be reassessed. The definition of Wiley is now revealed by comparative sequence analyses and genomic DNA (deoxyribonucleic acids) homology (Woes, 1987). Therefore, the study of bacterial

diversity in the recent years has involved a combination of phenotypic characterization, chemotaxonomy and genotypic characterization in order to lead to a stable bacterial taxonomy.

2.2.2 Culturable and nonculturable bacteria in soil

Classical studied of soil bacterial diversity before the advent of modern bacterial systematics, the study of bacterial diversity in soil was based on laboratory cultivation, which only the culturable bacteria in soil could be detected (Bakken, 1997). Alexander (1977) summarized the culturable bacterium genera in soil as the following ranges (percentage of total viable count): *Alcaligenes* (2-12%), *Agrobacterium* (up to 20%), *Arthrobacter* (5-60%), *Bacillus* (7-67%), *Flavobacterium* (2-10%), and *Pseudomonas* (3-15%). Bakken (1985) reported that the viable culturable cells of bacteria present in the soil samples were usually less than 5% of the total number of viable cells of bacteria while the majority of the viable nonculturable cells lacked the capacity to grow on the standard laboratory media. The viable culturable bacteria are those that can utilize the energy sources provided in the medium under the physical and chemical limitations of the growth medium. In addition, the bacteria generally selected for study are those that grow rapidly on the isolation medium. Thus, the viable nonculturable bacteria, which are the slow-growing bacteria or those that produce extremely small colonies, are commonly overlooked. It could be concluded that the bacteria isolated from the soil samples are the dominant strains that could be cultivated (Tate, 2000).

2.2.3 Methods used in the study of bacterial diversity in soil

Strategies for bacterial diversity analysis can be divided into two categories: (1) those relying on laboratory cultivation or incubation and (2) those based on the direct extraction and analysis of indicator molecules such as nucleic and fatty acids (Ogram and Feng, 1997). For the investigation of bacterial diversity in soil ecosystem, methods based on laboratory cultivation involved four steps: (1) separation of bacterial cells from soil particles, (2) dilution of the cells to an appropriate level, (3) growth of the cells on appropriate growth medium, and (4) characterization of the representative bacterium isolates. The greatest limitation of this approach is that relatively few species present in an environmental sample are readily cultivated (Pace *et al.*, 1986). The methods based on the direct extraction of indicator molecules such as DNA, RNA (ribonucleic acid), and phospholipids, provides data on the bacterial community that can overcome the disadvantages of the former approach.

Over the past decade, the most promising approach for the study of bacterial community involves the molecular techniques that are derived from 16S rRNA comparative sequence analysis (Marsh *et al*., 2000). The techniques commonly used are denaturing gel gradient electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP), which is based on analyzing 16S rDNA fragments obtained by PCR amplification of mixtures of bacterial genomes. DGGE is a technique based on the principle that the differences in base sequence influence the melting behavior. This technique employs a gradient of DNA denaturant. The DNA molecules with different sequences will stop migrating at different positions in the gel. The ARDRA technique involves the PCR

amplification of 16S rDNA from samples. The PCR products are digested with restriction enzymes. The fragments obtained are separated by electrophoresis in a sequencing polyacrylamide gel and visualized as band patterns with ethidium bromide. The T-RFLP technique is in principle similar to the ARDRA. However, there are some differences in that the PCR products are terminally labeled during the amplification process by the use of one fluorescent primer in the PCR reagent mixture. The labeled PCR products are digested with a restriction enzyme and subsequently assayed on an automated fluorescent DNA sequencer. This machine has high sensitivity and only one base difference in length can be detected. Hence, the T-RFLP technique is the most effective at discriminating between bacterial communities in a range of environments and high throughput is also available (Tiedje *et al*., 1999). T-RFLP has been used to assess the bacterial community of various environments such as compost (LaMontagne *et al*., 2002), arid grassland (Kuske *et al*., 2002), and wetland (Michelle *et al*., 2003).

Kuske *et al*. (2002) studied the comparison of soil bacterial communities in rhizospheres in the arid grassland at the Canyonlands National Park, Utah, U.S.A., using T-RFLP analysis. The soil DNA was extracted using a procedure that included incubation in SDS detergent lysis buffer at 70°C, followed by bead mill homogenization and ethanol precipitation. The soil DNA was purified for PCR by passage through Sephadex G-200 minicolumns prepared in 96-well plates. The eluted DNA was precipitated in sodium acetate and ethanol to concentrate for storage. The primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') were

used to amplify representatives of the total bacterial community. The forward primer pA was labeled with 6-carboxyfluorescein during synthesis. The PCR was performed under the following conditions: 94°C for 2 minutes; repeat of 35 cycles of 50°C for 30 seconds, 72°C for 1 minute, and 94°C for 10 seconds; and a final extension of 72°C for 5 minutes. The PCR products were purified with a QIAquick PCR cleanup kit (Qiagen, U.S.A.). The purified PCR amplicons were digested with two restriction enzymes (*Rsa*I and *Msp*I). The DNA fragments were separated by electrophoresis in denaturing 4% polyacrylamide gels using an ABI 377 DNA sequencer. The T-RFLP profiles were converted to binary data (presence or absence of a peak) for distance comparisons of soil bacterial communities using the Jaccard distance measure. Dendrograms were generated from Jaccard distance matrices using an unweighted paired group means analysis (UPGMA).

Though the T-RFLP has proven to be the most effective method for the assessment of bacterial community in soil, it also suffers from DNA extraction biases and PCR biases as with every PCR based methods. The most critical step in soil bacterial community study is the extraction of DNA from the soil samples. The DNA extraction from soil has three requirements that include high DNA yield, DNA free from inhibitors for subsequent molecular biology approaches and representative cell lysis of microorganisms within the samples. Unfortunately, it is very difficult to meet all of these requirements since soil extraction always suffers from humic substances that interfere with subsequent molecular biology approaches. This contaminant can inhibit *Taq* DNA

polymerase in PCR reactions, interfere with restriction enzyme digestion, reduce transformation efficiency and DNA hybridization specificity (Zhou *et al*., 1996). Tsai and Olson (1992) reported that their standard PCR reaction was inhibited by only 10 ng of humic acid present. In addition, the soil DNA extraction also suffers from incomplete cell lysis, DNA sorption to soil surfaces, and loss, degradation, or damage of DNA (Yeates *et al*., 1998). The biases in PCR reactions include: the database for primer sequences is largely of the culturable organisms (Amann *et al*., 1995); and PCR-induced chimeras from the PCR coamplification of mixed Genomes (Liesack *et al*., 1991).

An understanding of the bacterial diversity in hypersaline environments has increased greatly in the last decades by using a combination of laboratory cultivation and molecular biological techniques based on polymerase chain reaction (PCR) amplification of 16S rRNA genes from the biomass present in the samples. However, there were more studies of bacterial diversity in the hypersaline water environment than in the other hypersaline environments (Oren, 2002b).

For example, the study of types of some bacteria isolated from hypersaline soil collected from Alicante, Spain was based on laboratory cultivation. The bacterial cultivation was performed using spread plate technique with the media containing salt concentrations of 9, 50, 100, 200, and 250 g/L and incubated at 30°C for 15 days, then examined daily. Different colonies were randomly chosen for characterization in order to obtain the diversity data (**Quesada** *et al*., 1982).

Martinez-Murcia *et al.* (1995) studied the prokaryotic diversity in hypersaline ponds using the ARDRA technique. The 16S rDNA fragments of the

bacterial and archaeal from the salterns were amplified using the universal primers and subsequently digested with *Hinf*I and *Mbo*I. The products were separated by polyacrylamide gel electrophoresis. The patterns of each sample were compared. Similarity coefficients were calculated, and the similarity dendograms were generated to obtain the diversity data of the saltern ponds.

Litchfield and Gillevet (2002) studied the microbial diversity of the salterns in Shark Bay, Australia, and Eilat, Israel, based on the amplicon length heterogeneity (ALH) procedure. The 16S rDNA of each soil sample was extracted using the Fast DNA Spin Kit for Soil (Qbiogene, U.S.A.). For the water samples, the cell pellet was harvested by centrifugation prior to the extraction of 16S rDNA. The labeled fluorescent dye primer 6-FAM-5' 27F (5'-6- FAM-AGAGTTTGATCMTGGCTCAG-3') and unlabeled primer 355R (5'-GCTGCCTCCCGTAGGAGT-3") were used for the amplification of the 16S rDNA fragments for the *Bacteria*. The primers 6-FAM-5' 1HF (5'-6-FAM-ATTCCGGTTGATCCTGCCGG-3'), primer labeled 6-FAM fluorescent dye, and unlabeled H30-5R (5'-GTTACCCCACCGTCTACCT-3") were used for the amplification of the 16S rDNA fragments for the *Archaea*. The PCR was performed under the following conditions: 94°C for 5 minutes; repeat of 28 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and a final extension of 10 minutes at 72°C. The PCR products were separated on a 4.25% polyacrylamide gel using an ABI 377 Sequencer (Applied Biosystems, U.S.A.). Peaks were quantified based on its fluorescence intensity according to the ABI GeneScan Software.

Cytryn *et al.* (2000) studied of the diversity of *Archaea* in the Solar Lake, Sinai, Egypt, using the direct DNA extraction from the water samples. The archaeal primers, 21F (5'-TTCCGGTTGATCCYGCCGGA-3') and 958R (5'-YCCGGCGTTGAMTCCAATT-3') were employed for the amplification of 16S rDNA fragments. The PCR products were cloned and sequenced. The diversity of *Archaea* in the Solar Lake was assessed by phylogenetic analysis of detected sequences using the ARB program package.

Antón *et al.* (2000) assessed the halophilic bacterial community in the saltern ponds at Alicante, Spain, using the DGGE analysis. Bacterial cells were collected from the water samples by centrifugation. The 16S rDNA were amplified and subsequently separated in a polyacrylamide gel containing a linear gradient of DNA denaturants. The selected DGGE bands were excised from the gel, purified, reamplified, and partially sequenced. The sequences were analyzed by BLAST at the National Center for Biotechnology Information web page and aligned using the ARB program package.

To date the study of bacterial community in saline soil using the T-RFLP analysis has not been reported yet. Kuske *et al*. (2002) stated that no study has yet provided a complete survey of the bacterial community in a single soil sample. Thus, the bacterial community and community changes in soil in response to various environmental condition remain to be discovered.

2.2.4 Bacterial systematics: the basic information for bacterial diversity

Pre-prepared databases for grouped organisms allow new isolates to be catalogued and ordered into their appropriate taxa (Priest and Austin, 1993). Bacterial

systematics is important because it serves as a database about the history, properties and relationships of a particular organism. It also facilitates identification. In conventional bacterial systematics, bacterial species was characterized based on morphology and physiology such as pigmentation, staining properties, the presence or absence of spores, nutritional requirements, sugar fermentation, temperature and pH requirements (and tolerance), and the ability to grow in the presence of inhibitory compounds. These phenotypic characteristics were derived largely on behavioral properties of strains. This classification method may lead to misidentification and misclassification since members of a species may vary by as much as 40 percent in DNA homology (McKane and Kandel, 1996). Also, the over reliance placed on a small numbers of subjectively chosen properties and the properties examined were themselves inadequate for identification. (Stackbrandt and Goodfellow, 1991). Moreover, the conventional bacterial systematics did not reflect the evolutionary relationships between organisms. Methods for bacterial classification have changed dramatically in the past thirty years. Modern bacterial systematics has been recognized by microbiologists due to their need for exploring extreme environments, searching for the new products, and improving the identification methods, has recognized modern bacterial systematics.

2.3 Halophilic microorganisms

Halophilic microorganisms or "salt-loving" microorganisms live in environments with high salt concentration that would kill most other microbes. Halotolerant and halophilic microorganisms can grow in hypersaline environments, but only halophiles

specifically require at least 0.2 M of salt for their growth. Halotolerant microorganisms can only tolerate media containing <0.2 M of salt. Distinctions between different kinds of halophilic microorganisms are made on the basis of their level of salt requirement and salt tolerance.

Larsen (1962) proposed 4 groups of microorganisms inhabiting saline environments: nonhalophiles, those which grow best in the medium containing <2% salt (NaCl), and slight, moderate, and extreme halophiles as those which grow best in the medium containing 2-5%, 5-20%, and 20-30% salt (NaCl), respectively.

Kushner (1993) expanded Larsen's (1962) definition, and proposed the classification of microorganisms' response to salt (NaCl) in which they grow best. Five groups were defined: 1) non-halophilic microorganisms, <0.2 M (~1%) salt; 2) slight halophiles, 0.2-0.5 M (~1-3%) salt; 3) moderate halophiles, 0.5-2.5 M (~3-15%) salt; 4) borderline extreme halophiles, 1.5-4.0 M (~9-23%) salt; and 5) extreme halophiles, 2.5-5.2 M (~15-32%) salt. The halotolerant grow best in media containing <0.2 M (~1%) salt and also can tolerate high salt concentrations. This definition is widely referred to in many reports (Arahal and Ventosa, 2002; Ventosa *et al*., 1998b; and Yoon *et al*., 2003b).

2.3.1 Systematics of halophilic bacteria

There are several problems associated with the accuracy of conventional method for halophilic bacterium systematics (Kushner, 1993). First, the diverse physiology of halophilic bacteria is by no means constant because it is affected by salt concentration. The salt requirement and tolerance properties of the bacteria are highly variable and may vary according to the growth temperature and the nature of the nutrients

available (Ventosa *et al*., 1998b). Halophilic bacteria produce a variety of colonial characteristics from pigmented to non-pigmented according to the salt concentration of the media. Second, halophilic bacteria do not grow fast especially the group of extremely halophilic bacteria. Many of them need natural brines and a variety of nutrients such as pressed fish juices and milk for their growth, as well as yeast extract to support their growth. Third, some lots of peptone of the Difco brand, a common constituent in many biochemical test media, inhibited the growth of halophilic archaeobacteria while the Oxoid brand did not (Vreeland, 1993b). This was shown to be due to the high concentration of bile salts in the Difco Bacto-peptone, compounds to which halophilic archaeobacteria were especially sensitive.

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).

During the last two decades, there have been many attempts to provide a firm systematics base for halophilic bacteria. All of this work used different phenotypic feature tests and analytical systems to cluster the organisms. A successful taxonomy for halophiles has not be developed. Basically, the optimum growth conditions for all of the halophilic bacterial strains under study needed to be established prior to characterization steps. Unfortunately, this taxonomic methodology for the study of halophilic bacterial diversity is very time consuming and costly. Vreeland (1993b) recommended test

methodology for halophilic bacteria in that all tests and test media must be modified by the addition of salts and/or increasing incubation times to account for the requirements of halophiles (this reference book also provides more details on test methodology for halophiles). Presently, the determination of phenotypic and chemotaxonmic characterization along with 16S rRNA gene sequences are commonly used for halophilic bacterial systematics (Ihara *et al*., 1997; Stan-lotter *et al*., 2002; and Yoon *et al*., 2003a).

Historically, the taxonomy of halophilic bacteria was based upon a few phenotypic characteristics with little attention given to either biochemistry or phylogenic of the organisms (Vreeland, 1993b). Once nucleic acid techniques based on 16S rRNA genes were developed, it was revealed that the extremely halophilic archaeobacteria and the halophilic eubacteria have a different phylogenetic branch. Most extreme halophiles are archaeobacteria while the moderate and slight halophiles are members of archaeobacteria and eubacteria. Moderately and extremely halophilic bacteria are the most important group in hypersaline habitats, and receive much attention from microbiologists. There are few studies on slightly halophilic bacteria since the early studies have concentrated on particular habitats such as the Great Salt Lake, Dead Sea, Wadi Natrun, Lake Magadi, and solar salterns. These habitats have one or more harsh environmental conditions such as high salinity, high temperature, low oxygen availability, high nutrient availability, high light intensity, and extremely alkalinity. Only moderately and extremely halophilic bacteria survive and play a major ecological role. The slightly halophilic bacteria constitute a low proportion of the total microbial population (Rodriguez-Valera, 1988) in such habitats. In addition, slightly halophilic

bacteria are similar to bacteria present in common environments making them uninteresting for scientific studies when compared to moderately and extremely halophilic bacteria. Due to the scarcity of information concerning the taxonomy of slightly halophilic bacteria, only the revision on the taxonomy of extremely and moderately halophilic bacteria is available.

2.3.1.1 Extremely halophilic bacteria

The extremely halophilic bacteria (halobacteria) are members of the class *Halobacteria* (order *Halobacteriales* and family *Halobacteriaceae*) (Grant *et al.*, 2001). They are rods, coccus or a multitude of involution forms from disks to triangle. They require at least 1.5 M (~9%) NaCl for growth and lack muramic acid-containing peptidoglycan in the cell envelope. Their colonies are various shades of red due to the presence of C_{50} carotenoids (bacterioruberins). Their intracellular enzymes have a requirement for high levels of KCl, over 3 M and up to 5 M. Their cytoplasmic membrane is composed of phytanyl ether lipids. They are insensitive towards many antibiotics and occur in hypersaline habitats such as salt lakes, soda lakes, and salterns. The family *Halobacteriaceae* consists of 14 genera: *Haloarcula*, *Halobacterium*, *Halobaculum*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halorubrum*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, and *Natronorubrum*.

2.3.1.2 Moderately halophilic bacteria

Moderately halophilic bacteria are bacteria that require at least 0.5 M (~3%) NaCl for growth. They constitute very heterogeneous groups. The taxonomy of

moderately halophilic bacteria presented by Ventosa (1989) is divided into two groups: moderately halophilic eubacteria and moderately halophilic archaeobacteria.

1) Moderately halophilic eubacteria

In general, most halophiles within the *Bacteria* are moderate rather than extreme halophiles (Oren, 2002a). 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 *Vibrio costicola*. Species of gram-positive moderate halophiles are *Micrococcus halobius*, *Sporosarcina halophila*, *Marinococcus halobius,* and *Marinococcus albus*. Phototrophs include *Ectothiorhodospira vacuolata*, *Rhodospirillum salexigens*, and *Rhodospirillum salinarum* (DasSarma, www, 2001; Ventosa, 1989).

2) Moderately halophilic archaeobacteria

Generally, the halophilic *Archaea* are the extreme halophiles. The exception is the methanogens group (Oren, 2002a). Cytoplasmic membranes of methanogenic archaeobacteria contain phytanyl ether lipids as all archaeobacteria. They use methylotrophic substrates rather than carbon dioxide, acetate, and hydrogen, and are

strict anaerobic archaea (Zinder, 1993). Their intracellular salt concentration is somewhat higher than that of most bacteria, about 0.6 mol/L KCl, but is significantly lower than for the extremely halophilic archaeaobacteria. Valid species of moderately halophilic archaeobacteria are *Methanohalophilus mahii*, *Methanohalophilus zhilinae*, *Methanohalophilus halophilus* and *Methanohalophilus portucalensis* (Oren, 2002b).

2.3.2 Habitats of halophilic bacteria

Hypersaline environments originated from two sources: seawater and non-seawater. The former is called thalassohaline, while the latter is referred to as athalassohaline. Thalassohaline environments contain sodium chloride as the predominant salt. The pH of thalassohaline environments is usually near neutral to slightly alkaline. In contrast to thalassohaline environments, dominant ions in athalassohaline environments are potassium, magnesium, or sodium (Litchfield and Gillevet, 2002). Three hypersaline habitats: saline waters, saline soils, and salted foods have been studied intensively and are summarized as follows:

2.3.2.1 Saline water

The best known environment for halophilic bacteria is saline water. Water with salinity more than 3% is considered to be saline (De Dekker, 1983). Examples of saline water are the ocean, the sea, salt lake, and saltern. The diversity of halophilic bacteria present in some well-known saline water has been studied and can be summarized as follows:

1) The Dead Sea (at the border of Israel and Jordan)

The Dead Sea is the athalassohaline environment. It is a very deep lake (maximum depth about 320 m). The water temperature of the sea has negligible changes (21° to 36°C). NaCl constitutes 78% of the salt in the sea. The Dead Sea is slightly acidic. The dominant ions of the Dead Sea are Mg^{2+}, Na^{+}, and Cl^{-} (Javor, 1989).

Several studies of the diversity of halophilic bacteria in the Dead Sea can be summarized as follows. Extremely halophilic archaeobacteria are *Halobacterium* spp. (Nissenbaum, 1975), *Halococcus* spp. (Larsen, 1980), *Halobaculum gomorrense* gen. nov., sp. nov. (Oren *et al.*, 1995), *Haloarcula marismortui*, *Haloarcula vallismortis*, and *Haloferax volcanii* (DasSarma, www, 2001). Halophilic eubacteria (aerobes or facultative anaerobes) are in genera *Flavobacterium*, *Pseudomonas* (Volcani, 1944), *Chromobacterium* (Ventosa *et al*., 1989; Volcani, 1944), *Bacillus*, particularly *Bacillus marismortui* sp. nov. (Arahal *et al.*, 1999) and *Halomonas* (Huval *et al.*, 1988). Halophilic eubacteria (anaerobes) are in genera *Clostridium* (Oren, 1983), *Halobacteroides* (Oren *et al.*, 1984) and *Sporohalobacter* (Oren *et al.*, 1987).

2) The Great Salt Lake, Utah, U.S.A.

The Great Salt Lake in Utah, U.S.A, is the thalassohaline saline and relatively shallow (maximum depth 10 m) desert lake. The Great Salt Lake is slightly alkaline. It is an extremely saline with the salinity up to 33% (Javor, 1989). During the last decade, its salinity had been changed dramatically from 20% to 33% due to the construction of a causeway across the lake in 1959. The causeway separated the lake into two independent water bodies consisting of the north arm and the south arm. The salinity

of the south arm is only about 12% because it receives water from surrounding mountains while the salinity of the north arm is extremely saline (33% salinity). The major salt in the lake is NaCl accounted for 86% (w/w). The temperature of the lake varies greatly (-5° to 35°C) (Post, 1977).

The halophilic bacteria on the Great Salt Lake are divided into three groups: (1) archaeobacteria in genera *Halobacterium*, *Halococcus,* which were found in the north arm due to its high salinity (Post, 1977), and the species of *Halorhabdus utahensis* gen. nov., sp. nov. (Waino *et al*., 2000), and *Methanohalophilus muhii* (DasSarma, www, 2001); (2) eubacteria (aerobes or facultative anaerobes) in the species of *Pseudomonas halophila*, and *Halomonas variabilis* (Fendrich, 1988), *Chromohalobacter marismortui* (Ventosa *et al.*, 1989), *Halobacillus litoralis,* and *Halobacillus trueperi* (Spring *et al*., 1996); (3) eubacteria (anaerobes) in the species of *Haloanaerobium praevalens* (Zeikus *et al.*, 1983), and *Desulfocella halophila* gen. nov., sp. nov. (Brandt *et al.*, 1999).

3) The Solar Lake, Sinai, Egypt

The Solar Lake, on the Sinai coast of the Gulf of Aqaba, is an extremely small monomictic hypersaline lake and is a shallow lake (4-6 m deep). The lake is subject to density stratification, high solar intensity in the lower layer (hence, the name), high evaporation rate, and complex and intense microbial interactions in the water column and in the sediment (Cohen *et al.*, 1977). During the summer the water column is completely oxygenated but in the fall it becomes stratified. The salinity of the lake rises to 20% in the summer and fall because of the high evaporation rate. A wide gravel bar

(60 m) separates the lake from the Red Sea. The lake is fed by occasional rain showers and by seawater seepage from the Red Sea (Javor, 1989).

According to Cytryn *et al.* (2000), halophilic archaeal sequences isolated from the Solar Lake were studied via phylogenetic analysis of 16S rDNA sequences. Most of sequences were defined as 4 clusters. Cluster I belongs to the family *Halobacteriaceae*. Cluster II is phylogenetically located between genera *Methanobacterium* and *Methanococcus*. Clusters III and IX are remotely related to the genus *Thermoplasma*. Two moderately halophilic bacteria of the species *Desulfovibrio halophilus* and *Spirochaeta halophila* are found in the Solar Lake. Two sulfur-oxidizing bacteria of the species *Achromatium volutans* and *Beggiatoa alba* were also isolated from the Solar Lake (DasSarma, www, 2001).

4) Lakes at the Wadi Natrun

The Wadi Natrun is located below sea level in an arid region of the north central Egypt. In the north of the Wadi Natrun, there are several alkaline hypersaline lakes, which are sometimes completely dry. Wadi Natrun lakes have high evaporation rates. Water is replenished by underground seepage from the Nile River that passes through burdi (grass) swamps. The salinity of sediments along the distance of 2 – 100 m from the lakes varies from 3.1 to 8.6% (Javor, 1989). *Bacillus haloalkaliphilus*, a haloalkaliphilic gram-positive bacterium has been isolated from the lake (**Weisser and Trüper, 1985)**. This isolate tolerates up to 4 M NaCl, but grows best from 0.5 to 3 M NaCl. Additionally, phototrophic bacteria and alkalophilic halobacteria have also been isolated from Wadi Natrun lakes (Javor, 1989).

5) Inland saltern of La Mala, Granada, Spain

La Mala is 780 m above sea level with a slope of 2% (Ramos-Cormenzana, 1989). It is an athalassohaline environment in which salts accumulate from the saline groundwater near the soil surface. The well water that feeds the ponds differs from seawater in that the major ion has a lower content of chloride but a high content of Mg^{2+}, Ca^{2+}, and K^{+} than seawater. The total salinity of the well water is 18% (w/v). The bacterial isolates encountered in the inland saltern of La Mala belonged to the following genera: *Acinetobacter*, *Alteromonas*, *Flavobacterium*, *Halobacterium*, *Halomonas*, *Pseudomonas,* and *Vibrio*, (Del Moral *et al.*, 1987).

2.3.2.2 Saline soil

Saline soil is a common hypersaline habitat of halophilic bacteria. The discussion of saline soil in this section is limited to microbiological aspects while in the Section 1.2 provides general information of saline soil and saline soil in the northeast Thailand. The soil habitat is inherently heterogeneous and therefore a wide range of the salinity might be present in any one saline soil and even at different soil depths (Grant, 1991; สมศรี อรุณินท์, 2542ก). The salinity of saline soil is much more variable than saline waters (**Quesada** *et al.*, 1982). Saline soil environments have not been studied intensively (Ramos-Cormenzana, 1989). Therefore very little information is available. Many plants such as salt tolerant species and halophytes are adapted to grow in saline soil. They play various ecological roles involving nutrient cycle and plant-microbial interactions that are different for each saline soil (Rodriguez-Valera, 1993).

Rodriguez-Valera (1988) stated that there was an abundance of halophilic bacteria in saline soil and that the dominant types encountered in saline soil belong to genera of *Alcaligenes*, *Bacillus*, *Micrococcus,* and *Pseudomonas*. A case study of the rhizosphere soil near xerophytic plants growing in hypersaline soil (5 to 10.7% NaCl) near Alicante, showed that the range of salt concentrations allowing the growth of organisms isolated was not correlated with the soil salinity. The optimal growth of all isolates is at salt concentrations between 5 and 15% NaCl, but about half of strains also grow at 0.9% NaCl. Plating on agar media containing 10% salt resulted in mostly gram-positive rods. Gram-negative rods dominated between 10 and 20% salt and gram-positive cocci developed above 20% salt. Isolates of gram-positive bacteria belong to genera (in order of abundance) *Actinomyces*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Micrococcus*, *Nocardia*, *Planococcus,* and *Staphylococcus* (**Quesada** *et al.*, 1982).

2.3.3.3 Salted food

Food in nature is naturally a poor source of salt (sodium chloride). Food becomes salty when salt is added for the preservation as part of some processes or to make them more appealing. Salt has been used to preserve food for thousands of years. Another use of salt has been that of a flavor enhancer. Food preservation often uses salting in conjunction with smoking and curing. Examples of salted food are salted fish, salted hides, bacon, ham, sausage, lunchmeats, hot dogs, crackers, cheese, chips, soy sauce, paste, and sauerkraut.

Halomonas salina was isolated from fully cured wet and dry bachalao that contains about 19% salt (**Vilhelmsson** *et al.*, **1996).** *Pediococcus halophilus*

was the dominant bacterium at the end of the curing process of anchovies (**Villar** *et al.*, **1985). According to DasSarmas, www (2001), several halophilic bacteria are isolated from salted food. *Pseudomonas beijerinckii* has been isolated from salted beans preserved in brine. *Halomonas halodenitrificans* has been isolated from meat curing brines. *Vibrio costicola* has been isolated from Australian bacon. *Halobacterium* sp. and *Halococcus* sp. were isolated from** Thai fish sauce (Thongthai and Suntinanalert, 1991).

2.3.4 Adaptation of halophilic bacteria in response to high osmotic pressure

Availability of water is the most important prerequisite for life of any living cell. The ability of an organism to adapt to changes in external osmotic pressure (osmoadaptation) and the development of mechanisms to achieve this (osmoregulation) are fundamental to its survival (Csonka, 1989). In general, exposure of microorganisms to hypersaline environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell. This causes a reduction in turgor, dehydration of the cytoplasm and is consequently lethal. Halophilic bacteria have adapted during evolution (genotypic and phenotypic adaptation) to optimally grow in hypersaline environments. Therefore they are not stressed by these conditions (Imhoff, 1993). Their adaptation is genotypical while halotolerants adapt phenotypically (Russell, 1989). The degree of salt dependency and salt tolerance of microorganisms is distinguished by their levels of salt requirement and salt tolerance that reflects the differences in osmoadaptation to hypersaline environments.

There are three mechanisms available for adaptation of halotolerant and halophilic microorganisms to high-osmolarity environments: (1) the recognition of

osmotic imbalance by an osmosensor; (2) the accumulation of osmolytes or compatible solutes in response to the imposed pressure difference; and (3) the stabilization of macromolecules under the new intracellular conditions (Roberts, 2000).

2.3.4.1 Osmosensor and osmoregulation by bacteria

An osmosensor is likely to be macromolecules that undergo conformational transitions between "off" and "on" in response to changes in extracellular water activity (direct osmosensing) or resulting changes in cell structure (indirect osmosensing). Responses of bacteria to osmolarity shifts are on the genetic level (transcription) and the enzymatic level (RoeBler and Muller, 2001). This conserved mechanism is known as two component regulatory systems. These consist of a sensor protein, which detects the signal, and a regulator protein that binds DNA and controls gene expression. The two component regulatory systems that respond to the osmotic changes are the EnvZ/OmpR and the KdpD/KdpE. The EnvZ/OmpR has been found in the *Eubacteria* while the KdpD/KdpE has been found in the *Archaea* (RoeBler and Muller, 2001).

The EnvZ is a transmembrane histidine kinase that monitors osmolarity changes on both sides of the cytoplasmic membrane through the OmpR (Bartlett and Roberts, 2000). It functions as a dimer with a part in the cytoplasm and a part extending outside the membrane. It has three separate enzymatic activities.

The KdpD, a sensor kinase, is autophosphorylated under a decrease in turgor pressure condition. Subsequently, the phosphoryl group is transferred to the response regulator, KdpE, which then acts as a transcriptional activator for the *KdpABC*

operon encoding a primary ABC-type K^+ transporter. This allows the cell to counteract the stress by increasing the internal osmolarity through the accumulation of K^+ via the KdpABC ATPase (Poolman *et al*., 2002).

2.3.4.2 Accumulation of compatible solutes by halophilic bacteria

Microorganisms in hypersaline environments need to balance their cytoplasm with the osmotic pressure exerted by the external medium. An osmotic balance can be achieved by the accumulation of compatible solutes. The solutes can be accumulated at high concentrations without interfering with cellular processes (Brown, 1976)**.** However, when the turgor becomes too high, microorganisms need to excrete compatible solutes from their cytoplasm. Halophilic eubacteria accumulate compatible solutes by either *de novo* synthesis or uptake from the medium. **Compatible solutes can be classified into 2 groups: inorganic ions (intracellular concentrations of inorganic cations:** K^+, Mg^{2+}, and Na^+**) and organic solutes (betaines, ectoines, and glycine). The accumulation of inorganic ions,** salt-in cytoplasm strategy, is found in the *Archaea* of the family *Halobacteriaceae* and the *Bacteria* of the order *Haloanaerobiales*. **The accumulation of** specific organic solutes, organic solutes strategy, is found in all other species of halophilic bacteria (Da Costa *et al.*, 1998). **However, it may be a combined function of inorganic ions and organic solutes, since the inorganic ions are insufficient to provide osmotic balance with the external medium** (Ventosa *et al.*, 1998b).

The salt-in cytoplasm strategy is strictly confined to those environments in which salinity is high and relatively stables (Yancey *et al.*, 1982). The

major type of intracellular ion is K^+, which is less harmful for the enzymes in the cytoplasm than Na^+ intracellularly at high concentration. The intracellular ion concentrations can be varied among different species of halophilic bacteria and even within the same species by means of transport, synthesis or catabolism. However, the following trends are clear: (1) the intracellular K^+ concentration is generally higher than that outside; (2) the intracellular Na^+ concentration is generally lower than that in the medium; (3) the intracellular K^+ concentration increases with increasing external NaCl concentration in a nonlinear pattern **(Roberts, 2000).** The Kdp is the main transport system for K^+. The K^+ uptake via the Kdp system is osmotically regulated on the activity level as well as on the level of gene expression.

There are three classes of organic solutes in bacteria: (1) zwitterions (amino acids and derivatives including betaines, ectoine, proline, and beta-glutamate); (2) neutral solutes including carbohydrates such as sucrose, trehalose, mannosucrose, and polyhydric alcohols such as glycerol and inositol; and (3) anionic solutes where the negative charge is supplied by a carboxylate, phosphate or sulfate. Zwitterions are most common solutes in moderately halophilic bacteria while anionic solutes are the major class of osmolytes in extremely halophilic archaeobacteria (Roberts, 2000). Several osmotically regulated transporters are responsible for the uptake of organic solutes. For examples, in *E. coli*, the betaines are uptaked from the external medium by two osmotically regulated transporters, BetP and OpuP. The proline is accumulated from the external medium by two osmotically regulated transporters, ProP and ProU (Bartlett and Roberts, 2000; Wood, 1999). The effect of compatible solutes is

not only that they can be accumulated up to molar concentrations without inhibiting enzyme function, but that they are able to stabilize proteins. The ability to stabilize proteins under unfavorable conditions is also reflected by the fact that most of the organic solutes do not only confer increased tolerance towards higher osmolarity but also towards desiccation, freezing, and elevated temperatures. Thus, bacteria using organic solute strategy can tolerate fluctuations in ambient salinity far better than bacteria using the salt-in cytoplasm strategy. However, the disadvantage of organic solute synthesis is that it consumes more energy than the accumulation of K^+.

2.3.4.3 Stabilization of macromolecules

Since changes in water activity can also have a profound effect on protein stability and folding, cells also respond to changes in osmotic pressure with mechanisms to promote correct protein folding. This may involve the adaptation of their membrane, the accumulation of compatible solutes and the adaptation of macromolecules to high ionic strength. Extremely halophilic archaeobacteria possess typical *Archaea* lipids, which are genotypically adapted by having additional substitutions with negative charges such as sulfate on the surface of their membrane. This has the advantage that the negative charges on the polar headgroups are shielded by the high ionic concentration, preventing disruption of the lipid bilayers due to charge-repulsive forces and providing a charge stabilized lipid bilayers. Unlike extremely halophilic archaeobacteria, the moderately halophilic bacteria increase the amount of negative charges upon an increase of salt concentration in their growth medium.

Different solutes accumulated can have different effects on proteins and their structures and activities. Bacteria using the salt-in strategy have intracellular proteins with a higher proportion of acidic amino acids and a lower proportion of non-polar residues than proteins usually contain. On the contrary, since organic solutes act as osmoprotectants, there is no need for modification of a vast group of proteins when the salt concentration of the environment changes. Therefore bacteria using the organic solute strategy can tolerate fluctuations in ambient salinity far better than bacteria using the salt-in strategy. The latter are strictly confined to those environments in which the salinity of the environment is both high and relatively stable (Yancey *et al*., 1982), since many enzymes and other proteins require the continuous presence of high salt for activity and stability (Oren, 1999). In addition, all halophilic microorganisms contain potent transport mechanisms, generally based on Na^+/H^+ antiporters, to expel sodium ions from the interior of the cell (Oren, 2002c).

2.3.5 Applications of halophilic bacteria

Halophilic bacteria provide a high potential for biotechnological applications for at least two reasons: (1) their activities in natural environments with regard to their participation in biogeochemical processes of C, N, S, and P, the formation and dissolution of carbonates, the immobilization of phosphate, and the production of growth factors and nutrients (Rodriguez-Valera, 1993); and (2) their nutritional requirements are simple. The majority can use a large range of compounds as their sole carbon and energy source. Most of them can grow at high salt concentrations, minimizing the risk of

contamination. Moreover, several genetic tools developed for the nonhalophilic bacteria can be applied to the halophiles, and hence their genetic manipulation seems feasible (Ventosa *et al*., 1998b). The current industrial applications and the possibilities of their biotechnological applications are summarized as follows.

First, halophilic bacteria have the ability to produce compatible solutes, which are useful for the biotechnological production of these osmolytes. Some compatible solutes, especially glycine, betaines, and ectoines, may be used as stress protectants (against high salinity, thermal denaturation, desiccation, and freezing) and stabilizers of enzymes, nucleic acids, membranes and whole cells (Galinski, 1993). The industrial applications of these compounds in enzyme technology are most promising (Ventosa and Nieto, 1995). The other compatible solutes such as trehalose, glycerol, proline, ectoines, sugars, and hydroxyectoine from halophilic bacteria showed the highest efficiency of protection of lactate dehydrogenase against freeze-thaw treatment and heat stress. Ectoine was also the most effective freeze-stabilizing agent for phosphofructokinase (Wohlfarth *et al.*, 1989).

Secondly, halophilic bacteria produce a number of extra- and intra-cellular enzymes and antimicrobial compounds that are currently of commercial interest (Kamekura and Seno, 1990). Halophilic bacteria can produce enzymes that have optimal activity at high salinity, which is advantageous for harsh industrial processes. Several extracellular enzymes have been reported to be produce from halophilic bacteria such as amylases from *Micrococcus halobius* (Onishi, 1970) and *Halobacterium halobium* (Good and Hartman, 1970), nucleases from *Micrococcus varians* (Kamekura and Onishi, 1974a)

and *Bacillus* sp. (Onishi, *et al*., 1983) as well as proteases from *Halobacterium salinarum* (Norberg and Hofsten, 1969), *Bacillus* sp. (Kamekura and Onishi, 1974b) and *Pseudomonas* sp. (Makino *et al.*, 1981). A serine protease from an unidentified member of the family *Halobacteriaceae* showed high activity at 75-80°C in the presence of 25% (w/v) salt (Galinski and Tindall, 1992).

Third, halophilic bacteria produce a variety of hydroxyalkanoates, which are useful for thermally processed plastics (Vreeland, 1993a). *Haloferax mediterranei* produces exopolysaccharides (up to 3 g/L) with pseudoplastic behavior that are resistant to pH, heat, and shear. They show higher viscosity at dilute concentrations and elevated temperatures than commercial polymers such as xanthan gum (Galinski and Tindall, 1992; and Ventosa *et al*., 1998b).

Lastly, the application of halophilic bacteria in environmental biotechnology is possible for (1) the recovery of saline soil, (2) the decontamination of saline or alkaline industrial wastewater, and (3) the degradation of toxic compounds in hypersaline environments.

The use of halophilic bacteria in the recovery of saline soils is covered by the following hypotheses. The first hypothesis is that microbial activities in saline soil may favor the growth of plants resistant to soil salinity. The second hypothesis is based on the utilization of these bacteria as bio-indicators in saline wells. Indicator microorganisms can be selected by their abilities to grow at different salt concentrations. These organisms could indicated that well water could be used with producing low saline contamination of plants or soils which could be alleviated desertification of soil (Ramos-

Cormenzana, 1993). Last hypothesis is the application of halophilic bacterium genes using a genetic manipulation technique to assist wild type plants to adapt to grow in saline soil by giving them the genes for crucial enzymes that are taken from halophiles. The production of genetically modified plants has however been controversial.

Woolard and Irvine (1992) reported the utilization of a biofilm of a moderately halophilic bacterium isolated from a saltern at the Great Salt Lake, Utah, for the treatment of hypersaline wastewater containing phenol. By using a batch biofilm reactor, more than 99% of the phenol was removed from a waste containing 15% salt. Benzoate and other aromatic compounds could be degraded by *Pseudomonas halodurans* by cleavage of aromatic rings (Rosenberg, 1983). Hayes *et al.* (2000) stated that *Chromohalobacter marismortui* or *Pseudomonas beijerinckii*, moderately halophilic bacteria, isolated from a hypersaline spring in Utah could have utilized organophosphates as phosphorus sources for growth.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents

3.1.1 Chemicals and reagents for microbiological analysis

The microbiological media preparations are given in Appendix A as described by Atlas (1997a). The chemicals and reagents used for identification of bacterial isolates based on conventional method were Gram staining reagents, nitrate test reagents, Kovács' reagent, 1% solution of dimethyl-p-phenylenediamine dihydrochloride, 3% hydrogen peroxide, Methyl red test reagent, Voges-Proskauer reagents, McFarland scale 0.5, antibiotic disks (Oxoid, U.K.), and various sugars for testing acid from carbohydrate utilization. The preparation of these reagents is given elsewhere in section 3.7.1.3 and section 2 Appendix A (Beishier, 1991 and Smibert and Krieg, 1994).

3.1.2 Reagents for fatty acid extraction

The reagent preparations for fatty acid extraction are shown in section 3 Appendix A as described by Sasser (1990). A calibrated mixture and extracted fatty acid of *Stenotrophomonas* (*Xanthomonas*) *maltophilia* were purchased from the MIDI, U.S.A.

3.1.3 Reagents for nucleic acid analysis

The reagent preparations for nucleic acids analysis are given in Appendix A. The reagents used for DNA extraction were lysis buffer, 10% SDS, phenol, TE-saturated, chloroform:isoamyl alcohol (24:1), TE buffer, absolute ethanol, and 70% ethanol. The reagents used for amplification of 16S rDNA were PCR buffer without $MgCl_2$ (100 mM tris-HCl, pH 8.3; 500 mM KCl), $MgCl_2$ solution (25 mM $MgCl_2$ in sterile water), dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), primers, and *Taq* DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems, U.S.A.). The reagents used for sequencing were prepared as described in the manual of ABI PRISM^{TM} 3100 Genetic Analyzer (Applied Biosystems, U.S.A.).

3.2 Instrumentation

Instruments required for the determination of soil moisture content, pH, and salinity, and the determination of halophilic bacterial density and their phenotypic characteristics were located at the Instrument Buildings of the Centre for Scientific and Technological Equipment, Suranaree University of Technology, Thailand. Instruments required for the characterization of halophilic bacterium isolates using nucleic acid techniques and chemical analysis were located at the Laboratory Services Division, University of Guelph, Canada. Instruments required for the determination of soil texture, organic matter, total nitrogen, available phosphorus, and available potassium were located at the Office of Land Development Region 5, Thailand.

3.3 Site description

The study area, Nong Bo Reservoir, is located in the center of Korat Basin between latitude 16°01′ to 16°02′ North and longitude 103°00′ to 103°04′ East with the total area of approximately 320 hectares (2,000 rais). About 160 hectares (1,000 rais) is the catchment area. It is situated in Borabu District, Mahasarakham Province, Thailand. The geology consists of Korat soil (Paleustults) which is Red-Yellow-Podzolic soil (Mitsuchi *et al.*, 1986). This soil is directly and indirectly derived from the Mahasarakham Formation (formerly called the Salt Formation). The weather of the study area is tropical Savanna. The amount and distribution of rainfall are influenced by the depression storm that has erratic direction. The sampling sites were divided into 5 plots according to the difference in ecological systems (Figure 2) as described in Table 2.

Table 2. Details of 5 sampling plots at Nong Bo Reservoir.

Plot	Ecological system	Total area of each plot in hectares (unit in rais)	Area of each subplot in hectares (unit in rais)
1	Fishponds	11.2 (70)	3.7 (23.3)
2	The halophyte plot	8.0 (50)	2.6 (16.6)
3	The mixed-agricultural experimental plot	12.8 (80)	4.2 (26.6)
4	The halophyte experimental plot	5.1 (32)	1.7 (10.6)
5	The land where a salt storehouse was located	6.4 (40)	2.1 (13.3)

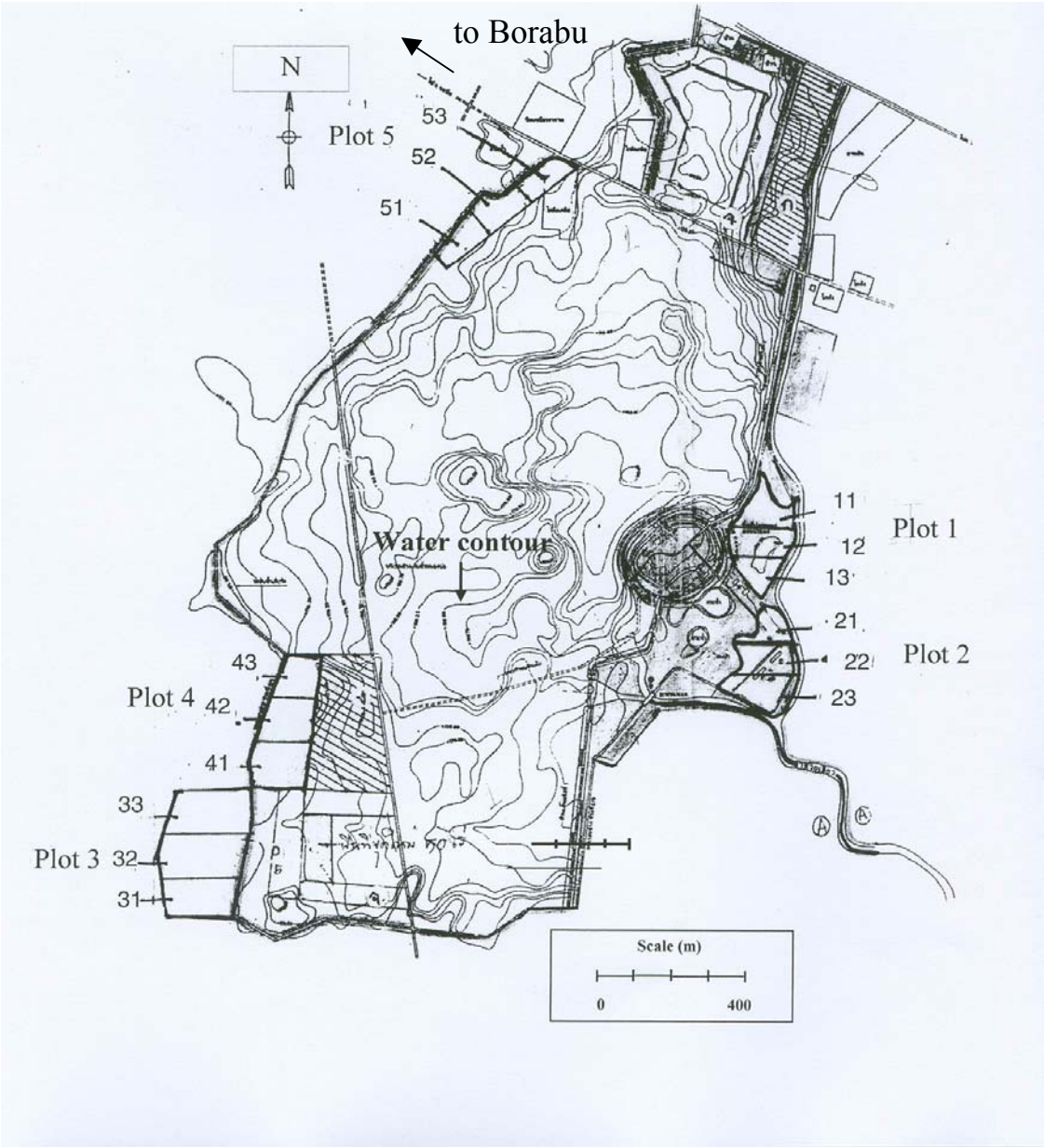


Figure 2. Location of sampling plots at Nong Bo Reservoir.

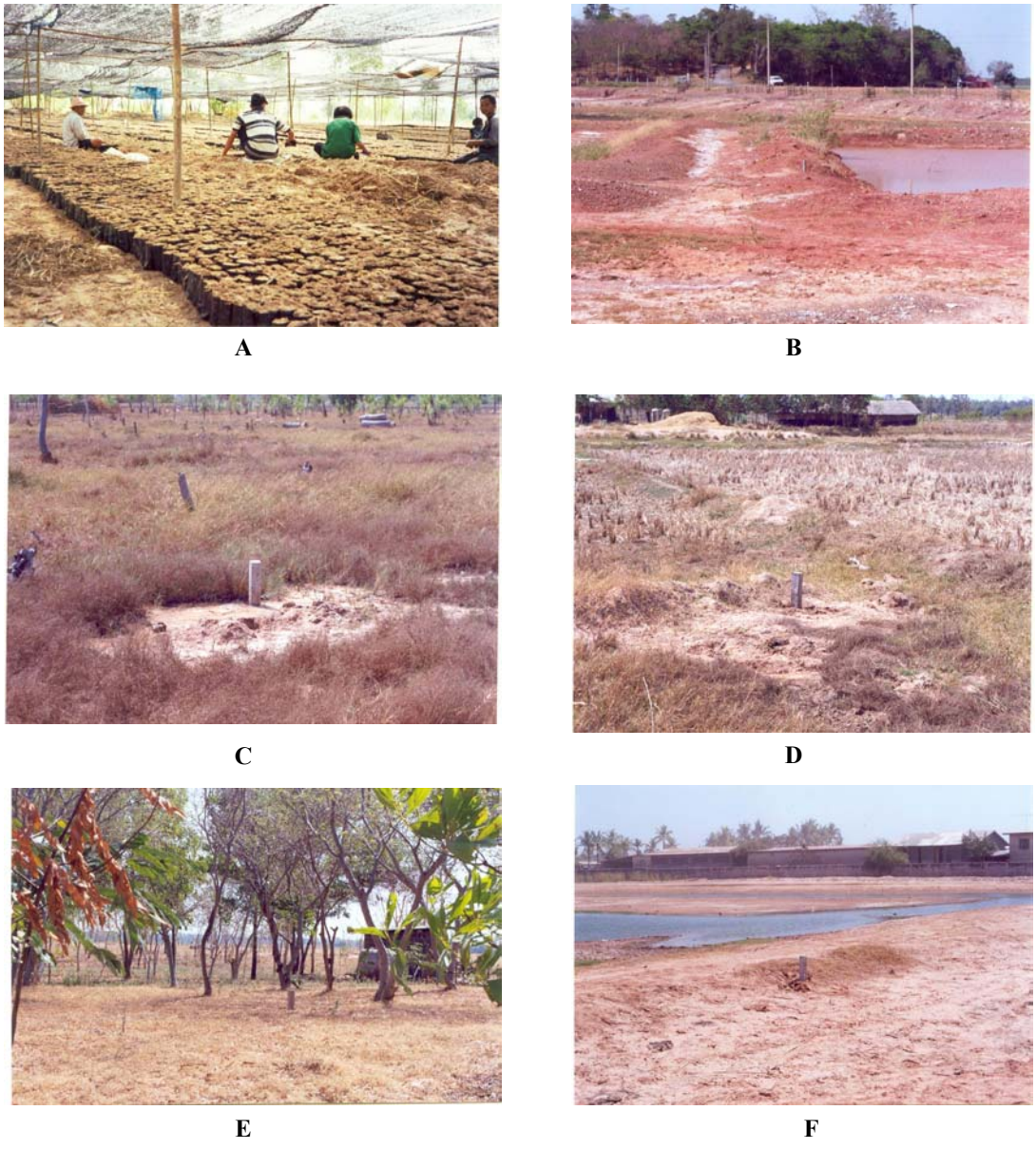


Figure 3. Study area at Nong Bo Reservoir: A, preparing for plantation; B, the sampling plot 1; C, the sampling plot 2; D, the sampling plot 3; E, the sampling plot 4; and F, the sampling plot 5. The pictures were taken in December 2001.

3.4 Saline soil sample collection

Three equal areas in each sampling plot were marked out for three subplots (Table 2). Soil samples were collected monthly for one year during June 2001 to May 2002. In all subplots, the samples were randomly taken at the depth of 50 cm using a hand auger. The auger bucket head has the diameter of 5 inches and length of 12 inches. Samples of the same subplot were mixed together to obtain the composite sample for each subplots. Then a portion (1.0 km) of the composite soil sample was collected and stored at 4°C for further examination.

3.5 Physical and chemical analysis of saline soil samples

Temperature of air and soil at the soil surface (6 cm) were determined and recorded on site. Soil moisture content, pH, and salinity were analyzed monthly at the Instrument Buildings of the Centre for Scientific and Technological Equipment, Suranaree University of Technology, Thailand. The soil texture (sand, silt, clay), organic matter, total nitrogen, available phosphorus, and available potassium of the soil samples were analyzed seasonally at the Office of Land Development Region 5 according to the collaboration. The reference for the determination methods of these soil properties is กองวิเคราะห์ดิน (2544). Methods for the determination of air temperature, soil temperature, soil moisture content, soil pH, and soil salinity are described as follows:

3.5.1 Determination of air and soil temperature

The thermometer was placed at the depth of 6.0 cm from the soil surface. The thermometer was held for a few minutes until the temperature was stable, then recorded as the soil temperature in the unit of °C. At the same time, the air temperature was determined and recorded in the units of °C.

3.5.2 Determination of soil moisture content

Soil moisture content has been expressed as the ratio of the mass of water present in a sample to the mass of the sample after it has been dried to constant weight. To determine this, the mass of the sample must be determined before and after the removal of water on the basis of oven-dry soil at 105°C. The procedures of the determination of soil moisture content are as follows:

1. The empty sample container was dried at 105°C for 24 hours or until weight becomes constant.

2. The empty sample container was allowed to cool in the desiccator. Then, the empty sample containers were weighed and recorded in the unit of grams.

3. About 50.0 g of sample were placed in the empty sample container. The container was weighed again, recorded was the weight of the wet soil plus container.

4. The moist soil in the container was dried in 105°C oven for 24 hours.

5. The empty sample container was removed from the oven and allowed to cool in the desiccator.

6. The container was weighed again, recorded was the weight of the dry soil plus container. The soil moisture content was calculated by using the following equation.

$$\text{Soil moisture content (\%)} = \frac{\text{(wet weight soil + tin) - (dry weight soil + tin)}}{\text{(dry weight soil + tin) – tin weight}} \times 100$$

3.5.3 Determination of soil pH

Soil pH is a measure of the activity of ionized H (H^+) in the soil solution. The pH of soil is potentiometrically measured in the supernatant suspension of 1: 2 soil: liquid (w/v) mixture. This liquid is made up of distilled water. The soil pH was measured with conductivity meter Model 4200 (Jenway, U.K.). The calibration of conductivity meter was obtained using KCl solutions according to the meter's instruction manual. For the determination of soil pH, twenty grams of air-dry soil was weighed into a 100 mL beaker. Then 20 mL of distilled water was added to each beaker and then stirred periodically with a glass rod for a period of 30 minutes. Soil suspension was let to stand for 10 minutes. After that, an electrode was inserted into the container and swirled slightly. The pH was read and recorded.

3.5.4 Determination of soil salinity

Soil salinity was measured using conductivity meter Model 4200 (Jenway, U.K.) with optional for the determination of salinity. The calibration of conductivity meter was obtained using KCl solutions according to the meter's instruction manual. For the determination of soil salinity, 50 g air-dry soil was weighed into a 250 mL beaker. 50 mL of deionized water was added to each beaker and then stirred periodically with a glass rod

for a period of 30 minutes. Soil suspension was let to stand for 10 minutes. After that, an electrode was inserted into the container and swirled slightly. The salinity was read and recorded in the units of percent.

3.6 Microbiological analysis of saline soil samples

Three groups of halophilic bacteria namely: slightly-, moderately-, and extremely halophilic bacteria in saline were enumerated and isolated using the halobacteria medium (section 1.1 Appendix A) containing 3%, 8%, and 25% NaCl, respectively, according to their salinity requirements (Kushner, 1993). The preparation procedures for these media are summarized in Appendix A. Forty-five g of soil were placed into the bottle containing 45.0 mL of 0.1% peptone water to make the 1:2 dilution. This was shook vigorously for two minutes. One milliliter of soil solution was transferred to the bottle containing 4.0 mL of 0.1% peptone water to make the 1:10 dilution. The bottle was vortexed. Then serial dilutions of $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$ were prepared. The dilutions of $1:10^4$, $1:10^5$, and $1:10^6$ were used for the enumeration of slightly- and moderately-halophilic bacteria. Dilutions of 1:2, 1:10, and $1:10^2$ were used for enumeration of extremely halophilic bacteria using the standard spread plate method. Each selected dilution of each soil sample was analyzed in duplicate. The plates were inverted and incubated for 3 to 7 days at 37^oC. Results were recorded as colony forming unit (CFU) per gram dry soil. Different colonies grown on media were selected and purified for further investigation.

3.7 Characterization of bacterial isolates

Once pure cultures of bacterial isolates were obtained, they were initially characterized by Gram stain examination and some cultural characteristics tests were conducted to obtain representative bacterial isolates. The re-cultivation of moderately halophilic bacterium isolates on halophiles moderate medium (section 1.3 Appendix A)was the additional procedure to screen the true moderately halophilic bacteria (Ramos-Cormenzana, 1993). Representative bacterial isolates were characterized by using three aspects for bacterial characterization: phenotypic characterization, chemical analysis, and genotypic characterization.

3.7.1 Phenotypic characterization

The phenotypic characteristics of all isolates studied were determined and compared to phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986; Grant *et al*., 2001; Kloos and Schleifer, 1986; Meyer, 1989; Palleroni, 1984; Schleifer, 1986; and Vreeland, 1984). The phenotypic features characterized in this study were as follows:

3.7.1.1 Morphological characteristics

Gram stain reaction, cell morphology and motility of bacterial isolates were examined as described by Cappuccino and Sherman (1999).

3.7.1.2 Cultural characteristics

Colony morphology of bacterial isolates was evaluated from first picked colonies from the original plate of soil dilution plate count.

3.7.1.3 Physiological characteristics

1) pH-, temperature-, and salt tolerance tests

Bacterial isolates were tested for growth at different pH tolerance (4, 5, 7, 9, and 11), temperature ranges (10, 25, 35, 45, and 50°C), and salt ranges (0, 3, 8, 15, 20, 25, and 32% NaCl), on halobacteria broth (section 1.1 Appendix A). For pH tolerance test, only the pH of the broth was varied while the temperature and NaCl concentration were fixed at 37°C and at appropriate NaCl concentration according to the requirement of each group of bacterial isolates to be tested. For temperature range test, only the temperature of the broth was varied while the pH and NaCl concentration were fixed at 7.2 and at appropriate NaCl concentration according to the requirement of each group of bacterial isolates to be tested. For salt range test, only the NaCl concentration of the broth was varied while the pH and temperature were fixed at 7.2 and 37°C. The inoculum size of 0.1 mL (approximate 10^8 CFU/mL) was aseptically transferred to 4.0 mL of halobacteria broth for each test. The optical density at the wavelength of 600 nm was used for evaluating bacterial growth.

2) Biochemical tests

Varieties of biochemical tests were conducted according to the standard determinative bacteriology procedure (Beishier, 1991 and Smibert and Krieg, 1994). The biochemical tests were as follows:

A. Nitrate reduction test

The inoculum size of 0.1 mL (approximate 10^8 CFU/mL) was inoculated to a nitrate reduction broth (section 1.9 Appendix A) and incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. Then 1.0 mL of solution A (section 2.7 Appendix A) was added to each culture tube, followed by 1.0 mL of solution B (section 2.7 Appendix A) and mixed thoroughly. The development of a red color indicated a positive test while no red color developed indicates a negative test. The result of a negative test was confirmed by adding small amounts of zinc powder. The true negative test was indicated by the development of the red color.

B. Methyl red test

The inoculum size of 0.1 mL (approximate 10^8 CFU/mL) was inoculated to the MRVP medium (section 1.7 Appendix) and incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. For result testing, 5 drops of the methyl red solution (section 2.6 Appendix A) was added directly to the broth. The development of a stable red color in the medium indicated a positive result.

C. Voges-Proskauer test

The inoculum size of 0.1 mL (approximate 10^8 CFU/mL) was inoculated to the MRVP medium and incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. For result testing, 1 mL of the broth was transferred to a clean test tube. The 0.6 mL of solution A (section 2.8 Appendix A) was added to the tube, followed by 0.2 mL of solution B (section 2.8 Appendix A). The tube was shook

gently to expose the medium to atmospheric oxygen and allowed to remain undisturbed for 10 to 15 minutes. A positive result was indicated by the development of a red color in 15 minutes or more but was not over 1 hour after reagents were added.

D. Indole formation

The inoculum size of 0.1 mL (approximate 10^8 CFU/mL) was inoculated to a tryptone broth (section 1.15 Appendix A) and incubated at 35°C for 48 hours to 1 week o r until bacterial growth was observed. For result testing, 1.0 mL of Kovács' reagent solution A (section 2.4 Appendix A) was added. The positive result indicated by a bright fuchsia red color at the interface of the reagent and the broth within seconds after adding the reagent.

E. Acid from carbohydrate utilization

Eight different sugars including L-arabinose, D-fructose, D-galactose, glucose, lactose, D-mannitol, D-mannose, and D-xylose, were used to test for acid production from carbohydrate utilization using the method modified from New Zealand Dairy Research Institute (1995). For preparation of inoculum, a pure culture of bacterial isolates to be tested was grown in halobacteria broth (section 1.1 Appendix A) until the log phase. A sterile cotton swab was dipped into the inoculum. The swab was streaked all over the surface of the halobacteria medium using the three dimension swab technique. The plate was incubated at 37°C for 48 hours to one week or until bacterial growth observed. Then, 5.0 mL of sterile phosphate buffer was added to the plate surface to dilute the bacterial cells. The cell suspension was transferred to new sterile vial and used for diluting to obtain a 4-6 McFarland unit suspension in 2 mL of sterile phosphate

buffer water as compared its turbidity to the 4-6 McFarland turbidity standards (section 2.5 Appendix A). Next, 200 µL of the 4-6 McFarland unit suspension was transferred to 6 mL of phenol red broth (section 1.11 Appendix A) and mixed thoroughly. The 6.8% concentration of each sugar were prepared by filtration through a 0.45 µm pore size membrane filter (Millipore, U.S.A.) onto the sterile container. The 50 µL of each sugar (for obtaining the final concentration of sugar at 2%) was distributed in each well of a sterile microtiter plate (recorded of which well contains which sugar). Then 120 µL of the phenol red broth containing bacterial suspension was added to each carbohydrate in the microtiter plate. The microtiter plate was incubated at 37°C for 48 hours to one week or until bacterial growth was observed. Acid production was indicated by the color of the broth changing from red to yellow.

F. Starch hydrolysis

A starch hydrolysis agar (section 1.12 Appendix A) plate was inoculated with each bacterium to be tested and incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. The plate was then flooded with iodine solution (section 2.2.3 Appendix A), then the result was observed. Blue color of the agar around the colony of the test organism indicated negative result (no starch hydrolysis), while a clear zone around the colony of the test organism indicated positive result (positive starch hydrolysis).

G. Gelatin hydrolysis Each bacterium to be tested was stabbed deep into the gelatin medium (section 1.10 Appendix A). The inoculated gelatin

medium was incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. Gelatin hydrolysis was indicated by the liquification of the medium after the tube was kept at 4°C for 20-30 minutes.

H. Hydrogen sulfide production

Each bacterium to be tested was stabbed deep into the lead acetate agar (section 1.4 Appendix A) slant and also streaked on the surface of the slant, then incubated at 37°C for 48 hours to 1 week or until bacterial growth was observed. Brownish color formed on the surface and along the line of the stab indicated the positive result.

3) Enzymatic tests

A. Catalase test

The bacterial cells were transferred to the surface of a glass slide. 1 or 2 drops of 3% hydrogen peroxide (section 2.3 Appendix A) were added over cells. Rapid appearance of gas bubbles indicated a positive result.

B. Oxidase test

The filter paper (Whatman no. 40) was placed into a petri dish and wet with 0.5 mL of 1% dimethyl-p-phenylenediamine dihydrochloride (section 2.1 Appendix A). Then, the bacterial cells were streaked onto the reagent zone of the filter paper. The development of a deep blue color at the inoculation site within 10 seconds indicated a positive result.

3.7.1.4 Antimicrobial sensitivity test

The susceptibility to antimicrobial agents was tested on Mueller-Hinton II agar (section 1.8 Appendix A) with antibiotic disks (Oxoid, U.K.) by the method of Bauer *et al*. (1966). The test was applied to only the isolates obtained from the soil dilution plating contained 25% NaCl. The antibiotics tested were penicillin, erythromycin, bacitracin chloramphenicol, streptomycin, tetracycline, vancomycin, and kanamycin. For inoculum preparation, the bacterial cells grown in halobacteria broth were diluted with sterile phosphate buffer to achieve 0.5 McFarland turbidity. The 0.5 McFarland suspension (approximate 10^8 CFU/mL) was streaked all over the surface of the Mueller-Hinton II agar (supplemented with 20% NaCl) using the three dimension swab technique. The antibiotic disks were placed on the inoculated plates using a sterile forceps. Six disks were placed on one 100 mm plate with spaced evenly, approximately 15 mm from the edge of the plate, and one disk was placed in the center of the plate. Each disc was pressed down gently to ensure contact with the medium. The incubated plate was let to dry for a few minutes at room temperature with the lid closed. Then it was incubated at 35^{o}C for 48 hours to 1 week or until bacterial growth was observed. At the end of incubation time, the diameter of each zone (including the diameter of the disk) was measured and recorded in mm. Then result was interpreted according to the critical diameters by comparing with the interpretive standards for dilution and disk diffusion susceptibility testing (Wood and Washington, 1995).

3.7.2 Chemical analysis

Chemical analysis was performed using the Microbial Identification System (MIDI system) (MIDI, U.S.A.), for the fatty acid methyl ester (FAME) analysis. This analysis

was carried out at the Laboratory Services Division, University of Guelph, Canada. Each bacterium to be tested was streaked on to the tryptic soy agar (section 1.12 Appendix A) supplemented with 5% NaCl, covering four quadrants of the medium and incubated at 35^{o}C for 24-48 hours. About 40 mg of cells were harvested from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate using a 4 mm loop. If the culture growth merged under the surface of the agar, a clean microscopic slide was used to scrape the cells and about 80 mg was taken. The cells were placed in a clean 13x100 culture tube. The cellular fatty acid of harvested bacterial cells was extracted using four steps: saponification, methylation, extraction, and base washing, as described by Sasser (1990) in the MIDI procedure manual.

In saponification step, 1.0 mL of reagent 1 (section 3.1 Appendix A) was added to each tube containing the harvested cells. The tube was tightly closed, then vortexed briefly and heated in a boiling water bath for 5 minutes. After that the tube was vigorously vortexed for 5-10 seconds and heated in a boiling water bath for 25 minutes to complete the 30 minutes heating.

In methylation step, the cooled tube was uncapped and 2 mL of reagent 2 (section 3.2 Appendix A) was added. The tube was capped and briefly vortexed, then heated for 10 ± 1 minutes at 80° ± 1°C.

In extraction step, 1.25 mL of reagent 3 (section 3.3 Appendix A) was added to the cooled tube. The tube was capped and gently tumbled on a clinical rotator for about 10 minutes. After that the tube was allowed to stand for a while to separate extracted fatty acid from the solvent. Then the bottom phase was pipetted out and discarded. At the final

step, base washing, 3 mL of reagent 4 (section 3.4 Appendix A) was added to the organic phase remaining in the tube. The tube was tumbled for 5 minutes. After that the tube was allowed to stand for a while to separate extracted fatty acid methyl esters from the base reagent. Then about 2/3 of the top phase above the interface was pipetted into a GC vial which was then capped and ready for analysis.

The extracted fatty acid methyl esters were analyzed by gas chromatography using the MIDI system as described by Haack *et al*. (1994). FAMEs were separated by gas chromatography on a fuse-silica capillary column with an HP 5890A chromatograph equipped with a flame ionization detector and a HP-3396 integrator (Hewlett-Packard Instrument Corporation, U.S.A.), using H_2 as the carrier gas at a flow rate of 15 mL/minute and at the temperature ranging from 170°C to 270°C, ramp rate 5°C per minute. Following the analysis, a rapid increase to 300°C was allowed to promote cleaning of the column during a hold of 2 minutes. A calibration mixture containing defined fatty acids (C_{11} – C_{12} straight chain, saturated, unsaturated, hydroxy, cyclopropane, iso and anteiso fatty acids) was used as a reference for comparison. *Stenotrophomonas* (*Xanthomonas*) *maltophilia* was used as a positive control. The samples of fatty acid composition reports and chromatograms of the calibration mix, the *Stenotrophomonas maltophilia*, and the reagent blank are given in Figures 1B-6B (Appendix B). The FAMEs analysis was also applied to 6 reference type strains of halophilic bacteria ordered from the American Type Culture Collection (ATCC), U.S.A.: *Bacillus halophilus* ATCC 49085, *Bacillus subtilis* ATCC 6051, *Halobacterium salinarum* ATCC 33171, *Halococcus saccharolyticus* ATCC 49257, *Micrococcus luteus*

ATCC 4698, and *Salinicoccus hispanicus* ATCC 49259. The media used for the cultivation of *Bacillus halophilus* ATCC 49085, *Halobacterium salinarum* ATCC 33171, *Halococcus saccharolyticus* ATCC 49257, *Salinicoccus hispanicus* ATCC 49259 are given in sections 1.5, 1.16, 1.2, and 1.5 of Appendix A, respectively. The halobacteria medium was used for the cultivation of *Bacillus subtilis* ATCC 6051 and *Micrococcus luteus* ATCC 4698.

For bacterial identification, cellular fatty acids were identified on the basis of equivalent chain length data which is a representation of a fatty acid's retention time as it relates to a series of FAMEs found in the calibration mixture. The MIDI system determined the area of each sample peak and converted to a percentage of the total area of all peaks. Microorganisms identification by the MIDI system is based on the similarity index which determined by the comparison of the unknown organism's FAME profile with those of the TSBA40 and the CLIN40 library database version 3.7 for 644 and 353 bacterial species, respectively. The similarity index (SI) was expressed on a numerical scale of 0 – 1.0. The higher the SI, the more likely the identification is correct. An interpretation of the SI value is as follows:

SI 0.6 or greater, with no second choice is an excellent identification

0.5 or greater, with second choice > 0.2 lower is a good identification

0.3-0.5 with no second choice is a good likelihood

< 0.3 with or without second choice means machine is guessing

After computer analysis, the fatty acid profile report and chromatographic peak were generated for each sample. An example of a fatty acid composition report and chromatogram was given in the Appendix B.

The dendogram was constructed by using cluster analysis techniques to produce an unweighted pair matching based on cellular fatty acid composition. From the dendogram, the relationship between samples can be evaluated by using the Euclidean Distance (ED) value. The manufacturer recommends that isolates with ED of 10-6 are the same species and that those less than 6 belong to the same subspecies or biotype.

The 2-D plot was generated by using the principal components analysis of the FAMEs profiles to group sample entries in a two-dimensional space. In this study, the Sherlock Library Generation Software of the MIDI system was used to generated the dendogram and the 2-D plot of the soil bacterial isolates and the ATCC reference type strains.

3.7.3 Genotypic characterization

Sequencing of 16S rDNA was used as a tool for genotypic characterization. This analysis was done at the Laboratory Services Division, University of Guelph, Canada. There are 4 major steps for genotypic characterization of halophilic bacterium isolates in this study: genomic DNA extraction, PCR amplification of 16S rDNA, sequencing of PCR amplicon, and 16S rDNA sequence analysis.

3.7.3.1 Genomic DNA extraction

Genomic DNA extraction from halophilic bacterium isolates was performed as described by Moore (1995). Two loops full of cells from the late

exponential phase were harvested and transferred into a microcentrifuge tube having 200 μL of TE buffer (section 4.5 Appendix A). The tube was vortexed and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded. The cells were washed again to obtain the clean cells. The clean cells were suspended in 200 μL of lysis buffer (section 4.2 Appendix A) and incubated at 37°C for 30 minutes. Then, 30 μL of 10% SDS and 10 μL of proteinase K (20 mg/mL) were added. The tube was gently mixed by hand and incubated at 37°C for 1 hour. An equal volume of phenol, TE saturated:chloroform (240 μL of each) was added; the tube was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube. Twice volume of cold absolute ethanol was added to the supernatant and gently mixed by hand to precipitate DNA. The tube was then centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was discarded. The precipitated DNA was washed with 600 μL of 70% ethanol, dried it at 37°C for 30 minutes., resuspended with 50 μL of TE buffer and kept overnight at 4°C to dissolve the precipitated DNA.

3.7.3.2 PCR amplification of 16S rDNA of bacterial isolates

The 16S rDNA of halophilic bacterium isolates were amplified by PCR using primers 27f, 21f and 1525r (Table 3).

The PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, U.S.A.) with a primary heating step for 2 minutes at 95°C, followed by 30 cycles of denaturation for 20 seconds at 95°C, annealing for 60 seconds at 55°C, and extension for 2 minutes at 72°C, then followed by a final extension step for 7

minutes at 72°C. Each 25 μL reaction mixture contained 2 μL of genomic DNA, 14.25 μL of MilliQ water, 2.5 μL of 10× buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 μL of $MgCl_2$ (25 mM), 2.5 μL of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.0 μL of each primer (20.0 pmoles/μL), and 0.25 μL of *Taq* DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems, U.S.A.). The PCR amplified products were examined by electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 μg/mL). A 100 bp DNA ladder (Promega, U.S.A.) was used to reference the size of the PCR products. The gel-separated PCR products were observed under short wavelength UV light (Figure 1F Appendix F). Size of the PCR products was approximately 1,500 bp.

Table 3. Oligonucleotide primers used for PCR amplification and sequencing of 16S rDNA.

Primer designation	Assay type	Sequence (5'–3')	Orientation	Reference
27f	PCR, sequencing	AGAGTTTGATCMTGGCTCAG	Forward	Lawson *et al.* (1996)
1525r	PCR	AAGGAGGTGWTCCARCC	Reverse	Lawson *et al*. (1996); Stan-Lotter *et al*. (2002)
21f	PCR, sequencing	TTCCGGTTGATCCYGCCGGA	Forward	Stan-Lotter *et al.* (2002)

The PCR products were purified prior to sequencing by using MultiScreen 96-well Filter Plates (Millipore, U.S.A.) according to the manufacture's protocol. Briefly, 250 μL of MilliQ water and then the PCR products were added to the

wells of the filter plate. The filter plate was placed on a vacuum manifold, and vacuumed at 20 inches of Hg for 10 minutes, or until the wells were completely empty, and appeared shiny. PCR products were then dissolved in 20 µL of MilliQ water and collected after shaking for 10 minutes on a plate shaker. The purified PCR products were then transferred to new microcentrifuge tubes and stored at -20°C until sequencing.

3.7.3.3 Sequencing of PCR amplicons

The purified DNA amplicons were sequenced using the ABI PRISM^{TM} 3100 Genetic Analyzer (Applied Biosystems, U.S.A.) according to the manufacture's instructions and using sequencing primers as described in Table 3. Approximately 20 ng of the purified PCR amplicons were used for a sequencing reaction. The sequences obtained were deposited to the GenBank® (NCBI, U.S.A.).

3.7.3.4 16S rDNA sequence analysis

The sequences obtained were edited manually and compared to nucleotides database provided by the National Center for Biotechnology Information using the BLAST (Basic Local Alignment Search Tool). The alignment scores and the percent sequence identity were determined for the closest identity of the sequences obtained. Further sequencing was conducted for isolates which had percent similar identity less than 97% using primer 490f. The sequences were assembled using Clustal W software version 1.82 (Thompson *et al.*, 1994) available at http://www.ebi.ac.uk.

The evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes and Cantor (1969). A phylogenetic tree was inferred by using the neighbour-joining method (Saitou and Nei, 1987) with the

software TREECON for Windows version 1.3b (Van de Peer and De Wachter, 1994). The stability relationships were evaluated by a bootstrap analysis of 100 data sets.

3.8 Maintenance of selected bacterial isolates for future application

For short-term preservation of bacterial cultures, bacterial isolates were subcultured to halobacteria agar slant containing salt concentration according to their requirement. For long-term preservation of bacterial cultures for future application, the cultures were preserved in 10% skimmed milk and kept at –80°C. For propagation procedure, the cultures were thawed for 3 minutes at room temperature and inoculated into halobacteria broth containing salt according to their requirement, then incubated at 35°C for at least 48 hours (for slightly- and moderately- halophilic bacteria) to 1 week (for extremely halophilic bacteria).

Bacterial isolates were also preserved for long-term preservation with the liquid dried method. For propagation procedure, 0.5–1.0 mL of halobacteria broth was transferred to the liquid dried culture. Several drops of suspension were transferred to halophilic bacteria broth and/or streaked onto the halobacteria agar plate, then incubated at 35°C for at least 48 hours (for slightly- and moderately-halophilic bacteria) to 1 week (for extremely halophilic bacteria).

3.9 Data analysis

The values of physical and chemical properties of soil samples of each plot were averaged from its subplots and interpreted by comparing to the guidelines for interpreting soil tests as described by กองจำแนกดิน (2516) (Table 10C Appendix C) and Soil Survey

Division Staff (1993) (Table 11C Appendix C). The densities of slightly-, moderately-, and extremely halophilic bacteria of each plot were averaged from its subplots then transformed to $\log_{10}$ to obtain the data of halophilic bacterial density. The relationship between bacterial numbers and some physical and chemical properties of soil samples: soil temperature, moisture content, pH, and salinity, was assessed by linear regression using the SPSS for Windows version 10.0.1 (SPSS, U.S.A.) The relationship between bacterial numbers and organic matter, soil texture, total nitrogen, available phosphorus and available potassium were analyzed and concluded. The correlation in slightly-, and moderately- halophilic bacterial numbers was also evaluated using the SPSS.

The results from the phenotypic characterization, the fatty acid analysis, and the 16S rDNA sequence analysis were combined and used to determine the identity for unknown halophilic bacterial isolates. The diversity of halophilic bacteria in saline soil at Nong Bo Reservoir was determined using the ultimate results of bacterial characterization.

The relationship of halophilic bacteria in saline soil at Nong Bo Reservoir was determined by means of a dendogram and 2-D plot based on fatty acid analysis, and a phylogenetic tree based on 16S rDNA sequence.

CHAPTER IV

RESULTS AND DISCUSSION

Saline soil samples were collected monthly from five sampling plots in the area of Nong Bo Reservoir, Borabu District, Mahasarakham Province, Thailand, from June 2001 to May 2002, to determine some physical and chemical properties of saline soil samples, and the density and diversity of halophilic bacteria inhabiting the saline soil. The results obtained are presented and discussed in the following sections.

4.1 Physical and chemical properties of saline soil samples

The physical and chemical properties of soil samples in each plot averaged from its subplots, are described in Tables 1C-9C Appendix C and Table 4.

An assessment of the correlation among some of the soil physical and chemical data using the SPSS indicates that a slight correlation exists between soil temperature and soil salinity with the correlation significant at the 0.05 level (Table 1D Appendix D). No correlation between soil salinity, temperature, moisture content and pH was found. The results obtained from the statistical analysis, which were analyzed from the average values of all sampling plots did not give any significant information. The physical and chemical properties of soil samples of each sampling plot (Figure 4) were also considered to ascertain the relationship among the soil moisture content, temperature, pH, and salinity.

Table 4. Results of particle size analysis of soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

Plot	Percentage of sand, silt, clay	Soil texture class	Type of soil matrix
1	68, 18, 13	Sandy loam	Moderately coarse
2	79, 15, 5	Loamy sand	Coarse
3	77, 16, 7	Loamy sand	Coarse
4	73, 21, 6	Loamy sand	Coarse
5	76, 13, 11	Sandy loam	Moderately coarse

The average rainfall from June 2001 to May 2002 was 1,705 mm (personal communication, เจ้าหน้าที่ประจำโครงการส่งน้ำและบำรุงรักษาลุ่มน้ำเสียวใหญ่). Mitsuchi, *et al*. (1986) stated that the annual rainfall of 1,500 to 2,000 mm could be expected to provide enough water in soil. Nevertheless, the soil moisture content still becomes a major problem for plantations in the area of Nong Bo Reservoir. This is due to the sandy loam and loamy sand nature of the soil texture, which have low water holding capacity. The large pores of sandy soil increase the movement of any materials moving through it, including air, water, and microorganisms (Maier and Pepper, 2000). This causes rapid and inconsistent changes in soil moisture content, pH, salinity, and temperature, which were also reported by สมศรี อรุณินท์ (2542ก).

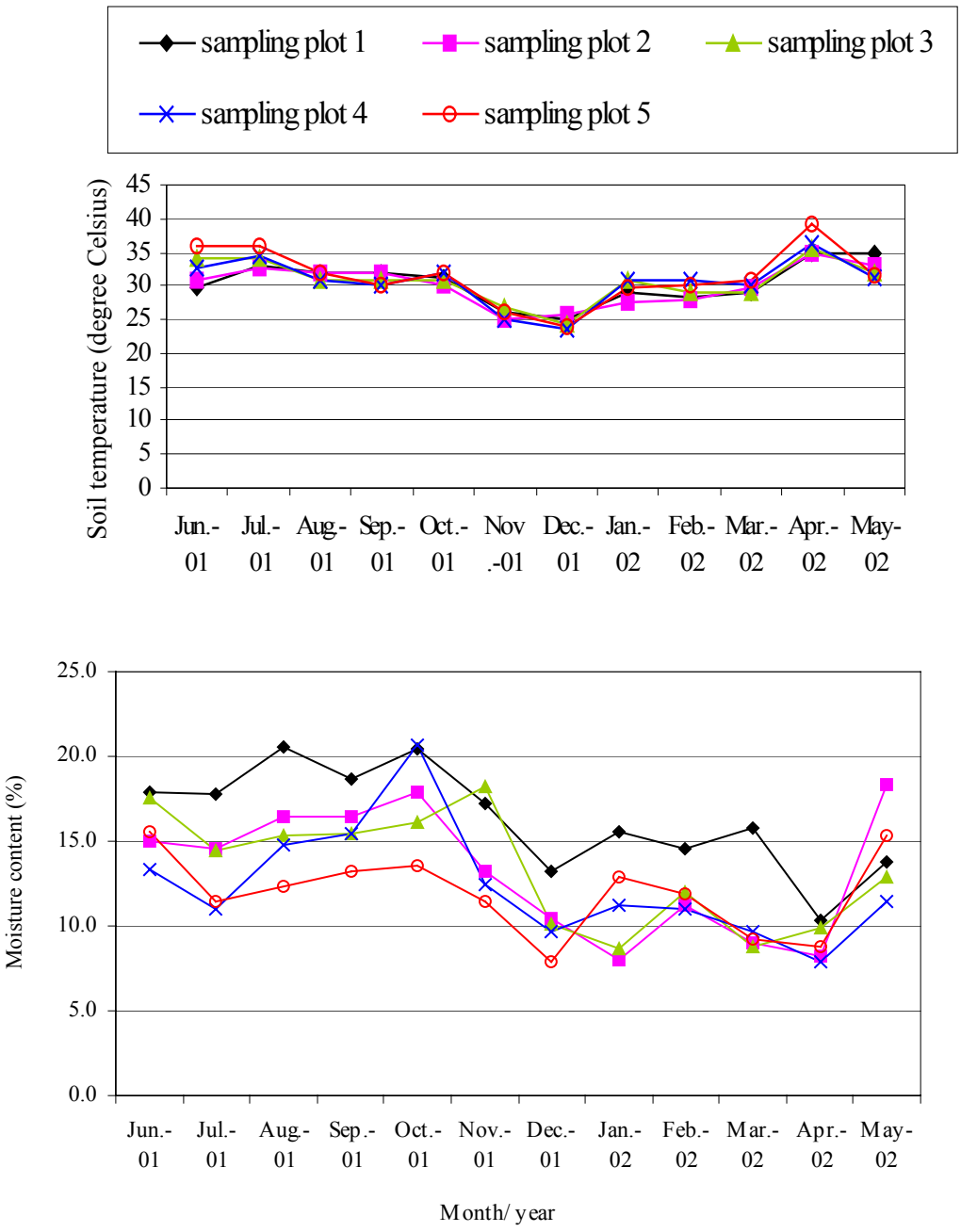
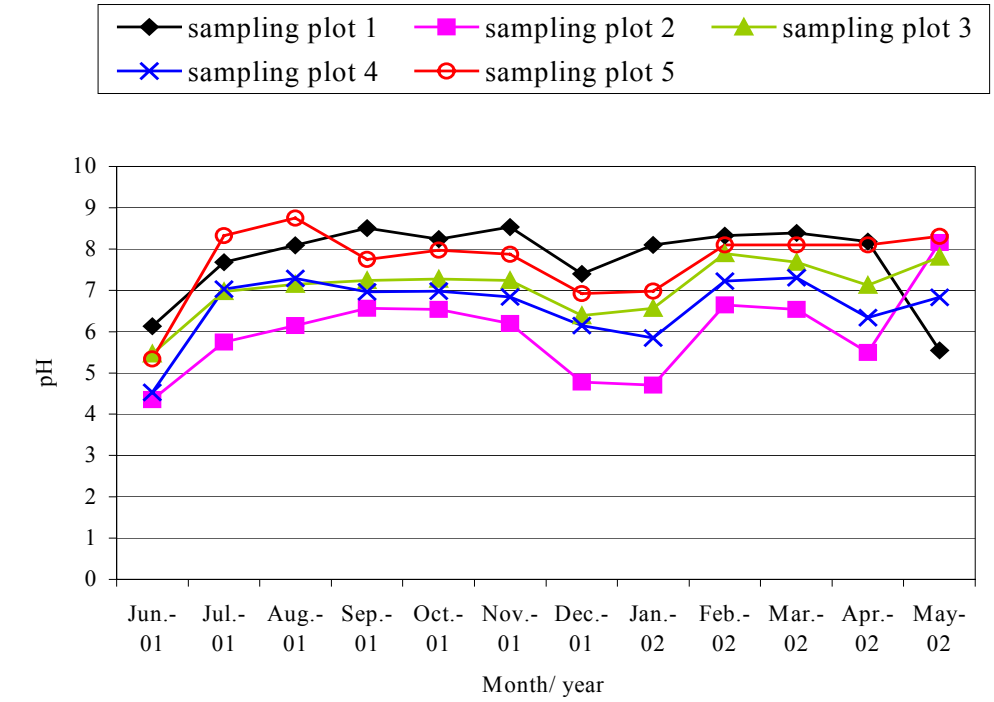
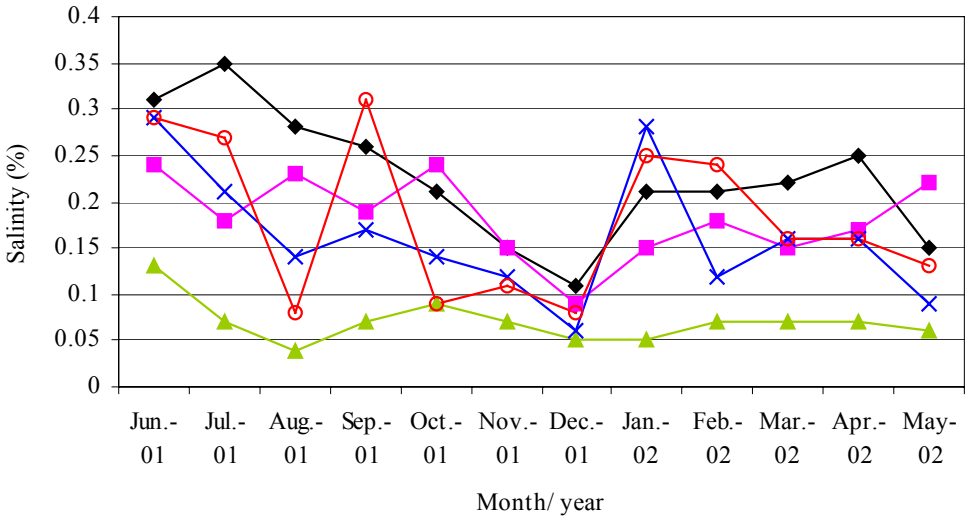


Figure 4. Soil temperature (A), moisture content (B), pH (C), and salinity (D) of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.



C



D

Figure 4. (Continued)

From Figure 4, the values of soil temperature, moisture content, and pH in all sampling plots varied in the same trend. The soil salinity of four sampling plots: the land of fishponds, the halophyte plot of the Department of Land Development, the halophyte experimental plot of Rajapruek Institute Foundation, and the land where a salt storehouse was located, was highly fluctuate and considered to be slightly saline, according to the interpretation of total salt content (%) in soil described by Landon (1991). Only the soil of the mixed-agricultural experimental plot of Rajabhat Institute Mahasarakham was non-saline and the salinity was quite stable.

The organic matter, total nitrogen, available phosphorus, and available potassium of soil samples of each sampling plot are shown in Figures 5-8. The organic matter, total nitrogen, available phosphorus, and available potassium in four sampling plots: the land of fishponds, the halophyte plot of the Department of Land Development, and the halophyte experimental plot of Rajapruek Institute Foundation varied in the same trend.

The ranges (minimum to maximum) of physical and chemical properties of saline soil samples were observed to obtain a big picture of the soil physical and chemical properties of the study area (Table 5).

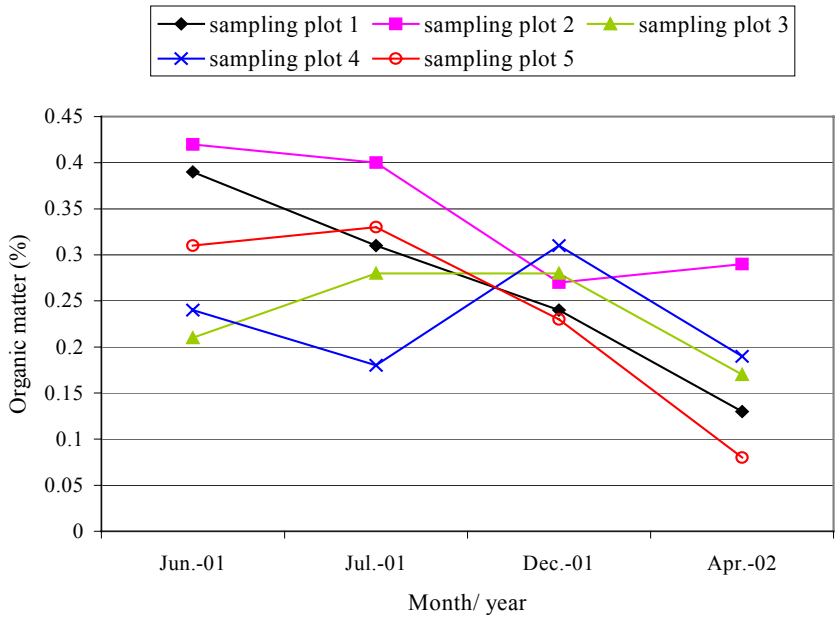


Figure 5. Organic matter of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

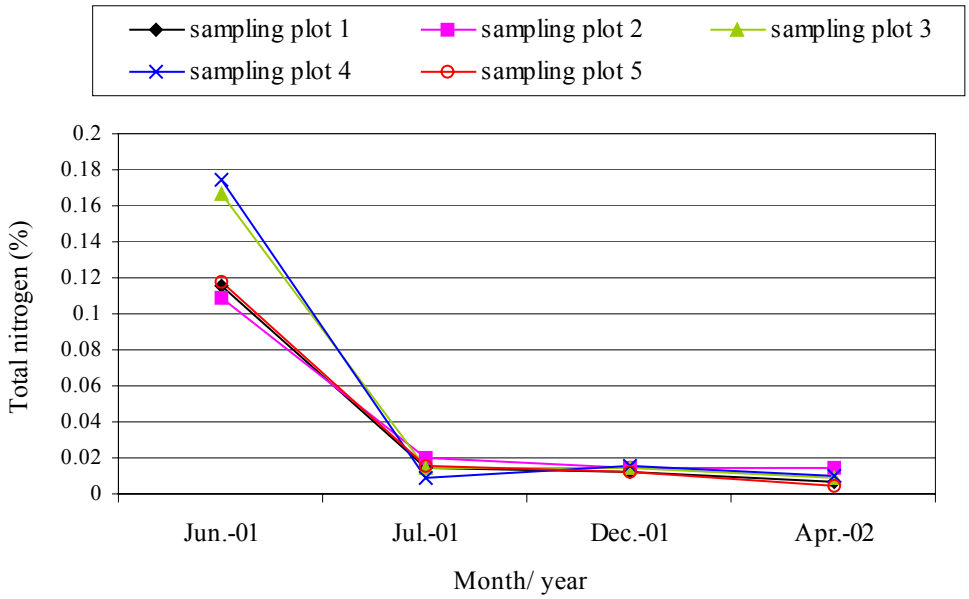


Figure 6. Total nitrogen of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

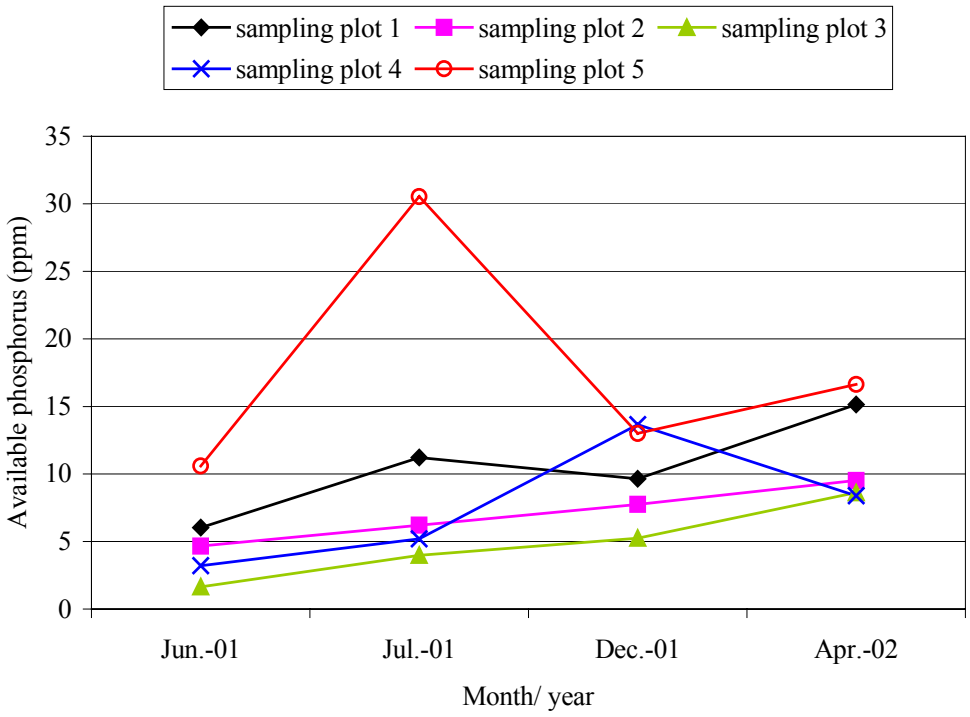


Figure 7. Available phosphorus of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

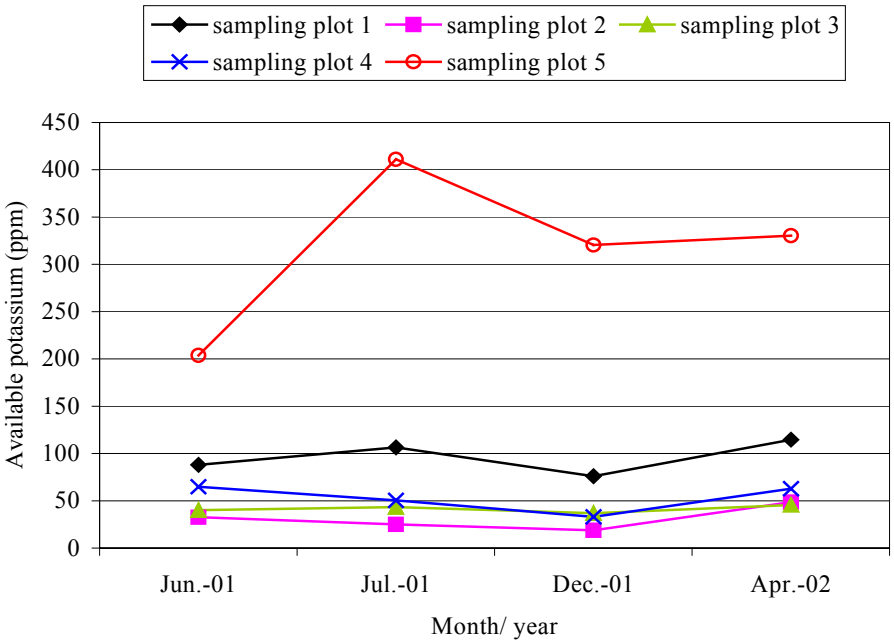


Figure 8. Available potassium of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

Table 5. Physical and chemical properties of saline soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

Parameter	Value	Interpretation
Moisture content (%)	8.05-20.67	Very dry-dry
pH	4.35-8.75	Moderately acidic-alkaline
Salinity	0.04-0.35	Non-saline-slight saline
Soil texture class	Loamy sand-sandy loam	Moderately coarse-coarse
Organic matter (%)	0.13-0.42	Extremely low
Total nitrogen (%)	0.005-0.175	Extremely low-high
Available phosphorus (ppm)	1.65-30.54	Extremely low-high
Available potassium (ppm)	18.75-411.08	Extremely low-high

These results indicate that the soil salinity seems not a major problem at present. In addition, the other soil properties, soil texture and plant nutrients promote the plant stress. The implementation results of plantations in the area of Nong Bo Reservoir took 13 years to reduce the salinity problem in both soil and water at Nong Bo Reservoir since the severe problems arose in 1980. From personal communication to คุณ วรวุฒิ วรวิเศษ (เจ้าหน้าที่ประจำสถานีพัฒนาที่ดินจังหวัดมหาสารคาม), after planting, the plants need a good deal of care for 4-5 years for survival. The slow success in the

rehabilitation of Nong Bo Reservoir is due to the fact that several soil problems including high fluctuation of soil salinity, low fertility, low moisture content, and soil texture would be integrated to promote plant stress. Thus the integrative approaches should be considered to assist the replantation approach for the achievement of restoring the saline soil at Nong Bo Reservoir. In addition, the growers need to have an understanding of which plants are suitable for planting in the area and the sensitivity of these plants at different stages of growth.

4.1 Density of halophilic bacteria in saline soil

Due to the overlap of salt requirements and the differences of salt tolerance range among the different strains of halophilic bacteria, choosing the salt concentration of media for isolation of individual groups of halophilic bacteria from the environmental samples is difficult. However if the salt concentration of media is chosen from the optimum salt range of an individual group of halophilic bacteria, it can enhance the growth of the majority population of each group. Hence, in this section, the slightly-, moderately-, and extremely-halophilic bacteria refer to bacteria that grew on media containing 3%, 8%, and 25% NaCl, respectively. The density of the three groups of bacteria, obtained from soil samples at Nong Bo Reservoir (Figure 9 and Tables 13C-15C Appendix C) can be summarized as follows:

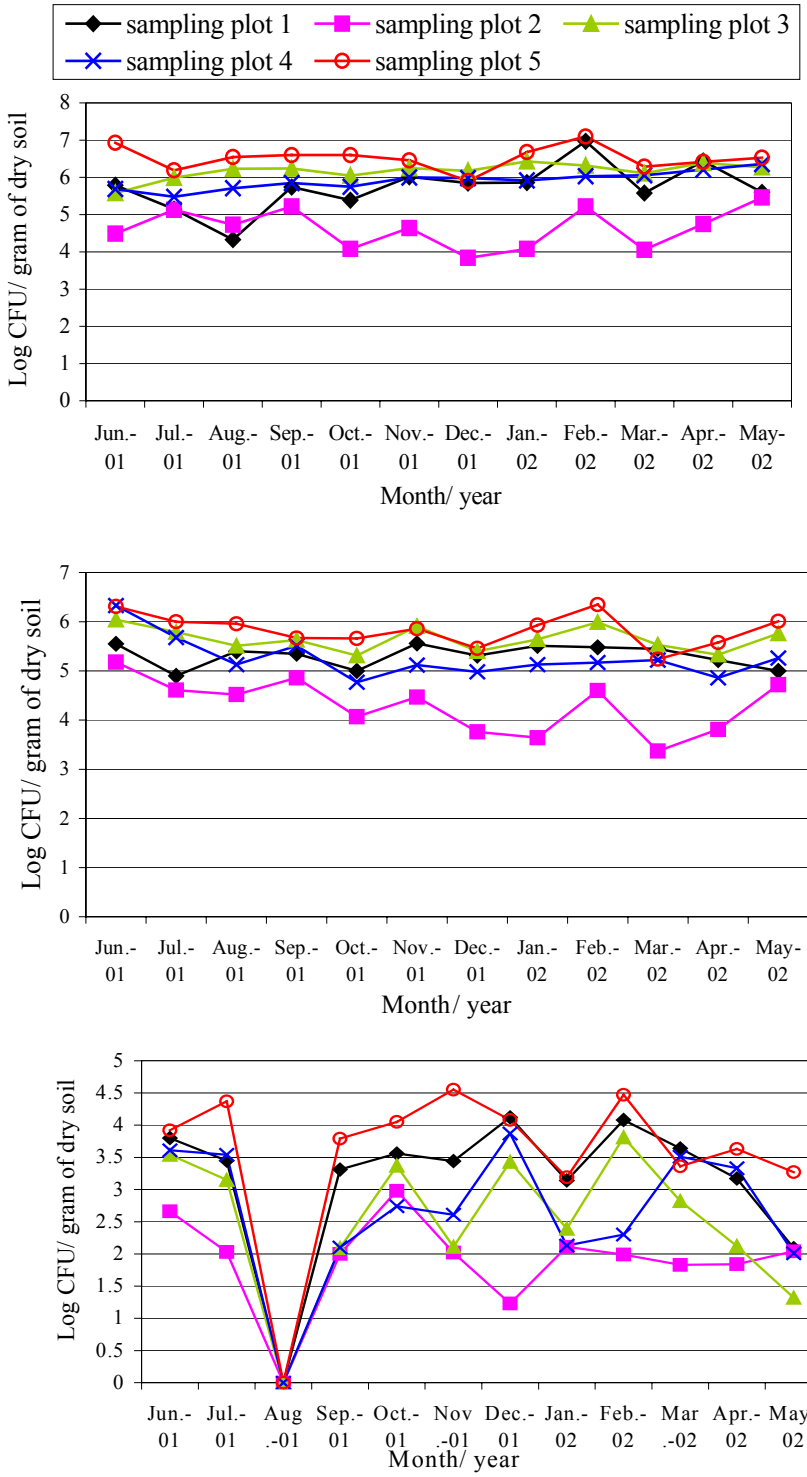


Figure 9. Density of slightly-, moderately-, and extremely halophilic bacteria (A, B, and C, respectively) of soil samples collected at Nong Bo Reservoir from June 2001 to May 2002.

From statistical analysis, there was no correlation among the three groups of halophilic bacteria (Table 2D Appendix D). The relationship between the density of the three groups of halophilic bacteria was analyzed using the graphs shown in Figure 9. The slightly halophilic bacteria occurred at the highest density in all the sampling plots and throughout the time of the study, followed by the moderately halophilic bacteria and the extremely halophilic bacteria. These results indicate that the halophilic bacterial density was markedly decreased as the salt concentration of the media increased. The slightly- and moderately halophilic bacteria were more abundant than the extremely halophilic bacteria. These results are in agreement with those reported by **Quesada** *et al*. (1982), and **Rodriguez-Valera** (1988) in that saline soil appeared to yield largely moderately halophilic and halotolerant bacteria rather than the extremely halophilic bacteria. These phenomena are mainly due to the low level of salt content in saline soil, which does not support the growth of extremely halophilic bacteria.

Moreover, the variation in the average counts of slightly halophilic bacteria was more correlated to the moderately halophilic bacteria than the extremely halophilic bacteria. An explanation for this phenomenon is that the slightly- and moderately-halophilic bacteria colonize different niches to the extremely halophilic bacteria (Oren, 1993). It may also be due to the fact that the slightly- and moderately halophilic bacteria are both descended from the *Eubacteria* while the extremely halophilic bacteria are the *Archaea*. The physiological and genotypical adaptations for living in the hypersaline environments among the *Eubacteria* and the *Archaea* are different. In addition, the levels of salt requirements for growth of the slightly- and moderately halophilic bacteria are closer than those required for the extremely

halophilic bacteria. Also the growth of extremely halophilic bacteria requires relatively high NaCl (at least 9% NaCl) and the majority of them require magnesium ion (Mg^{2+}) for their growth while the growth of slightly- and moderately-halophilic bacteria do not strict to this ion (Grant *et al*., 2001).

The highest density of slightly-, moderately-, and extremely-halophilic bacteria was found in the land where a salt storehouse was located and was enumerated at 7.10, 6.35, and 4.47 log CFU/g of dry soil, respectively. The reason for this result may due to this plot historically being used as a salt storehouse. Various kinds of salts may be left over and support the growth of halophilic bacteria. The halophyte plot of the Department of Land Development shows the lowest density of slightly-, moderately-, and extremely-halophilic bacteria and was enumerated at 3.84 and 3.37 log CFU/g of dry soil and not found (at soil dilution of 1:2). The sharpest drop of the extremely halophilic bacterial density to not found (at soil dilution of 1:2) was observed during August in all sampling plots which was due to all the sampling plots were flooded in this month. The flood could be removed or diluted the magnesium ion in soil that required for the growth of extremely halophilic bacteria. However, in September, the extremely halophilic bacteria was recovered with the lowest density was 2.00 log CFU/g of dry soil at the mixed-agricultural experimental plot of Rajabhat Institute Mahasarakham. This result is due to the extremely halophilic bacteria that survived after flooding could be reproduced as the magnesium ion and the other trace elements required for their growth was continually released from the beneath rock salt dome.

Statistically, there is no correlation between slightly halophilic bacteria and any physical/chemical characteristics of the soil samples (Table 3D Appendix D). The density of moderately halophilic bacteria has insignificantly low correlation to soil pH,

moisture content, and soil temperature but has significantly good correlation to soil salinity (Table 4D Appendix D). The density of extremely halophilic bacteria had insignificantly low correlation to soil pH, moisture content, salinity, and soil temperature (Table 5D Appendix D). Since the statistical analysis did not provide any significant information, the interaction among the variation of halophilic bacterial density and the physical/chemical properties of saline soil samples was analyzed using the graphs in Figures 4-9. The relationship between the density of halophilic bacteria and the physical/chemical properties of soil was significant in two of the sampling plots, the halophyte plot of the Department of Land Development and the land where a salt storehouse was located.

Basically, the number of microorganisms found in a soil often increases as the soil organic matter content increases (Maier and Pepper, 2000). When comparing between the organic matter content and the halophilic bacterial density of the halophyte plot of the Department of Land Development and the land where a salt storehouse was located, the results reveal an inverse relationship. In the halophyte plot of the Department of Land Development, the lowest density of three groups of halophilic bacteria was observed although the organic matter was markedly higher than the land where a salt storehouse was located. The lowest soil pH (5.99 in average), rather high salinity (0.18% in average), and highest percentage of sand was also observed in this plot. The moderately acidic soil was unfavorable for growth of halophilic bacteria since their optimum pH was 7 (results obtained from the pH range tests in this study). The low soil pH also affected the solubility of nutrients such as phosphorus, calcium, and magnesium (Landon, 1991) which is in agreement with the result of low available phosphorus. These factors may indirectly influence the

halophilic bacterial population. In addition, the high percentage of sand reduces the surface areas for the attachment of bacteria to the soil particles, consequently reduces the bacterial population in soil.

In the land where a salt storehouse was located, the organic matter was rather lower than in the halophyte plot of the Department of Land Development but had the highest density of the three groups of halophilic bacteria. The average pH of this plot was 7.7. The percentage of sand was a bit lower than the halophyte plot of the Department of Land Development. In addition, various kinds of salts may be existed as the area was used as a salt storehouse. All of the soil properties of this plot may support growth of halophilic bacteria more than the halophyte plot of the Department of Land Development. The highest density of halophilic bacteria in this plot correlated to the highest available phosphorus (17.7 ppm in average) when compared to the other sampling plots (4.8-10.5 ppm). These results may due to the halophilic bacteria involved in phosphate solubilization as reported by Ramos-Cormenzana (1993). The role of halophilic bacteria in phosphate solubilization is significant in improving soil fertility. Since phosphorus is a plant limiting nutrients and is largely controlled by biological processes (Nautiyal *et al*., 2000).

From these findings, it seems that a variety of soil properties influenced the density of halophilic bacteria and not any one specific property such as the salinity or the organic matter. These results were not markedly different from many former reports dealing with bacterial diversity in saline soil studied by **Quesada** *et al*. (1982) and Zahran *et al*. (1992). The reasons for these results are not only due to the heterogeneity of the soil habitat and the markedly variable changes of some soil physical and chemical properties in space and time but also the complex interrelated

effects of the soil physical and chemical properties to the microbial density and activities. In relation to these results, it is important to consider the organic matter content and the related factors such as rhizosphere soil or root-free soil addition, along with the other soil physical and chemical characteristics, when studying the factors affecting the soil microbial distribution (El-Abyad *et al*., 1979).

4.2 Diversity of halophilic bacteria in saline soil

Saline soil samples collected at Nong Bo Reservoir, Borabu District, Mahasarakham Province, Thailand, from June 2001 to May 2002, yielded diverse groups of halophilic bacteria. It appears that the low salt concentrations of the media used for culturing resulted in the high density and diversity of halophilic bacteria (Figure 10). At first selection, a total of 240 representative bacterial isolates were obtained from the soil dilution plate during the whole period of soil sample collection. The re-cultivation of the moderately halophilic bacteria on halophiles moderate medium containing 18% NaCl (section 1.3 Appendix A) was a second selection step taken to screen the true moderately halophilic bacteria. This step is important for reiteration of bacterial isolates in the group of moderately halophilic bacteria due to the fact that extremely halophilic bacteria and halotolerant eubacteria may interfere in the isolation of the desirable moderately halophilic bacteria (Ramos-Cormenzana, 1993). Twenty-two of 110 moderately halophilic bacterium isolates could be grown on this medium. The summarization of the number of halophilic bacterium isolates selected from this study is described in Table 6.

A **B** **C**

Figure 10. Diverse bacterial colonies form cultured from soil samples on halobacteria medium containing 3% (A), 8% (B), and 25% (C) NaCl concentration, respectively.

Table 6. Summarization of the number of halophilic bacterium isolates selected from this study.

Group of halophilic bacteria	Number of isolates from first selection	Number of isolates from second selection	Number of isolates used for characterization
Slightly halophilic bacteria	115	-	115
Moderately halophilic bacteria	110	22	22
Extremely halophilic bacteria	15	-	15
Total	240	22	152

A total of 152 representative bacterial isolates were selected for the determination of the diversity of halophilic bacteria in saline soil at Nong Bo Reservoir, Mahasarakham Province, Thailand, from June 2001 to May 2002. The phenotypic characteristics alone (Appendix E) was not enough to differentiate the bacterial isolates and can lead to serious identification problems. The main reason for this is that the standardization of a conventional methodology was difficult, especially when it was applied to halophilic bacteria which their growth characteristic highly dependent on many factors such as NaCl concentrations, temperature, pH, and medium composition. In addition, many of the extremely halophilic bacteria are biochemically unreactive, and thus limited the phenotypic data (Grant *et al*., 2001). Fritze (2002) recommended that phenotypic characterization results cannot and should not be directly compared without full background knowledge of the precise conditions used for a particular test. This can be particularly true for the group of Gram-positive endospore-forming bacteria that were formerly classified as the genus *Bacillus* but have now been reclassified based upon phylogenetic diversity into 6 RNA groups and separate linages (Stackebrandt and Swiderski, 2002). Therefore, fatty acid analysis and 16S rDNA sequence analysis were also used to ensure the accuracy for the halophilic bacterium characterization reported in this study.

4.3.1 Phenotypic characteristics of halophilic bacterium isolates

The 152 bacterial isolates were subjected to the following phenotypic characteristic tests: morphologic characteristics, cultural characteristics, catalase, oxidase, pH tolerance, salt tolerance, temperature tolerance, motility, indole production, gelatin liquefaction, nitrate reduction, hydrolysis of starch, hydrogen sulfide production, and carbohydrate utilization. The antimicrobial susceptibility test was applied to representative bacterial isolates in the group of extremely halophilic bacteria since this test was used for identification of extremely halophilic bacteria. The results obtained from phenotypic feature tests showed an extremely high diversity as detailed in Tables 1E-4E Appendix E. These results showed differences in phenotypic properties even among the same group causing the difficulties in their identification by phenotypic traits.

Once halophilic bacteria were isolated from saline soil samples, they were presumptively separated to slightly-, moderately-, and extremely-halophilic bacteria, according to 3%, 8%, and 25% of NaCl concentrations of the media used for their isolation from soil by using the standard spread plate method. However, practically, this approach is ineffective for this purpose because of the optimum and tolerant NaCl range of halophilic bacteria is critical. Therefore, in order to classify the halophilic bacterium isolates using their response to NaCl according to the classification of halophilic bacteria proposed by Kushner (1993), the halophilic bacterium isolates were grouped by using the results of salt tolerant test. On this basis, of the 152 bacterial isolates, 76, 34, 36, and 6 isolates were grouped as the halotolerant bacteria, slightly halophilic bacteria, moderately halophilic bacteria, and extremely halophilic bacteria (or the extremely halophilic archaeaobacteria), respectively. The percentage of these numbers is given in Figure 11.

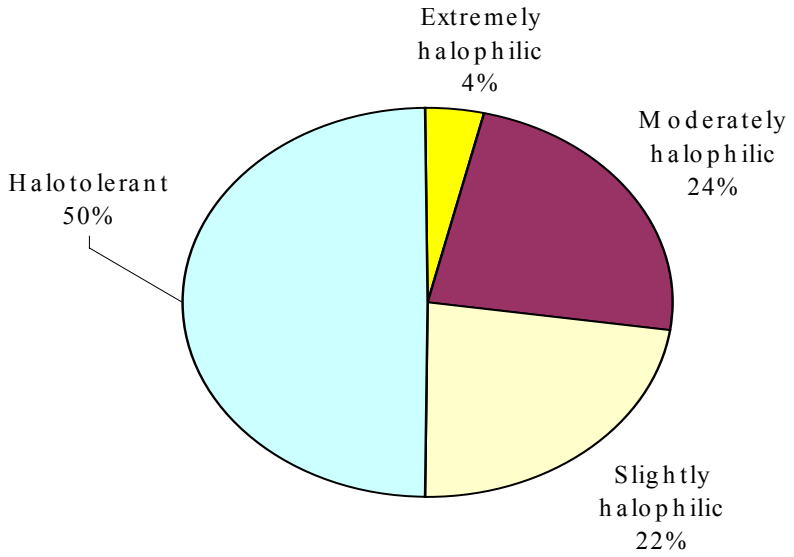


Figure 11. Percentage of different groups of halophilic bacteria inhabiting saline soil at Nong Bo Reservoir.

These results can be correlated to the density results of halophilic bacteria described in the previous section. The phenotypic characteristics of the 38 representative bacterial isolates in 38 identified species obtained from the ultimate result of bacterial characterization are summarized in Tables 7 to 9. Morphologically, the halophilic bacterium isolates found were Gram-positive endospore-forming rod, Gram-negative rod, and Gram-positive cocci, abundantly density in sequential. This result is due to the spore-forming bacteria may likely to be dispersed widely by wind blown dust and migratory

birds (Jones and Grant, 1999). The bacteria in sporulation stage are more resistant to the environmental changes than the vegetative stage. In addition, the teichoic acids found in the cell walls of Gram-positive bacteria provide the negative charges of the cell surface as a whole thus increasing the mobility and transport of these bacteria in the environments (Reynolds and Pepper, 2000). Generally, the pH value at 7 is the optimum for growth of several halophilic bacterium species. The slightly- and moderately-halophilic bacterium isolates were able to utilize various kinds of carbon source compared to extremely halophilic bacteria. The ability to produce acid from the utilization of L-arabinose, D-fructose, D-galactose, glucose, lactose, D-mannitol, D-mannose, and D-xylose was found largely in the slightly- and moderately halophilic bacterium isolates. Many *Bacillus* spp. had the ability to utilize starch. Some of halophilic bacterium isolates especially the *Bacillus* had the ability to produce hydrogen sulfide from cysteine. The majority of all halophilic bacterium isolates had the ability to reduce nitrate to nitrite. All of the extremely halophilic bacterium isolates were resistant to penicillin, erythromycin, bacitracin, chloramphenicol, streptomycin, tetracycline, vancomycin, and kanamycin. On the basis of results obtained from the phenotypic characteristics of the halophilic bacterium isolates and the results of available phosphorus in Section 4.1, they presumably play an important and substantial role in the biogeochemical cycles. In addition, Chanway (2002) and Kloepper (1993) reported that the *Bacillus* spp. were plant growth promoting bacteria. They promoted plant growth by both direct and indirect mechanisms. In direct mechanisms, the *Bacillus* spp. involved nitrogen fixation on or in root or shoot tissue, production of plant growth regulators such as auxins, cytokinins, and giberellins, phosphorus solubilization, inhibition of ethylene synthesis in the inoculated plants,

increased root permeability, increase nitrate reductase activity and nitrate assimilation, and sulfur oxidation. For indirect mechanisms, the *Bacillus* spp. involved the affecting of the other factors in the rhizosphere such as suppression of disease-causing microorganisms and induction of plant systemic resistance to pathogens, which resulted in enhanced plant growth. Therefore, the halophilic bacterium isolates obtained from this study could provide a high potential in promoting plant growth. These bacterial isolates may also applied to promote the growth of plant in Nong Bo Reservoir for the purpose of saline soil recovery.

Table 7. Phenotypic features of 20 bacterial isolates in different species that were isolated from the media containing 3% NaCl.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Cell shape	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	C	R	R
Endospores produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Cell size (μm)	0.5× 1-2	0.8× 2-5	0.3× 2-4	0.5× 1-2	0.3× 2	0.5× 1-3	0.5× 1-2	0.8× 2-3	1.0× 2-3	0.5× 1-2	0.6× 2-3	0.5× 1-2	0.5× 2-5	1.0× 2-5	0.5× 1-2	0.8× 2	0.5× 2-10	0.5× 0.5	0.3× 2	0.5× 0.5-1
Colony color	C	C	C	P	PY	C	P	Y	C	P	W	PY	PO	PY	PO	O	W	Y	PY	PY
Motility	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+
pH range	5-9	7-9	7-9	7-9	7-9	7-9	7-9	5-9	7-9	7-9	5-11	5-9	7-9	7	7-9	7-11	7-11	7	4-11	5-11
Salt range (% NaCl)	0-3	0-3	0-8	0-8	0-3	0-3	0-8	0-8	0-3	0-8	0-8	0-15	0-8	0-15	0-20	0-20	0-3	0-8	0-20	0-8
Temperature range (°C)	10-45	10-35	25-45	10-45	10-50	25-45	10-45	10-45	10-50	10-50	10-50	10-45	10-45	25-50	10-50	25-35	25-35	25-50	10-50	10-45
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
MR test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	+	-	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+
Acid production from:																				
D-mannose	-	-	+	-	-	-	+	+	-	+	-	+	+	+	+	-	-	-	-	+
Lactose	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-
D-fructose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-
D-galactose	+	-	+	+	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-
D-xylose	+	-	-	+	-	+	+	-	-	+	+	+	-	+	-	-	-	-	-	-
L-arabinose	+	-	-	+	-	+	-	+	-	+	+	+	+	+	-	-	-	-	-	+
D-mannitol	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-	weak -
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+
Gelatin	+	+	-	-	+	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-
Starch	-	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+	-	+	+
H_2S production	-	+	-	-	+	+	-	+	+	+	+	-	-	+	-	-	+	+	-	-

Note: Species (taxa identified indicates in parenthesis): 1, A7aA (*B. bataviensis*); 2, A11at (*B. cereus*); 3, AIIV (*B. circulans*); 4, A1n1 (*B. firmus*); 5, A7Y (*B. flexus*); 6, A9aT (*B. licheniformis*); 7, A12aj (*B. marisflavi*); 8, A9an (*B. megaterium*); 9, A9ay (*B. mycoides*); 10, AIIau (*B. pumilus*); 11, Aiaz (*B. subtilis*); 12, A5au (*B. vallismortis*); 13, A8e (*B. vietnamensis*); 14, A6aP (*Geobacillus caldoxylosilyticus*); 15, A1q (*Halobacillus salinus*); 16, A11S (*Halobacillus trueperi*); 17, A11as (*Nocardiopsis dassonvillei*); 18, A11aR (*Staphylococcus saprophyticus*); 19, A12ajj (*Marinobacter aquaeolei*); and 20, A12ar (*Pseudomonas stutzeri*). Symbols: +, positive; -, negative; R, rod; C, cocci; C, cream; O, orange; P, pink; PO, pale orange; PY, pale yellow; W, white; and Y, yellow.

Table 8. Phenotypic features of 11 bacterial isolates in different species that were isolated from the media containing 8% NaCl.

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Gram reaction	-	+	+	+	-	+	+	+	-	-	-
Cell shape	R	R	R	R	R	C	R	R	R	R	R
Endospores produced	-	+	+	+	-	-	+	+	-	-	-
Cell size (μm)	0.8×1	0.25×1-2	0.5×1-3	0.5×3	0.3×1-2	0.5×0.5	0.6×2	0.5×2-7	0.8×2	0.5×1-2	0.5×1
Colony color	C	PY	PO	C	PB	Y	PO	C	C	W	PB
Motility	+	+	+	+	+	-	+	+	+	+	-
pH range	5-9	7-9	5-7	7-9	4-9	5-7	7-9	7-9	5-11	4-11	4-9
Salt range (% NaCl)	0-15	0-15	0-15	0-15	0-20	0-15	0-15	0-15	0-20	0-20	0-25
Temperature range (°C)	10-50	25-35	10-45	25-45	10-50	10-45	25-35	10-50	10-50	10-50	10-50
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	-	-	+	+	+	+	+
MR test	-	-	-	-	-	-	-	+	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	-	+	-	-	-	+	+
Acid production from:											
D-mannose	-	+	-	-	+	+	-	+	-	+	-
Lactose	-	+	-	-	+	-	-	+	-	+	-
D-fructose	-	+	+	-	+	+	-	+	-	+	-
D-galactose	-	+	-	-	+	+	-	+	-	+	-
D-xylose	-	-	-	-	+	+	-	+	-	+	-
L-arabinose	-	-	-	-	+	+	+weak	+	-	+	-
D-mannitol	+	-	-	+	+	+	+	-	-	+	-
Glucose	+	+	+	-	+	+	+	+	+	+	+weak
Gelatin	-	-	-	+	-	-	+	-	-	-	+
Starch	-	+	+	-	-	-	-	+	-	-	-
H_2S production	-	-	+	-	-	+	-	-	-	-	-

Note: Species (taxa identified indicates in parenthesis): 1, B11ax (*Halomonas desiderata*); 2, B7W (*Oceanobacillus iheyensis*); 3, B10aFF (*B. aquimaris*); 4, B8ax (*B. halodenitrificans*); 5, B9aB (*Haererehalobacter ostenderis*); 6, B8aN (*Staphylococcus gallinarum*); 7, B3au1 (*Halobacillus litoralis*); 8, B8az1 (*Vigribacillus pantothenticus*); 9, B3az (*Deleya pacifica*); 10, B11ab (*Halomonas nitritophilus*); and 11, B9av (*Halomonas ventosae*). Symbols: +, positive; -, negative; R, rod; C, cocci; C, cream; PB, pale brown; PO, pale orange; PY, pale yellow; and W, white.

Table 9. Phenotypic features of 7 bacterial isolates in different species that were isolated from the media containing 25% NaCl.

Characteristics	1	2	3	4	5	6	7
Gram reaction	+	-	-	-	+	-	+
Cell shape	C	R	R	R	C	R	R
Endospores produced	-	-	-	-	-	-	+
Cell size (μm)	1.5×1.5	1×1-2	0.5×2	0.5×2.5	1.0×1.0	0.5×2	0.5×2-3
Colony color	PO	P	P	Red	Red	C	W
Motility	-	+	+	+	-	+	-
pH range	7-9	7-9	7-9	7-9	7-9	7-9	5-9
Salt range (% NaCl)	15-25	8-25	8-25	15-25	15-25	0-25	3-25
Temperature range (°C)	35	35	35	35	35	25-45	35
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
MR test	NG	NG	NG	NG	NG	-	-
VP test	NG	NG	NG	NG	NG	-	-
Indole test	NG	NG	NG	NG	NG	-	-
Nitrate reduction	+	+	+	+	+	+	-
Acid production from:							
D-mannose	+	-	-	-	-	-	+
Lactose	-	-	-	-	-	-	-
D-fructose	+	+	+	+	+	-	-
D-galactose	-	-	-	-	-	-	-
D-xylose	+weak	-	-	-	-	+	-
L-arabinose	+weak	-	-	-	-	-	-
D-mannitol	-	-	-	-	+	-	+
Glucose	+	+	+	+	+	+	+
Gelatin	+	+	+	-	NG	-	-
Starch	-	+	+	-	NG	-	-
H_2S production	+	+	+	NG	NG	-	-
Sensitivity to antibiotics:							
Penicillin	-	-	-	-	-	-	+
Erythromycin	-	-	-	-	-	+	+
Chloramphenicol	-	-	-	-	-	+	-
Streptomycin	-	-	-	-	-	-	-
Tetracycline	-	-	-	-	-	-	+
Vancomycin	-	-	-	-	-	-	+
kanamycin	-	-	-	-	-	-	+

Note: Species (taxa identified indicates in parenthesis): 1, C12C (*Natronococcus occultus*); 2, C7h1 (*Halobacterium volcanii*); 3, C7h2 (*Haloferax mediterranei*); 4, C12h (*Halobacterium sodomense*); 5, C12h1 (*Halococcus tibetense*); 6, C12j (*Halomonas elongata*); and 7, C12K (Halophilic bacterium MBIC3303). Symbols: +, positive; -, negative; R, rod; C, cocci; C, cream; P, pink; PO, pale orange; W, white; NG, no growth.

4.3.2 Cellular fatty acid profiles of halophilic bacterium isolates

Prior to fatty acid extraction, bacterial isolates were tested for their capability to grow on the trypticase soy broth agar (TSBA) (section 1.14 Appendix A) and blood agar (purchased from the Carr-Scarborough Microbiologicals, U.S.A.), the standard growth media recommended by the MIDI for fatty acid analysis. The halophilic bacterium isolates exhibited no growth and/or low growth in these media. Thus, the tryptic soy agar medium was used for halophilic bacterium cultivation (Yoon *et al*., 2001). All halophilic bacterium isolates were tested for the optimal salt concentration that promote growth of the majority of bacterial isolates. The results indicated that 83%, 69%, and 64% of slightly-, moderately-, and extremely halophilic bacteria were grown on TSA (section 1.13 Appendix A) supplemented with 5%, 7%, and 8% NaCl, respectively. Therefore, the TSA agar with 5% NaCl and incubation temperature at 35^{o}C for 24-48 hours was employed for cultivation of halophilic bacteria in this study. With this culture condition, only 142 bacterial isolates and three reference type strains: *Salinicoccus hispanicus* ATCC 49259, *Bacillus subtilis* ATCC 6051, and *Micrococcus luteus* ATCC 4698 ATCC, were grown and had enough cells for fatty acid analysis. The other 10 bacterial isolates and

three reference type strains: *Halobacterium salinarum* ATCC 33171, *Halococcus saccharolyticus* ATCC 49257, and *Bacillus halophilus* ATCC 49085, did not have enough cells (40 mg) and/or did not produce enough fatty acids suitable for analysis. Noticeably, the failure to obtain adequate level of fatty acids was found in some isolates of the moderately halophilic bacteria and all isolates in the group of extremely halophilic bacteria. This result was due to low amount of fatty acids produced in certain halophilic bacteria group especially in extremely halophilic bacteria (Kamekura, 1993). The study of Kuchta and Russell (1994) showed that total fatty acid synthetase was 82% inhibited in culture medium with 3 M NaCl. A similar explanation for this result is that the maximum fatty acid synthase activity occurs in the presence of low concentration of sodium chloride and is 90% inhibited in the presence of 4 M NaCl (Hochstein, 1988).

Presently, the MIDI system does not contain a library for halophilic bacteria. Bacterial identification of this study was based on two databases for aerobic bacteria namely: TSBA40 and CLIN40. These two databases are the closet database for halophilic bacteria. However, when compared the identity of bacterial isolates to the bacterial species of the databases, 12 and 9 bacterial species of 37 identified species were in the database of TSBA40 and CLIN40, respectively. The MIDI's identification was listed with a confidence measurement (similarity index [SI]) on a scale of no match to 0.965. Compared to lengthy ultimate result of bacterial identification, the MIDI system correctly identified 64 of 142 (45.0%) bacterial isolates to the correct species level and 122 of 142 (85.9%) to the correct genus or the related genus level, and 20 of 142 (14.1%) were can not be identified. According to these findings, it cannot be recommended at this time for

the routine identification of halophilic bacteria until a library of halophilic bacterium isolates is established in the MIDI database. The data and isolates obtained in this study can be used to develop the database in the MIDI system library for future identification of halophilic bacteria.

The 32 representative bacterial isolates from 32 identified species of the ultimate identification results and three reference type strains of the ATCC were used to compare the fatty acid profiles (Table 10). The results of fatty acid profiles revealed that all Gram-positive bacterium isolates determined had a cellular fatty acid profile with large amounts of branched fatty acids; and that major fatty acids were C15:0 iso and C15:0 anteiso. In contrast, branched chain fatty acids appeared only very low amounts in Gram-negative bacterium isolates, while saturated fatty acids (C16:0) and unsaturated fatty acid (C18:1 w7c) were the predominant fatty acids. The fatty acids profile of bacterial isolates in this study was in agreement with the study of Ivanova *et al*. (1999), Yoon *et al*. (2001), and Yoon *et al*. (2003a). The explanation for high amounts of branched chain fatty acids in halophilic bacteria is because of their contribution in preserving the membrane integrity of halophilic bacteria under hypersaline environments (Kaneda, 1991). To date there is lack of studies on the function of saturated and unsaturated fatty acids of membrane component. The first study of cellular fatty acid composition in Gram-negative moderately halophilic bacteria was only related to the influence of salt concentration on the cellular fatty acid composition (Valderrama *et al*., 1998).

Table 10. Fatty acid profiles of representative bacterial isolates from 32 identified species and 3 reference strains that had been done using the MIDI system. Values given are percentage of total fatty acid. Fatty acids of less than 3% were omitted from this table.

Isolate codes with identified species	Fatty acid (%)																					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
A6U1 (*B. aquimaris*)									45.0	27.7					3.6							4.2
A7aA (*B. bataviensis*)							4.6		65.9	9.5		4.8										
A11at (*B. cereus*)			6.1			4.4			33.0	5.3	6.3	5.9		8.6							12.6	
Aiiv (*B. circulans*)							15.9	28.4	33.3			10.3			4.4							
A7[y] (*B. flexus*)						3.6	3.3		28.9	38.7	2.7		12.6		3.6							3.9
A1n1 (*B. firmus*)									24.2	38.7			3.7		9.3							7.7
A9aT (*B. licheniformis*)									36.1	31.9	4.1	4.0		12.5	8.8							
A12aj (*B. marisflavi*)									34.2	39.6		2.9			7.6							
B8ax (*B. halodenitrificans*)							7.8		10.1	57.7		5.8			11.5							
A9an (*B. megaterium*)						4.1	14.7		40.0	36.7	5.5											
A9ay (*B. mycoides*)				6.7		6.6			31.1	5.6	5.9	4.1		5.4								
Aiiau (*B. pumilus*)									49.3	27.3				4.5	4.2							
Aiaz (*B. subtilis*)									19.1	40.7	4.6	3.5		13.3	15.3							
B. subtilis ATCC 6015									15.9	40.6	4.8	3.0		13.6	17.7							
A5au (*B. vallismortis*)									26.7	37.5	3.9			15.4	10.6							
A8e (*B. vietnamensis*)									6.6	52.0		4.5			16.2							7.4
A6aP (*Geobacillus caldoxylosilyticus*)									27.3	33.6	10.9		5.9								8.3	
A11S (*Halobacillus trueperi*)									11.6	45.9		4.7		6.1	20.8							3.2
A1q (*Halobacillus salinus*)									5.9	57.4		4.8			25.3							
B3au1 (*Halobacillus litoralis*)									9.4	51.9	5.8	7.1		3.0	18.0							
B7w (*Oceanobacillus iheyensis*)							5.78		37.5	31.0		8.3		4.4	8.4							

Table 10. (Continue)

Isolate codes with identified species	Fatty acid (%)																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
B8az1 (*Vigribacillus pantothenticus*)									15.9	52.4				3.0	20.9							
A11aR (*Staphylococcus saprophyticus*)									10.7	54.8				6.2	15.3		3.6					
B8aN (*Staphylococcus gallinarum*)				21.9	15.0				9.2	22.8				7.6	7.4							
Mcrococcus luteus ATCC 4698					3.0	9.0			12.0	61.8	3.4				4.5							
Salinicoccus hispanicus ATCC 49259						3.2			33.6	24.7	6.1	3.4		6.7	3.5							
A11as (*Nocardiopsis dassonvillei*)										4.9	5.3	27.8				8.1	3.9		15.0		3.5	
B9aB (*Haererehalobacter ostenderis*)		6.4									28.8							45.6		5.0	3.9	
B3az (*Deleya pacifica*)		5.1									25.1							42.5		9.5	9.5	
A12ajj (*Marinobacter aquaeolei*)	6.1	6.6									25.2		9.3						13.3		14.4	
B11ax (*Halomonas desiderata*)	3.1	6.8									27.6							40.9		3.5	13.3	
C12j (*Halomonas elongata*)		6.1									25.0							43.9		10.5	3.4	
B11<ab> (*Halomonas nitritophilus*)		5.1									19.3							5.2			10.0	
B9av (*Halomonas ventosae*)		8.0									20.5							48.1			15.9	
A12ar (*Pseudomonas stutzeri*)	7.1										22.5							30.7			26.9	

Note: Fatty acid identified as: 1, C12:0; 2, C12:0 3OH; 3, C13:0; 4, C13:0 iso; 5, C13:0 anteiso; 6, C14:0; 7, C14:0 iso; 8, C15:0; 9, C15:0 iso; 10, C15:0 anteiso; 11, C16:0; 12, C16:0 iso; 13, C16:1 w11c; 14, C17:0 iso; 15, C17:0 anteiso; 16, C17:1 w8c; 17, C18:0; 18, C18:1 w7c; 19, C18:1 w9c; 20, C19:0 cyclo w8c; 21, Sum in feature 3; and 22, Sum in feature 4. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C16:1 w7c and/or C15:0 iso 2OH. Summed feature 4 contained iso-C17 : 1 I and/or anteiso-C17 : 1 B.

The organisms used for comparing the fatty acid profiles (Table 9) were also used to determine their relatedness using the constructed dendogram and 2-D plot (Figures 12 and 13). Only 27 of 32 bacterial isolates and one (*Micrococcus luteus* type strain ATCC 4698) of three reference type strains were marked at the dendogram and 2-D plot printout. The failure of the other isolates was the low peak (less than 5,000) of fatty acids produced (personal communication, the MIDI, U.S.A.).

4.3.3 Genotypic characteristics of halophilic bacterium isolates

For 16S rDNA sequence analysis, the genomic DNA was extracted from the 152 bacterial isolates. The data from phenotypic feature tests and fatty acid analysis were used for choosing the primer for PCR amplification of 16S rDNA. All of the bacterial isolates of the slightly- and moderately halophilic bacteria, and the 4 bacterial isolates of the extremely halophilic bacteria which were identified as in the group of *Eubacteria* were successfully amplified with primers 27f and 1525r. The rest of extremely halophilic bacterium isolates that were identified as *Archaea* were successfully amplified with primers 21f and 1525r.

The sequences of an approximately 600 to 1,500 bp portion of the 16S rDNA were obtained for 152 bacterial isolates. The sequences obtained were edited manually and compared to nucleotides database provided by the National Center for Biotechnology Information using the BLAST (Basic Local Alignment Search Tool). The percent sequence identity were determined for the closest identity of the sequences obtained. The DNA-DNA cross-hybridization of more than 70% has been suggested to

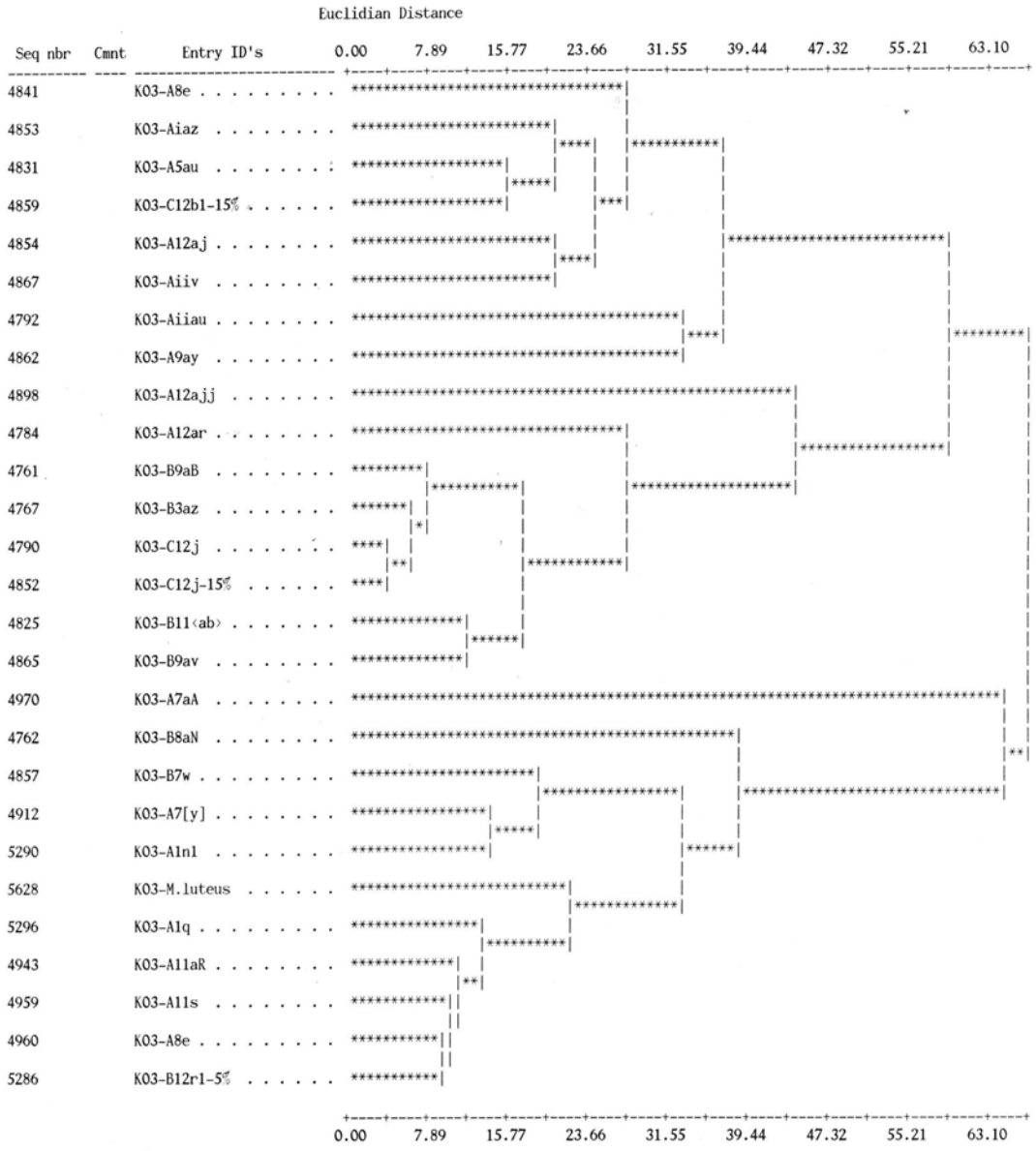


Figure 12. Dendogram showing the clustering of 27 bacterial isolates studied and 1 reference type strain (*Micrococcus luteus* ATCC 4698, sequence number 5628) based on fatty acid profiles analyzed by the MIDI system.

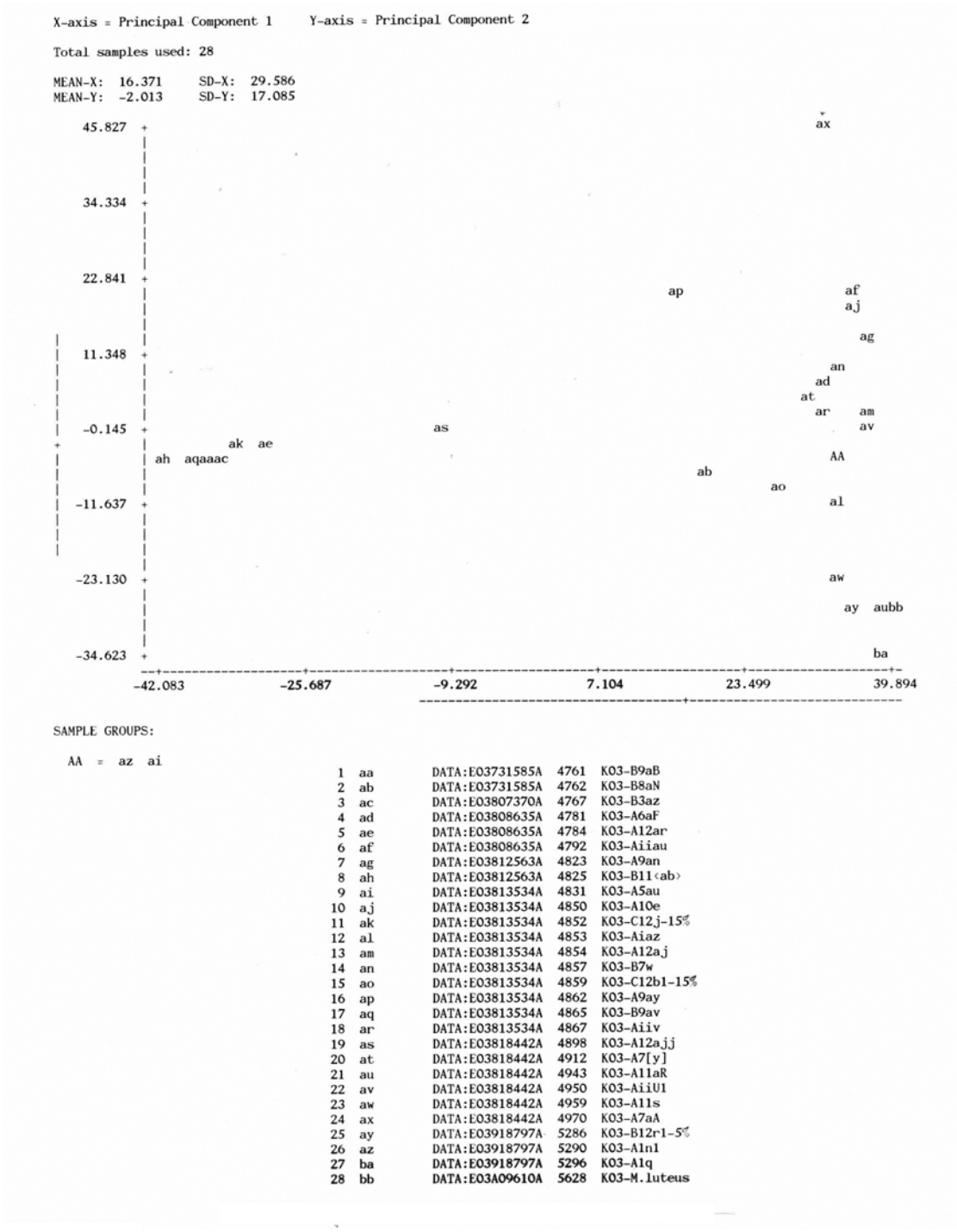


Figure 13. 2-D plot showing the clustering of 27 bacterial isolates studied and 1 reference type strain *Micrococcus luteus* ATCC 4698 (bb) based on fatty acid profiles analyzed by the MIDI system.

indicate that the two bacteria belong to the same species (Wayne *et al*., 1987) because at these levels of cross-hybridization, degree of a 16S rDNA sequence similarity is higher than 97% (Stackebrandt and Göbel, 1994). Thus the sequence similarity of 97% is used as a cutoff for bacterial characterization using the 16S rDNA sequence analysis in this study.

Comparisons between sequences of the isolates studied and the sequences available in the GenBank database exhibited high sequence similarity with the majority exceeding 97%. Further sequencing was conducted for isolates which had percent similarity less than 97% using primer 490f (5'-TGCCAGCAGCCGCGGTAATAC-3') designed from the sequences obtained in this study. Only two isolates (C12b1 and C12K) had the similarity lower than 97% though their sequences obtained were 1497 bp. The first match for these 2 isolates was Halophilic bacterium MBIC3303 (96%) and the second match was *Bacillus* sp. (93%). In addition, with its phenotypic features, these isolates belonged to the genus *Bacillus* and the identity given from the MIDI was *Vigribacillus* sp. Based on the combination of bacterial characterization used in this study, these 2 isolates were belonged to *Bacillus* sp. The result of 16S rDNA sequence similarity between these 2 isolates and the other *Bacillus* sp. also confirms this identity result. The percent similarity for each isolate compared to the first match of the BLAST search is shown in Table 5E (Appendix E). Of the 152 sequences obtained, 38 unique sequence types were identified.

The percentage of 16S rDNA sequence similarity among the representatives of the genus *Bacillus* and related genera obtained in this study is

78-98% (Table 11). The percentage of 16S rDNA similarity among the sequences that belonged to the genera *Halomonas* is 94-96%. The identity of 38 unique sequences based on phylogenetic analysis of 16S rDNA sequence and the 16S rDNA sequence information of 6 reference strains studied is given in Table 12. From observation of electrophenograms within the same identified species, there was no difference in the 16S rDNA sequence of the isolates which differed in the degree of halophilic (Figures 2F and 3F Appendix F), for example.

The 38 unique sequences together with the 16S rDNA sequences of 6 reference type strains: *B. halophilus* DSM 4771, *B. subtilis* ATCC 6051, *Halobacterium salinarum* DSM 3754T, *Halococcus saccharolyticus* ATCC 49259, *Micrococcus luteus* DSM 20030, and *Salinicoccus hispanicus* DSM 5352, were used to generate a phylogenetic tree using software TREECON for Windows version 1.3b (Van de Peer and De Wachter, 1994) and the algorithm of Jukes and Cantor (1969).

The phylogenetic tree generated is depicted in Figure 14. The phylogenetic tree shows 3 distinct phylogenetic clusters: Cluster I Gram-positive bacteria, Cluster II Proteobacteria, and Cluster III Archaeobacteria, which reflect the evolutionary relationships among prokaryotes.

Based on the combined phenotypic, chemotaxonomic, and genotypic data, 152 bacterial isolates were identified to 16 genera and 38 different species. The results of the bacterial identification for the 152 representative bacterial isolates are summarized in Table 5E (Appendix E). The 150 bacterial isolates were identified as belonging to the certain species level. Only 2 bacterial isolates (C12b1 and C12K)

were identified to the genus level. The genus *Bacillus* was the majority group and accounted for 105 of 152 isolates (68.0%).

Table 11. 16S rDNA sequence similarity of *Bacillus* and related genera.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1																							
2	89																						
3	89	94																					
4	86	91	91																				
5	88	91	93	90																			
6	87	91	92	91	93																		
7	86	92	93	89	92	93																	
8	91	92	91	90	92	90	90																
9	88	92	91	91	90	89	91	91															
10	89	97	94	92	93	91	92	92	93														
11	88	91	93	89	94	96	80	91	90	91													
12	85	91	91	99	90	91	89	90	91	91	91												
13	88	91	91	92	92	91	93	90	93	92	92	91											
14	89	93	91	91	90	90	92	92	95	94	91	90	94										
15	87	93	90	92	90	90	92	92	95	95	85	92	95	98									
16	87	97	92	92	90	91	93	93	93	96	82	92	92	94	94								
17	88	89	89	84	87	84	85	89	87	88	86	84	87	87	86	84							
18	89	93	91	92	92	91	90	91	92	93	92	90	91	93	92	92	87						
19	92	89	87	88	89	87	90	92	88	89	89	87	87	88	90	91	85	88					
20	94	91	91	78	90	80	78	92	81	91	89	79	80	82	80	81	87	91	83				
21	84	80	80	88	80	88	91	84	89	81	88	86	88	87	90	91	78	79	96	83			
22	90	91	92	89	92	89	88	94	88	91	90	89	90	90	89	89	88	90	88	90	81		
23	88	89	89	90	89	87	89	94	89	89	87	89	88	88	91	91	87	89	91	79	91	90	

Note: Species (taxa identified indicates in parenthesis): 1, *Bacillus halophilus* DSM 4771; 2, B10aFF (*Bacillus aquaemaris*); 3, A7aA (*Bacillus bataviensis*); 4, A11at (*Bacillus cereus*); 5, AIIV (*Bacillus circulans*); 6, A7y (*Bacillus flexus*); 7, A1n1 (*Bacillus firmus*); 8, B8ax (*Bacillus halodenitrificans*); 9, A9aT (*Bacillus licheniformis*); 10, A12aj (*Bacillus marisflavi*); 11, A9an (*Bacillus megaterium*); 12, A9ay (*Bacillus mycoides*); 13, AIIau (*Bacillus pumilus*); 14, AIaz (*Bacillus subtilis*); 15, A5au (*Bacillus vallismortis*); 16, A8e (*Bacillus vietnamensis*); 17, C12K (Halophilic bacterium MBIC3303, *Bacillus* sp.); 18, A6aP (*Geobacillus caldoxylosilyticus*); 19, B3AU1 (*Halobacillus litoralis*); 20, A1q (*Halobacillu ssalinus*); 21, A11S (*Halobacillu strueperi*); 22, B7w (*Oceanobacillus iheyensis*); 23, B8az (*Virgibacillus pantothenticus*).

Table 12. Summary information of 16S rDNA sequence determination.

Cluster	Isolate codes/ reference strains	Closest relative	Length of sequences	% similarity	Accession number from GenBank	information of organisms used for sequences comparison	
						Accession number	References
I	B10aFF	*Bacillus aquimaris*	1471	98	AY647307	AF483625	Yoon *et al.* (2003b)
	A7aA	*Bacillus bataviensis*	1401	97	AY647284	AJ542507	Heyrman *et al.* (2004)
	A11at	*Bacillus cereus*	742	99	AY647292	AE017013	Ivanova *et al.* (2003)
	AIIV	*Bacillus circulans*	962	97	AY647299	AY043084	Ince Yilmaz (2003)
	A1n1	*Bacillus firmus*	644	98	AY647280	AJ509007	Muscillo *et al.* (2003)
	A7y	*Bacillus flexus*	801	97	AY647285	AB021185	Goto *et al.* (2000)
	B8ax	*Bacillus halodenitrificans*	1267	98	AY647304	AB021186	Goto *et al.* (2000)
	B. halophilus DSM4771		1517			AJ243920	Schlesner *et al.* (2001)
	A9aT	*Bacillus licheniformis*	791	97	AY647288	AF276309	Nazina *et al.* (2000)
	A12aj	*Bacillus marisflavi*	1500	97	AY647294	AF483624	Yoon *et al.* (2003b)
	A9an	*Bacillus megaterium*	668	99	AY647287	AY030338	Venkateswaran *et al.* (2003)
	A9ay	*Bacillus mycoides*	837	99	AY647289	AY373357	Ding and Chen (2003)
	AIIau	*Bacillus pumilus*	822	99	AY647298	AB098578	Hiraishi *et al.* (2003)
	AIaz	*Bacillus subtilis*	836	99	AY647297	AY162133	Bodour *et al.* (2003)
	B. subtilis ATCC 6051		636			AF443053	Pannucci *et al.* (2002)
	A5au	*Bacillus vallismortis*	722	99	AY647282	AB021198	Goto *et al.* (2000)
	A8e	*Bacillus vietnamensis*	682	99	AY647286	AB099708	Noguchi *et al.* (2003)
	A6aP	*Geobacillus caldoxylosilyticus*	1085	97	AY647283	AJ489326	Marchant *et al.* (2002)
	B3AU1	*Halobacillus litoralis*	796	97	AY647300	X94558	Spring *et al.* (1996)
	A1q	*Halobacillus salinus*	904	97	AY647281	AF500003	Yoon *et al.* (2003a)
	A11S	*Halobacillus trueperi*	910	98	AY647293	AJ310149	Swiderski (2003)
	C12K	Halophilic bacterium MBIC3303	1497	96	AY647316	AB015022	Hamada (2003)
	B7w	*Oceanobacillus iheyensis*	1455	98	AY647302	AP004595	Lu *et al.* (2001)
	B8az	*Virgibacillus pantothenticus*	753	97	AY642594	AB039331	Huang *et al.* (2003)
	Micrococcus luteus DSM 20030		1418			AJ536198	Swiderski (2003)
	A11as	*Nocardiopsis dassonvillei*	1026	97	AY647291	AF479269	Andrzej (2003)

Table 12. (Continued)

Cluster	Isolate codes/ reference strains	Closest relative	Length of sequences	% similarity	Accession number from GenBank	information of organisms used for sequences comparison	
						Accession number	Reference
	Salinicoccus hispanicus DSM 5352		1508			AY028927	Yoon *et al.* (2003c)
	B8aN	*Staphylococcus gallinarum*	788	97	AY647303	D83366	Takahashi (2003)
	A11aR	*Staphylococcus saprophyticus*	674	99	AY647290	Z26902	MacLean and Carter (2003)
II	B3az	*Deleya pacifica*	599	98	AY647301	L42616	Dobson and Franzmann (2003)
	B9aB	*Haererehalobacter ostenderis*	1210	97	AY647305	U78786	Austin *et al.* (2003)
	B11ax	*Halomonas desiderata*	1435	97	AY647309	X92417	Berendes *et al.* (1996)
	C12j	*Halomonas elongata*	684	99	AY647315	X67023	Gauthier *et al.* (1992)
	B11ab	*Halomonas nitritophilus*	1427	97	AY647308	AJ309564	Gilvanova *et al.* (2003)
	B9av	*Halomonas ventosae*	1413	98	AY647306	AY268080	Martinez-Canovas *et al.* (2003)
	A12ajj	*Marinobacter aquaeolei*	1053	97	AY647295	AJ000726	Huu *et al.* (1999)
	A12ar	*Pseudomonas stutzeri*	658	99	AY647296	AY364327	Kong (2003)
III	C12h	*Halobacterium sodomense*	673	99	AY647314	D13379	Kamekura and Seno (1993)
	C7h1	*Halobacterium volcanii*	723	98	AY647310	K00421	Gupta *et al.*(1983)
	C7h2	*Haloferax mediterranei*	550	99	AY647311	D11107	Kamekura and Seno (1992)
	C12h1	*Halococcus tibetense*	628	98	AY647313	AF435112	Fan (2003)
	C12C	*Natronococcus occultus*	854	98	AY647312	Z28378	McGenity and Grant (1993)
	Halobacterium salinarum DSM 3754T		1736			AJ496185	Gruber *et al.* (2003)
	Halococcus saccharolyticus ATCC49259		1472			AB004876	Ventosa *et al.* (1999)

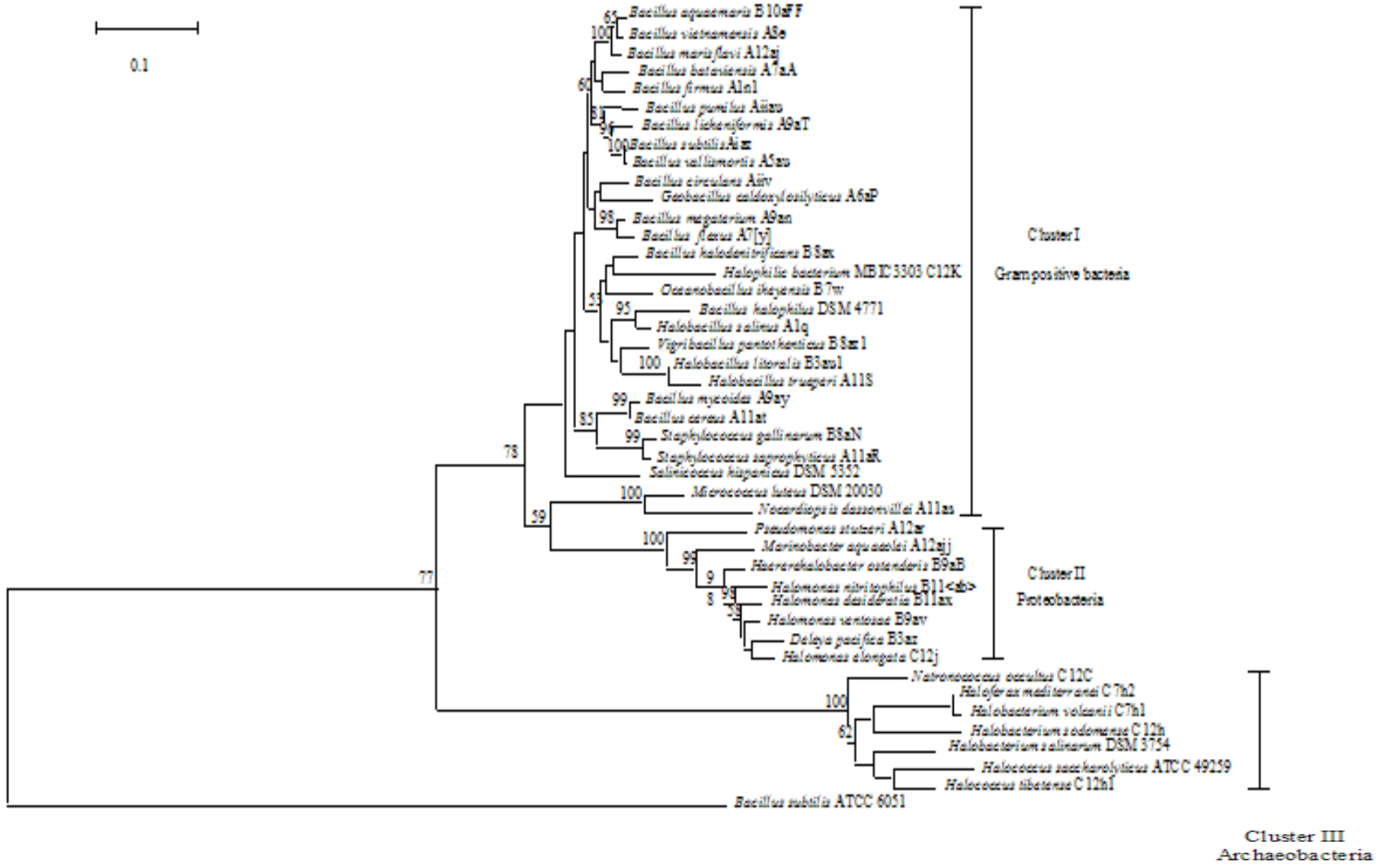


Figure 14 Phylogenetic tree of bacterial and archaeal sequences obtained from the cultures that isolated from saline soil at Nong Bo Reservoir, Mahasarakham, Thailand. The scale bar indicates 0.1substitutions per nucleotide position.

Among the genus *Bacillus*, *B. firmus* was found to be the most common with the number of identified isolates totaling 20 of the 105 isolates (19.0%) of the identified *Bacillus* species. This was followed by *B. subtilis* that accounted for 18 of the 105 isolates (17.1%) of the identified *Bacillus* species. *B. aquimaris, B. licheniformis,* and *B. pumilus*, were relatively common in the soil studied while *B. bataviensis, B. cereus*, *B. circulans*, *B. flexus*, *B. halodenitrificans, B. megaterium*, *B. marisflavi*, *B. mycoides*, *B. vallismortis*, and *B. vietnamensis* were represented with a lower occurrence. The percentage of identified species inhabiting saline soil at Nong Bo Reservoir collected from June 2001 to May 2002 is illustrated in Figure 15.

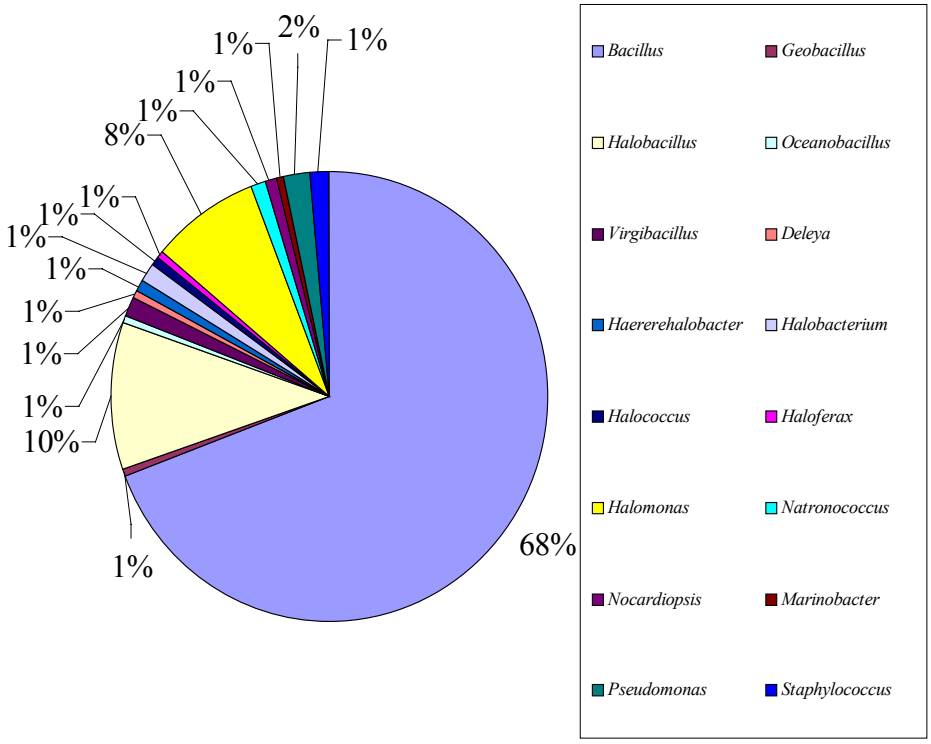


Figure 15. Percentage of identified species inhabiting saline soil at Nong Bo Reservoir.

From Figure 15, it is clear that the genus *Bacillus* and related genera were the dominant species colonized in saline soil at Nong Bo Reservoir. These results are the same as the previous studies that *Bacillus* spp. presented in hypersaline soil (**Quesada** *et al*.**,** 1982; **Rodriguez-Valera,** 1988; and Zahran *et al*., 1992). In addition, **Rodriguez-Valera** (1988) and Quesada *et al*. (1983) stated that the bacteria of hypersaline soil were halophilic representatives of normal soil bacteria while those of hypersaline water seem to be halophilic representatives of marine bacteria.

Of 38 different identified species, 33 species (86.8%) are in the domain *Eubacteria* and 5 species (13.2%) are in the domain *Archaea*. One interesting feature of this finding is that the extremely halophilic archaeobacteria were isolated from the soil samples which had low salinity with the average salinity ranging from only 0.05 to 0.35%, and was extremely variable with some soil samples having the salinity reach 0.8%. Javor (1989) found that the ability of the extremely halophilic archaeobacteria to grow in ≤ 2.5 M (15%) NaCl depends on the strains, the temperature, and other salts in solution. These results imply that the extremely halophilic archaeobacteria in the study area have adapted to the special characteristics of the environment in which they are established. The reason for the occurrence of the extremely halophilic archaeobacteria in the soil samples, which have salt content far less than their requirement, may due to the heterogeneity of the soil habitat causing a sufficiently high concentration of salts in some local microhabitat sufficient to support their growth (**Quesada** *et al*., 1982). Moreover, due to the differences in soil genesis, and stratigraphy of a particular soil, the specific kinds of salts generated may also support their growth. In the case of extremely halophilic archaeobacteria, NaCl is the most important salt type for their growth although KCl can replace NaCl to certain extent (Kushner and

Kamekura, 1988). These results coincide with the report of Furukawa (2542) that the Mahasarakham Formation, the rock salt beds of the study area mainly composed of NaCl while the other salts such as KCl, $MgCl_2$, $CaCl_2$, $CaSO_4$, and $CaCO_3$ were found in the lesser amounts. However, these results are not so surprising since the occurrence of the extremely halophilic archaeobacteria in hypersaline soil has been reported previously by Ihara *et al*., (1997) and Lizama *et al*. (2001).

All isolates identified in this research belonged to the species which have been isolated from many other hypersaline environments such as salt-affected soil (**Quesada *et al*.,** 1982), Thai fish sauce (Thongthai and Suntinanalert, 1991), the inland salterns of La Mala, Granada, Spain saltern ponds (Del Moral ***et al*.,** 1987), the Dead Sea (Kushner, 1988), the Solar Lake, Sinai, Egypt (Cytryn ***et al*.,** 2000), Vietnamese fish sauce, sea water from the Gulf of Mexico (Noguchi ***et al*.,** 2003), and the Yellow Sea in Korea (Yoon ***et al*.,** 2003b).

CHAPTER IV

CONCLUSION AND FUTURE PERSPECTIVE

A study of halophilic bacterial diversity in saline soil was conducted at Nong Bo Reservoir, Mahasarakham Province, Thailand from June 2001 to May 2002. Saline soil samples were collected monthly from five sampling plots: the land of the fishponds, the halophyte plot of the Department of Land Development, the mixed-agricultural experimental plot, the halophyte experimental plot of Rajapruek Institute Foundation and the land where a salt storehouse was located. The soil samples were analyzed to determine some physical and chemical properties, and the density and diversity of halophilic bacteria inhabited the saline soil. The density of slightly-, moderately-, and extremely halophilic bacteria were enumerated using the halobacteria medium containing 3%, 8%, and 25% NaCl, respectively. Different colonies grown on media were selected and purified for the investigation of halophilic bacterial diversity. The halophilic bacterium isolates were characterized by using the combined phenotypic, chemotaxonomic and genotypic data.

The results of physical and chemical properties of soil samples reveal two main soil problems, extremely low fertility with the average organic matter ranging from 0.13-0.42% and the high percentage of sand. At present, the soil is non saline to slightly saline. The average soil salinity is 0.04-0.35%. Although this level of soil salinity seems not a

serious stress for plant growth, the problems either the poor growth or death of plants still remain.

The highest halophilic bacterial density was found in the sampling plot at the land where salt storehouse was located, although their organic matter and total nitrogen content are somewhat lower than the other sampling plots. The highest density of slightly-, moderately-, and extremely halophilic bacteria was accounted for 7.10, 6.35, and 4.47 log CFU/g of dry soil, respectively. The lowest density of slightly- and moderately halophilic bacteria was found in the halophyte plot of the Department of Land Development, and was accounted for 3.84 and 3.37 log CFU/g of dry soil, respectively while the extremely halophilic bacteria was not found (at soil dilution of 1:2). The reasons for these results are that the land where salt storehouse was located may has the left over of the various kinds of salts from the salt production, also the soil pH near neutral could support the growth of halophilic bacteria.

Regarding the study of halophilic bacterial diversity, the phenotypic characterization alone was unreliable when applied to halophilic bacteria since their growth were affected by many factors such as NaCl concentrations, temperature, pH, and media recipes. In addition, many of extremely halophilic bacteria are biochemically unreactive, and thus limited the phenotypic data. The fatty acid analysis by the MIDI system can be a useful supplement and reference method, but cannot be recommended at this time for the routine identification of halophilic bacteria until a library of halophilic bacterium isolates is established in the MIDI database. The data and isolates obtained in

this study can be used to develop the database in the MIDI system library for future identification of halophilic bacteria.

The 16S rDNA sequence analysis is the reliable method for halophilic bacterium identification. However, a polythetic view should be considered to obtain the stable and reliable characterization of halophilic bacteria. Therefore, the combination of phenotypic characterization, fatty acid analysis and 16S rDNA sequence analysis were used for halophilic bacterium characterization in this study.

Based on the combined phenotypic, chemotaxonomic, and genotypic data, 152 bacterial isolates were identified to 16 genera and 38 different species. The identified species were *Bacillus firmus*, *B. subtilis, B. pumilus*, *B. licheniformis*, *B. aquimaris, B. bataviensis, B. cereus*, *B. circulans*, *B. flexus*, *B. halodenitrificans, B. megaterium*, *B. marisflavi*, *B. mycoides*, *B. vallismortis*, *B. vietnamensis*, *Deleya pacifica*, *Geobacillus caldoxylosilyticus, Halobacillus litoralis*, *Halobacillus salinus*, *Halobacillus trueperi*, Halophilic bacterium MBIC3303 (*Bacillus* sp.), *Haererehalobacter ostenderis, Halobacterium volcanii*, *Halobacterium sodomense*, *Halococcus tibetense, Haloferax mediterranei*, *Halomonas desideratia*, *Halomonas elongata*, *Halomonas nitritophilus, Halomonas ventosae*, *Marinobacter aquaeolei*, *Natronococcus occultus*, *Nocardiopsis dassonvillei, Oceanobacillus iheyensis, Pseudomonas stutzeri, Staphylococcus* saprophyticus, *Staphylococcus gallinarum*, and *Vigribacillus pantothenticus*.

These results reveal a high diversity of halophilic bacteria in saline soil at Nong Bo Reservoir, Mahasarakham Province, Thailand. The predominant organisms colonized in the soil were halotolerant and halophilic eubacteria. The extremely halophilic

archaeobacteria were also able to grow in the soil, although the soil salinity was far less than their salt requirement (9% NaCl). This may imply that these organisms are capable of surviving under diverse ecological conditions.

The results of phenotypic characterization reveal the ability of halophilic bacterium isolates in utilizing a wide variety of nutrient sources including carbon, nitrogen and sulfur. Resistant to penicillin, erythromycin, bacitracin, chloramphenicol, streptomycin, tetracycline, vancomycin and kanamycin are shown in all extremely halophilic bacterium isolates. From their physiology, they presumably play an important and substantial role in the biogeochemical cycles.

From this study we may not gain the true microbial community structure because the microbial community may contain both viable culturable and nonculturable bacteria. In addition, the progress in defining ecological roles of halophilic bacteria is realized best by using classical culturing and molecular approaches in concert. Therefore, apart from the laboratory cultivation method used for the study of halophilic bacterial density in this study, the direct DNA extraction from saline soil samples and analysis of nucleic acids using the T-RFLP technique have been tried to evaluate the true microbial community of the saline soil at Nong Bo Reservoir (Appendix G). The work could not be completed due to the problems in humic acid contamination in the extracted DNA and the limitation of time and financial support. However, a number of procedures have been tried to extract DNA from saline soil, which would be useful for future study of microbial diversity in saline soil.

There are a number of challenges involved in describing microbial dynamics in saline soil habitats that remain unsolved. This study provides the basic information for the study of halophilic bacteria in saline soil including the methodology for studying halophilic bacterial density and diversity. In addition, the information of the 16S rDNA sequences obtained from this study also provides information for the design of specific oligonucleotides primers and probes hybridizing to the 16S rRNA gene sequences of halophilic bacteria for the detection and monitoring changes of halophilic bacteria in the environments.

Since saline soil at Nong Bo Reservoir is a complex problem, the integrative and interdisciplinary approaches for their solution are necessary. The halophilic bacterium isolates obtained from this study can be used for variety of biotechnological applications including: the recovery of saline soil, increasing crop yield in saline soil and production of specific bacterium metabolites such as enzymes, antimicrobial compounds, and pharmaceutical compounds. The reasons for their high potential applications including: their role in biogeochemical cycles in saline soil, the majority of them are plant growth-promoting bacteria, the flexibility of their growth in the diverse ecological conditions and their ability to grow on the culture media. There are more to study regarding saline soil recovery in order to achieve the goal of Nong Bo Reservoir rehabilitation and sustainable conservation which was proposed by the National Center for Genetic Engineering and Biotechnology, Thailand. Examples of the future studies are the metabolic diversity of halophilic bacteria in saline soil, the capability of the halophilic bacterium isolates to promote plant growth, growth of plants inoculated with the inoculum of halophilic

bacterium isolates in the greenhouse and field conditions, the development of molecular techniques for the study of the microbial communities of normal saline soil and saline soil that have been inoculated with the inoculum of halophilic bacterium isolates, and any specific kind of plants that their growth could be promoted by using the inoculum of halophilic bacterium isolates.

In conclusion, the saline soil at Nong Bo Reservoir is colonized by diverse halophilic bacteria. All the information obtained from this study along with the halophilic bacterium isolates is useful for the study of their biotecthnological application and for sustainable conservation of saline soil.

REFERENCES

กองจำแนกดิน. (2516). **Soil Interpretation for Thailand.** กรุงเทพฯ: กรมพัฒนาที่ดิน กระทรวงพัฒนาการแห่งชาติ.

กองวิเคราะห์ดิน. (2544). **วิธีวิเคราะห์ดินทางเคมี.** กรุงเทพฯ: กรมพัฒนาที่ดิน.

ชัยนาม ดิสถาพร. (2542). การปลูกป่าเพื่อป้องกันการแพร่กระจายดินเค็ม. ใน กรมพัฒนาที่ดิน**. เอกสารคู่มือเจ้าหน้าที่ของรัฐเรื่องดินเค็ม** (นน. 233-239). กรุงเทพฯ: กระทรวงเกษตรและสหกรณ์.

นเรศ สัตยารักษ์, สมเกียรติ จันทร์มหา, เจตต์ จุลวงษ์, ปกรณ์ สุวานิช, และ ธวัช จาปะเกษตร์. (2542). อิทธิพลของชั้นเกลือหินที่ต่อน้ำใต้ดินในภาคอีสาน. ใน กรมพัฒนาที่ดิน**. เอกสารคู่มือเจ้าหน้าที่ของรัฐเรื่องดินเค็ม** (นน. 145-173). กรุงเทพฯ: กระทรวงเกษตรและสหกรณ์.

สมศรี อรุณินท์. (2542ก). การปรับปรุงดินเค็มและดินโซดิก. ใน กรมพัฒนาที่ดิน**. เอกสารคู่มือเจ้าหน้าที่ของรัฐเรื่องดินเค็ม** (นน. 19-29). กรุงเทพฯ: กระทรวงเกษตรและสหกรณ์.

สมศรี อรุณินท์. (2542ข). แผนพัฒนาเศรษฐกิจและสังคมแห่งชาติฉบับที่ ๘ (พ.ศ. 2540 - 2544) กับโครงการพัฒนาพื้นที่ดินเค็ม. ใน กรมพัฒนาที่ดิน**. เอกสารคู่มือเจ้าหน้าที่ของรัฐเรื่องดินเค็ม.** (นน. 1-5). กรุงเทพฯ: กระทรวงเกษตรและสหกรณ์.

Alexander, M. (1977). Introduction to soil microbiology. Quoted in J.D. van Elsas, J.T. Trevors, and E.M.H. Wellington. (1997). **Modern Soil Microbiology** (pp. 47-59). New York: Marcel Dekker.

Amann, R., Springer, N., Ludwig, W., Görtz, H-D., and Schleifer, K.H. (1991). Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. **Nature**. 351: 161-164.

Andrzej, G., Huang, Y. and Liu, Z. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Antón, J., Rosselló-Mora, R., Rodriguez-Valera, F., and Amann, R. (2000). Extremely halophilic Bacteria in crystallizer ponds from solar salterns. **Appl. Environ. Microbiol.** 66(7): 3052-3057.

Arahal, D.R., Marquez, M.C., Volcani, B.E., Schleifer, K.H., and Ventosa, A. (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. **Int. J. Syst. Bacteriol.** 49(2): 521-530.

Arahal, D.R. and Ventosa, A. (2002). Moderately halophilic and halotolerant species of *Bacillus* and related genera. In R. Berkeley, M. Heyndrickx, N. Logan, and P. De Vos (eds.). **Applications and Systematics of *Bacillus* and Relatives** (pp. 83-99). Oxford: Blackwell.

Arunin, S. (1987). Management of saline and alkaline soil in Thailand. Quoted in F. Ghassemi, A.J. Jakeman, and H.A. Nix (eds.). (1995). **Salanisation of Land and Water Resources** (p. 445). Wallingford Oxon: CAB International.

Arunin, S. (1992). Strategies for utilizing salt-affected lands in Thailand. Quoted in F. Ghassemi, A.J. Jakeman, and H.A. Nix (eds.). (1995). **Salanisation of Land and Water Resources** (p. 445). Wallingford Oxon: CAB International.

Atlas, R.M. (1997a). **Handbook of Microbiological Media** (2nd ed.). Boca Raton: CRC Press.

*Atlas, R.M. (1997b). **Principles of Microbiology** (2nd ed.). Boston: Wm.C.Brown.*

Austin, D.J., Connor, T.H. and Shimmin, L.C. 2003. **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Bakken, L.R. (1985). Separation and purification of bacteria from soil. **Appl. Environ. Microbiol.** 49 (6): 1482-1487.

Bakken, L.R. (1997). Culturable and nonculturable bacteria in soil. In J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington. **Modern Soil Microbiology** (pp. 47-59). New York: Marcel Dekker.

Bartlett, D.H. and Roberts, M.F. (2000). Osmotic stress. In J. Lederberg (ed.). **Encyclopedia of Microbiology** (Vol. III) (2nd ed., pp. 502-516). San Diego: Academic Press.

Bauer, A.W., Serries, J.C., Turck, M., and Kirby, W.M. (1966). Antibiotic susceptibility testing by a standard single disc method. **Am. J. Clin. Path.** 45: 493-496.

Beishier, L. (1991). **Microbiology in Practice** (5th ed.). New York: Harper Collins.

Berendes, F., Gottschalk, G., Heine-Dobbernack, E., Moore, E.R.B. and Tindall, B.J. (1996). *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and

denitrifying bacterium isolated from a municipal sewage works. **Syst. Appl. Microbiol.** 19: 158-167.

Bodour, A.A., Drees, K.P. and Maier, R.M. (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. **Appl. Environ. Microbiol.** 69(6): 3280-3287.

Brady, N.C. and Weil, R.R. (1999). ***The Nature and Properties of Soils****. New Jersey: Prentice Hall.*

Brandt, K.K., Patel, B.K., and Ingvorsen, K. (1999). *Desulfocella halopila* gen. nov., sp. nov., a halophilic, fatty-acid-oxidixing, sulfate-reducing bacterium isolated from sediments of the Great Salt Lake. **Int. J. Syst. Bacteriol.** 49(1): 441-448.

Bresler, E., McNeal, B.L., and Carter, D.L. (1982). Saline and sodic soils. Quoted in D.L. Spark (ed.). (1995). ***Environmental Soil Chemistry*** *(p. 230). California: Academic Press.*

Brown, A. D. (1976)**.** Microbial water stress. **Bacteriol. Rev.** 40: 803-846.

Chanway, C.P. (2002). Plant growth promotion by *Bacillus* and relatives. In R. Berkeley, M. Heyndrickx, N. Logan, and P. De Vos (eds.). **Applications and Systematics of *Bacillus* and Relatives** (pp. 219-232). Oxford: Blackwell.

Cappuccino, J.G. and Sherman, N. (1999). **Microbiology: A Laboratory Manual**. California: Benjamin/Cummings Science.

Claus, D. and Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. II) (pp. 1105–1139). Baltimore: Williams & Wilkins.

Cohen, Y., Krumbein, W. E., Goldberg, M., and Shilo, M. (1977). Solar Lake (Sinai) I: physical and chemical limnology. Quoted in B. Javor (ed.). (1989). **Hypersaline Environments**: **Microbiology and Biogeochemistry** (p. 205). Berlin: Springer-Verlag.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. **Microbiol. Rev.** 53: 121-147.

Cytryn, E., Minz, D., Oremland, R.S., and Cohen, Y. (2000). Distribution and diversity of Archaea corresponding to the limnological cycle of a hypersaline stratified lake (Solar Lake, Sinai, Egypt). **Appl. Environ. Microbiol.** 66(8): 3269-3276.

Da Costa, M.S, Santos, H., and Galinski, E.A. (1998). An overview of the role and diversity of compatible solutes in bacteria andarchaea. **Adv. Biochem. Eng. Biotechnol.** 61: 117-53

DasSarma, S. (2001). Halophiles. **Encyclopedia of Life Science** [On-line]. Available: http://www.zdna2.umbi.umd.edu

De Dekker, P. (1983). Australian salt lakes: their history, chemistry, and biota-a review. Quoted in R.H. Vreeland, and L.I. Hochstein (eds.). (1993). ***The Biology of Halophilic Bacteria*** *(p. 4). Boca Raton: CRC Press.*

Del Moral, A., Quesada, E., and Ramos-Cormenzana, A. (1987). Distribution and types of bacteria isolated from an inland saltern. **Ann. Inst. Pasteur Microbiol**. 138(1): 59-66.

Denariaz, G., Payne, W.J., and Le Gall, J. (1989). A halophilic denitrifier, *Bacillus halodenitrificans* sp. nov. **Int. J. Syst. Bacteriol.** 39(2): 145-151.

Ding, Y. and Chen, S. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Dobson, S.J. and Franzmann, P.D. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Dunbar, J., Ticknor, L.O. and Kuske, C.R. *(2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities.* ***Appl. Environ. Microbiol.*** *67(1): 190-197.*

El-Abyad, M.S., Ismail, I.K., and Rizk, M.A. (1979). Ecological studies of the microflora of saline Egyptian soils. Quoted in H.H., Zahran, A.M., Moharram, and H.A. Mohammad. (1992). Some ecological and physiological studies on bacteria isolated from salt-affected soils of Egypt. **J. Basic Microbiol.** 32(6): 405-413.

Fan, H., Xue, Y. and Ma, Y. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Fendrich, C. (1988). *Halovibrio variabilis* gen. nov. sp. nov., *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid eubacterium from Great Salt Lake, Utah. **Syst. Appl. Microbiol. 11: 36-43.**

Fritze, D. (2002). *Bacillus* identification- traditional approaches. In R. Berkeley, M. Heyndrickx, N. Logan, and P. De Vos. (eds.). **Applications and Systematics of *Bacillus* and Relatives** (pp. 100-122). Oxford: Blackwell.

Furukawa, H. (2542). *การละลายของชั้นเกลือต่อลักษณะทางธรณีสัณฐานในภาคตะวันออกเฉียงเหนือ*. แปลโดย อรุณี ยูวะนิยม. *ใน กรมพัฒนาที่ดิน. **เอกสารคู่มือเจ้าหน้าที่ของรัฐเรื่องดินเค็ม*** *(นน. 177-181). กรุงเทพฯ: กระทรวงเกษตรและสหกรณ์.*

Galinski, E.A. (1993). Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. **Experientia**. 49: 487-496.

Galinski E.A. and Tindall, B.J. (1992). Biotechnological prospects for halophiles and halotolerant micro-organisms. In R.A. Herbert and R.J. Sharp (eds.). **Molecular Biology and Biotechnology of Extremophiles** (pp. 76-108). Glasgow: Blackie & Son.

Gauthier, M.J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P., and Bertrand, J.C. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. **Int. J. Syst. Bacteriol.** 42(4): 568-576.

Ghassemi F., Jakeman, A.J., and Nix, H.A (eds.). (1995). **Salanisation of Land and Water Resources.** Wallingford Oxon: CAB International.

Gilvanova, E.A., Paavilainen, S.K., Makela, M.J., Korpela, T.K. and Usanov, N.G. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Good, V.A. and Hartman, P.A. (1970). Properties of the amylase from *Halobacterium halobium*. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Goto, K., Omura, T., Hara, Y. and Sadaie, Y. (2000). Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. **J. Gen. Appl. Microbiol.** 46: 1-8.

Grant, W.D. (1991). General view of halophiles. Quoted in A. Ventosa, J.J. Nieto and A. Oren. (1998). Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev.** 62(2): 504-544.

Grant, W.D., Kamekura, M., McGenity, T.J., and Ventosa, A. (2001). Order I *Halobacteriales* Grant and Larsen 1989b,495vp. In D.R. Boone, R.W Calstenholz, and G.M. Garrity (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. I) (2nd ed., pp. 294-334). Berlin: Springer-Verlag.

Gruber, C., Radax, C. and Stan-Lotter, H. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Gupta, R., Lanter, J.M. and Woese, C.R. (1983). Sequence of the 16S ribosomal RNA from *Halobacterium volcanii*, an archaebacterium. **Science.** 221: 656-659.

Haack, S.K., Garchow, H., Odelson, D.A., Forney, L.J., and Klug, M.J. (1994). Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. **Appl. Environ. Microbiol.** 60: 2483-2493.

Hamada, T. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Hayes, V.E., Ternan, N.G., and McMullan, G. (2000). Organophosphonate metabolism by a moderately halophilic bacterial isolate. ***FEMS Microbiol. Lett****. 186(2): 171-175.*

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G., and Logan, N. A. (1998). *Virgibacillus* : a new genus to accommodate *Bacillus pantothenticus*. **Int. J. Syst. Bacteriol.** 48: 99-106.

Heyrman, J., Vanparys, B., Logan, N.A., Balcaen, A., Rodriguez-Diaz, M., Felske, A., and De Vos, P. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. **Int. J. Syst. Evol. Microbiol.** 54: 47-57.

Hiraishi,A., Narihiro,T., and Yamanaka,Y. (2003). Microbial community dynamics during start-up operation of flowerpot-using fed-batch reactors for composting of household biowaste. **Environ. Microbiol.** 5(9): 765-776.

Hochstein, L.I. (1988). The physiology and metabolism of the extremely halophilic bacteria. In F. **Rodriguez-Valera (ed.). Halophilic Bacteria (Vol. I) (pp. 68-79).** Boca Raton: CRC Press.

Huang, Z., Cui, Z., Ishii, M. and Igarashi, Y. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Huu, N.B., Denner, E.B., Ha, D.T., Wanner, G. and Stan-Lotter, H. (1999). *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. **Int. J. Syst. Bacteriol.** 49: 367-375.

Huval, J. H., Latta, R., Wallace, R., Kushner, D.J., and Vreeland, R.H. (1995). Description of two new species of *Halomonas*: *Halomonas israelensis* sp. nov. and *Halomonas canadensis* sp. nov. **Can. J. Microbiol**. 41: 1124-1131.

Ihara, K., Watanabe, S., and Tamura, T. (1997). ***Haloarcula argentinensis*** **sp. nov. and** ***Haloarcula mukohataei*** **sp. nov., two new extremely halophilic archaea collected in Argentina. Int. J. Syst. Bacteriol.** 47: 73-77.

Imhoff, J.F. (1993). Osmotic adaptation in halophilic and halotolerant microorganisms. In R.H. Vreeland, and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 211-212). Boca Raton: CRC Press.

Ince Yilmaz, E. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Ivanova1, E.P., Vysotskii, M.V., Svetashev, V.I., Nedashkovskaya1, O.I., Gorshkova1, N.M., Mikhailov, V.V., Yumoto, N., Shigeri, Y., Taguchi, T., and Yoshikawa, S. (1999). Characterization of *Bacillus* strains of marine origin. **Internatl. Microbiol.** 2: 267–271.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M., Ehrlich, D.S.D., Overbeek, R., and Kyrpides, N. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. **Nature.** 423 (6935): 87-91.

Javor, B (ed.). (1989). **Hypersaline environments: Microbiology and Biogeochemistry.** Berlin: Springer-Verlag.

Jones, B.E. and Grant, W.D. (1999). Microbial diversity and ecology of the Soda Lakes of East Africa. In C.R. Bell, M. Brylinsky and P. J-Green (eds). **Proceedings of the 8th International Symposium on Microbial Ecology**. Halifax: Atlantic Canada Society for Microbial Ecology.

Jukes, T. H. and Cantor, C. R. (1969). Evolution of protein molecules. In H. N. Munro (ed.). **Mammalian Protein Metabolism** (pp. 21–132). New York: Academic Press.

Kamekura, M. (1993). Lipids of extremely halophiles. In R.H. Vreeland and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (p. 135-139). Boca Raton: CRC Press.

Kamekura, M. (1998). Diversity of extremely halophilic bacteria. **Extremophiles**. 2(3): 289-295.

Kamekura M. and Onishi, H. (1974a). Halophilic nucleases from a moderately halophilic *Micrococcus varians*. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Kamekura M. and Onishi, H. (1974b). Protease formation by a moderately halophilic *Bacillus* strain. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Kamekura, M. and Seno,Y. (1990). A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. **Biochem. Cell. Biol.** 68(1): 352-359.

Kamekura, M. and Seno, Y. (1992). Nucleotide sequences of 16S rRNA encoding genes from halophilic archaea *Halococcus morrhuae* NRC 16008 and *Haloferax mediterranei* ATCC 33500. **Nucleic Acids Res.** 20 (13): 3517.

Kamekura, M. and Seno, Y. (1993). Partial sequence of the gene for a serine protease from a halophilic archaeum *Haloferax mediterranei* R4, and nucleotide sequences of 16S rRNA encoding genes from several halophilic archaea. **Experientia.** 49(6-7): 503-513.

Kaneda, T. (1991). Iso and anteiso fatty acids in bacteria: biosynthesis, function, and taxonomic significance. **Microbiol. Rev**. 55(2): 288-302.

Kaurichev, I.S. (1980). Practicas de Edafologia. Quoted in R.H. Vreeland, and L.I. Hochstein (eds.). (1993). **The Biology of Halophilic Bacteria** (p. 4). Boca Raton: CRC Press.

Kloepper, J.W. (1993). Plant growth-promoting rhizobacteria as biological control agents. In R.B. Metting (ed.). **Soil Microbial Ecology**: **Applications in Agricultural and Environmental Management** (pp. 255-274). New York: Marcel Dekker.

Kloos, W.E. and Schleifer, K.H. (1986). Genus IV *Staphylococcus*. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. II) (pp. 1013–1034). Baltimore: Williams & Wilkins.

Kong, Y., Zhao, J., and Shi, D. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Kuchta, T. and Russell, N.J. (1994). Glycinebetaine stimulates, but NaCl inhibits, fatty acid biosynthesis in the moderately halophilic eubacterium HX. **Arch. Microbiol.** 161: 234-238.

Kushner, D.J. (1988). What is the "true" internal environment of halophilic and other bacteria?. **Can. J. Microbiol**. 34: 482-486.

Kushner, D.J. (1993). Growth and nutrition of halophilic bacteria. In R.H. Vreeland and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 87-89). Boca Raton: CRC Press.

Kushner, D.J. and Kamekura, M. (1988). Physiology of halophilic eubacteria. In F. **Rodriguez-Valera (ed.). Halophilic Bacteria (Vol. I) (pp. 68-79).** Boca Raton: CRC Press.

Kuske, C.R., Ticknor, L.O., Miller, M.E., Dunbar, J.M., Davis, J.A., Barns, S.M., and Belnap, J. (2002). Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland.Rhizospheres of Three Plant Species and the Interspaces in an Arid Grassland. **Appl**. **Environ**. **Microbiol**. 68(4): 1854─1863.

LaMontagne, M.G., Michel, F.C.Jr., Holden, P.A., and Reddy, C.A. (2002). Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. **J**. **Microbiol**. **Methods**. 49: 255─264.

Landon, J.R. (1991). **Booker Tropical Soil Manual**. Essex: Longman.

Larsen, H. (1962). Halophilism. Quoted in R.H. Vreeland and L.I. Hochstein (eds.). (1993). **The Biology of Halophilic Bacteria** (p. 87). Boca Raton: CRC Press.

Larsen, H. (1980). Ecology of hypersaline environment. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Lawson, P.A., Deutch, C.E., and Collins, M.D. (1996). Phylogenetic characterization of a novel salt-tolerant Bacillus species: description of *Bacillus dipsosauri* sp. nov. **J. Appl. Bacteriol**. 81: 109-112.

Liesack, W. and Stackebrandt, E. (1991). Occurrence of a novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. **J. Bacteriol.** 174: 5072-5087.

Litchfield, C.D and Gillevet, P.M. (2002). Microbial diversity and complexity in hypersaline environments: a preliminary assessment. ***J. Ind. Microbiol. Biotechnol****. 28(1): 48-55.*

Lizama, C, Monteoliva-Sanchez M, Prado B, Ramos-Cormenzana A, Weckesser J, and Campos V. (2001). Taxonomic study of extreme halophilic archaea isolated from the "Salar de Atacama", Chile. **Syst. Appl. Microbiol.** 24(3): 464-74.

Lu, J., Nogi, Y., and Takami, H. (2001). *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. **FEMS Microbiol. Lett.** 205(2): 291-297.

Maas, E.V. (1990). Crop salt tolerance. In K.K. Tanji (ed.). ***Agricultural Salinity Assessment and Management*** *(pp. 262-304). New York: American Society of Civil Engineers.*

MacLean, I.A. and Carter, P.E. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Maier, R.M. and Pepper, I.L. (2000). Terrestrial environments. In R.M. Maier, I.L. Pepper, and C.P. Gerba (eds.). **Environmental Microbiology** (pp. 61-89). San Diego: Academic Press.

Makino, K., Koshikawa, T., Nishira, T.H., Ichikawa, T., and Kondo, M. (1981). Studies on protease from marine bacteria. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Marchant, R., Banat, I.M., Rahman, T.J., and Berzano, M. (2002). The frequency and characteristics of highly thermophilic bacteria in cool soil environments. **Environ. Microbiol.** 4 (10): 595-602.

Marsh, T. L., Saxman, P., Cole, J., and Tiedje, J. (2000). Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. **Appl**. **Environ**. **Microbiol**. 66(8): 3616–3620.

Martinez-Canovas, M.J., Llamas, I., Quesada, E. and Bejar, V. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Martinez-Murcia, A.J., Acinas, S.G., and Rodriguez-Valera, F. (1995). Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. **FEMS Microbiol**. **Ecol**. 17: 247-256.

McGenity, T.J. and Grant, W.D. (1993). The haloalkaliphilic archaeon (archaebacterium) *Natronococcus occultus* represents a distinct lineage within the *Halobacteriales*, most closely related to the other haloalkaliphilic lineage (*Natronobacterium*). **Syst. Appl. Microbiol.** 16: 239-243.

McKane, L. and Kandel, J (eds.). 1996. **Microbiology: Essentials and Applications**. New York: McGraw-Hill.

Meyer, J. (1989). Genus *Nocardiopsis* Meyer 1976, 487^{AL}. In S.T., Williams, M.E., Sharpe, and J.G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. IV) (pp. 2562-2565). Baltimore: Williams & Wilkins.

Michelle, M.L., Mary, A.V., and Tracey, A.S. (2003). **Preliminary Assessment of Microbial Communities and Biodegradation of Chlorinated Volatile Organic Compounds in Wetlands at Cluster 13, Lauderick Creek Area, Aberdeen Proving Ground, Maryland.** Maryland: the U.S. Army.

Miller, D. N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C. (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. **Appl. Environ. Microbiol.** 65(11): 4715-4724.

Mitsuchi, M., Wichaidit, P., and Jeungnijnirund, S. (1986). **Outline of soils of the northeast plateau, Thailand: their characteristics and constraints. Technical**

paper No. 1. Khon-Kaen: Agricultural Development Research Center in the northeast.

Moore, D.D. (1995). Preparation and analysis of DNA. In F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds). **Current Protocols in Molecular Biology (pp. 2-1 to 2-12).** New York: John Wiley.

Muscillo, M., Marianelli, C., De Carolis, E., Sali, M., Riccardi, C., Mansi, A., Papacchini, M., and La Rosa, G. T. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Nautiyal, C.S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., and Verma, D. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. **FEMS Microbol. Lett.** 182: 291-296.

Nazina, T.N., Turova, T.P., Poltaraus, A.B., Novikova, E.V., Ivanova, A.E., Grigor'ian, A.A., Lysenko, A.M., and Beliaev, S.S. (2000). Physiological and phylogenetic diversity of thermophilic spore-forming hydrocarbon-oxidizing bacteria from oil fields. **Mikrobiologia**. 69(1): 113-119.

New Zealand Dairy Research Institute. (1995). Identification of Lactobacilli. Starters and Microbiology Section, New Zealand Dairy Reasearch Institute. **Method BJC.1-Identification of Lactobacilli.** Issue 3, 12 October 1995: 1-18.

Nissenbaum, A. (1975). The microbiology and biogeochemistry of the Dead Sea. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp. No. 29.** 49: 290-300.

Noguchi, H., Uchino, M., Shida, O., Takano, K., Nakamura, L.K., and Komagata, K. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Norberg, P. and Hofsten, B.V. (1969). Proteolytic enzymes from extremely halophilic bacteria. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp No**. 49 (pp. 289-309). Cambridge: Elsevier.

Ogram, A. and Feng, X. (1997). Methods of soil microbial community analysis. In C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter. **Manual of Environmental Microbiology** (pp. 422-430). Washington D.C.: ASM Press.

Onishi, H. (1970). Halophilic amylase from a moderately halophilic *Micrococcus*. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). (1989). **FEMS Symp No. 49** (pp. 289-309). Cambridge: Elsevier.

Onishi, H., Mori, T., Takeuchi, S., Tani, K., Kobayashi, T., and Kamekura, M. (1983). Halophilic nucleases of a moderately halophilic *Bacillus* sp.; production, purification, and characterization. Quoted in M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). **FEMS Symp No. 49** (pp. 289-309). Cambridge: Elsevier.

Oren, A. (1983). *Clostridium lortetii* sp. nov. a halophilic obligatory anaerobic bacterium producing endospores with attached gas vacuoles. Arch. Microbiol. 136: 42-48.

Oren, A. (1993). Ecology of extremely halophilic microorganisms. In R. H. Vreeland and L. I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 25-53). Boca Raton: CRC Press.

Oren, A. (1999). Microbiology and biogeochemistry of halophilic microorganisms-an overview. In A. Oren (ed.). **Microbiology and Biogeochemistry of Hypersaline Environments** (pp. 1-9). Boca Raton: CRC Press.

Oren, A. (2002a). Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. **J**. **Ind**. **Microbiol**. **Biotechnol**. 28(1): 56-63.

Oren, A. (2002b). **Halophilic microorganisms and their environments**. Boston : Kluwer Academic.

Oren, A. (2002c). Molecular ecology of extremely halophilicarchaea and bacteria. **FEMS Microbiol. Ecol.** 39: 1-7.

Oren, A., Gurevich, P., Gemmell, R.T., and Teske, A. (1995). *Halobaculum gomorrense* gen. nov., sp. nov., a novel extremely halophilic archaeon from the Dead Sea. **Int. J. Syst. Bacteriol.** 45(4): 747-754.

Oren, A., Pohla, H., and Stackebrandt, E. (1987). Transfer of *Clostridium lortetii* comb. nov., and description of *Sporohalobacter marismortui* sp. nov. **Syst. Appl. Microbiol**. 9: 239-246.

Oren, A., Weisburg, A.W.G., Kessel, M., and Woese, C.R. (1984). *Halobacteroides halobius* gen. nov., sp. nov., a moderately halophilic anaerobic bacterium from the bottom sediments of the Dead Sea. **Syst. Appl. Microbiol**. 5: 58-70.

Pace, N. R., Stahl, D. A., Lane, D. J., and Olsen, G. J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. **Adv. Microb. Ecol.** 9: 1-55.

Palleroni, N.J. (1984). Genus I *Pseudomonas* Migula 1894, 237^{AL}. In N.R. Krieg and J.G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. I) (pp. 142-175). Baltomore: Williams & Wilkins.

Pannucci, J., Okinaka, R.T., Sabin, R., and Kuske, C.R. (2002). *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. **J. Bacteriol.** 184(1): 134-141.

Poolman, B. Blount, P. Folgering, J.H.A., Friesen, R.H.E. Moe, P.C., and Heide, T. van der. (2002). How do membrane proteins sense water stress?. **Molec. Microbiol.** 44(4): 889–902.

Post, F.J. (1977). The microbial ecology of the Great Salt Lake. Quoted in J. Barbara (ed.). (1989). **Hypersaline Environments: Microbiology and Biogeochemistry** (p. 273). Berlin: Springer-Verlag.

Priest, F. and Austin, B. (1993). **Modern Bacterial Taxonomy** (2nd ed.). London: Chapman & Hall.

Quesada, E., Ventosa, A., Rodriguez-Valera, F., and Ramos-Cormenzana, A. (1982). Types and properties of some bacteria isolated from hypersaline soils. **J. Appl. Microbiol.** 53: 155-161.

Quesada, E., Ventosa, A., Rodriguez-Valera, F., Magias, L., **and** Ramos-Cormenzana, A. (1983). Numerical taxonomy of moderately halophilic Gram negative bacteria from hypersaline soils. **J. Gen. Microbiol.** 129: 2649-2657.

Ramos-Cormenzana, A. (1989). Ecological distribution and biotechnological potential of halophilic microorganisms. In M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). **FEMS Symp. No. 49** (pp. 289-309). Cambridge: Elsevier.

Ramos-Cormenzana, A. (1990). Biotechnology applications of the halophilic bacteria. **Eur. Congr. Biotechnol.** 5: 600-603.

Ramos-Cormenzana, A. (1993). Ecology of moderately halophilic bacteria. In R.H. Vreeland and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 55-86). Boca Raton: CRC Press.

Reynolds, K.A. and Pepper, I.L. (2000). Microorganisms in the environment. In R.M. Maier, I.L. Pepper, and C.P. Gerba (eds.). **Environmental Microbiology** (pp. 7-41). San Diego: Academic Press.

Rimwanich, S. and Suebsiri, B. (1984). Nature and management of problem soils in Thailand. Quoted in D.L. Spark. (1995). **Environmental Soil Chemistry** (p. 230). California: Academic Press.

Robert, M.F. (2000). Osmoadaptation and osmoregulation in archaea. **Frontiers in Bioscience**. 5: 796-812.

Rodriguez-Valera, F. (1988). Characteristics and microbial ecology of hypersaline environments. In F. Rodriguez-Valera (ed.). **Halophilic Bacteria** (Vol. I) (pp. 3-30). Boca Raton: CRC Press.

Rodriguez-Valera, F. (1993). Introduction to saline environments. In R.H. Vreeland and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 1-12). Boca Raton: CRC Press.

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

Russell, N.J. (1989). Adaptive modifications in membranes of halotolerant and halophilic microorganisms. **J**. **Bioenerg**. **Biomembr**. 21(1): 93-113.

Rosenberg, A. (1983). Pseudomonas halodurans sp. nov., a halotolerant bacterium. **Arch. Microbiol.** 136: 117-123.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. **Molec. Biol. Evol.** 4: 406-425.

Sasser, M. (1990). **Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids.** Newark: MIDI.

Schleifer, K.H. (1986). Family I Micrococcaceae Prevot 1961, 31^{AL}. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. II) (pp.995–1010). Baltimore: Williams & Wilkins.

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

Smibert, R.M. and Krieg, N.R. (1994). Phenotypic characterization. In P., Gerhardt, R.G. E., Murray, W.A., Wood, and N.R., Krieg (eds.). **Manual of Methods for General Bacteriology.** Washington, D.C.: American Society for Microbiology.

Soil Survey Division Staff. (1993**). Soil Survey Manual. U.S. Department of Agriculture Handbook 18.** Washington, D.C.: Government Printing Office, *Soil Conservation Service, U.S. Department of Agriculture.*

Spark, D.L. (1995). **Environmental Soil Chemistry**. California: Academic Press.

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

Stackebrandt, E., and Göbel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and t_{65} rRNA sequence analysis in the present species definition in bacteriology. **Int**. **J**. **Syst**. **Bacteriol**. 44: 846–849.

Stackebrandt, E. and Goodfellow, M. (1991). Introduction. In Stackebrandt, E. and Goodfellow, M. (eds.). **Nucleic Acid in Bacterial Systematics** (pp. x-xxix). New York: John Willey & Sons.

Stackebrandt, E. and Swiderski, J. (2002). From phylogeny to systematics: the dissection of the genus *Bacillus*. In R. Berkeley, M. Heyndrickx, N. Logan, and P. De Vos (eds.). **Applications and Systematics of *Bacillus* and Relatives** (pp. 8-22). Oxford: Blackwell.

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

Subbarao, G.V. and Johansen, C. (1999). Strategies and scope for improving salinity tolerance in crop plants. In M. Pessarakli (ed.). **Handbook of Plant and Crop Stress** (pp. 1069-1088). New York: Marcel Dekker.

Swiderski, J. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Takahashi,T., Kaneko, M., Mori, Y., Kikuchi, N., and Hiramune, T. (2003). **Entrez Nucleotide** [On-line]. Available: http://www.ncbi.nlm.nih.gov/

Tasker, R. (1990). Salt in the wound. Quoted in F. Ghassemi, A.J. Jakeman, and H.A. Nix (eds.). (1995). **Salanization of Land and Water Resources** (p. 453). Wallingford Oxon: CAB International.

Tate, R.L. (2000). **Soil Microbiology** (2nd ed.). New York: John Wiley.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. **Nucleic Acids Res**. 22: 4673-4680.

Thongthai, C. and Suntinanalert, P. (1991). Halophiles in Thai fish sauce (Nam Pla), In **General and Applied Aspects of Halophilic Microorganisms. Proceeding of The FEMS-NATO Advanced Research Workshop** (pp. 381-387). New York: Plenum Press.

Tiedje, J. M., Asuming-Brempong, S., Nűsslein, K., Marsh, T. L., and Flynn, S. J. (1999). Opening the black box of soil microbial diversity. **Appl**. **Soil Ecol**. 13: 109–122.

Tsai, Y.L. and Olson, B.H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. ***Appl. Environ. Microbiol***. 58(7): 2292-2295.

Valderrama, M.J., Monteoliva-Sanchez, M., Quesada, E., and Ramos-Cormenzana, A. (1998). Influence of salt concentration on the cellular fatty acid composition of the moderately halophilic bacterium *Halomonas salina*. **Res Microbiol.** 149(9): 675-9.

Van de Peer, Y. and De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. **Comput. Applic. Biosci.** 10: 569-570.

Venkateswaran, K., Hattori, N., La Duc, M.T., and Kern, R. (2003). ATP as a biomarker of viable microorganisms in clean-room facilities. **J. Microbiol. Methods.** 52 (3): 367-377.

Ventosa, A. (1989). Taxonomy of halophilic bacteria. In M.S. Da Costa, J.C. Duarte, and R.A.D. Williams (eds.). **FEMS Symp. No. 49** (pp. 269-279). Cambridge: Elsevier.

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

Ventosa, A., Gutierrez, M.C., Kamekura, M., and Dyall-Smith, M.L. (1999). Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to *Haloterrigena turkmenica* gen. nov., comb. Nov. **Int. J. Syst. Bacteriol.** 49: 131-136.

Ventosa, A., Marquez, M.C., Garabito, M.J., and Arahal, D.R. (1998a). Moderately halophilic gram-positive bacterial diversity in hypersaline environments. **Extremophiles.** 2(3): 297-304.

Ventosa, A. and Nieto, J.J. (1995). Biotechnological applications and potentialities of halophilic microorganisms. **World J. Microbiol. Biotechnol.** 11: 85-94.

Ventosa, A., Nieto, J.J., and Oren, A. (1998b). Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev.** 62(2): 504-544.

Ventosa, A., **Quesada, E., Rodriguez-Valera, F.,** Ruiz-Berraquero, F., **and** Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram negative rods. **J. Gen. Microbiol.** 128: 1959-1968.

Vilhelmsson, O., Hafsteinsson, H., and Kristjánsson, J.K. (1996). Isolation and characterization of moderately halophilic bacteria from fully cured salted cod (bachalao). **J. Appl. Bacteriol.** 81: 95-103.

Villar, M., De Ruiz Holgado, A.P., Sanchez, J.J., Trucco, R.E., and Oliver, G. (1985). Isolation and characterization of *Pediococcus halophilus* from salted anchovies (*Engraulis anchoita*). **Appl. Environ. Microbiol.** 49: 664-666.

Volcani, B.E. (1944). The microorganisms of the Dead Sea. Quoted in A. Ventosa, J.J. Nieto and A. Oren. (1998). Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev.** 62(2): 504-544.

Vreeland, R.H. (1984). Genus Halomonas Vreeland, Litchfield, Martin, and Elliot 1980, 494^{VP}. In N.R. Krieg and J.G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology** (Vol. I) (pp. 341-343). Baltomore: Williams & Wilkins.

Vreeland, R.H. (1993a). Biology of halophilic bacteria, Part I. Research priorities and biotechnological potential for the 1990s. **Experientia.** 49(6): 471-472.

Vreeland, R.H. (1993b). Taxonomy of halophilic bacteria. In R.H. Vreeland and L.I. Hochstein (eds.). **The Biology of Halophilic Bacteria** (pp. 105-134). Boca Raton: CRC Press.

Waino, M., Tindall, B.J., and Ingvorsen, K. (2000). *Halorhabdus utahensis* gen. nov., sp. nov., an aerobic, extremely halophilic member of the archaea from Great Salt Lake, Utah. **Int. J. Syst. Evol. Microbiol.** 50: 183-190.

Waisel, Y (ed.). (1972). **Biology of Halophytes**. New York: Academic Press.

Watson, R.J. and Blackwell, B. (2000). Purification and characterization of a common soil component which inhibits the polymerase chain reaction. **Can. J. Microbiol**. 46: 633-642.

Wayne, L.G., Brenner, D.J., Colwell, R.R. , Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P.,

and **Trüper**, H.G. (1987). Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. **Int. J. Syst. Bacteriol.** 37: 463–464.

Weisser, J., and Trüper, H. G. (1985). Osmoregulation in a new haloalkaliphilic *Bacillus* from the Wadi Natrun (Egypt). **Syst. Appl. Microbiol.** 6: 7-11.

Wiley, E.O. (1978). **Phylogenetics: The Theory and Practice of Phylogenetic Systematics**. New York: John Wiley.

Woese, C.R. (1987). Bacterial evolution. **Microbiol. Rev.** 51: 221-271.

Wohlfarth, A., Severin, J., and Galinski, E. A. (1989). Screening for compatible solutes of halophilic bacteria. **FEMS Symp. No. 49** (p.421). Cambridge: Elsevier.

Wood, G.L. and Washington, J.A. (1995). Antibacterial susceptibility tests: dilution and disk diffusion methods. In P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, and R.H. Yolken (eds.). **Manual of Clinical Microbiology** (6th ed., pp. 1327-1336). Washington: ASM Press.

Wood, J.M. (1999). Osmosensing by bacteria: signal and membrane-based sensors. **Microbiol. Mol. Biol. Rev.** 63(1): 230-262.

Woolard, C.R. and Irvine, R.L. (1992). Biological treatment of hypersaline wastewater by a biofilm of halophilic bacteria. Quoted in A. Ventosa, J.J. Nieto, and A. Oren, (1998). Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev.** 62(2): 504-544.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero, G.N. (1982). Living with water stress: evolution of osmolyte systems. **Science.** 217: 1214-1222.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., and Veal, D.A. (1998). Methods for microbial DNA extraction from soil for PCR amplification. **Biological Procedures Online**. 1: 40–47.

Yoon, J.H., Choi, S.H., Lee, K-C., Kho, Y. H., Kang, K.H., and Park, Y.H. (2001). *Halomonas marisflavi* sp. nov., a halophilic bacterium isolated from the Yellow Sea in Korea. **Int. J. Syst. Evol. Microbiol.** 51: 1171-1177.

Yoon, J.H., Kang, K.H., and Park, Y.H. (2003a). *Halobacillus salinus* sp. nov., isolated from a salt lake on the coast of the East Sea in Korea. **Int. J. Syst. Evol. Microbiol.** 53: 687-693.

Yoon, J.H., Kim, I.G., Kang, K.H., Oh, T.K., and Park, Y.H. (2003b). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. **Int. J. Syst. Evol. Microbiol.** 53: 1297-1303.

Yoon, J.H., Lee, K-C., Weiss, N., Kang, K.H., and Park, Y.H. (2003c). *Jeotgalicoccus halotolerans* gen. nov., sp. nov. and *Jeotgalicoccus psychrophilus* sp. nov., isolated from the traditional Korean fermented seafood jeotgal. **Int. J. Syst. Evol. Microbiol.** 53: 595-602.

Zahran, H.H., Moharram, A.M., and Mohammad, H.A. (1992). Some ecological and physiological studies on bacteria isolated from salt-affected soils of Egypt**. J. Basic Microbiol.** 32(6): 405-413.

Zeikus, J.G., Hegge, P.W., Thomson, T.E., and langworthy, T.A. (1983). Isolation and description of *Haloanaerobium praevalens* gen. nov., and sp. nov., and obligately

anaerobic halophile common to Great Salt Lake sediments. **Curr. Microbiol.** 9: 225-234.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. **Appl Environ Microbiol**. 62(2): 316–322.

Zinder, S.H. (1993). Physiological Ecology of Methanogens. In J.G. Ferry (ed.). **Methanogens: Ecology, Physiology, Biochemistry and Genetics** (pp. 115-120). London: Chapman & Hall.

APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENT PREPARATIONS

1. Culture media for growth of halophilic bacteria

The medium was autoclaved for 15 minutes at 15 psi pressure at 121°C unless stated. The culture media were prepared as described by Atlas (1997a).

1.1 Halobacteria medium

Composition per liter:

Agar	15.0-10.0 g
$MgSO_4 \cdot 7H2O$	10.0 g
Casein hydrolysate	5.0 g
KCl	5.0 g
Disodium citrate	3.0 g
KNO_3	1.0 g
Yeast extract	1.0 g
$CaCl_2 \cdot 6H_2O$	0.2 g

pH 7.2 – 7.4 at 25°C

Preparation of medium: 30.0, 80.0, and 250.0 g of NaCl were supplemented for the cultivation of slightly-, moderately-, and extremely halophilic bacteria. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. The halobacteria broth was prepared by using the same method for preparation of halobacteria medium without adding the agar.

1.2 Halobacterium medium (ATCC medium 213)

Composition per liter:

NaCl	250.0 g
$MgSO_4 \cdot 7H_2O$	10.0 g
KCl	5.0 g
Tryptone	2.5 g
Yeast extract	10.0 g
$CaCl_2 \cdot 6H_2O$	0.2 g
Agar	20.0 g

pH 7.2 – 7.4 at 25°C

Preparation of medium: All components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the cultivation and maintenance of *Halococcus saccharolyticus* ATCC 49257.

1.3 Halophiles moderate medium (Ventosa *et al*., 1982)

Composition per liter:

Sodium chloride	178.0 g
Agar	24.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Sodium bromide	0.23 g
Sodium bicarbonate	0.06 g
Potassium chloride	2.0 g
Ferric chloride	trace
Yeast extract	10.0 g

Glucose	1.0 g
Proteose peptone no. 3	5.0 g
$CaCl_2 \cdot 2H_2O$	0.36 g

pH 7.2 ± 0.2 at 25°C

Preparation of medium: All components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the re-cultivation of moderately halophilic bacterial isolates.

1.4 Lead acetate agar

Composition per liter:

Proteose peptone	5.0 g
Peptone	15.0 g
Glucose	1.0 g
Lead acetate	0.2 g
Sodium thiosulfate	0.08 g
Agar	15.0 g

pH 6.6 ± 0.2 at 25°C

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. After autoclaving, the tubes were allowed to cool in a slanted position. This medium was used for the observation of hydrogen sulfide production.

1.5 Moderate halophilic medium (ATCC medium 1708)

Composition per liter:

NaCl	81.0 g
$MgCl_2 \cdot 6H_2O$	7.0 g
$MgSO_4 \cdot 7H_2O$	9.6 g
$CaCl_2 \cdot 2H_2O$	0.36 g
KCl	2.0 g
$NaHCO_3$	60.0 mg
NaBr	26.0 mg
Proteose Peptone No. 3	5.0 g
Yeast extract	10.0 g
Glucose	1.0 g

pH 7.1 ± 0.1 at 25°C

Preparation of medium: All components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the cultivation and maintenance of *Bacillus halophilus* ATCC 49085 and *Salinicoccus hispanicus* ATCC 49259.

1.6 Motility test medium

Composition per liter:

Tryptone	10.0 g
Agar	5.0 g

pH 7.2 ± 0.2 at 25°C.

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the observation of motility.

1.7 MRVP medium (Methyl Red Voges-Proskauer medium)

Composition per liter:

Peptone	5.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g

pH 7.5 ± 0.2 at 25°C.

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the differentiation of bacteria based on acid production (Methyl Red test) and acetoin production (Voges-Proskauer reaction).

1.8 Mueller-Hinton II agar

Composition per liter:

Casein hydrolysate	17.5 g
Beef extract	2.0 g
Starch	1.5 g
Agar	17.0 g

pH 7.3 ± 0.1 at 25°C.

Preparation of medium: 200.0 g of NaCl was supplemented to obtain 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for antimicrobial disc diffusion susceptibility testing by the Bauer-Kirby method of extremely halophilic bacterial isolates.

1.9 Nitrate reduction broth

Composition per liter:

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g

pH 6.9 ± 0.2 at 25°C.

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the differentiation of bacteria based on their ability to reduce nitrate to nitrite or form nitrogen gas.

1.10Nutrient gelatin

Composition per liter:

Gelatin	120.0 g
Beef extract	3.0 g
Peptone	5.0 g

pH 6.8 ± 0.2 at 25°C.

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all

components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the differentiation of bacteria based on their ability to liquefy gelatin.

1.11Phenol red broth

Composition per liter:

Proteose peptone	10.0 g
Phenol red	0.018 g

pH 7.4 ± 0.2 at 25°C.

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved for 15 minutes at 13 psi pressure at 118°C. This medium was used as a broth base for the differentiation of bacteria based on their ability to ferment carbohydrate.

1.12Starch hydrolysis agar

Composition per liter:

Soluble starch	20.0 g
Beef extract	3.0 g
Tryptose	10.0 g
Agar	15.0 g

pH 7.4 ± 0.2 at 25°C

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. This medium was used for the differentiation of bacteria based on amylase production.

1.13Tryptic soy agar (TSA)

Composition per liter:

Tryptone	17.0 g
Peptic digest of soybean meal	3.0 g
Dextrose	2.5 g
Disodium hydrogen phosphate	2.5 g

pH 7.3 ± 0.2 at 25°C

Preparation of medium: 50.0, 70.0, 80.0, and 150.0 g of NaCl were supplemented to obtain 5%, 7%, 8%, and 15% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved.

1.14Trypticase soy broth agar (TSBA)

Composition per liter:

Trypticase soy broth	30.0 g
Agar	15.0 g

pH 7.3 ± 0.2 at 25°C

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components

were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved.

1.15Tryptone broth

Composition per liter:

Tryptone	10.0 g

pH 7.5 ± 0.2 at 25°C

Preparation of medium: 30.0, 80.0, and 200.0 g of NaCl were supplemented to obtain 3%, 8%, and 20% NaCl concentrations. Then all components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved.

1.16Van Neil's yeast medium (ATCC medium 217)

Composition per liter:

NaCl	250.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Yeast extract	0.0 g
Agar	20.0 g

pH 7.0 – 7.2 at 25°C

Preparation of medium: All components were added to distilled water and brought volume up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved. Mixed thoroughly and gently heated until dissolved. This medium was used for the cultivation and maintenance of *Halobacterium salinarum* ATCC 33171.

2. Reagents for conventional method of bacterial characterization

The reagents for conventional method of bacterial characterization were prepared as described by Cappuccino and Sherman (1999).

2.1 1% dimethyl-p-phenylenediamine dihydrochloride

Dimethyl-p-phenylenediamine dihydrochloride 1.0 g was dissolved in 100.0 mL. This reagent was kept in the refrigerator.

2.2 Gram staining reagents

2.2.1 Ammonium oxalate crystal violet

Crystal violet 2.0 g was dissolved in 20.0 mL of ethanol 95%. Then, the mixture was transferred to 80.0 mL of 1% ammonium oxalate solution and mixed throughly.

2.2.2 Safranin O

Safranin O 0.25 g was dissolved in 10.0 mL of 95% ethanol. Then the mixture was diluted with 90.0 mL of distilled water

2.2.3 Iodine solution

Iodine 2.0 g and potassium iodide 4.0 g were dissolved in distilled water and made up volume to 600.0 mL. This solution was kept in the dark place.

2.3 3% hydrogen peroxide diluted

The 40% H_2O_2 7.5 mL was diluted with distilled water to 100.0 mL.

2.4 Kovacs' reagent

The *p*-dimethylaminobenzaldehyde 5.0 g was dissolved in 75.0 mL of amyl alcohol at 50°C. The mixture was added to 25.0 mL of concentrated hydrochloric acid. This reagent was kept in the dark bottle and stored in a refrigerator.

2.5 McFarland turbidity standards

1% solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 were prepared and mixed together in various proportions as detailed in Table 1A to form a turbid suspension.

Table 1A. Preparation of McFarland turbidity standards (Cappuccino and Sherman, 1999).

McFarland scale number	Amount of 1% $BaCl_2$ (mL)	Amount of 1% H_2SO_4 (mL)	Corresponding approximate density of bacteria (million/mL)
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1,200
5	0.5	9.5	1,500
6	0.6	9.4	1,800
7	0.7	9.3	2,100
8	0.8	9.2	2,400
9	0.9	9.1	2,700
10	1.0	9.0	3,000

2.6 Methyl red solution

Methyl red 0.1 g was dissolved in 300.0 mL of 95% ethanol and made up volume to 500.0 mL with distilled water.

2.7 Nitrate test reagents

Nitrate test reagents composed of solution A and B. For preparation of solution A, sulfanilic acid 0.8 g was dissolved in 1000.0 mL of 5 N acetic acid (1 part of glacial acetic acid in 2.5 parts of distilled water). For preparation of solution B, dimethyl-α-napthylamine 5.0 g was dissolved in 1000.0 mL of 5 N acetic acid.

2.8 Voges-Proskauer test solution

This solution was composed of two solution: solution A and B. Solution A was prepared by diluting 10.0 g of alpha-napthol in 100.0 mL of 95% ethanol. Solution B was prepared by diluting 20.0 g of KOH in 100.0 mL of distilled water.

3. Reagents for fatty acid analysis

3.1 Reagent 1: 45 g of sodium hydroxide was added to 150 mL of methanol and 150 mL distilled water in the fume hood.

3.2 Reagent 2: 325 mL of 6.0 N hydrochloric acid was gently added to 275 mL of methanol in the fume hood.

3.3 Reagent 3: 200 mL of hexane was gently added to 200 mL of methyl-tert-butyl ether in the fume hood.

3.4 Reagent 4: Dissolved 10.8 g of sodium hydroxide in 900 mL of distilled water.

4. Reagents for nucleic acid analysis

4.1 Loading buffer: 0.25% bromphenol blue, 0.25% Xylene cyanol FF and 40% (w/v) sucrose was diluted in MilliQ water.

4.2 Lysis buffer: 50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA

4.3 Phenol, TE saturated

Dissolved phenol was transferred to 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8

4.5 TE buffer: Ten mL of 1 M Tris-HCl, pH 8.0 was mixed with 0.2 mL of 0.5 M EDTA, pH 8.0 and made up volume to 1.0 L.

APPENDIX B

EXAMPLES OF FATTY ACID COMPOSITION REPORT AND FATTY ACID CHROMATOGRAM

Sherlock Version: 3.10 DATA:E03728489A 28-JUL-03 12:08:27

ID: 1 QC-CAL-MIX(300110 Date of run: 28-JUL-03 11:44:10
Bottle: 1 CALIBRATION [TSBA40]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.656	404022672	0.026	. . .	7.000	SOLVENT PEAK	. . .	< min rt	
2.588	50636	0.028	1.280	9.000	9:0	5.09		
3.055	108252	0.028	1.195	10.000	10:0	10.16	Peak match -0.0007	
3.699	57774	0.029	1.123	11.000	11:0	5.10	Peak match 0.0020	
3.832	23853	0.030	1.113	11.155	10:0 2OH	2.09	Peak match -0.0061	
4.060	11979	0.031	1.097	11.419	10:0 3OH	1.03	Peak match 0.0046	
4.559	122035	0.031	1.064	12.000	12:0	10.20	Peak match -0.0009	
5.644	63720	0.034	1.017	13.000	13:0	5.09	Peak match 0.0016	
6.948	131967	0.038	0.980	14.000	14:0	10.16	Peak match -0.0005	
8.424	68129	0.040	0.952	15.000	15:0	5.10	Peak match -0.0010	
8.747	28809	0.043	0.948	15.200	14:0 2OH	2.14	Peak match 0.0027	
9.204	14525	0.044	0.942	15.484	Sum In Feature 2 . .	1.07	Peak match 0.0028	14:0 3OH/16:1 ISO I
10.036	137915	0.043	0.933	16.000	16:0	10.10	Peak match -0.0020	
11.721	70448	0.044	0.920	17.000	17:0	5.09	Peak match -0.0011	
12.117	29304	0.046	0.918	17.230	16:0 2OH	2.11	Peak match 0.0027	
13.443	142318	0.047	0.913	18.000	18:0	10.21	Peak match -0.0016	
15.159	71514	0.046	0.911	19.000	19:0	5.12	Peak match 0.0017	
16.862	141423	0.048	0.912	20.000	20:0	10.13		
*******	14525	. . .	. . .	. . .	SUMMED FEATURE 2 . .	1.07	12:0 ALDE ?	unknown 10.928
*******	. . . . .	. . .	. . .	. . .	. . . . .	. . .	16:1 ISO I/14:0 3OH	14:0 3OH/16:1 ISO I

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
404022672	1274602	1274602	100.00	1273143	0	. . . . .	. . . . .

GOOD PEAK MATCHING: PEAK POSITION MATCHING ERROR (RMS) IS 0.0026.

TSBA40 [Rev 4.10] MIDI Calibration Mix 1 . . . . . 0.999
CLIN40 [Rev 4.0] MIDI Calibration Mix 1 . . . . . 0.999

Figure 1B. Fatty acid composition report of calibration mixture analyzed by the MIDI system.

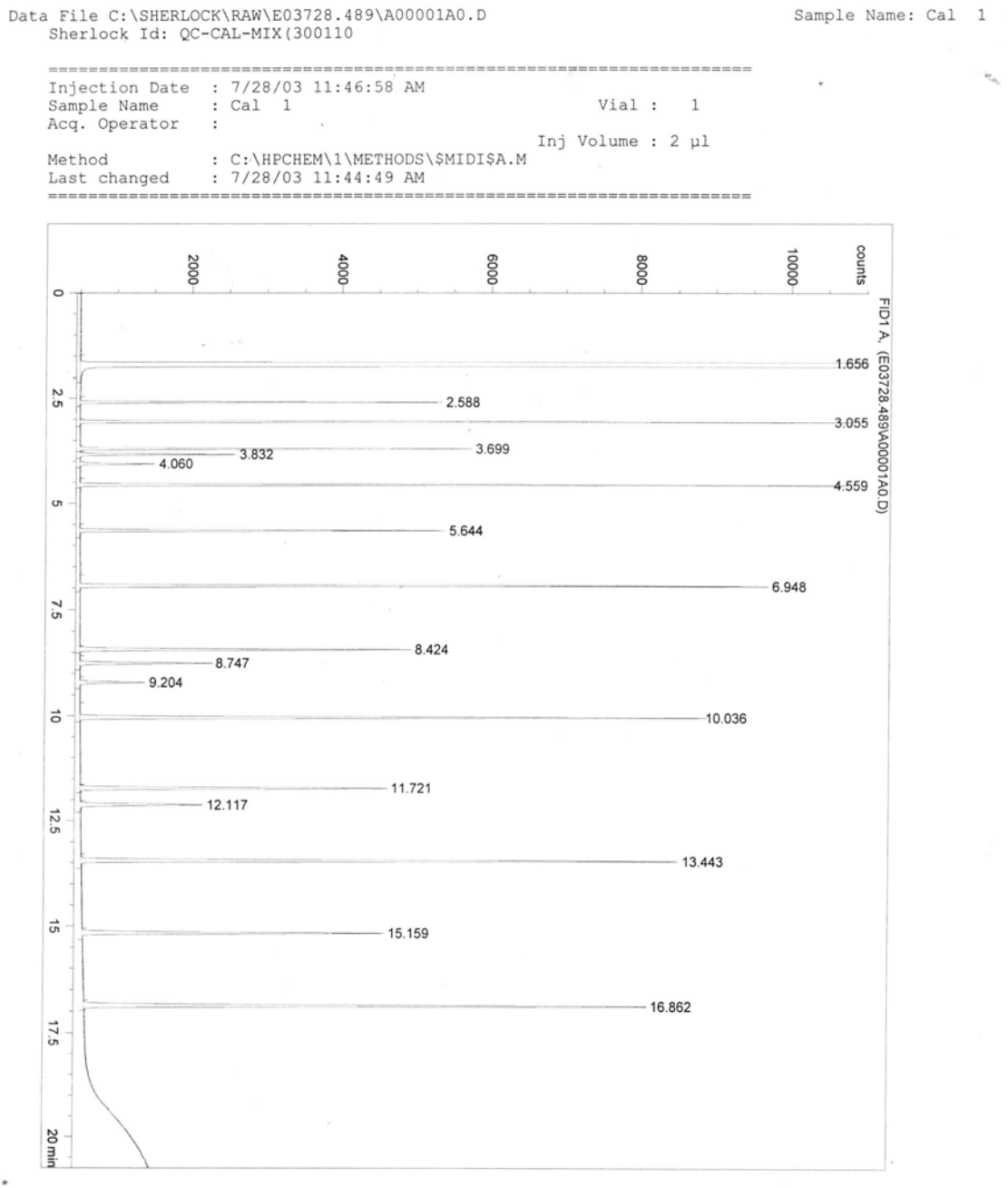


Figure 2B Fatty acid chromatogram of calibration mixture analyzed by the MIDI system.

Sherlock Version: 3.10 DATA:E03725524A 25-JUL-03 15:46:58

ID: 4739 QC-STEN-MALTO Date of run: 25-JUL-03 14:52:17
Bottle: 3 SAMPLE [TSBA40]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.650	396989112	0.034	. . .	7.001	SOLVENT PEAK	. . .	< min rt	
1.924	3429	0.079	. . .	7.587	. . . . .	. . .	< min rt	
2.022	2611	0.071	. . .	7.795	. . . . .	. . .	< min rt	
2.275	2215	0.037	. . .	8.341	. . . . .	. . .	< min rt	
3.052	2922	0.029	1.193	10.000	10:0	0.70	ECL deviates 0.000	Reference -0.009
3.443	15646	0.029	1.156	10.606	11:0 ISO	3.65	ECL deviates 0.000	Reference -0.008
3.832	738	0.041	1.124	11.157	10:0 2OH	0.17	ECL deviates 0.004	
4.061	1050	0.034	1.109	11.422	10:0 3OH	0.24	ECL deviates -0.000	
4.384	7001	0.032	1.090	11.797	unknown 11.799 . . .	1.54	ECL deviates -0.002	
4.533	1257	0.042	. . .	11.969	. . . . .	. . .		
4.654	7995	0.033	1.075	12.089	11:0 ISO 3OH	1.73	ECL deviates -0.000	
5.224	3005	0.040	1.050	12.612	13:0 ISO	0.64	ECL deviates -0.002	Reference -0.009
6.236	14875	0.038	1.015	13.453	12:0 3OH	3.05	ECL deviates -0.001	
6.451	2371	0.038	1.009	13.617	14:0 ISO	0.48	ECL deviates -0.002	Reference -0.009
6.824	570	0.038	0.998	13.903	14:1 w5c	0.11	ECL deviates 0.002	
6.950	21074	0.038	0.995	13.999	14:0	4.23	ECL deviates -0.001	Reference -0.008
7.109	17501	0.039	0.991	14.108	13:0 ISO 3OH	3.50	ECL deviates -0.001	
7.234	1136	0.045	0.988	14.192	13:0 2OH	0.23	ECL deviates -0.002	
7.561	5192	0.040	0.980	14.413	15:1 ISO F	1.03	ECL deviates -0.002	
7.877	193756	0.039	0.974	14.627	15:0 ISO	38.07	ECL deviates 0.004	Reference -0.004
8.005	28426	0.040	0.971	14.713	15:0 ANTEISO	5.57	ECL deviates 0.000	Reference -0.008
8.430	1836	0.042	0.962	15.000	15:0	0.36	ECL deviates 0.000	Reference -0.008
9.440	3319	0.046	0.946	15.627	16:0 ISO	0.63	ECL deviates 0.000	Reference -0.008
9.673	16967	0.042	0.942	15.772	16:1 w9c	3.23	ECL deviates -0.002	
9.747	66546	0.044	0.941	15.817	Sum In Feature 3 . .	12.64	ECL deviates -0.005	16:1 w7c/15 iso 2OH
10.040	36877	0.044	0.937	15.999	16:0	6.97	ECL deviates -0.001	Reference -0.009
10.741	21907	0.044	0.928	16.416	ISO 17:1 w9c	4.10	ECL deviates -0.000	
11.104	16335	0.044	0.924	16.631	17:0 ISO	3.05	ECL deviates 0.001	Reference -0.008
11.258	825	0.046	0.922	16.723	17:0 ANTEISO	0.15	ECL deviates -0.000	Reference -0.009
11.380	685	0.042	0.921	16.795	17:1 w8c	0.13	ECL deviates 0.003	
12.961	1893	0.046	0.907	17.717	Sum In Feature 5 . .	0.35	ECL deviates -0.003	18:2 w6,9c/18:0 ANTE
13.048	9617	0.046	0.907	17.768	18:1 w9c	1.76	ECL deviates -0.001	
13.139	5014	0.047	0.906	17.821	18:1 w7c	0.92	ECL deviates -0.002	
13.446	2557	0.050	0.904	17.999	18:0	0.47	ECL deviates -0.001	Reference -0.011
14.167	2437	0.054	. . .	18.420	. . . . .	. . .		
14.535	1782	0.047	0.899	18.634	19:0 ISO	0.32	ECL deviates 0.000	Reference -0.011
18.840	672	0.058	. . .	21.155	. . . . .	. . .	> max rt	
*******	66546	. . .	. . .	. . .	SUMMED FEATURE 3 . .	12.64	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c
*******	1893	. . .	. . .	. . .	SUMMED FEATURE 5 . .	0.35	18:2 w6,9c/18:0 ANTE	18:0 ANTE/18:2 w6,9c

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
396989112	513113	509419	99.28	495556	14	0.002	0.009

TSBA40 [Rev 4.10] Stenotrophomonas . . . . . 0.905 (Xanthomonas, Pseudomonas)
S. maltophilia* . . . . . 0.905 (Xanthomonas, Pseudomonas)
CLIN40 [Rev 4.0] Stenotrophomonas . . . . . 0.503 (Xanthomonas, Pseudomonas)
S. maltophilia . . . . . 0.503 (Xanthomonas, Pseudomonas)

Figure 3B. Fatty acid composition report of *Stenotrophomonas* (*Xanthomonas*) *maltophilia* analyzed by the MIDI system.

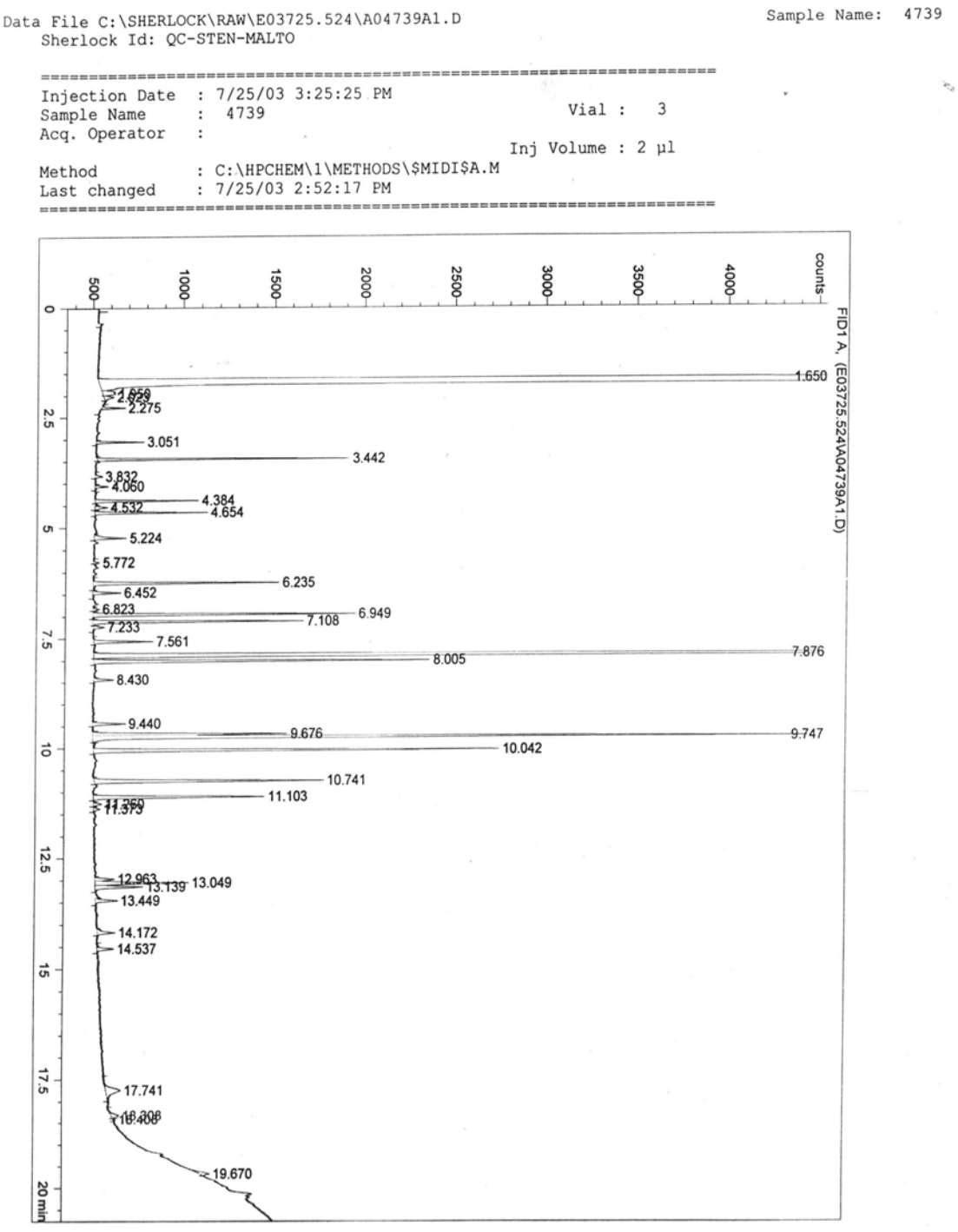


Figure 4B. Fatty acid chromatogram of *Stenotrophomonas* (*Xanthomonas*) *maltophilia* (positive control) analyzed by the MIDI system.

Sherlock Version: 3.10 DATA:E03725524A 25-JUL-03 14:52:17

ID: 4738 QC-REAGENT-BLANK Date of run: 25-JUL-03 14:29:05
Bottle: 2 SAMPLE [TSBA40]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
0.415	2392	0.010	. . .	4.353	. . . . .	. . .	< min rt	
0.498	4993	0.134	. . .	4.530	. . . . .	. . .	< min rt	
0.697	5641	0.153	. . .	4.956	. . . . .	. . .	< min rt	
1.655	367508880	0.033	. . .	7.003	SOLVENT PEAK	. . .	< min rt	
1.832	2064	0.038	. . .	7.383	. . . . .	. . .	< min rt	
1.915	1549	0.056	. . .	7.559	. . . . .	. . .	< min rt	
1.962	893	0.040	. . .	7.660	. . . . .	. . .	< min rt	
2.103	473	0.003	. . .	7.962	. . . . .	. . .	< min rt	

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
367508880	0	0	0.00	0	0	. . . . .	. . . . .

* QUESTION ANALYSIS: TOTAL AREA LESS THAN 50000. CONCENTRATE AND RE-RUN.

*** ANALYSIS NOT GOOD ENOUGH FOR LIBRARY SEARCH ***

Figure 5B The fatty acid composition report of reagent blank analyzed by the MIDI system.

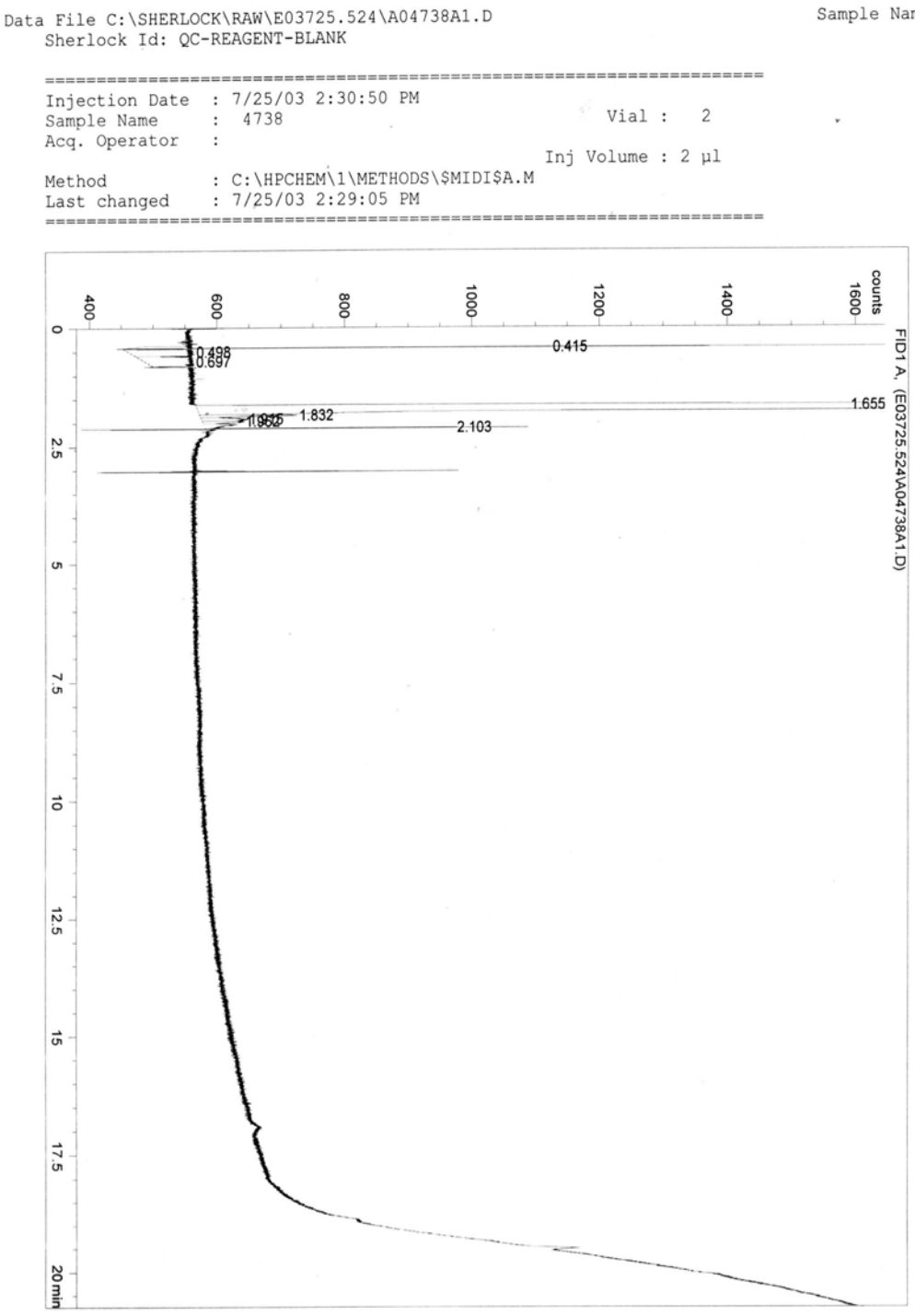


Figure 6B. The fatty acid chromatogram of reagent blank (negative control) analyzed by the MIDI system.

APPENDIX C

PHYSICAL AND CHEMICAL PROPERTIES, AND HALOPHILIC BACTERIAL DENSITY OF SALINE SOIL SAMPLES

Table 1C. Soil temperature of 5 sampling plots at Nong Bo Reservoir recorded on the soil sampling date from June 2001 to May 2002.

Month/ year	Soil temperature (°C)						
	1	2	3	4	5	Average	S.D.*
Jun.-01	29.7	30.7	34.0	32.7	36.0	32.6	2.53
Jul.-01	33.0	32.7	34.0	34.3	36.0	34.0	1.30
Aug.-01	32.0	32.0	31.0	31.0	32.0	31.6	0.55
Sep.-01	32.0	32.0	31.0	30.3	30.0	31.1	0.93
Oct.-01	31.2	30.0	31.0	32.0	32.0	31.2	0.83
Nov.-01	26.0	25.0	27.0	25.0	26.0	25.8	0.84
Dec.-01	25.0	25.7	24.3	23.7	24.0	24.5	0.81
Jan.-02	29.0	27.7	31.0	30.7	29.7	29.6	1.34
Feb.-02	28.3	28.0	29.0	30.7	30.0	29.2	1.14
Mar.-02	29.0	29.7	29.0	30.0	30.7	29.7	0.72
Apr.-02	34.7	34.7	35.7	36.3	39.3	36.1	1.89
May-02	34.7	33.0	32.0	31.3	31.7	32.5	1.36
Average	30.38	30.1	30.75	30.67	31.45		
S.D.*	3.12	3.01	3.15	3.47	4.23		

Note: S.D.* is the standard deviation from the mean value of 30.67.

Table 2C. Moisture content of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Moisture content (%)						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	17.9	15.05	17.51	13.31	15.52	15.86	1.88
Jul.-01	17.74	14.56	14.45	11.00	11.5	13.85	2.72
Aug.-01	20.56	16.41	15.34	14.76	12.28	15.87	3.03
Sep.-01	18.72	16.4	15.45	15.50	13.22	15.86	1.98
Oct.-01	20.47	17.84	16.14	20.67	13.53	17.73	3.01
Nov.-01	17.21	13.21	18.22	12.42	11.41	14.49	3.03
Dec.-01	13.19	10.47	10.09	9.66	7.84	10.25	1.93
Jan.-02	15.60	8.05	8.65	11.23	12.92	11.29	3.11
Feb.-02	14.55	11.21	11.99	10.97	11.93	12.13	1.42
Mar.-02	15.82	9.00	8.75	9.69	9.23	10.50	3.00
Apr.-02	10.35	8.25	9.91	7.84	8.81	9.03	1.07
May-02	13.75	18.3	12.89	11.47	15.36	14.35	2.62
Average	16.32	13.23	13.28	12.38	11.96		
S.D.*	3.05	3.73	3.39	3.39	2.42		

Note: S.D.* is the standard deviation from the mean value of 13.43.

Table 3C. pH of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	pH						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	6.13	4.35	5.46	4.53	5.34	5.16	0.73
Jul.-01	7.68	5.75	6.97	7.03	8.32	7.15	0.96
Aug.-01	8.09	6.15	7.15	7.29	8.75	7.49	0.99
Sep.-01	8.51	6.56	7.24	6.96	7.75	7.40	0.75
Oct.-01	8.24	6.54	7.27	6.98	7.97	7.40	0.70
Nov.-01	8.53	6.20	7.24	6.84	7.87	7.34	0.90
Dec.-01	7.40	4.78	6.39	6.15	6.92	6.33	0.99
Jan.-02	8.10	4.70	6.56	5.85	6.98	6.44	1.27
Feb.-02	8.32	6.65	7.89	7.22	8.10	7.64	0.69
Mar.-02	8.39	6.54	7.68	7.31	8.10	7.60	0.72
Apr.-02	8.18	5.50	7.12	6.34	8.10	7.05	1.15
May-02	5.55	8.16	7.81	6.83	8.31	7.33	1.15
Average	7.76	5.99	7.07	6.61	7.71		
S.D.*	0.96	1.06	0.68	0.80	0.91		

Note: S.D.* is the standard deviation from the mean value of 7.03.

Table 4C. Salinity of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Salinity (%)						
	1	2	3	4	5	Average	S.D.*
Jun.-01	0.31	0.24	0.13	0.29	0.29	0.25	0.07
Jul.-01	0.35	0.18	0.07	0.21	0.27	0.22	0.10
Aug.-01	0.28	0.23	0.04	0.14	0.08	0.15	0.10
Sep.-01	0.26	0.19	0.07	0.17	0.31	0.20	0.09
Oct.-01	0.21	0.24	0.09	0.14	0.09	0.15	0.07
Nov.-01	0.15	0.15	0.07	0.12	0.11	0.12	0.03
Dec.-01	0.11	0.09	0.05	0.06	0.08	0.08	0.02
Jan.-02	0.21	0.15	0.05	0.28	0.25	0.19	0.09
Feb.-02	0.21	0.18	0.07	0.12	0.24	0.16	0.07
Mar.-02	0.22	0.15	0.07	0.16	0.16	0.15	0.05
Apr.-02	0.25	0.17	0.07	0.16	0.16	0.16	0.06
May-02	0.15	0.22	0.06	0.09	0.13	0.13	0.06
Average	0.23	0.18	0.07	0.16	0.18		
S.D.*	0.07	0.04	0.02	0.07	0.09		

Note: S.D.* is the standard deviation from the mean value of 0.16.

Table 5C. Particle size analysis of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Percentage of sand, silt, clay				
	1	2	3	4	5
Jun.-01	76.1, 5.5, 18.4	73.0, 20.0, 6.9	72.0, 19.0, 9.2	48.5, 43.2, 8.3	72.6, 9.5, 18.0
Jul.-01	62.1, 24.7, 13.2	83.9, 13.3, 2.8	79.8, 14.7, 5.5	82.3, 14.2, 3.5	75.8, 15.9, 8.3
Dec.-01	67.4, 22.3, 10.3	83.4, 13.7, 2.9	80.8, 15.1, 4.0	80.9, 14.2, 4.9	78.8, 14.3, 6.9
Apr.-02	67.6, 20.6, 11.8	79.1, 13.1, 6.8	77.2, 13.4, 9.45	79.6, 10.4, 6.6	75.8, 13.5, 10.7
Average	68.3, 18.3, 13.4	79.9, 15.0, 4.9	77.5, 15.6, 7.0	72.8, 20.5, 5.8	75.8, 13.3, 11.0
S.D.*	5.8, 8.7, 3.5	5.0, 3.3, 2.3	3.9, 2.4, 3.7	16.3, 15.2, 2.1	2.5, 2.7, 4.9

Note: S.D.* is the standard deviation from the mean value of 78.84, 16.53, 8.42 (value in sequent of % sand, % silt, % clay).

Table 6C. Organic matter of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Organic matter (%)						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	0.39	0.42	0.21	0.24	0.31	0.31	0.09
Jul.-01	0.31	0.40	0.28	0.18	0.33	0.30	0.08
Dec.-01	0.24	0.27	0.28	0.31	0.23	0.27	0.03
Apr.-02	0.13	0.29	0.17	0.19	0.08	0.17	0.08
Average	0.27	0.35	0.24	0.23	0.24		
S.D.*	0.11	0.08	0.05	0.06	0.11		

Note: S.D.* is the standard deviation from the mean value of 0.26.

Table 7C. Total nitrogen of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Total nitrogen (%)						
	1	2	3	4	5	Average	S.D.*
Jun.-01	0.116	0.109	0.167	0.175	0.118	0.137	0.03
Jul.-01	0.015	0.020	0.014	0.009	0.016	0.0149	0.00
Dec.-01	0.012	0.014	0.014	0.016	0.012	0.0134	0.00
Apr.-02	0.007	0.015	0.009	0.010	0.005	0.009	0.00
Average	0.04	0.04	0.05	0.05	0.04		
S.D.*	0.05	0.05	0.08	0.08	0.05		

Note: S.D.* is the standard deviation from the mean value of 0.5.

Table 8C. Available phosphorus of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Available phosphorus (ppm)						
	1	2	3	4	5	Average	S.D.
Jun.-01	6.04	4.67	1.65	3.22	10.60	5.24	3.42
Jul.-01	11.23	6.21	3.99	5.20	30.54	11.43	11.03
Dec.-01	9.65	7.75	5.26	13.66	13.01	9.87	3.54
Apr.-02	15.15	9.52	8.63	8.39	16.63	11.66	3.92
Average	10.52	7.04	4.88	7.62	17.70		
S.D.*	3.78	2.08	2.91	4.56	8.91		

Note: S.D.* is the standard deviation from the mean value of 9.55.

Table 9C. Available potassium of soil samples collected from 5 sampling plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Available potassium (ppm)						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	88.00	32.58	40.17	64.92	203.75	85.88	69.40
Jul.-01	106.50	25.08	43.33	50.50	411.08	127.30	161.53
Dec.-01	75.92	18.75	36.92	33.17	320.42	97.04	126.66
Apr.-02	114.75	48.67	45.58	62.75	330.32	120.41	120.59
Average	96.29	31.27	41.50	52.84	316.39		
S.D.*	17.59	12.90	3.77	14.57	85.37		

Note: S.D.* is the standard deviation from the mean value of 107.66.

Table 10C. Guidelines for interpreting soil tests as described by กองจำแนกดิน (2516).

Rank / Soil parameter	Extremely low	Low	Moderate	High	Extremely high
pH	4.5	4.5-6.0	6.0-7.5	7.5-8.5	>8.5
Organic matter (%)	<0.5	0.5-1.0	1.5-2.5	2.5-3.5	>3.5
Total nitrogen (%)	<0.025	0.050-0.075	0.075-0.125	0.125-0.175	>0.175
Available phosphorus (ppm)	<3.0	3.0-10.0	10-15	15-45	>45
Available potassium (ppm)	<30	30-60	60-90	90-120	>120

Table 11C. *Guidelines for interpreting type of soil matrix using the soil texture class (Soil Survey Division Staff, 1993).*

Soil texture class	Type of soil matrix
Sand, loamy sand	Coarse
Sandy loam	Moderately coarse
Loam, silt loam, and silt	Medium
Clay loam, sandy clay loam, and silty clay loam	Moderately fine
Sandy clay, silty clay, and clay	Fine

Table 12C. Density of slightly halophilic bacteria reported as colony forming units (CFU) per gram of dry soil of soil samples collected from 5 sampling plots at plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Slightly halophilic bacteria (Log CFU/g of dry soil)						
	1	2	3	4	5	Average	S.D.*
Jun.-01	5.79	4.49	5.58	5.69	6.93	5.70	0.87
Jul.-01	5.15	5.13	5.99	5.48	6.19	5.59	0.48
Aug.-01	4.33	4.73	6.23	5.71	6.55	5.51	0.95
Sep.-01	5.74	5.22	6.24	5.85	6.60	5.93	0.52
Oct.-01	5.39	4.08	6.05	5.75	6.60	5.57	0.95
Nov.-01	6.02	4.64	6.25	6.00	6.46	5.87	0.71
Dec.-01	5.85	3.84	6.18	5.98	5.90	5.55	0.96
Jan.-02	5.86	4.08	6.43	5.92	6.68	5.79	1.02
Feb.-02	6.97	5.22	6.32	6.03	7.10	6.33	0.76
Mar.-02	5.58	4.06	6.11	6.06	6.29	5.62	0.91
Apr.-02	6.44	4.75	6.39	6.21	6.42	6.04	0.73
May-02	5.60	5.46	6.27	6.36	6.53	6.04	0.48
Average	5.73	4.64	6.17	5.92	6.52		
S.D.*	0.65	0.54	0.23	0.24	0.32		

Note: S.D.* is the standard deviation from the mean value of 5.80.

Table 13C. Density of moderately halophilic bacteria reported as colony forming units (CFU) per gram of dry soil of soil samples collected from 5 sampling plots at plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Moderately halophilic bacteria (Log CFU/g of dry soil)						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	5.55	5.18	6.04	6.33	6.31	5.88	0.50
Jul.-01	4.90	4.61	5.79	5.68	6.00	5.40	0.61
Aug.-01	5.40	4.52	5.51	5.13	5.96	5.30	0.53
Sep.-01	5.35	4.86	5.63	5.51	5.67	5.40	0.33
Oct.-01	5.00	4.07	5.31	4.77	5.66	4.96	0.60
Nov.-01	5.56	4.47	5.91	5.12	5.86	5.38	0.60
Dec.-01	5.31	3.76	5.4	4.98	5.46	4.98	0.71
Jan.-02	5.51	3.64	5.64	5.13	5.93	5.17	0.90
Feb.-02	5.48	4.60	5.99	5.17	6.35	5.52	0.69
Mar.-02	5.45	3.37	5.53	5.22	5.24	4.96	0.90
Apr.-02	5.22	3.81	5.33	4.86	5.58	4.96	0.69
May-02	5.00	4.72	5.76	5.26	6.01	5.35	0.53
Average	5.31	4.30	5.65	5.26	5.84		
S.D.*	0.23	0.56	0.25	0.42	0.33		

Note: S.D.* is the standard deviation from the mean value of 5.27.

Table 14C. Density of extremely halophilic bacteria reported as colony forming units (CFU) per gram of dry soil of soil samples collected from 5 sampling plots at plots at Nong Bo Reservoir from June 2001 to May 2002.

Month/ year	Extremely halophilic bacteria (Log CFU/g of dry soil)						
	1	**2**	**3**	**4**	**5**	**Average**	**S.D.***
Jun.-01	3.8	2.66	3.54	3.61	3.92	3.51	0.50
Jul.-01	3.45	2.03	3.15	3.54	4.37	3.31	0.85
Aug.-01	0.00	0.00	0.00	0.00	0.00	0.00	0
Sep.-01	3.31	2.00	2.09	2.1	3.79	2.66	0.83
Oct.-01	3.56	2.98	3.37	2.74	4.05	3.34	0.51
Nov.-01	3.44	2.02	2.11	2.61	4.55	2.95	1.06
Dec.-01	4.12	1.23	3.43	3.87	4.08	3.35	1.21
Jan.-02	3.14	2.11	2.39	2.13	3.19	2.59	0.53
Feb.-02	4.08	1.99	3.81	2.3	4.47	3.33	1.11
Mar.-02	3.64	1.83	2.82	3.51	3.36	3.03	0.74
Apr.-02	3.17	1.84	2.12	3.33	3.63	2.82	0.79
May-02	2.09	2.04	1.32	2.01	3.27	2.15	0.70
Average	3.15	1.89	2.51	2.65	3.56		
S.D.*	1.12	0.73	1.09	1.07	1.21		

Note: S.D.* is the standard deviation from the mean value of 2.75.

APPENDIX D

RESULTS OF STATISTICAL ANALYSIS

Table 1D. Output of correlation analysis of physical and chemical properties of saline soil samples.

		Moisture	pH	Salinity	Soil temperature
Moisture	Pearson correlation	1	0.031	0.32	0.111
	Significant (2-tailed)	.	0.923	0.31	0.732
	N	12	12	12	12
pH	Pearson correlation	0.031	1	-0.378	0.01
	Significant (2-tailed)	0.923	.	0.226	0.975
	N	12	12	12	12
Salinity	Pearson correlation	0.32	-0.378	1	0.611
	Significant (2-tailed)	0.31	0.226	.	0.035
	N	12	12	12	12
Soil temperature	Pearson correlation	0.111	0.01	0.611	1
	Significant (2-tailed)	0.732	0.975	0.035	.
	N	12	12	12	12

Note: N = The number of observation

Table 2D. Output of correlation analysis of three groups of halophilic bacteria of saline soil samples.

		Slightly halophilic bacteria	**Moderately** halophilic bacteria	Extremely halophilic bacteria
Slightly halophilic bacteria	Pearson correlation	1	0.254	0.168
	Significant (2-tailed)	.	0.426	0.602
	N	12	12	12
Moderately halophilic bacteria	Pearson correlation	0.254	1	0.034
	Significant (2-tailed)	0.426	.	0.917
	N	12	12	12
Extremely halophilic bacteria	Pearson correlation	0.168	0.034	1
	Significant (2-tailed)	0.602	0.917	.
	N	12	12	12

Note: N = The number of observation

Table 3D. Output of linear regression analysis of slightly halophilic bacteria and physical and chemical properties of saline soil samples.

Variable entered	Correlation coefficient	F-test	Standardized coefficient	T-test	Significant
Soil temperature	0.125	0.158	0.125	0.398	0.699
Moisture	0.238	0.599	-0.238	-0.774	0.457
pH	0.248	0.656	0.248	0.81	0.437
Salinity	0.009	0.001	0.009	0.029	0.978

Table 4D. Output of linear regression analysis of moderately halophilic bacteria and physical and chemical properties of saline soil samples.

Variable entered	Correlation coefficient	F-test	Standardized coefficient	T-test	Significant
Soil temperature	0.124	0.156	0.124	0.395	0.701
Moisture	0.459	2.669	0.459	1.634	0.133
pH	0.398	1.888	-0.398	-1.374	0.199
Salinity	0.613	6.009	0.613	2.451	0.034

Table 5D. Output of linear regression analysis of extremely halophilic bacteria and physical and chemical properties of saline soil samples.

Variable entered	Correlation coefficient	F-test	Standardized coefficient	T-test	Significant
Soil temperature	0.156	0.250	-0.156	-0.500	0.628
Moisture	0.212	0.473	-0.212	-0.688	0.507
pH	0.311	1.067	-0.311	-1.033	0.326
Salinity	0.140	0.199	0.140	0.446	0.665

APPENDIX E

RESULTS OF HALOPHILIC BACTERIAL CHARACTERIZATION

Table 1E. Comparison of phenotypic characteristics of 109 slightly halophilic bacterium isolates to their closest relative species from reference sources.

Isolate code / Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a	% a
Gram reaction	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	N-100	N-100
Cell shape	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	R-100	C-100	R-100	R-100
Endospores produced	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	P-100	N-100	N-100	N-100
Colony color	C-100	NI	NI	NI	NI	NI	PY-75	NI	NI	NI	NI	NI	NI	NI	NI	O-100	W-100	D	Y-100	NI
Motility	P-100	P-50	P-60	NI	NI	P-79	P-75	NI	N-100	NI	NI	NI	NI	NI	NI	P-100	NI	N-100	P-100	P-100
optimum pH	7-100	NI	NI	NI	NI	NI	7-100	NI	NI	NI	NI	NI	NI	NI	7-100	NI	NI	NI	7-100	NI
optimum NaCl (%)	NI	NI	NI	NI	NI	NI	3-100	NI	NI	NI	NI	NI	NI	NI	3-100	10-100	NI	NI	5-100	NI
optimum Temp. (°C)	30-100	NI	NI	NI	NI	NI	30-100	NI	NI	NI	NI	NI	NI	50-100	35-100	NI	NI	NI	30-100	35-100
Catalase	P-100	P-100	P-100	P-100	NI	P-100	P-100	P-100	P-100	NI	NI	+	NI	P-100	P-100	P-100	NI	P-100	P-100	P-100
Oxidase	NI	D	D	NI	NI	D	N-25	D	N-0	N-62	P-50	+	NI	P-100	P-100	P-100	NI	N-100	P-100	P-100
VP test	N-100	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	P-100	NI	NI	NI	NI	NI	NI
Indole test	N-100	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	P-100	NI	NI	NI	NI	NI	NI
Nitrate reduction	P-100	NI	NI	P-100	NI	NI	NI	NI	NI	NI	NI	NI	NI	P-100	N-100	NI	NI	N- 0	NI	NI
Acid production from:																				
D-mannose	P- 0	NI	P-80	NI	NI	NI	P-75	NI	NI	N-69	N-78	NI	NI	NI	P-100	NI	NI	P-100	NI	NI
Lactose	P-100	N-0	P-60	N-60	NI	NI	P-75	NI	NI	N-85	N-68	NI	NI	P-100	NI	NI	NI	NI	NI	NI
D-fructose	P-100	NI	P-80	NI	NI	NI	P-50	NI	NI	N-85	N-85	NI	NI	P-100	P-100	P-67	NI	NI	NI	NI
D-galactose	NI	NI	NI	NI	NI	NI	P-75	NI	NI	N-77	N-95	NI	NI	P-100	P-100	P-100	NI	N-100	NI	NI
D-xylose	N-100	NI	NI	NI	NI	NI	P-75	NI	NI	P-69	P-79	NI	NI	P-100	NI	N-100	NI	N-100	NI	NI
L-arabinose	N-100	NI	NI	NI	NI	NI	N-50	NI	NI	P-85	P-85	NI	NI	P-100	NI	NI	NI	NI	NI	NI
D-mannitol	P-100	NI	NI	P-85	NI	NI	P-50	NI	NI	P-77	P-85	NI	NI	NI	P-100	N-100	NI	D	NI	NI
Glucose	P-100	NI	NI	NI	NI	NI	P-100	NI	NI	P-85	P-89	NI	NI	P-100	P-100	P-100	NI	NI	NI	NI
Gelatin	P-100	NI	NI	D	NI	NI	NI	NI	NI	NI	NI	+	NI	NI	P-100	P-67	P-100	NI	NI	N-100
Starch	NI	NI	NI	NI	NI	NI	N-100	NI	NI	NI	NI	+	NI	N- 0	N-100	N-100	NI	NI	NI	P-100
H_2S production	N-100	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	-	NI	NI	NI	NI	NI	NI	NI	NI
Number of identified isolates	1	2	5	20	5	14	4	5	1	13	19	2	4	1	1	6	1	1	1	3

Symbol: % a, indicates the characteristic of their closest relative species and percentage of isolates having similarity character to the compared reference species; P, positive; N, negative; R, rods; C, coccus; C, cream; O, orange; P-Y, pale yellow; W, white; Y, yellow; NG, no growth; D, differs among strains; and V, variable reaction. The isolate codes and the references of their closest relative species were taken from: 1. *B. bataviensis*, Heyrman *et al.* (2004); 2. *B. cereus*, Claus and Berkeley (1986); 3. *B. circulans*, Claus and Berkeley (1986); 4. *B. firmus*, Claus and Berkeley (1986); 5. *B. flexus*, Claus and Berkeley (1986); 6. *B. licheniformis*, Claus and Berkeley (1986); 7. *B. marisflavi*, Yoon *et al.* (2003); 8. *B. megaterium*, Claus and Berkeley (1986); 9. *B. mycoide*, Claus and Berkeley (1986); 10. *B. pumilus*, Claus and Berkeley (1986) ; 11. *B. subtilis*, Claus and Berkeley (1986); 12. *B. vallismortis*; 13. *B. vietnamensis*; 14. *Geobacillus caldoxylosilyticus*, Marchant *et al.* (2002); 15. *Halobacillus salinus*, Yoon *et al.* (2003); 16. *Halobacillus trueperi*, Spring *et al.* (1996); 17. *Nocardiopsis dassonvillei*, Meyer (1989); 18. *Staphylococcus saprophyticus*, Kloos and Schleifer (1986); 19. *Marinobacter aquaeolei*, Huu *et al.* (1999); and 20. *Pseudomonas stutzeri*, Palleroni (1984).

Table 2E. Comparison of phenotypic characteristics of 28 moderately halophilic bacterium isolates to their closest relative species from reference sources.

Isolate code / Characteristics	1	2	3	4	5	6	7	8	9	10	11
	%a	%a	%a	%a	%a	%a	%a	%a	%a	%a	%a
Gram reaction	N-100	P-100	P-100	P-100	NI	P-100	P-100	P-100	N-100	N-100	N-100
Cell shape	R-100	R-100	R-100	R-100	NI	C-100	R-100	R-100	R-100	R-100	R-100
Endospores produced	N-100	P-100	P-100	P-100	NI	N-100	P-100	P-100	N-100	N-100	N-100
Colony color	NI	Cr-0	PO-100	Cr-100	NI	Y-100	O-100	NI	NI	NI	NI
Motility	P-100	P-100	P-71	P-100	NI	N-100	P-100	P-100	P-100	NI	NI
optimum pH	NI	7-100	7-100	7-100	NI	NI	NI	NI	NI	NI	NI
optimum NaCl (%)	NI	3-100	3-71	3-100	NI	NI	10- 67	NI	NI	NI	NI
optimum Temp. (^{o}C)	NI	30-100	35-71	38-100	NI	35-100	35-100	NI	NI	NI	NI
Catalase	P-100	P-100	P- 71	NI	NI	P-100	P-100	P-100	P-100	P-100	P-100
Oxidase	P-100	V	N-57	NI	NI	N-100	P-100	P-100	P-100	NI	NI
VP test	NI	P-100	NI	N-100	NI	NI	NI	NI	NI	NI	NI
Indole test	NI	P-100	NI	N-100	NI	NI	NI	NI	N-100	NI	NI
Nitrate reduction	P-100	N-100	NI	P-100	NI	P-100	NI	V	N-100	NI	NI
Acid production from:											
D-mannose	NI	P-100	N-57	N-100	NI	P-100	NI	P-100	NI	NI	NI
Lactose	NI	N-0	N-86	N-100	NI	NI	NI	V	NI	NI	NI
D-fructose	NI	P-100	P-71	N-100	NI	NI	NI	P-50	NI	NI	NI
D-galactose	NI	N- 0	N-86	N-100	NI	P-100	N-100	P-50	NI	NI	NI
D-xylose	NI	N-100	N-86	NG-100	NI	P-100	P-78	NI	NI	NI	NI
L-arabinose	NI	NI	N-86	NG-100	NI	P-100	NI	P-50	NI	NI	NI
D-mannitol	NI	NI	N-43	P-100	NI	P-100	NI	N-100	NI	NI	NI
Glucose	NI	P-100	P-100	P-0	NI	P-100	P-100	P-100	NI	NI	NI
Gelatin	N-100	NI	NI	P-100	NI	NI	P-100	NI	NI	NI	NI
Starch	N-100	NI	P-71	N-100	NI	NI	N-100	NI	NI	NI	NI
H_2S production	NI	N-100	NI	NI	NI	NI	NI	NI	NI	NI	NI
Number of identified isolates	2	1	7	1	1	1	9	2	1	2	1

Symbol: % a, indicates the characteristic of their closest relative species and percentage of isolates having similarity character to the compared reference species; P, positive; N, negative; R, rods; C, coccus; Cr, cream; O, orange; PO-pale orange; Y, yellow; NG, no growth; D, differs among strains; and V, variable reaction. The data of the closest relative species were taken from: 1. *Halomonas desiderata*, Berendes *et al.* (1996); 2. *Oceanobacillus iheyensis*, Lu *et al.* (2001); 3. *B. aquimaris*, Yoon *et al.* (2003); 4. *B. halodenitrificans*, Denariaz *et al.* (1989); 5. *Haererehalobacter ostenderis*; 6. *Staphylococcus gallinarum*, Kloos and Schleifer (1986); 7. *Halobacillus litoralis*, Spring *et al.* (1996); 8. *Vigribacillus pantothenticus*, Heyndrickx *et al.* (1998); 9. *Deleya pacifica*, Holt *et al*. (1994); 10. *Halomonas nitritophilus*; and 11. *Halomonas ventosae*.

Table 3E. Comparison of phenotypic characteristics of 15 extremely halophilic bacterium isolates to their closest relative species from reference sources.

Isolate code / Characteristics	1	2	3	4	5	6	7
	%a	%a	%a	%a	%a	%a	%a
Gram reaction	V-0	N-100	N-100	N-100	P-100	N-100	P-100
Cell shape	C-100	R-100	R-100	R-100	C-100	R-100	R-100
Endospores produced	N-100	N-100	N-100	N-100	N-100	N-100	P-100
Colony color	PO-100	NI	Pink-100	Red-100	Red-0	Cr-100	NI
Motility	N-100	P-100	P-100	P-100	N-100	P-71	NI
optimum pH	NI	NI	7-100	NI	NI	NI	NI
optimum NaCl (%)	20-50	NI	NI	10-100	25-100	3-100	NI
optimum Temp. (°C)	35-100	NI	50- 0	40-ND	35-100	NI	NI
Catalase	P-100	P-100	P-100	P-100	P-100	P-100	P-100
Oxidase	P-50	P-100	P-100	P-100	P-100	P-100	NI
VP test	NI	NI	NI	NI	NI	D	NI
Indole test	NI	P-0	P-0	N-100	NI	D	NI
Nitrate reduction	P-100	P-100	P-100	P-100	P-100	P-100	D
Acid production from:							
D-mannose	NI	NI	NI	N-100	NI	NI	NI
Lactose	NI	NI	NI	N-100	NI	P-71	NI
D-fructose	NI	NI	P-100	P-100	NI	NI	NI
D-galactose	NI	NI	NI	N-100	NI	NI	NI
D-xylose	NI	NI	NI	P-0	NI	NI	NI
L-arabinose	NI	NI	NI	N-100	NI	P-71	NI
D-mannitol	NI	NI	NI	N-100	NI	P-57	NI
Glucose	NI	P-100	P-100	P-100	P-100	P-71	NI
Gelatin	P-50	N-100	P-100	N-100	NI	D	NI
Starch	N-50	N-0	P-100	N-100	NI	N-71	NI
H_2S production	P-50	P-100	N-100	NI	NI	N-86	NI

Table 3E. (Continued)

Characteristics \ Isolate code	1	2	3	4	5	6	7
	%a	%a	%a	%a	%a	%a	%a
Sensitivity to antibiotics:							
Penicillin	NI	N-100	NI	NI	NI	D	NI
Erythromycin	NI	NI	NI	NI	NI	NI	NI
Chloramphenicol	N-100	N-100	NI	NI	NI	D	NI
Streptomycin	N-100	N-100	NI	NI	NI	N-86	NI
Tetracycline	N-100	N-100	NI	NI	NI	D	NI
Vancomycin	NI	NI	NI	NI	NI	NI	NI
kanamycin	NI	NI	NI	NI	NI	NI	NI
Number of identified isolates	2	1	1	1	1	7	2

Symbol: % a, indicates the characteristic of their closest relative species and percentage of isolates having similarity character to the compared reference species; P, positive; N, negative; R, rods; C, coccus; Cr, cream; NG, no growth; D, differs among strains; and V, variable reaction. The data of the closest relative species were taken from: 1. *Natronococcus occultus*, Grant *et al*. (2001); 2. *Halobacterium volcanii*, Grant *et al*. (2001); 3. *Haloferax mediterranei*, Grant *et al*. (2001); 4. *Halobacterium sodomense*, Grant *et al*. (2001); 5. *Halococcus tibetense*, Grant *et al*. (2001); 6. *Halomonas elongata*, Ventosa *et al*. (1998); and 7. *Bacillus* sp., Claus and Berkeley (1986).

Table 4E. Results of pH-, temperature-, and salt- tolerance tests of the 152 halophilic bacterium isolates isolated from saline soil at Nong Bo Reservoir, Mahasarakham Province, Thailand.

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
1. A9ax	*Bacillus aquimaris*	7	35	0	0-8	T
2. A7g	*Bacillus aquimaris*	7	35	0-8	0-15	T
3. A8a4	*Bacillus aquimaris*	7-9	25-35	0	0-8	T
4. Ape	*Bacillus aquimaris*	7	25	0	0-8	T
5. A10e	*Bacillus aquimaris*	7	35	3	0-15	S
6. A6u1	*Bacillus aquimaris*	7-9	25-35	0-3	0-15	T
7. AIIu1	*Bacillus aquimaris*	7-9	25-35	0-3	0-5	T
8. B10aFF	*Bacillus aquimaris*	7	25	0	0-15	T
9. A7aA	*Bacillus bataviensis*	7	35	0-3	0-8	T
10. A11at	*Bacillus cereus*	7	35	3	0-3	S
11. A10ab	*Bacillus cereus*	7	35	0-3	0-8	T
12. AIIV	*Bacillus circulans*	7-9	25-35	0-3	0-8	T
13. A8aq	*Bacillus circulans*	7-9	25-35	0-3	0-15	T
14. A10aq	*Bacillus circulans*	7-9	25-35	0-3	0-8	T
15. A5[aq]	*Bacillus circulans*	7-9	25-35	0-3	0-15	T
16. A1x	*Bacillus circulans*	5-9	25-35	0-3	0-8	T
17. A10[ajj]	*Bacillus firmus*	7	35	0-3	0-8	T
18. A8ak1	*Bacillus firmus*	7	35	0-3	0-3	T
19. A12ao	*Bacillus firmus*	7	35	0-8	0-8	T
20. AIIao	*Bacillus firmus*	7	25-35	0-3	0-8	T
21. A8av$_1$	*Bacillus firmus*	7-9	25-35	0-3	0-8	T
22. A$_{12}$a$_2$	*Bacillus firmus*	7-9	25-35	0-3	0-8	T
23. A$_6$f	*Bacillus firmus*	7	35	0-8	0-8	T
24. A$_{12}$f	*Bacillus firmus*	7	25-35	0-3	0-3	T
25. AIa	*Bacillus firmus*	7	35	0-3	0-8	T

Table 4E. (Continued)

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
26. App	*Bacillus firmus*	7-9	35	0-3	0-3	T
27. AIIp	*Bacillus firmus*	7	35	0-3	0-8	T
28. A1z	*Bacillus firmus*	7	25-35	3	0-8	S
29. A6x	*Bacillus firmus*	7	35	3	3-8	S
30. AIIah	*Bacillus firmus*	7	25-45	0	0-8	T
31. A8ah	*Bacillus firmus*	7	35-45	0	0-8	T
32. A11aI	*Bacillus firmus*	7	35	0	0-3	T
33. A1h	*Bacillus firmus*	7	25-35	3	0-8	S
34. Apj	*Bacillus firmus*	7	25	0-8	0-8	S
35. A1n1	*Bacillus firmus*	7	35	0	0-8	T
36. A8ai	*Bacillus firmus*	7	25-35	0	0-8	T
37. A8p	*Bacillus flexus*	7	10-35	0-3	0-3	T
38. A9s	*Bacillus flexus*	7	35	0-3	0-8	T
39. A11y	*Bacillus flexus*	7	25-45	0-3	0-8	T
40. A7y	*Bacillus flexus*	7-9	35-45	3	0-3	S
41. B6e	*Bacillus flexus*	7	35-45	0	0-15	T
42. B8ax	*Bacillus halodenitrificans*	7	35-45	3	0-20	S
43. AIat	*Bacillus licheniformis*	7-9	35	8	0-8	M
44. A8[aa]	*Bacillus licheniformis*	7	35	0-3	0-8	T
45. Apaw	*Bacillus licheniformis*	7	35-45	3	0-8	S
46. A5aG	*Bacillus licheniformis*	7	35-45	0-3	0-8	T
47. A6aF	*Bacillus licheniformis*	7	35-45	3	0-8	S
48. A7aF	*Bacillus licheniformis*	7	35	3	0-8	S
49. A9af1	*Bacillus licheniformis*	7	35	0-3	0-3	T
50. AaT	*Bacillus licheniformis*	7	35	0	0-3	T
51. A8aV	*Bacillus licheniformis*	7	35-45	3	0-8	S
52. A8aT	*Bacillus licheniformis*	7	35-45	8	0-8	M
53. A9aT	*Bacillus licheniformis*	7	35	0	0-3	T

Table 4E. (Continued)

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
54. B7aV	*Bacillus licheniformis*	7	35-45	8	0-8	M
55. B2r	*Bacillus licheniformis*	7	45	3	0-15	S
56. B7aW	*Bacillus licheniformis*	7-9	35-45	3-8	0-15	S
57. A12aj	*Bacillus marisflavi*	7-9	25-35	0	0-8	T
58. A7[q]	*Bacillus marisflavi*	7	35	0	0-15	T
59. C10k	*Bacillus marisflavi*	7-9	35	0	0-8	T
60. AIIy	*Bacillus megaterium*	7	35	0-3	0-8	T
61. A6V	*Bacillus megaterium*	7	35	0	0-15	T
62. A12ag	*Bacillus megaterium*	7	35	0-3	0-8	T
63. AIag	*Bacillus megaterium*	7	35	0	0-8	T
64. A9an	*Bacillus megaterium*	7	35	0	0-8	T
65. A9ay	*Bacillus mycoides*	7	35	0-3	0-3	T
66. A8y	*Bacillus pumilus*	7	35	3	0-8	S
67. A8aD	*Bacillus pumilus*	7	35	3	0-8	S
68. A8aD1	*Bacillus pumilus*	7	35	3	0-8	S
69. A5at	*Bacillus pumilus*	7	35	3	0-8	S
70. A9aa	*Bacillus pumilus*	7	35	8	0-8	M
71. AIIau	*Bacillus pumilus*	9	35-45	0	0-3	T
72. Apau1	*Bacillus pumilus*	7	35-45	0	0-8	T
73. A1ar	*Bacillus pumilus*	9	35	0-3	0-15	T
74. A6ar	*Bacillus pumilus*	7-9	35	3	0-8	S
75. A5af1	*Bacillus pumilus*	7	35	3	0-8	S
76. A7af	*Bacillus pumilus*	7	35	0-3	0-3	T
77. A5[ac]	*Bacillus pumilus*	7-9	35	3	0-15	S
78. A7[ac]	*Bacillus pumilus*	7-9	35	3	0-15	S
79. A12q	*Bacillus subtilis*	7-9	35	0-3	0-15	T
80. ApaA1	*Bacillus subtilis*	7-9	35	3	0-8	S
81. A7aA1	*Bacillus subtilis*	9	35	8	0-8	M
82. A12[ab]	*Bacillus subtilis*	7	35	0	0-8	T

Table 4E. (Continued)

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
83. A9[ab]	*Bacillus subtilis*	7	35-45	0	0-8	T
84. A7[ab]	*Bacillus subtilis*	7-9	35-45	8	0-8	M
85. A8[ab]	*Bacillus subtilis*	7	35-45	3	0-15	S
86. A8ab2	*Bacillus subtilis*	5-7	35	0	0-8	T
87. A9ab2	*Bacillus subtilis*	7-9	35	0	0-15	T
88. A7ab1	*Bacillus subtilis*	7-9	35	3	0-8	S
89. A8<ab>	*Bacillus subtilis*	5-7	35	3	0-3	S
90. A10aE	*Bacillus subtilis*	7	35	3	0-15	S
91. A7aDD	*Bacillus subtilis*	7	25	0-8	0-8	T
92. A9aDD	*Bacillus subtilis*	7	35-45	3	0-8	S
93. AIaz	*Bacillus subtilis*	7	35	3-8	0-8	S
94. A2az	*Bacillus subtilis*	7	35	0-8	0-8	T
95. A8az1	*Bacillus subtilis*	7	35	8	0-8	M
96. A9aB1	*Bacillus subtilis*	7	35	0-8	0-8	T
97. A1az	*Bacillus vallismortis*	7-9	35	0-8	0-8	T
98. A6aJ	*Bacillus vallismortis*	7	25-35	8	0-8	M
99. A5au	*Bacillus vallismortis*	7	35	0-8	0-15	T
100. A8e	*Bacillus vietnamensis*	9	25-35	0-8	0-8	T
101. A11b	*Bacillus vietnamensis*	7	35	3	0-8	T
102. Apa	*Bacillus vietnamensis*	7	35	0-3	0-15	T
103. A1c	*Bacillus vietnamensis*	7-9	35	0-3	0-3	T
104. A6aP	*Geobacillus caldoxylosilyticus*	7	45-50	0-3	0-15	T
105. A9ajj	*Halobacillus litoralis*	7-9	35	3-8	0-15	M
106. A7aM	*Halobacillus litoralis*	5-7	35-50	3	0-15	S
107. A10au	*Halobacillus litoralis*	7	35	8	0-8	M
108. A9au	*Halobacillus litoralis*	7-9	35	8	0-15	M
109. B7aD	*Halobacillus litoralis*	7	35	8	0-15	M
110. B3au1	*Halobacillus litoralis*	7-9	35	8-20	0-25	M
111. B7ab	*Halobacillus litoralis*	7	45	8	3-20	M

Table 4E. (Continued)

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
112. B_9aq_1	*Halobacillus litoralis*	7	35	8	0-15	M
113. B12r1	*Halobacillus litoralis*	7	35	3-8	3-8	M
114. A1q	*Halobacillus salinus*	7	25-35	3	0-8	S
115. A11s1	*Halobacillus trueperi*	7-9	25-35	8	0-20	M
116. A11s	*Halobacillus trueperi*	7-9	25-35	8	0-20	M
117. A1u	*Halobacillus trueperi*	7	35	3	0-15	S
118. A5y	*Halobacillus trueperi*	7	35	3	0-8	S
119. A_1w	*Halobacillus trueperi*	7	35	3-8	0-15	M
120. $B_8[ac]$	*Halobacillus trueperi*	7	35	3-8	0-20	M
121. $C_{12}b_1$	*Halophilic bacterium MBIC3303*	7	35	8-20	0-25	M
122. $C_{12}k$	*Halophilic bacterium MBIC3303*	7	35	8-20	0-25	M
123. B_7W	*Oceanobacillus iheyensis*	7-9	35	0-3	0-20	T
124. B_8aZ_1	*Vigribacillus pantothenticus*	7	35-45	0-3	0-15	T
125. B_8aZ	*Vigribacillus pantothenticus*	7	35-45	0-3	0-15	T
126. $A_{11}as$	*Nocardiopsis dassonvillei*	7	35	0-3	0-3	T
127. B_8aN	*Staphylococcus gallinarum*	5-7	25-35	0-3	0-15	T
128. $A_{11}aR$	*Staphylococcus saprophyticus*	7	45-50	3	0-8	S
129. B_3aZ	*Deleya pacifica*	7	35	8	0-20	M
130. B_9aB	*Haererehalobacter ostenderis*	7	25-35	3-8	0-20	M
131. A_1ax	*Halomonas desiderata*	7-9	35	3-8	0-20	M
132. $B_{11}ax$	*Halomonas desiderata*	5-7	35	3-8	0-20	M
133. $B_{12}aq_1$	*Halomonas elongata*	7	35	3-8	0-15	M
134. C12a	*Halomonas elongata*	7	35	15	8-25	M
135. C10b	*Halomonas elongata*	7	35	8	0-25	M
136. Cd	*Halomonas elongata*	9	35	15	8-25	M
137. C10j	*Halomonas elongata*	7	35	15	0-25	M

Table 4E. (Continued)

Isolate code	Identified species	Optimum pH	Optimum temp. (°C)	Optimum NaCl (%)	NaCl range (%)	Degree of halophilic*
138. $C_{12}j$	*Halomonas elongata*	7	35	8	0-25	M
139. $C_{12}O_1$	*Halomonas elongata*	7	35	8	0-25	M
140. Apax	*Halomonas nitritophilus*	7	35	8	0-20	M
141. $B_{11}ab$	*Halomonas nitritophilus*	7	35	3-8	0-20	M
142. B_9av	*Halomonas ventosae*	7	35	3-15	0-25	M
143. A12ajj	*Marinobacter aquaeolei*	7-9	35	0-8	0-20	T
144. A11aD	*Pseudomonas stutzeri*	5-7	35	0	0-8	T
145. A12ar	*Pseudomonas stutzeri*	5-7	35	0	0-8	T
146. A12ar1	*Pseudomonas stutzeri*	5-7	35	0	0-8	T
147. C12h	*Halobacterium sodomense*	7	35	20	15-25	E
148. C7h1	*Halobacterium volcanii*	7	35	15	8-20	E
149. $C_{12}h_1$	*Halococcus tibetense*	7	35	15	15-25	E
150. C_7h_2	*Haloferax mediterranei*	7	35	15-20	8-25	E
151. $C_{12}c$	*Natronococcus occultus*	9	35	25	15-25	E
152. $C_{12}n_1$	*Natronococcus occultus*	7-9	35	15-20	15-20	E

Note: *Degree of halophilic of the bacterium isolates was interpreted by comparing to the classification of microorganisms' response to salt (NaCl) described by Kushner (1993). Symbols for degree of halophilic: E, extremely halophilic (15-32 % NaCl); M, moderately halophilic (3-15 % NaCl); S, slightly halophilic (1-3 % NaCl); and T, halotolerant (<1 % NaCl)

Table 5E. Identification results of halophilic bacteria in saline soil at Nong Bo Reservoir. When identified by the combination of phenotypic characteristics, fatty acid profiles, and 16S rDNA sequences, the bacterial species belonged to the same species obtained from 16S rDNA sequence analysis.

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
1. A1ax	*Pseudomonas* sp.	0.380	*Halomonas desiderata*	97
2. Apax	*Pseudomonas* sp.	0.405	*Halomonas nitritophilus*	97
3. A9ax	*Micrococcus* sp.	0.017	*Bacillus aquimaris*	97
4. A12aj	*Bacillus marinus*	0.531	***Bacillus marisflavi***	97
5. A9ajj	*Bacillus* sp.	0.503	*Halobacillus litoralis*	97
6. A10[ajj]	*No growth*		*Bacillus firmus*	97
7. A12ajj	*Pseudomonas nautica*	0.226	*Marinobacter aquaeolei*	98
8. A8ak1	*Bacillus* sp.	0.072	*Bacillus firmus*	97
9. A12ao	*Brevibacillus* sp.	0.379	*Bacillus firmus*	97
10. AIIao	*Bacillus* sp.	0.130	*Bacillus firmus*	97
11. A7g	*Bacillus* sp.	0.130	*Bacillus aquimaris*	98
12. A8av1	*Bacillus* sp.	0.642	*Bacillus firmus*	97
13. A8a4	*Bacillus* sp.	0.365	*Bacillus aquimaris*	98
14. A12a2	*Brevibacillus* sp.	0.122	*Bacillus firmus*	97
15. A8e	*Brevibacillus brevis*	0.702	*Bacillus vietnamensis*	98
16. Ape	*Bacillus* sp.	0.559	*Bacillus aquimaris*	98
17. A1c	*Brevibacillus brevis*	0.405	*Bacillus vietnamensis*	97
18. A6f	*Brevibacillus* sp.	0.080	*Bacillus firmus*	97
19. A12f	*Brevibacillus* sp.	0.055	*Bacillus firmus*	97
20. A10e	*Brevibacillus* sp.	0.642	*Bacillus aquimaris*	98
21. A11b	*Bacillus* sp.	0.409	*Bacillus vietnamensis*	97
22. Apa	*Bacillus* sp.	0.400	*Bacillus vietnamensis*	98
23. Aia	*Bacillus* sp.	0.208	*Bacillus firmus*	97

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S Rrna gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
24. App	*Bacillus* sp.	0.156	*Bacillus firmus*	97
25. A8p	*Bacillus flexus*	0.401	*Bacillus flexus*	97
26. AIIp	*Bacillus* sp.	0.024	*Bacillus firmus*	98
27. A9s	*Bacillus flexus*	0.635	*Bacillus flexus*	97
28. A11s1	*Brevibacillus* sp.	0.617	*Halobacillus trueperi*	99
29. A11s	*Bacillus* sp.	0.678	*Halobacillus trueperi*	98
30. A1q	*Bacillus coagulans*	0.527	***Halobacillus salinus***	99
31. A12q	***Bacillus subtilis***	*0.735*	***Bacillus subtilis***	98
32. A7[q]	*Bacillus marinus*	0.813	*Bacillus marisflavi*	98
33. A1u	*Brevibacillus* sp.	0.670	*Halobacillus trueperi*	97
34. $A_{6}u_{1}$	*Brevibacillus* sp.	0.580	*Bacillus aquimaris*	98
35. AIIu1	*Bacillus marinus*	0.647	*Bacillus aquimaris*	97
36. A8y	*Bacillus pumilus*	0.867	*Bacillus pumilus*	97
37. A11y	*Bacillus flexus*	0.880	*Bacillus flexus*	97
38. AIIy	*Bacillus megaterium*	0.833	*Bacillus megaterium*	98
39. A5y	*Bacillus* sp.	0.812	*Halobacillus trueperi*	97
40. AIIV	*Bacillus circulans*	0.308	*Bacillus circulans*	97
41. A6V	*Bacillus megaterium*	0.526	*Bacillus megaterium*	98
42. A1w	*Paenibacillus* sp.	0.689	*Halobacillus trueperi*	97
43. A7y	*Bacillus flexus*	0.913	*Bacillus flexus*	97
44. A12ag	*Bacillus megaterium*	0.606	*Bacillus megaterium*	99
45. AIag	*Bacillus megaterium*	0.606	*Bacillus megaterium*	99
46. A_{8}aq	No match		*Bacillus circulans*	98
47. A_{10}aq	*Bacillus circulans*	0.332	*Bacillus circulans*	98
48. A_{5}[aq]	*Bacillus circulans*	0.312	*Bacillus circulans*	99
49. ApaA_{1}	*Bacillus lentimorbus*	0.412	*Bacillus subtilis*	98

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
50. A7aA	*Bacillus* sp.	0.502	*Bacillus bataviensis*	97
51. A7aA1	*Bacillus subtilis*	0.688	*Bacillus subtilis*	99
52. A12[ab]	*Bacillus subtilis*	0.690	*Bacillus subtilis.*	99
53. A9[ab]	*Bacillus subtilis*	0.832	*Bacillus subtilis*	98
54. A7[ab]	*Bacillus subtilis*	0.737	*Bacillus subtilis*	98
55. A8[ab]	*Bacillus subtilis*	0.806	*Bacillus subtilis*	98
56. A8ab2	*Bacillus subtilis*	0.829	*Bacillus subtilis*	98
57. A_9ab_2	*Bacillus subtilis*	0.829	*Bacillus subtilis*	98
58. A10ab	*Bacillus cereus*	0.567	*Bacillus cereus*	99
59. A_7ab_1	***Bacillus subtilis***	0.818	***Bacillus subtilis***	98
60. A8<ab>	*Bacillus* sp.	0.438	*Bacillus subtilis.*	99
61. A8aD	*Bacillus* sp.	0.319	*Bacillus pumilus*	98
62. A_8aD_1	*Bacillus pumilus*	0.409	*Bacillus pumilus*	98
63. A11aD	*Pseudomonas stutzeri*	0.826	*Pseudomonas stutzeri*	99
64. A10aE	*Bacillus* sp.	0.352	*Bacillus subtilis*	98
65. A7aDD	***Bacillus subtilis***	0.729	*Bacillus subtilis*	98
66. A9aDD	***Bacillus subtilis***	0.811	*Bacillus subtilis*	99
67. A1z	*Bacillus firmus*	0.268	*Bacillus firmus*	97
68. A1az	***Bacillus subtilis***	0.916	*Bacillus vallismortis*	97
69. AIaz	***Bacillus subtilis***	0.745	*Bacillus subtilis*	99
70. A2az	***Bacillus subtilis***	0.893	*Bacillus subtilis*	98
71. A9ay	*Bacillus mycoides*	0.157	*Bacillus mycoides*	99
72. AIat	***Bacillus subtilis***	0.745	*Bacillus licheniformis*	98
73. A5at	*Bacillus pumilus*	0.769	*Bacillus pumilus*	99
74. A11at	*Bacillus cereus*	0.210	*Bacillus cereus*	98
75. A6aJ	*Bacillus subtilis*	0.887	*Bacillus vallismortis*	98

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
76. A8az1	*Bacillus subtilis*	0.740	*Bacillus subtilis*	98
77. A9aB1	*Bacillus subtilis*	0.757	*Bacillus subtilis*	98
78. A9aa	*Bacillus pumilus*	*0.754*	*Bacillus pumilus*	98
79. A8[aa]	*Bacillus licheniformis*	*0.965*	*Bacillus licheniformis*	98
80. Apaw	*Bacillus licheniformis*	*0.646*	*Bacillus licheniformis*	98
81. A11as	*Nocardiopsis dassonvillei*	*0.557*	*Nocardiopsis dassonvillei*	98
82. A7aM	*Paenibacillus gordonae*	*0.513*	*Halobacillus litoralis*	97
83. A6x	*Bacillus* sp.	*0.161*	***Bacillus firmus***	97
84. A1x	*Bacillus circulans*	*0.527*	*Bacillus circulans*	97
85. A5aG	*Bacillus licheniformis*	*0.587*	*Bacillus licheniformis*	98
86. A6aF	*Bacillus licheniformis*	*0.807*	*Bacillus licheniformis*	98
87. A7aF	*Bacillus licheniformis*	*0.762*	*Bacillus licheniformis*	98
88. A9an	*Bacillus megaterium*	*0.591*	*Bacillus megaterium*	99
89. A11aR	*Staphylococcus cohnii*	*0.721*	*Staphylococcus saprophyticus*	98
90. A6aP	*Bacillus* sp.	*0.043*	*Geobacillus caldoxylosilyticus*	97
91. A10au	*Paenibacillus apiarius*	*0.560*	*Halobacillus litoralis*	98
92. A9au	*Bacillus* sp.	*0.341*	*Halobacillus litoralis*	97
93. AIIau	*Bacillus pumilus*	*0.697*	*Bacillus pumilus*	99
94. A5au	*Bacillus subtilis*	*0.893*	***Bacillus vallismortis***	99
95. Apau1	*Brevibacillu* sp.	*0.552*	*Bacillus pumilus*	99
96. A1ar	*Bacillus pumilus*	*0.710*	*Bacillus pumilus*	99
97. A6ar	*Bacillus pumilus*	*0.581*	*Bacillus pumilus*	98
98. A12ar	*Pseudomonas stutzeri*	*0.895*	*Pseudomonas stutzeri*	99
99. A12ar1	*Pseudomonas stutzeri*	*0.840*	*Pseudomonas stutzeri*	99
100. AIIah	*Bacillus* sp.	*0.516*	*Bacillus firmus*	97

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
101. A8ah	*Bacillus* sp.	*0.361*	*Bacillus firmus*	98
102. A5af1	*Bacillus* sp.	*0.160*	*Bacillus pumilus*	99
103. A7af	*Bacillus pumilus*	*0.745*	*Bacillus pumilus*	99
104. A9af1	*Bacillus* sp.	*0.157*	*Bacillus licheniformis*	97
105. A11aI	*Bacillus* sp.	0.368	*Bacillus firmus*	97
106. A1h	*Bacillus firmus*	0.312	*Bacillus firmus*	97
107. Apj	*Bacillus firmus*	0.307	*Bacillus firmus*	97
108. A1n1	*Bacillus firmus*	0.425	*Bacillus firmus*	98
109. A8ai	No growth		*Bacillus firmus*	97
110. AaT	*Bacillus licheniformis*	0.527	***Bacillus licheniformis***	97
111. A8aV	*Bacillus subtilis*	0.520	*Bacillus licheniformis*	97
112. A8aT	*Bacillus licheniformis*	0.076	*Bacillus licheniformis*	97
113. A9aT	*Bacillus licheniformis*	0.471	*Bacillus licheniformis*	98
114. A5[ac]	*Bacillus pumilus*	0.795	*Bacillus pumilus*	98
115. A7[ac]	*Bacillus pumilus*	0.840	*Bacillus pumilus*	98
116. B11ax	***Pseudomonas diminuta***	0.359	*Halomonas desiderata*	97
117. B7aD	*Bacillus* sp.	0.540	*Halobacillus litoralis*	97
118. B7aV	*Bacillus licheniformis*	0.760	*Bacillus licheniformis*	98
119. B7W	*Bacillus circulans*	0.237	***Oceanobacillus iheyensis***	98
120. B10aFF	*Bacillus* sp.	0.676	*Bacillus aquimaris*	98
121. B2r	*Bacillus licheniformis*	0.589	*Bacillus licheniformis*	98
122. B7aW	*Bacillus licheniformis*	0.693	*Bacillus licheniformis*	97
123. B8ax	*Bacillus circulans*	0.296	*Bacillus halodenitrificans*	98
124. B9aB	*Pseudomonas diminuta*	0.134	*Haererehalobacter ostenderis*	97
125. B8aN	*Staphylococcus gallinarum*	0.064	*Staphylococcus gallinarum*	97

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
126. B6e	*Bacillus flexus*	0.371	*Bacillus flexus*	97
127. B8aZ1	*Bacillus* sp.	0.450	*Vigribacillus pantothenticus*	97
128. B12aq1	***Pseudomonas diminuta***	0.098	*Halomonas elongata*	98
129. B3au1	No growth		*Halobacillus litoralis*	97
130. B7ab	No growth		*Halobacillus litoralis*	97
131. B8aZ	*Bacillus coagulans*	0.322	*Vigribacillus pantothenticus*	97
132. B9aq1	*Bacillus halodenitrificans*	0.210	*Halobacillus litoralis*	97
133. B12r1	*Bacillus* sp.	0.700	*Halobacillus litoralis*	97
134. B3aZ	*Pseudomonas* sp. - SI = 0.198	0.198	***Deleya pacifica***	98
135. B8[ac]	*Bacillus* sp.	0.452	*Halobacillus trueperi*	98
136. B11ab	*Pseudomonas* sp.	0.255	*Halomonas nitritophilus*	98
137. B9av	*Pseudomonas* sp.	0.185	*Halomonas ventosae*	97
138. C12a	*Pseudomonas diminuta*	0.216	*Halomonas elongata*	97
139. C10b	*Pseudomonas diminuta*	0.151	*Halomonas elongata*	98
140. C12b1	*Vigribacillus* sp.	0.240	**Halophilic bacterium MBIC3303**	96
141. C12c	No growth		*Natronococcus occultus*	97
142. Cd	***Pseudomonas diminuta***	0.301	*Halomonas elongata*	98
143. C7h1	No growth		***Halobacterium volcanii***	98
144. C7h2	No growth		***Haloferax mediterranei***	98
145. C12h	No growth		***Halobacterium sodomense***	97
146. C12h1	No growth		***Halococcus tibetense***	98
147. C10j	***Pseudomonas diminuta***	0.121	*Halomonas elongata*	98
148. C12j	***Pseudomonas diminuta***	0.051	*Halomonas elongata*	99
149. C12n1	No growth		*Natronococcus occultus*	97

Table 5E. (Continued)

Isolate code	Fatty acid analysis		16S rRNA gene sequence analysis	
	Identified species	Similarity index (SI)	Identified species	Similarity (%)
150. C10k	*Bacillus megaterium*	0.262	*Bacillus marisflavi*	98
151. C12k	*Vigribacillus* sp.	0.228	Halophilic bacterium MBIC3303	96
152. C12O1	***Pseudomonas diminuta***	0.182	*Halomonas elongata*	98

An interpretation of the SI value is as follows:

SI 0.6 or greater, with no second choice is an excellent ID

0.5 or greater, with second choice > 0.2 lower is a good ID

0.3-0.5 with no second choice is a good likelihood

< 0.3 with or without second choice means machine is guessing

APPENDIX F

EXAMPLES OF GEL ELECTROPHORESIS OF AMPLIFIED BACTERIAL 16S rDNA AND ELECTROPHENOGRAMS OF DNA SEQUENCES

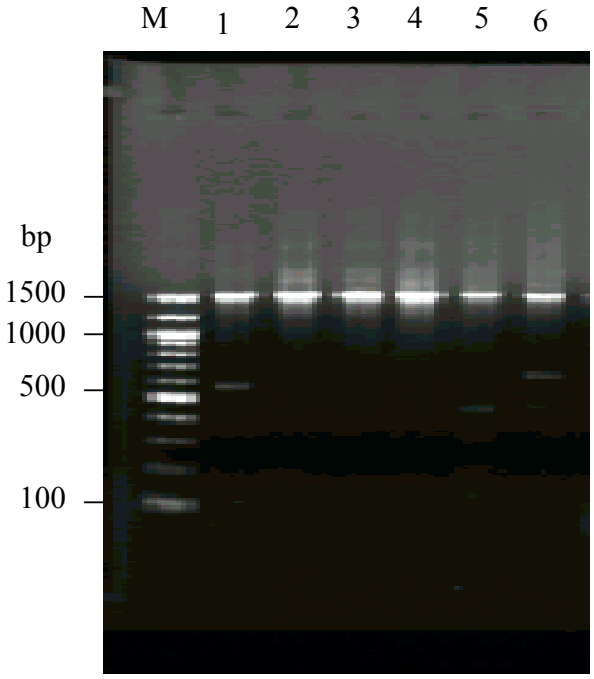


Figure 1F. Gel electrophoresis of **PCR** products obtained from the amplification of bacterial DNA using primers 27f and 1525r. Lanes: M, 100 bp DNA ladder (Promega) as a molecular weight marker; 1, bacterial isolate A_{12}ajj; 2, A_6X; 3, Ape; 4, A_9aT; 5, Apaw; and 6, A_8aD_1.

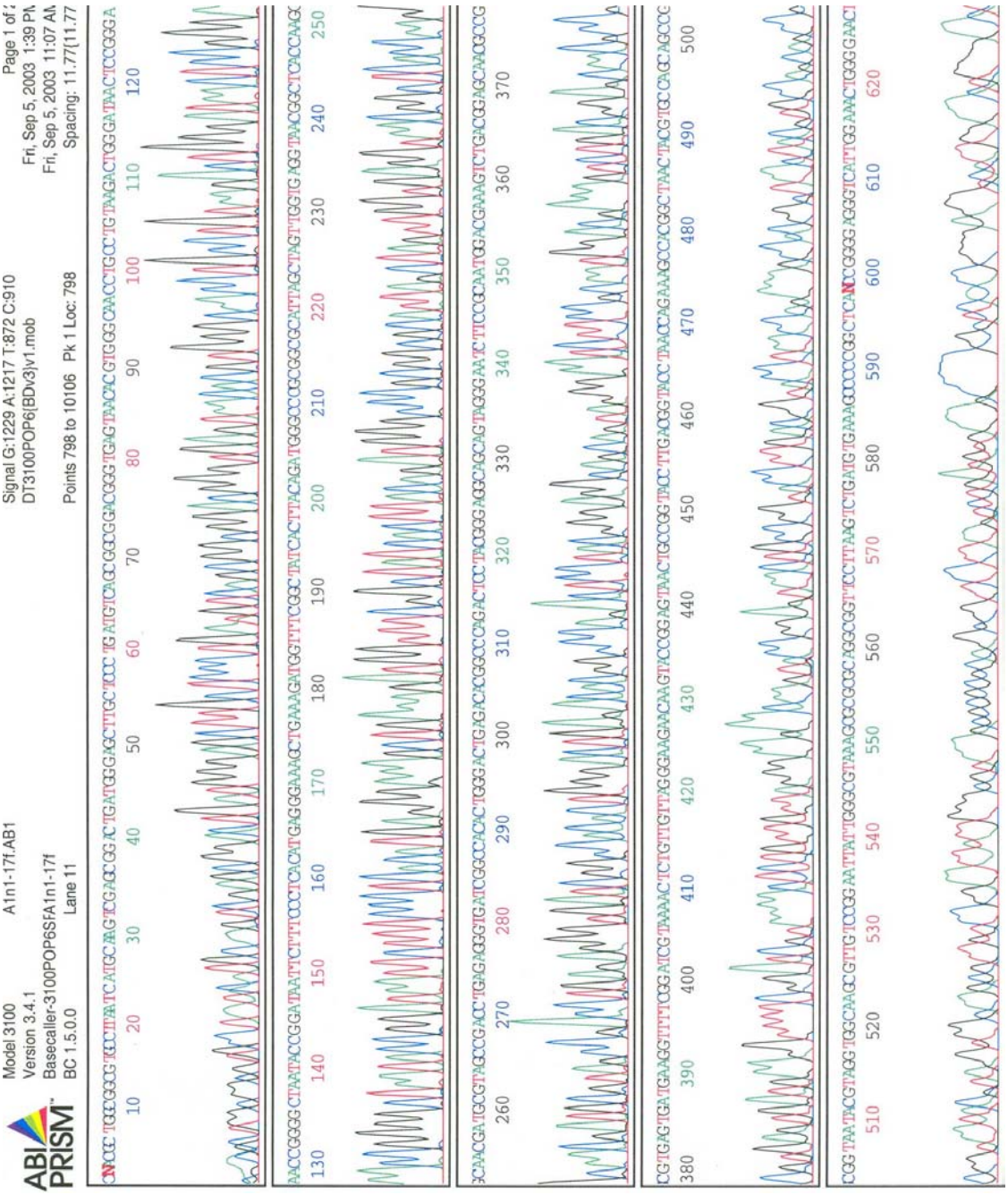


Figure 2F. The sequence electrophenogram of halophilic bacterium isolate code A1n1 which belonged to *Bacillus firmus*.

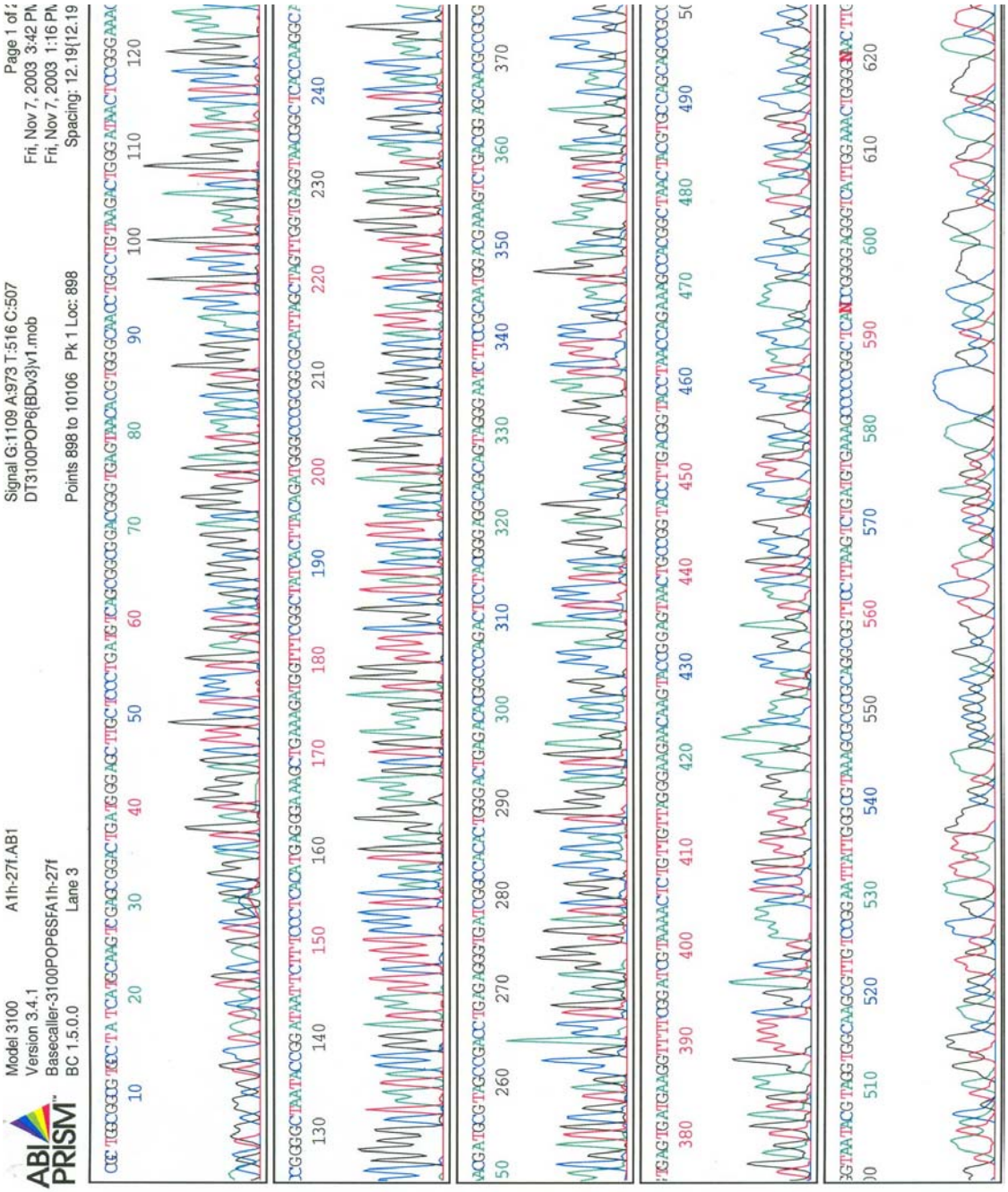


Figure 3F. The sequence electrophenogram of halophilic bacterium isolate code A1h which belonged to *Bacillus firmus*.

APPENDIX G

PRELIMINARY STUDY OF DIRECT DNA EXTRACTION FROM SALINE SOIL FOR THE EVALUATION OF MICROBIAL COMMUNITIES USING T-RFLP TECHNIQUE

1. Introduction

Over the last three decades, the study of soil microbial communities relied on molecular analysis due to low percentage of microorganisms has been brought into culture (Pace, 1986). Three techniques, DGGE, ARDRA, and T-RFLP, are commonly used for the study of microbial communities. Among these techniques, T-RFLP is the most effective technique at discriminating between bacterial communities in a range of environments (Tiedje *et al*., 1999). For the application of T-RFLP technique, microbial DNAs are directly extracted from soil samples.

To date, a number of methods for DNA extraction from soil have been proposed. However, the efficacy of each protocol influences by soil types (Zhou *et al*., 1996). In addition, the extracted DNA should have high DNA yield, be free from inhibitors for subsequent molecular biology approaches, and be derived from the representative of microorganisms of the samples. This research aimed to obtain the method of direct DNA extraction from saline soil for evaluating microbial communities using T-RFLP technique.

2. Materials

2.1 UltraClean soil DNA isolation kit (MoBio Laboratories, U.S.A.)

2.2 QIAamp DNA blood mini kit (Qiagen, U.S.A.)

2.3 Mini bead beater (BioSpec Products, U.S.A.)

2.4 AutoSeq G-50 (Amersham Biosciences, U.S.A)

2.5 Sephadex G200 (Amersham Biosciences, U.S.A)

2.6 Chemicals and reagents: absolute ethanol, chloroform:isoamyl alcohol (24:1), CTAB (2% w/v CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 8), isopropanol, lysis buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8), lysozyme, phenol:chloroform:isoamyl alcohol (24:24:1) saturated in TE buffer, proteinase K 20 mg/mL, and 20% SDS.

2.7 Saline soil samples collected from Nong Bo Reservoir in summer (May), rainy season (October), and winter (December), of the year 2001.

3. Methods

A total of 8 different procedures were evaluated for DNA extraction from saline soil samples (Table 1G) as follows:

Procedure 1: DNA was extracted from 0.5 g of soil samples using the UltraClean soil DNA isolation kit (MoBio Laboratories) according to the manufacture's instruction.

Procedure 2: This procedure was modified from the first procedure in which 10 minutes incubation at 70°C of the chemical treatment step was performed as an additional step for completing the microbial cell lysis.

Table 1G. Physical, enzymatical, and chemical treatments for DNA extraction procedures of 15 saline soil samples.

Procedure	Amount of soil sample (g)	Physical treatment	Enzymatical treatment	Chemical treatment	DNA purification	References
1	0.5	Bead beating	-	SDS	UltraClean Spin column	
2	0.5	Bead beating, high temperature	-	SDS	UltraClean Spin column	
3	10	-	+	SDS-phenol	UltraClean Spin column and QIAamp Spin column	Moore (1995)
4	10	-	+	SDS-phenol	QIAamp Spin column	Moore (1995)
5	0.5	-	+	SDS-phenol	Ethanol precipitation and AutoSeq G-50 column	Moore (1995) and Miller *et al.* (1999)
6	0.5	-	+	SDS-phenol	Isopropanol precipitation and AutoSeq G-50 column	Moore (1995) and Miller *et al.*(1999)
7	10	-	+	SDS-phenol	Isopropanol precipitation and AutoSeq G-50 column	Moore (1995) and Miller *et al.* (1999)
8	0.5	Freeze-thaw	+	SDS-phenol, and chloroform extraction	Isopropanol precipitation and sephadex G200	**Dunbar, Ticknor, and Kuske** (2001)

Procedure 3: This procedure was modified from the protocol described by Moore (1995), the protocol used for DNA extraction from pure culture of halophilic bacteria isolated from saline soil. The reagents of the UltraClean soil DNA isolation kit (MoBio Laboratories) were used for DNA extraction from soil samples as described in the following steps.

1. Ten g of soil sample were transferred to a 50 mL centrifuge tube. Ten mL of lysis buffer containing lysozyme 20 mg/mL, 200 µL of of Proteinase K (20

mg/mL), and 80 μL of 20% SDS were transferred to soil samples and gently tumbled on a clinical rotator for 3 hours at 37°C. Then, 10.3 mL of phenol/chloroform/isoamyl alcohol (25:24:1 V/V/V) were added to the mixture and vortexed.

2. The mixture was centrifuged for 10 minutes at 6,000 rpm, 4°C. The supernatant was transferred to a new tube. An equal volume of ethanol was added to the supernatant and incubated at -20°C for 30 minutes to precipitate DNA, then centrifuged for 10 minutes at 6,000 rpm and 4°C.

3. The supernatant was discarded. The DNA pellet was dried at room temperature for 30 minutes. Then, ninety-five μL of bead buffer, 30 μL of solution S1, 100 μL of solution IRS, and 125 μL of solution S2 were added to the DNA pellet and vortexed briefly. The mixture was incubated at 4°C for 10 minutes. Then, seven hundred μL of solution S3 was then added to the mixture and vortexed briefly.

4. The mixture was loaded onto a spin filter, and centrifuged at 12,000 rpm for 1 minute. The flow through was discarded.

5. Two hundred μL of solution S4 were added onto the spin filter and centrifuged for 30 seconds at 12,000 rpm. This step was done twice. The spin filter was centrifuged again for 1 minute and opened the lid to dry the DNA pellet for 10 minutes.

6. Thirty μL of solution S5 were added to the center of the white filter membrane, left for 5 minutes at room temperature then centrifuged for 30 seconds.

7. The filtrate was pipetted back to the white filter and repeated the step 8. The spin filter was discarded and the filtrate (DNA) was ready for furtherapplication.

Procedure 4: The protocol of the procedure 4 was almost the same as procedure 3. Only the high speed centrifuge at 14,000 rpm of procedure 4 was employed at the enzymatical and chemical steps.

Procedure 5: This procedure was modified from the protocol described by Moore (1995) and Miller *et al.* (1999). It was different from the procedure 3 in that only 0.5 g of soil sample were used for the extraction, and the higher ratio of reagents to soil sample was used in the enzymatical and chemical steps. Steps of the procedure 5 are as follows:

1. Soil sample (0.5 g) were transferred to a new microcentrifuge tube. Four hundred and fifty μL of lysis buffer containing lysozyme 15 mg/mL was added to the microcentrifuge tube, then briefly vortexed, and incubated at 37°C for 2 hours. During the incubation period, each tube was gently inverted every 30 minutes.

2. One hundred μL of Proteinase K (20 mg/mL), and 150 μL of 20% SDS were added to the soil sample mixture then briefly vortexed, and incubated at 37°C for 2 hours with mixing every 30 minutes. The sample was then centrifuged for 5 minutes at 12,000 rpm. Then, the supernatant was transferred to a new microcentrifug tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, V/V/V) was added to the supernatant, and gently mixed.

3. The mixture was centrifuged for 5 minutes at 12,000 rpm, 4°C. The supernatant was transferred to a new microcentrifug tube. An equal volume of absolute ethanol was added to the supernatant and incubated at -20°C for 30 minutes to precipitate DNA.

4. The mixture was centrifuged for 5 minutes at 12,000 rpm, 4°C. The DNA

pellet was washed with 600 μL of 70% ethanol. The supernatant was discarded. The precipitated DNA was dried for 30 minutes at room temperature.

5. The precipitated DNA was resuspended in 50 μL of TE buffer, and kept overnight at 4°C for completely dissolved of DNA.

Procedure 6: Protocol using in the procedure 6 is quite similar to the procedure 5. The different between procedures 6 to procedure 5 is that chloroform:isoamyl alcohol (24:1, v/v) was added as the additional step after phenol extraction. In addition, ethanol precipitation was substituted by isopropanol and incubated at –20°C for 30 minutes.

Procedure 7: The procedure 7 is similar to procedure 6 but 10 g of soil sample were used, and the solution in each step was proportionally added to the soil when the volume increased.

Procedure 8: This procedure was modified from the protocol described by **Dunbar *et al.* (2001)**. CTAB was used as an extraction buffer and three freeze-thaw cycles were also performed to ensure cell lysis. The protocol for procedure 8 is described as follows:

1. Soil sample (0.5 g) were transferred to a new microcentrifuge tube. Then 1.0 mL of CTAB containing lysozyme (15 mg/mL) and 5 μL of Proteinase K (20 mg/mL) were added to the soil sample, and mixed. The soil suspension was incubated at 37°C for 2 hours with gently mixed by inverting tube several times every 30 minutes. Then one hundred and fifty μL of 20% SDS were added to the soil suspension, and briefly vortexed. The mixture was incubated at 70°C for 2 hours. The sample was taken out for vortexing every 30 minutes.

2. The mixture was subjected to three freeze-thaw cycles consisting of 5 minutes in liquid N_2 followed by 5 minutes in a 70°C water bath, then centrifuged for 10 minutes at 12,000 rpm.

3. The supernatant was transferred to a new microcentrifuge tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, V/V/V) was added to the supernatant, and gently vortexed then centrifuged for 5 minutes at 12,000 rpm, 4°C.

4. An equal volume of chloroform/isoamyl alcohol (24:1 V/V) was added to the mixture, and gently vortexed, then centrifuged for 5 minutes at 12,000 rpm, 4°C.

5. The supernatant was transferred to a new microcentrifuge tube. An equal volume of isopropanol was added to the supernatant and incubated at -20°C for 30 minutes, then centrifuged for 10 minutes at 12,000 rpm, 4°C to precipitate DNA.

6. The DNA pellet was washed with 600 μL of 70% ethanol. The mixture was centrifuged for 5 minutes at 12,000 rpm, 4°C. The supernatant was discarded. The precipitated DNA was dried for 30 minutes at room temperature.

7. The DNA pellet was resuspended in 50 μL of TE buffer, and kept overnight at 4°C for completely dissolved of DNA. The DNA was detected using 1% agarose gel electrophoresis, and was quantified using the machine Molecular Devices and the software SOFTmax®Pro, version 3.1.2 (Molecular Devices, U.S.A.).

8. Extracted DNA was purified using sephadex G200. About 100 μL of sephadex G200 was pipetted and transfer to the microtiter plate. Three hundred and forty μL of MilliQ water were added to each well. The resin was allowed to stand at room temperature for 30 minutes to saturated rehydrate. Then 15 μL of extracted DNA was transferred to the well and centrifuged at 2,300 rpm for 5 minutes. The

purified DNA was collected and transferred to the new tube, and ready to use for PCR amplification.

4. Results and discussion

The DNA extraction using the UltraClean Soil DNA Isolation Kit (the procedure 2) gave the higher yield than procedure 1 when 10 minutes incubation at 70°C was performed at the chemical treatment step. However, the PCR amplification of the DNA extracted using the procedure 1 and 2 was unamplified due to the low DNA concentration. In the attempt to recover higher DNA from soil sample, the procedure 3 was carried out with increasing the amount of soil sample from 0.5 g to 10 g. The color of extracted DNA from the procedure 3 was dark brown and the DNA concentration spectronically measured at wavelength 260 nm was less than 0. In addition, the DNA from the procedure 3 was unamplified with the primers 27f and 1525r (Lawson *et al.*, 1996).

The procedure 4 was conducted by using the high speed centrifugation (14,000 rpm) in order to protect the contamination of soil particles to the phenol extraction step. However, the color of extracted DNA was very brown. Therefore, the purification of extracted DNA was performed using the QIAamp DNA blood mini kit (Qiagen) and the UltraClean Soil DNA Isolation Kit. The QIAamp DNA blood mini kit was conducted according to the manufacture's instructions. The UltraClean Soil DNA Isolation Kit was conducted with the suggestion of the MoBio Laboratories (U.S.A.). Unfortunately, the PCR amplification was unsuccessfully.

When extracted using the protocol 5, the DNA concentration was 0.058 to 0.261 μg/mL but no DNA band was detected in agarose gel of all samples. In addition,

white pellets were precipitated in the DNA of all samples, and the PCR amplification was unsuccessfully. The unsuccessful amplification may be due to unpurify of DNA template so the procedure 6 was used to purify the extracted DNA. The amount of soil sample used in the procedure 6 was much lower than the procedure 7, and the DNA quality obtained from the procedure 6 was much better than the DNA extracted from the procedure 7. This result is support the study of Tsai and Olson (1992) in that the major drawback of chemical treatment leads to the release of humic and fulvic acids resulting in coextracted DNA with humic substances. Therefore, the large amount of soil sample was used for DNA extraction, the more humic substances were released to coextract with the DNA. This would be summarized that physical treatment should be considered as the first step for cell lysis when the large amount of soil sample is applied. Alternatively, chemical treatment can be employed after separating soil particles from the sample.

The DNA concentration (14 of 15 soil samples) obtained from the procedure 8 was the highest (0.000 to 0.568 μg/mL). In addition, the DNA color was not very brown like the other procedures. This could be the result of removing the contaminants from crude DNA extract by the CTAB (Zhou *et al*., 1996). In addition, three freeze-thaw steps may promote the complete cell lysis. The PCR amplification was also performed with the spiking technique. But it was unsuccessful. This might be resulted from low DNA concentration and inhibitors contaminated in the DNA extracts. Tsai and Olson (1992) reported that their standard PCR reaction was inhibited by only 10 ng of humic acid presented. In addition, Watson and Blackwell (2000) stated that in the presence of the inhibitors, PCR amplification would be successful when the microbial population of soil sample exceeds about 10^3–10^4 cells

per gram. To date, the efficacy of diverse chemical treatments for microbial cell lysis remain unknown. In addition, soil extraction always suffers from incompleteness of cell lysis and DNA sorption to soil surfaces, and loss or degradation or damage of DNA (Yeates *et al*., 1998).

5. Conclusion

The potential application of direct DNA extraction from non-saline soil using the UltraClean soil DNA isolation kit has been reported by the MoBio Laboratories (U.S.A.). from this study, the kit could not provided the good quality of DNA for PCR amplification of the T-RFLP technique. The direct DNA extraction from saline soil using the procedure 8 which the CTAB was used as the extraction buffer at high temperature (70°C), as well as the physical treatment of three cycles of freeze-thaw. After phenol–chloroform and chloroform extraction, and isopropanol precipitation, the highest DNA concentration was achieved. But the PCR amplification was still unsuccessful. The reason for this failure result is doubtful. Inhibitors in the DNA extracts may be involved. Therefore, the study of DNA extraction and purification procedures is required for the successful PCR amplification and T-RFLP application.

Curriculum Vitae

1) **Name:** Mrs. Kannika Chookietwattana (Wongvutiyan)

2) **Date of birth:** 13 September 1968

3) **Education:**

1990 B.Sc. (Public Health, Major Sanitary Science) Mahidol University, Thailand

1993 M.Sc. (Environmental Science) Kasetsart University, Thailand

1998 M.Sc. (Environmental Pollution Control) University of Leeds, UK.

4) **Scholarships/Awards:**

1990-1992 Thailand Ministry of University Affairs scholarship for master degree study at Kasetsart University, Thailand

1996-1997 The Foreign and Commonwealth Office (FCO)/The British Council for master degree study at University of Leeds, UK.

5) **Contact address:** Faculty of Technology, Mahasarakham University,
Muang, Mahasarakham 44000, Thailand
Telephone 66 43 721-728 Faximile 66 43 743-135
E-mail address karb90@yahoo.com

6) **Work experience since graduating:**

Lecturer at School of Environmental Technology, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Mahasarakham, Thailand (May 1993 – present)