อนุกรมวิธานของแบคทีเรียที่ย่อยสลายไซแลนและลักษณะเฉพาะของไซแลเนส จากสายพันธุ์ที่คัดเลือกได้

นางสาว เสาวภา เขียนงาม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชเคมีและผลิตภัณฑ์ธรรมชาติ
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

TAXONOMY OF XYLANOLYTIC BACTERIA AND CHARACTERIZATION OF XYLANASE FROM SELECTED STRAINS

Miss Saowapar Khianngam

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Chemistry and Natural Products
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2010

Thesis Title	TAXONOMY OF XYLANOLYTIC BACTERIA AND CHARACTERIZATION OF XYLANASE FROM SELECTED STRAINS
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เสาวภา เขียนงาม: อนุกรมวิธานของแบคทีเรียที่ย่อยสลายไซแลนและลักษณะเฉพาะของไซแลเนสจากสายพันธุ์ที่คัดเลือกได้ (TAXONOMY OF XYLANOLYTIC BACTERIA AND CHARACTERIZATION OF XYLANASE FROM SELECTED STRAINS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.สมบูรณ์ ธนาสุภวัฒน์ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.วรรณพ วิเศษสงวน, Jung-Sook Lee, Ph. D., 192 หน้า

การคัดแยกสายพันธุ์แบคทีเรียที่สร้างไซแลเนสจากดินที่เก็บในประเทศไทยจำนวน 45 ตัวอย่าง พบว่าสามารถแยกแบคทีเรียที่มีคุณสมบัติตามต้องการได้ 70 ไอโซเลต จากผลการศึกษาลักษณะทางฟีโนไทป์ และผลทางอนุกรมวิธานเคมี รวมทั้งการวิเคราะห์ลำดับเบสของ 16S rRNA gene ของสายพันธุ์ตัวแทน สามารถแบ่งแบคทีเรียที่แยกได้เป็น 16 กลุ่ม โดยเป็นแบคทีเรียแกรมบวก 61 สายพันธุ์ สกุล *Bacillus* 25 สายพันธุ์ *Paenibacillus* 24 สายพันธุ์ *Cohnella* 4 สายพันธุ์ *Isoptericola* และ *Jonesia* อย่างละ 2 สายพันธุ์ *Microbacterium* 3 สายพันธุ์ และ *Nocardioides* 1 สายพันธุ์ โดยแบคทีเรียแกรมลบพบสกุลละ1 สายพันธุ์ คือ สกุล *Acinetobacter Aeromonas, Blastobacter, Ensifer, Pseudomonas, Sphingobacterium, Sphingomonas, Stenotrophomonas* และ *Zobellella* ผลการพิสูจน์เอกลักษณ์ของแบคทีเรียที่แยกได้ พบว่าเป็น *Bacillus licheniformis* 4 สายพันธุ์, *Paenibacillus barengoltzii* 3 สายพันธุ์, *B. subtilis* subsp. *subtilis*, *B. niabensis*, *B. cereus*, *Isoptericola variabilis*, *Jonesia denitrificans* และ *Microbacterium natoriense* สปีชีส์ละ 2 สายพันธุ์ *B. nealsonii*, *P. macerans*, *P. timonensis* , *P. montaniterrae*, *P. dendritiformis*, *Nocardioides simplex*, *Acinetobacter junii*, *Aeromonas enteropelogenes*, *Ensifer adhaerens*, *Pseudomonas stutzeri*, *Stenotrophomonas maltophilia* และ *Zobellella denitrificans* สปีชีส์ละ 1 สายพันธุ์ พบว่า *Bacillus* sp. 2 สายพันธุ์ *Paenibacillus* sp. 12 สายพันธุ์ *Cohnella* sp. 4 สายพันธุ์ *Microbacterium* sp. *Blastobacter* sp. *Sphingobacterium* sp. *Sphingomonas* sp. สปีชีส์ละ 1 สายพันธุ์ เป็นแบคทีเรียสายพันธุ์ใหม่ โดยลำดับเบสของ 16S rRNA gene คล้ายคลึงกับของแบคทีเรียตัวแทนด้วย 96.0-98.8 เปอร์เซ็นต์ และมีผลการศึกษาลักษณะทางฟีโนไทป์ รวมถึงผลทางอนุกรมวิธานเคมีบางประการที่แตกต่างกัน แบคทีเรียแกรมบวก รูปร่างแท่งสร้างสปอร์ ได้ถูกเสนอเป็นแบคทีเรียสายพันธุ์ใหม่ซึ่ง ได้แก่ *Paenibacillus thailandensis* sp. nov., *P. nanensis* sp. nov., *P. xylanisolvens* sp. nov., *Cohnella thailandensis* sp. nov, *C. xylanilytica* sp. nov. และ *C. terrae* sp. nov. *Bacillus* สายพันธุ์ P2-3 สามารถผลิตไซแลเนสได้สูงสุดเมื่อเปรียบเทียบกับแบคทีเรียสายพันธุ์อื่น ดังนั้นจึงนำมาทำการศึกษาต่อ ในการผลิตไซแลเนส พบว่าซังข้าวโพดบดเป็นสารตั้งต้นที่ดีที่สุด การศึกษาหาสภาวะที่เหมาะสมของส่วนประกอบอาหารและพีเอช สามารถเพิ่มการผลิตไซแลเนสได้ 2 เท่า เมื่อเปรียบเทียบกับอาหารสูตรเดิม การศึกษาลักษณะบางประการของไซแลเนสภายหลังการทำให้บริสุทธิ์บางส่วน พบว่า เอนไซม์มีน้ำหนักโมเลกุลเท่ากับ 17.7 กิโลดาลตัน ทำงานได้ดีที่ 60 องศาเซลเซียส และพีเอช 6 มีความเสถียรคงเหลือสูงกว่าร้อยละ 50 ที่ 30-40 องศาเซลเซียส และพีเอช 3-11 กิจกรรมเอนไซม์เพิ่มขึ้นเมื่อมีการเติม Ca^{2+}, Mg^{2+}, Mn^{2+}, DTT หรือ β-Me ที่ระดับ 1 มิลลิโมลาร์ ในขณะที่ถูกยับยั้งเพียงเล็กน้อยด้วย Fe^{2+}, PMSF และ SDS ที่ระดับความเข้มข้นเดียวกัน ไซแลเนสที่ได้สามารถย่อย Oat spelt xylan ได้ดีที่สุด โดยไม่สามารถย่อย β-glucan, carboxymethylcellulose และ pectin

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ ลายมือชื่อนิสิต เสาวภา เขียนงาม

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

497 6961633: MAJOR PHARMACEUTICAL CHEMISTRY AND NATURAL PRODUCTS
KEYWORDS: TAXONOMY/ XYLANASE/ BACTERIA

SAOWAPAR KHIANNGAM: TAXONOMY OF XYLANOLYTIC BACTERIA AND CHARACTERIZATION OF XYLANASE FROM SELECTED STRAINS. THESIS ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D., THESIS CO-ADVISOR: WONNOP VISESSANGUAN, Ph.D., JUNG-SOOK LEE, Ph.D., 192 pp.

Seventy isolates of xylanase-producing bacteria were isolated from 45 samples of soil collected in Thailand. These bacteria were divided into sixteen groups based on their phenotypic and chemotaxonomic characteristics including 16s rRNA gene sequences of the representative strains. Sixty-one strains were Gram-positive rods belonged to *Bacillus* 25 isolates, *Paenibacillus* 24 isolates, *Cohnella* 4 isolates, *Isoptericola* and *Jonesia* each of 2 isolates, *Microbacterium* 3 isolates and *Nocardioides* 1 isolate. Each isolate of Gram-negative rods, was belonged to *Acinetobacter, Aeromonas, Blastobacter, Ensifer, Pseudomonas, Sphingobacterium, Sphingomonas, Stenotrophomonas* and *Zobellella*. They were identified as *Bacillus licheniformis* 4 isolates, *Paenibacillus barengoltzii* 3 isolates; each of 2 isolates was *B. subtilis* subsp. *subtilis, B. niabensis, B. cereus, Isoptericola variabilis, Jonesia denitrificans* and *Microbacterium natoriense*; and each of 1 isolate was *B. nealsonii, P. macerans, P. timonensis, P. montaniterrae, P. dendritiformis, Nocardioides simplex, Acinetobacter junii, Aeromonas enteropelogenes, Ensifer adhaerens, Pseudomonas stutzeri, Stenotrophomonas maltophilia* and *Zobellella denitrificans*. In addition, the novel species of *Bacillus* 2 isolates, *Paenibacillus* 12 isolates, *Cohnella* 4 isolates and each isolate of *Microbacterium, Blastobacter, Sphingobacterium, Sphingomonas* were identified based on the differential phenotypic, chemotaxonomic characteristics and 16S rRNA gene sequences similarity (96.0-98.8%). The Gram-positive rod-shaped, spore forming bacteria, *Paenibacillus thailandensis* sp. nov., *P. nanensis* sp. nov., *P. xylanisolvens* sp. nov., *Cohnella thailandensis* sp. nov, *C. xylanilytica* sp. nov. and *C. terrae* sp. nov. were proposed. *Bacillus* sp. P2-3 produced the highest xylanase activity, when compared to other isolates. Thus, P2-3 was selected for further study. Corn cob was found to be the most preferred substrate for xylanase production. After optimization of medium composition and pH, the yield was increased about 2 times when compared with the initial medium. The partially purified xylanase from P2-3 had molecular weight of 17.7 kDa. The enzyme had a maximal activity at 60 °C and pH 6. Stability remained more than 50% at 30–40 °C and pH 3-11. The xylanase activity was activiated by the addition of 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, DTT, and β-Me. In contrast, the xylanase activity was slightly inhibited by Fe^{2+}, PMSF and SDS. The partially purified xylanase had the highest hydrolytic activity toward Oat spelt xylan, but no activity toward β-glucan, carboxymethylcellulose and pectin.

Field of Study: Pharmaceutical Chemistry and Natural Products — Student's Signature Saowapar Khiannggam
Academic Year 2010 — Advisor's Signature Somboon Tanasupawat
Co-Advisor's Signature

ACKNOWLEDGEMENTS

I would like to express the deepest gratefulness to my thesis advisor, Associate. Professor Dr. Somboon Tanasupawat, for his suggestions, comments, and patience, understanding and kindly accepted me as his graduate student. He's my great teacher. All of his advice is very valuable for my life and future career.

My deepest gratitude also to my thesis co-advisors, Dr. Wonnop Visessanguan. He is a scientist actively engaged in research and development. Besides teaching, pushing, stimulating suggestions throughout my work, he has given me opportunities to do research in field of enzymology. During my research at KCTC, I had great deal help from Dr. Jung-Sook Lee, Mr. Keun Chul Lee and Dr. Kwang Kyu Kim. They kindly provided me the laboratory facilities, chemicals and microbiological media. They also corrected the manuscripts.

I have been fully indebted to my former advisor, Associate Professor Dr. Ancharida Acharacharanya, for her supporting, consulting and suggestions. Moreover, she introduced and recommended me to Dr. Somboon, my supervisor.

I also would like to thank Associate Professor Dr. Nongluk Sriubolmas for serving as thesis committee chairperson and Associate Professor Dr. Pintip Pongpech Associate Professor Dr. Ancharida Acharacharanya Assistant Professor Dr. Warangkana Warisnoicharoen and Dr. Somporn Moonmangmee for serving as thesis committee members and their recommendations for the research.

The Royal Golden Jubilee Ph.D. program, 2007 is gratefully acknowledged.

I can never forget the help provided to me by my friends at Dr. Somboon's lab, KCTC and BIOTEC, especially Mrs. Preenapha, Miss Jureeporn, Mr. Dnai and Miss Woranuch, for all their kind helps, suggesting and being the best friendship.

Special thank is directed to Maj. Songdat for their understanding and sharing the stressful moments with me and helping me whenever I needed.

Lastly, and most importantly, I wish to thank my family for their greatest love, encouragement, help and support me to overcome all the challenges and difficulties in this work. I dedicate this thesis to them.

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LIST OF ABBREVATIONS

α	=	Alpha
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
B	=	Beta
BSA	=	Bovine serum albumin
°C	=	Degree celsius
Mm	=	miliimeter
Ca^{2+}	=	Calcium ion
CCD	=	Central composite design
CCM	=	Czech Collection of Microorganisms
CCUG	=	Culture Collection, University of Göteborg, Sweden
CH_3Cl	=	Chloroform
CIP	=	Pasteur Institute Collection, Biological resource Center of Pasteur Institute (CRBIP)
DAP	=	Diaminopimelic acid
DDBJ	=	DNA Data Bank of Japan
DNase	=	Deoxyribonuclease
DSM	=	Deutsche Sammlung von Mikroorganismen
EDTA	=	Disodiumethylenediaminetetraacetate
EMBL	=	European Molecular Biology Laboratory
FAME	=	Fatty acid methyl ester
Fe^{2+}	=	Iron ion
FPLC	=	Fast protein liquid chromatograph
g	=	Gram
G+C	=	Guanine-plus-cytosine
GenBank	=	National Institute of Health genetic sequence database
h	=	Hour
HCl	=	Hydrochloric acid
H_2O	=	water
H_2O_2	=	Hydrogen peroxide
HPLC	=	High performance liquid chromatrography
HPTLC	=	High performance thin layer chromatograhphy

H_2S	=	Hydrogen sulphide
IAM	=	IAM Culture Collection, Center for Cellular and Molecular Research
JCM	=	Japan Collection of Microorganisms
KACC	=	Korean Agricultural Culture Collection
KCTC	=	Korean Collection for Type Culture, Korea
K^+	=	Potassium ion
kDa	=	kilo Dalton
K_2HPO_4	=	Potassium phosphate
KOH	=	Potassium hydroxide
L	=	Liter
LMG	=	Laboratorium voor Microbiologie, Univversiteit Gent
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
meso-DAP	=	*meso*-Diaminopimelic acid
M	=	molar
Min	=	Minute
nt	=	nucleotide
µg	=	Microgram
mg	=	Milligram
Mg^{2+}	=	Magnesium ion
µl	=	Microliter
ml	=	Milliliter
Mn^{2+}	=	Manganese ion
µm	=	Micrometer
mm	=	Millimeter
mM	=	Millimole
MR	=	Methyl red
MW	=	Molecular weight
Na^+	=	Sodium ion
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NBRC	=	NITE Biological Resource Center

NCIMB	=	National Collections of Industrial, Marine and Food Bacteria
nm	=	Nanometer
nov.	=	Novel
%	=	Percent
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PBD	=	Placket-Burman design
PCR	=	Polymerase chain reaction
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PMSF	=	Phenylmethylsulfonyl fluoride
rDNA	=	Ribosomal deoxynucleic acid
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
sec	=	Second
SEM	=	Scanning electron microscope
SDS	=	Sodium dodesylsulfate
sp.	=	Species
SSC	=	Standard sodium citrate
TAE	=	Tris-acetate EDTA
TBE	=	Tris-borate EDTA
TCA	=	Trichloroacetic acid
TEM	=	Transmission electron microscope
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
UV-Vis	=	Ultraviolet-Visible
V_0	=	Void volume
V_e	=	Elution volume
VP	=	Voges-Proskaüer test
v/v	=	volume / volume
v/w	=	volume / weigh
w/v	=	weigh / volume

CHAPTER I
INTRODUCTION

Lignocellulose, the most abundant renewable organic compounds in nature, comprises average 40% cellulose, 33% hemicellulose and 23% lignin by dry weight (Sa-Pereira *et al*., 2002). Xylan is the most abundant of the hemicelluloses which are heteropolysaccharides having a linear backbone of β-1,4-linked xylopyranose residues that often have side chains of O-acetyl, arabinosyl and methylglucuronosyl substituents (Rawashdeh *et al*., 2005). The complete hydrolysis of xylan requires the combined action of various enzymes such as endoxylanase (endo-1,4-β-xylanase, 1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8), β-xylosidase (1,4-β-D-xylan xylohydrolase, E.C. 3.2.1.37) and several accessory enzymes to hydrolyse substituted xylan. The endoxylanase attacks internal xylosidic linkages on the backbone and the β-xylosidase releases xylosyl residues by endwise attack of xylooligosaccharide (Wong *et al*., 1988).

Xylanolytic enzymes occur widely in bacteria, yeasts and fungi. Many microorganisms are known to produce different type of xylanases. The nature of the enzymes varies between different organisms. Among xylan degrading bacteria, the strains of *Aeromonas*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Microbacterium*, *Paenibacillus*, *Ruminococcus* and *Streptomyces* have been reported (Rapp and Wagner, 1986). In addition, xylanase-producing bacteria show optimal activity at different values of pH and temperature. Several extracellular xylanases from bacteria have been studied and characterized *e.g*., *Bacillus firmus* is capable of growth at pH 10-12 and at above 55 ºC (Tseng *et al*., 2002), *B. thermantarcticus*, a thermophillic bacterium growth at 80 ºC (Lama *et al*., 2004) including *B. coagulans* (Wong *et al*., 1988), *B. circulans* (Kyu *et al*., 1994), *B. pumilus* (Duarte *et al*., 2000), *B. subtilis* (Yuan *et al*., 2005), and *B. polymyxa* (Sandhu and Kennedy, 1984). Recently the novel species of *Paenibacillus, P. montaniterrae, P. septentrionalis, P. siamensis* (Khianngam *et al*., 2009), *P. woosongensis* (Lee and Yoon, 2008), *P. soli* (Park *et al*., 2007), *P. cellulosilyticus* (Rivas *et al*., 2006), *P. panacisoli* (Ten *et al*., 2006), *P. xylanilyticus* (Rivas *et al*., 2005), *P. barcinonensis* (Sánchez *et al*., 2005), *P. favisporus* (Valazquez *et al*., 2004); *Microbacterium, M. paludicola* (Park *et al*., 2006), *M. xylanilyticum* (Kim *et al*., 2005) and *M. ulmi* (Rivas *et al*., 2004);

Cellulomonas, C. terrae (An *at al*., 2005), *C. xylanticus* (Rivas *et al*., 2004); *Xylanibacterium ulmi* (Rivas *et al*., 2004) and *Xylanibacter oryzae* (Ueki *et al*., 2006) were proposed as xylanase producer.

In recent years, xylanases have received attractable research interest due to their potential for industrial applications, *e. g.* pretreatment of pulp to boost the bleaching process (Viikari *et al*., 1994), pretreatment of forage crops and other lignocellulosic biomasses to improve nutrient utilization, flour improvement for bakery products, saccharification of hemicellulosic wastes (Gilbert and Hazlewood, 1993), pulp and fiber processing (Yang *et al*, 1995), clarification of juices and wines, extraction of plant oils and coffee (Kulkarni and Shendye, 1999; Uma and Chandra, 2000). However, such applications require xylanase (s) with particular properties, *e.g.* active under high temperature and/or alkaline condition. Bacterial xylanases are generally higher thermostable than fungal xylanases. Most xylanases from fungi have pH optima between 4.5 and 5.5, while bacterial xylanases active at alkaline pH have been reported from *Bacillus* and *Streptomyces* strains (Blanco and Zueco, 1999). Most of industrial processes are carried out at high temperature, so that thermostable enzymes would give an advantage. Thailand is located in the tropical area that is hot and humid, additionally, the relatively diverse soil types and natural high biodiversity of this region which is highly conducive for microbial growth. This work deals with the screening and identifying the xylanolytic bacteria including the optimization, purification and characterization of xylanase of the selected strain isolated from soils.

Research objectives

The main objectives of this present study are as followed:

1. To isolate, screen and identify the xylanolytic bacteria based on the phenotype and genotypic characteristics.
2. To purify and characterize the xylanase of the selected strain.

CHAPTER II

LITERATURE REVIEW

Source of xylan

Lignocellulose is the major component of biomass, comprising around half of the plant matter produced by photosynthesis (also called photomass) and representing the most abundant renewable organic resource in soil. It consists of three types of polymers, cellulose, hemicellulose and lignin (Figure 2.1) that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages. Cellulose and hemicelluloses are macromolecules constructed from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and proportions of these compounds vary between plants.

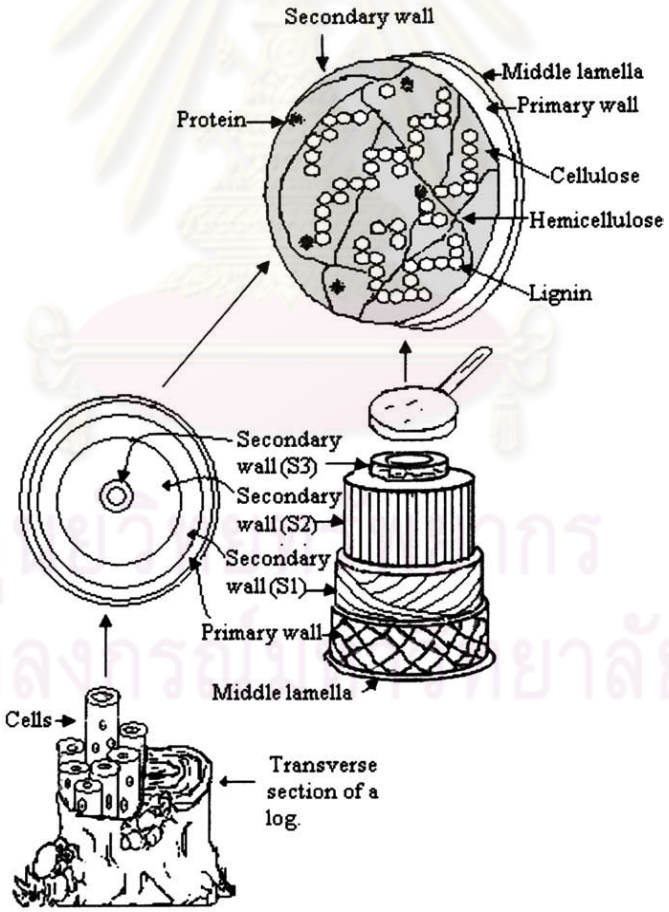

Figure 2.1 Composition of lignocellulosic residues (Sánchez, 2009).

Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellobiose. These form long chains (or elemental fibrils) linked together by hydrogen bonds and van der Waals forces. Cellulose usually is present as a crystalline form and a small amount of non-organized cellulose chains forms amorphous cellulose. Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-*O*-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds. Lignin is a complex polyphenolic polymer. It is linked to both hemicelluloses and cellulose (Sánchez, 2009).

Hemicelluloses are low-molecular-weight polysaccharides and usually considered to be structural polysaccharides. Hemicellulose are heteroglycans and one of the three major naturally plant biomass. Together with cellulose and lignin, hemicellulose built up the supporting material in plant cell wall. It consists of 20-30% hemicellulosic materials which are heterogeneous polysaccharides found in association with cellulose. Those from woody plant are built up from relatively few sugar residues, the most common of which are D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-*O*-methylglucuronic acid, D-galacturonic acid and glucuronic acid. The variety of sugar residues of hemicelluloses from grasses and cereals is smaller; D-xylose, L-arabinose, D-glucose and D-galactose are the most common. In contrast to wood hemicelluloses, however, there is a great variety of linkages and abundance of branching types in graminaceous hemicelluloses, depending on the species and the tissue within a signal species, as well as on the age of the tissue.

Hemicelluloses are usually named according to the main sugar residues in the backbone, such as xylan, glucomannans, galactans and glucans. Xylan is a major polymeric component of the hemicellulose fraction of plant cell wall and is the second most abundant renewable resource with a high potential for degradation to useful end products. Xylans of many plant materials are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic, and *p*-coumaric acids. The frequency and composition of branches are dependent on the source of xylan. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to *O*-2

and/or *O*-3 of xylose residues, and also by oligomeric side chains containing arabinose, xylose, and sometimes galactose residues (Figure 2.2) (Saha, 2003).

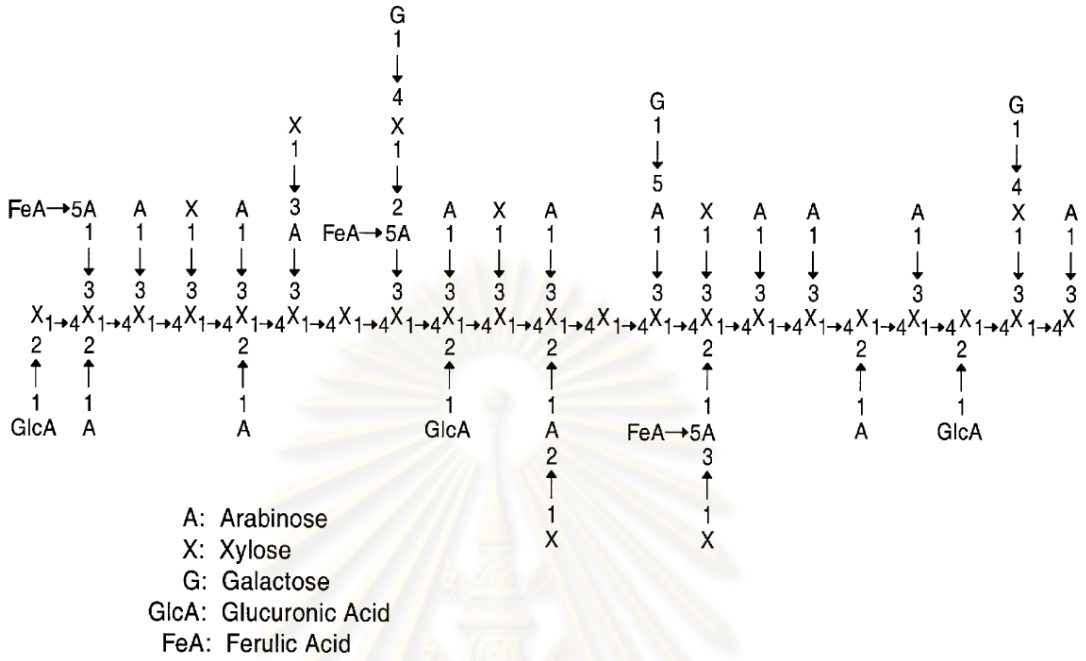

Figure 2.2 Schematic structure of corn fiber xylan (Saha, 2003)

Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 30-35% of the total dry weight. Xylan is the major hemicellulose in hardwood from angiosperms, but is less abundant in softwood from gymnosperms; it accounts for approximately 15-30% and 7-12% of the total dry weight, respectively (Beg *et al*., 2001).

Xylan from hardwood

The xylan from hardwood is *O*-acetyl-4–*O*-methylglucuronoxylan as shown in Figure 2.3. This polysaccharide consists of at least 70 β-xylopyranose residues [average degree of polymerization (DP) between 150 and 200], linked by β-1,4-glycosidic bonds. Every tenth xylose residue carries a 4-*O*-methylglucuronic acid attached to the 2 position of xylose. Hardwood xylans are highly acetylated (e.g., birchwood xylan contains more than 1 mol of acetic acid per 2 mol of xylose). Acetylation is more frequent at the C-3 than at the C-2 position. The presence of these acetyl groups is responsible for the partial solubility of xylan in water. These acetyl

groups are readily removed when xylan is subjected to alkali extraction (Beg *et al*., 2001).

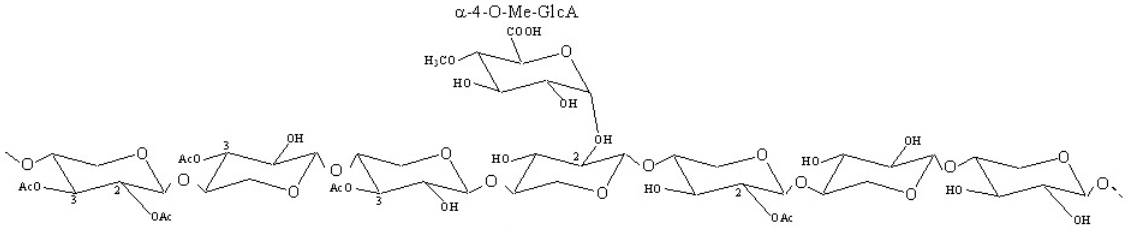

Figure 2.3 Composition of *O*-acetyl-4-*O*-methylglucuronoxylan (hardwood xylan). Numbers indicate the carbon atoms at which substitution take place. Ac, Acetyl group; α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid (Sunna and Antranikian, 1997).

Xylan from softwood

Xylans from softwood are composed of arabino-4-*O*-methylglucuroxylans as shown in Figure 2.4. They have a higher 4-*O*-methylglucuronic acid content than do hardwood xylans. The 4-*O*-methylglucuronic acid residues are attached to the C-2 position. Softwood xylans are not acetylated, and instead of an acetyl group they have α-L-arabinofuranose units linked by α-1,3-glycosidic bonds at the C-3 position of the xylose. The arabinosyl substituents occur on almost 12% of the xylosyl residues (Wong *et al*. 1988). The ratio of β-D-xylopyranose, 4-*O*-methyl-α-D-glucuronic acid and L-arabinofuranose is 100:20:13. Softwood xylans are shorter than hardwood xylans, with a DP between 70 and 130. They are also less branched.

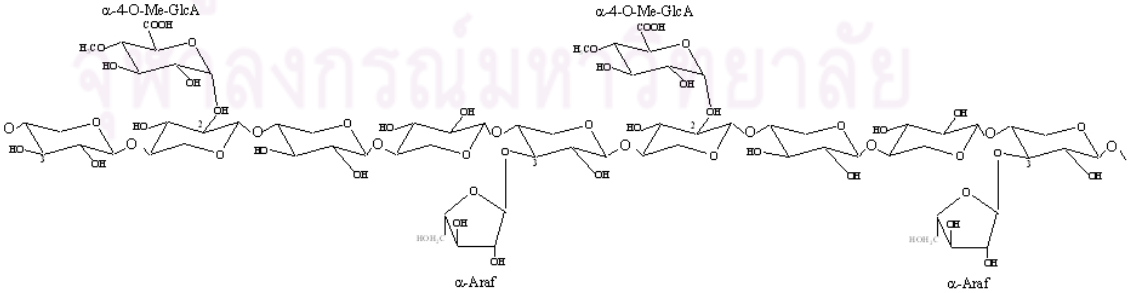

Figure 2.4 Composition of arabino-4-*O*-methylglucuronoxylan (softwood xylan). Numbers indicate the carbon atoms at which substitutions take place. α-Araf; α-arabinofuranose; α-4-*O*-Me-GlcA: a-4-*O*-methylglucuronic acid (Zimbo and Timell, 1997).

Xylan from grass

The xylan of grasses is also arabino-4-*O*-methylglucuronoxylan, degree of polymerization 70. It has less 1,2-linked 4-*O*-methyl-α-D-glucuronic acid than does hardwood xylan but does have a large contene of L-arabinofuranosyl side chain shown in Figure 2.5. there are linked to C-2 or C-3, or both, of the *β-D*-xylanopyranose main-chain residues. In addition, such xylans contain 2.5% by weight of *O*-acetyl groups linked to C-2 or C-3 of the xylopyranose units. Moreover, 6% of the arabinosyl side chains are themselves substituted at position 5 with feruloyl groups, while 3% are substituted with *p*-coumaroyl residues. The relative proportions of the various components of grass arabinoxylans vary from species to species and from tissue to tissue within a single species. The reader should note that the ester-linked substituents may be partially or completely lost from substrates prepared by solubilization in alkali. It should also be noted that in native lignocellulosic materials some or all of the feruloyl substituents may engage in covalent cross-linking of xylan molecules with lignin or with other xylan molecules (Coughlan *et al*., 1993).

Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain and in the side chain. The common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronysyl residues. Homoxylans, on the other hand, consist exclusively of xylosyl residues. This type of xylan is not widespread in nature and has been isolated from esparto grass, tobacco stalks, and guar seed husk. Xylans with β-1,3-linked backbone have been reported in marine algae. The mixed link of β-1,3- and β-1,4-xylans are found in seaweed such as *Palmeria palmate* (Beg *et al*., 2001).

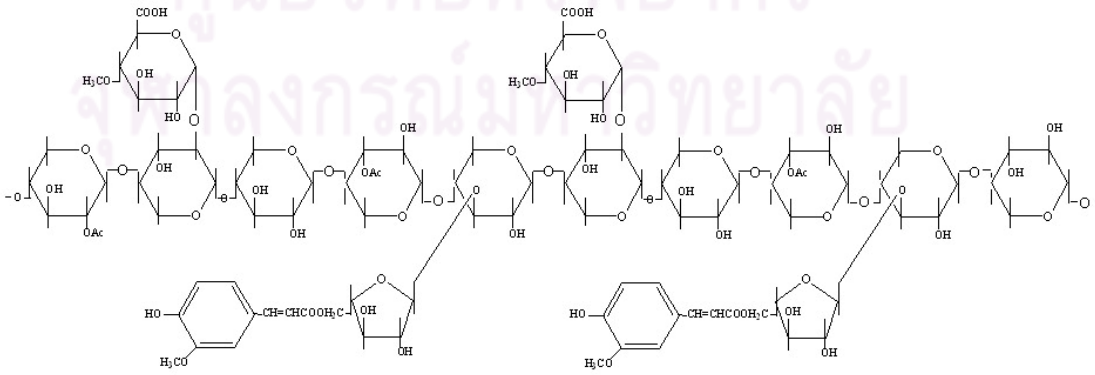

Figure 2.5 Composition of a typical cereal arabino-4-*O*-methylglucuronoxylan (Coughlan *et al*., 1993).

Xylans from different sources, such as grasses, cereals, softwood, and hardwood, differ in composition. Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrouronic acid. Wheat arabinoxylan contains 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose. Corn fiber xylan is one of the complex heteroxylans containing β-(1,4)-linked xylose residues. It contains 48–54% xylose, 33–35% arabinose, 5–11% galactose, and 3–6% glucuronic acid (Saha, 2003).

Xylanolytic enzymes

Several hydrolytic enzymes with diverse specificity and mode of action are required to complete hydrolysis of xylan which is heterogeneity and complex structure. Xylan degrading enzymes are usually composed of the following hydrolytic enzymes: β-1,4-endoxylanase (1,4-β-D-xylan xylohydrolase, E.C. 3.2.1.8), β-xylosidase (1,4-β-D-xylan xylohydrolase, E.C. 3.2.1.37), α-L-arabinofuranosidase, *α*-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Figure. 2.6), which are necessary for hydrolyzing various substituted xylans. Table 2.1 lists the enzymes involved in the degradation of xylan and their modes of action. The endo-xylanase attacks the main chains of xylans, and *β*-xylosidase hydrolyzes xylooligosaccharides to xylose. The *α*-arabinofuranosidase and *α*-glucuronidase remove the arabinose and 4-*O*-methyl glucuronic acid substituents, respectively, from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and *p*-coumaric acid (*ρ*-coumaric acid esterase). All theses enzymes act cooperatively to convert xylan to its constituent sugar. Heteroxylans contain different substituent groups in the backbone and side chain. Therfore, the degradation of such a complex polysaccharide may involve synergistic action between the different components of the xylanolytic enzyme system (Saha, 2003).

Table 2.1 Enzymes involved in the hydrolysis of complex heteroarabinoxylans (Saha, 2003)

Enzyme	Mode of action
Endo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolyzes β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans
ρ-Coumaric acid esterase	Hydrolyzes ρ-coumaryl ester bonds in xylans

Endo-1,4-β-xylanase

The 1,4-β-D-xylan xylohydrolase (β-1,4-endoxylanase, E.C. 3.2.1.8) cleaves the internal glycosidic linkage of the heteroxylan backbone (Figure. 2.6), resulting in a decreased DP of the substrate. The attack of the substrate is not random, and the bonds to be hydrolyzed depend on the nature of the substrate such as length and degree of branching of the substrate or the presence of substituents. During the early course of hydrolysis of xylan, the main products formed are xylooligosaccharides. As hydrolysis proceeds, these oligosaccharides will be further hydrolyzed to xylotriose, xylobiose and xylose. Endo-acting xylanases have been differentiated according to the end products released from the hydrolysis of xylan such as xylose, xylobiose and xylooligosaccharide. Xylanases are produced by many species of fungi and bacteria (Collins *et al*., 2005).

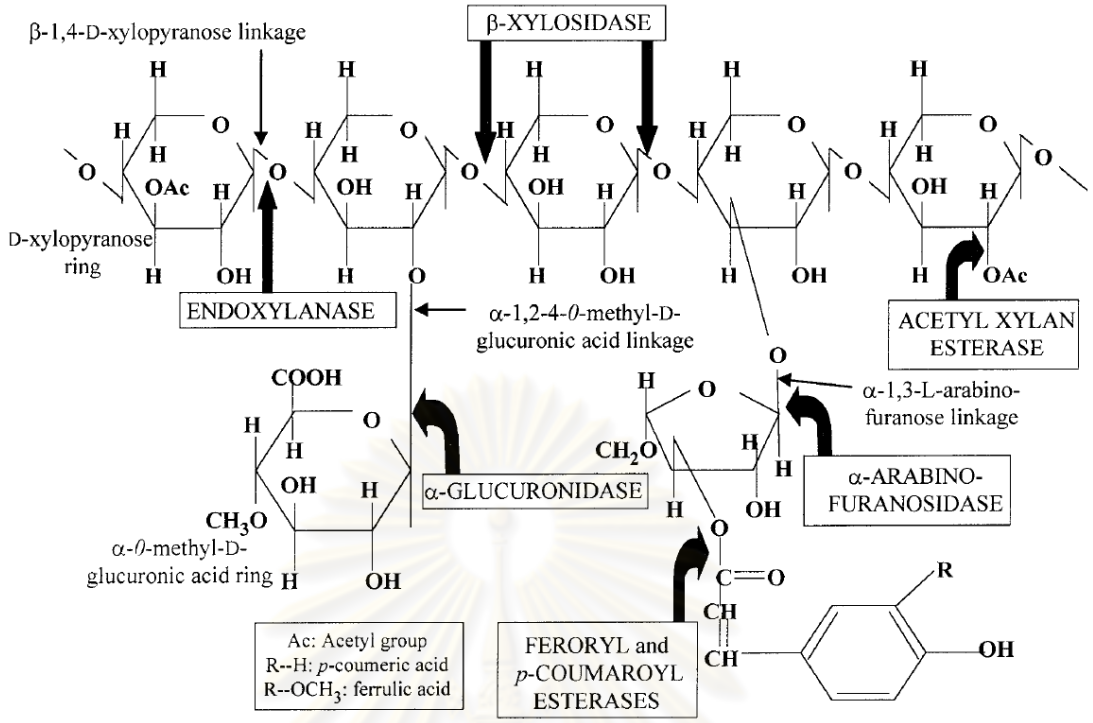

Figure 2.6 A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg *et al*., 2001).

β-Xylosidase

β-D-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) can be classified according to their relative affinities for xylobiose and larger xylooligosaccharides. Xylobiases and exo-1,4-β-xylanases can be recognized as distinct entities, but will be treated as xylosidases, that hydrolyze small xylooligosaccharides and xylobiose, releasing β-D-xylopyranosyl residues from the non-reducing terminus (Figure. 2.6)**.** An important role attributed to β-xylosidases comes into play after the xylan has suffered a number of successive hydrolyzes by xylanase. This reaction leads to the accumulation of short oligomers of β-D-xylopyranosyl, which may inhibit the endoxylanase. β-xylosidase then hydrolyzes these products, removing the cause of inhibition, and increasing the efficiency of xylan hydrolysis (Polizeli *et al*., 2005).

Acetylxylan esterase

Acetylxylan esterase (EC 3.1.1.6) removes the O-acetyl groups from positions 2 and/or 3 on the β-D-xylopyranosyl residues of acetyl xylan (Figure. 2.6). This enzyme was a late discovery, probably because the alkaline extraction frequently

employed with highly acetylated xylans, like those in hardwoods, tends to strip the acetyls from the xylan. Acetylxylan plays an important role in the hydrolysis of xylan, since the acetyl side-groups can interfere with the approach of enzymes that cleave the backbone, by steric hindrance, and their elimination thus facilitates the action of endoxylanases (Polizeli *et al*., 2005).

Arabinase

Arabinase removes L-arabinose residues substituted at positions 2 and 3 of the β-D-xylopyranosyl. There are two types with distinct modes of action: exo-α-L-arabinofuranosidase (EC 3.2.1.55) which degrades *p*-nitrophenyl-α-L-arabinofuranosides and branched arabinans (Figure. 2.6), and endo-1,5-α-L-arabinase (EC 3.2.1.99) which only hydrolyzes linear arabinans. Most arabinases investigated so far are of the exo type (Polizeli *et al*., 2005).

α-Glucuronidase

α-Glucuronidase (EC 3.2.1.-) hydrolyzes the α-1,2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Figure. 2.6). Some microorganisms exhibit their maximum activity only in the presence of short glucuronoxylan substrates. However, the substrate specificity varies with the microbial source, and some glucuronidases are able to hydrolyze the intact polymer. It has also been noted that acetyl groups close to the glucuronosyl substituents can partially hinder the α-glucuronidase activity (Polizeli *et al*., 2005).

Ferulic acid esterase and *p*-coumaric acid esterase

Ferulic acid esterase (EC 3.1.1.-) and *p*-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds on xylan; the first one cleaves between arabinose and ferulic acid sidegroups, while the second one cleaves between arabinose and *p*-coumaric acid (Polizeli *et al*., 2005).

Xylanolytic microorganisms

Xylanases catalyze the hydrolysis of xylans. These enzymes are produced mainly by microorganisms and take part in the breakdown of plant cell walls, along with other enzymes that hydrolyze polysaccharides, and also digest xylan during the germination of some seeds (e.g. in the malting of barley grain). Xylanases also can be found in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Polizeli *et al*., 2005).

Several microorganisms including fungi and bacteria have been reported to be readily hydrolyzing xylans by synthesising 1,4-β-D endoxylanases (E.C.3.2.18) and β-xylosidases (EC.3.2.1.37). Xylanases have been reported in *Bacillus*, *Streptomyces* and other bacterial genera that do not have any role related to plant pathogenicity. Since the introduction of xylanases in paper and pulp and food industries there have been many reports on xylanases from both bacterial and fungal microflora (Subramaniyan and Prema, 2002).

Bacterial xylanases

Bacteria just like in the case of many industrial enzymes fascinated the researchers for alkaline thermostable xylanase producing trait. Noteworthy members producing high levels of xylanase activity at alkaline pH and high temperature are *Bacillus* spp. *Bacillus circulans* was reported xylanase with an activity of 400 IU/ml. It had optimum activity at pH 7 and 40% of activity was retained at pH 9.2. *Streptomyces cuspidosporus* produced 40-49 U/ml in xylan medium. *Bacillus* sp. strain NCL 87-6-10 produced 93 U/ml of xylanase in the zeolite induced medium which was more effective than Tween 80 medium. Another *Bacillus* sp. *Bacillus circulans* AB 16 produced 19.28 U/ml of xylanase when grown on rice straw medium. *Streptomyces* sp. QG-11-3 was found to be producing xylanase (96 U/ml). *Rhodothermus marinus* was found to be producing thermostable xylanases of approximately 1.8-4.03 IU/ml. The strict thermophilic anaerobe *Caldocellum saccharolyticum* possesses xylanases with optimum activities at pH values 5.5-6.0 and at temperature 70 ºC (Subramaniyan and Prema, 2002).

Fungal xylanases

There has been increased usage of xylanase preparations having an optimum pH<5.5 produced invariably from fungi. The optimum pH for xylan hydrolysis is around 5 for most of the fungal xylanases although they are normally stable at pH 3-8. Most of the fungi produce xylanases, which tolerate temperatures below 50 ºC. In general, with rare exceptions, fungi reported to be producing xylanases have an initial cultivation pH lower than 7. Nevertheless it is different in the case of bacteria. The pH optima of bacterial xylanases are in general slightly higher than the pH optima of fungal xylanases. In most of the industrial applications, , the low pH required for the optimal growth and activity of xylanase necessitates additional steps in the subsequent stages which make fungal xylanases less suitable. *Trichoderma viride* was reported xylanase activity (188.1 U/ml, optimum pH 5.2). Similar to *T.viride*, *T. reesei* was also known to produce higher xylanase activity approximately 960 IU/ml. Like *Trichoderma* spp., *Schizophillum commune* is also one of the high xylanase producers with a xylanase activity of 1244 U/ml. Among white rot fungi, a potent plant cell wall degrading fungus *Phanerochaete chrysosporium* produced a xylanase activity of 15-20 U/ml in the culture medium. *Aspergillus niger* sp. showed only 76.60 U/ml of xylanase activity after 5.5 days of fermentation (Subramaniyan and Prema, 2002).

Furthermore, a wide variety of bacteria, fungi, yeasts, and actinomycetes are known to produce xylan-degrading enzymes (Table 2.2).

Table 2.2 Characteristics of xylanase from microorganisms (Beg *et al*., 2001)

Microorganism	Molecular weight (kDa)	Optimum		Stability	
		pH	Temperature (°C)	pH	Temperature (°C)
Bacteria					
Acidobacterium capsulatum	41	5	65	3–8	20–50
Bacillus sp. W–1	21.5	6	65	4–10	40
Bacillus circulans WL–12	15	5.5–7	–	–	–
Bacillus stearothermophilus T–6	43	6.5	55	6.5–10	70
Bacillus sp. strain BP-23	32	5.5	50	9.5–11	55
Bacillus sp. strain BP-7	22–120	6	55	8–9	65
Bacillus polymyxa CECT 153	61	6.5	50	–	–
Bacillus sp. strain K-1	23	5.5	60	5–12	50–60
Bacillus sp. NG-27	–	7, 8.4	70	6–11	40–90
Bacillus sp. SPS-0	–	6	75	6–9	85
Bacillus sp. strain AR-009	23, 48	9–10	60–75	8–9	60–65
Bacillus sp. NCIM 59	15.8, 35	6	50–60	7	50
Cellulomonas fimi	14–150	5–6.5	40–45	–	–
Cellulomonas sp. N.C.I.M. 2353	22, 33, 53	6.5	55	–	–
Micrococcus sp. AR-135	56	7.5–9	55	6.5–10	40
Staphylococcus sp. SG-13	60	7.5, 9.2	50	7.5–9.5	50
Thermoanaerobacterium sp. JW/SL–YS 485	24–180	6.2	80	–	–
Thermotoga maritima MSB8	40, 120	5.4, 6.2	92–105	–	–
Fungi					
Acrophialophora nainiana	17	6	50	5	50
Aspergillus niger	13.5–14.0	5.5	45	5–6	60
Aspergillus kawachii IFO 4308	26–35	2–5.5	50–60	1–10	30–60
Aspergillus nidulans	22–34	5.4	55	5.4	24–40
Aspergillus fischeri Fxn1	31	6	60	5–9.5	55
Aspergillus sojae	32.7, 35.5	5, 5.5	60, 50	5–8, 5–9	50, 35

Table 2.2 (continued)

Microorganism	Molecular weight (kDa)	Optimum		Stability	
		pH	Temperature (°C)	pH	Temperature (°C)
Aspergillus sydowii MG 49	30	5.5	60	–	–
Cephalosporium sp.	30, 70	8	40	8–10	–
Fusarium oxysporum	20.8, 23.5	6	60, 55	7–10	30
Geotrichum candidum	60–67	4	50	3–4.5	45
Paecilomyces varioti	20	4	50	–	–
Penicillium purpurogenum	33, 23	7, 3.5	60, 50	6–7.5, 4.5–7.5	40
Thermomyces lanuginosus DSM 5826	25.5	7	60–70	5–9	60
Thermomyces lanuginosus–SSBP	23.6	6.5	70–75	5–12	60
Trichoderma harzianum	20	5	50	–	40
Trichoderma reesei	20, 19	5–5.5, 4–4.5	45, 40	3–8.5, 2.5–8.5	–
Yeast					
Aureobasidium pullulans Y-2311–1	25	4.4	54	4.5	55
Cryptococcus albidus	48	5	25	–	–
Trichosporon cutaneum SL409	–	6.5	50	4.5–8.5	50
Actinomycete					
Streptomyces sp. EC 10	32	7–8	60	–	–
Streptomyces sp. B–12–2	23.8–40.5	6–7	55–60	–	–
Streptomyces T7	20	4.5–5.5	60	5	37–50
Streptomyces thermoviolaceus OPC–520	33, 54	7	60–70	–	–
Streptomyces chattanoogensis CECT 3336	48	6	50	5–8	40–60
Streptomyces viridisporus T7A	59	7–8	65–70	5–9	70
Streptomyces sp. QG-11-3	–	8.6	60	5.4–9.2	50–75
Thermomonospora curvata	15–36	6.8–7.8	75	–	–

Industrial applications

Xylanolytic enzymes from microorganism have attracted a great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes, such as food, feed, and pulp and paper industries. Xylanases have shown an immense potential for increasing the production of several useful products in a most economical way. The main possibilities are the production of SCPs, enzymes, liquid or gaseous fuels, and solvents and sugar syrups, which can be used as such or as feed stock for other microbiological processes (Beg *et al*., 2001):

Currently, the most promising application of xylanases is in the prebleaching of kraft pulps. Enzyme application improves pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps. Xylanases are also useful in yielding cellulose from dissolving pulps for rayon production and biobleaching of wood pulps.

Depression in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity. Incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency.

The efficiency of xylanases in improving the quality of bread has been seen with an increase in specific bread volume. This is further enhanced when amylase is used in combination with xylanase.

Xylan is present in large amounts in wastes from agricultural and food industries. Hence, xylanases are used for conversion of xylan into xylose in waste water. The development of an efficient process of enzymatic hydrolysis offers new prospects for treating hemicellulosic wastes.

Xylanase treatment of plant cells can induce glycosylation and fatty acylation of phytosterols. Treatment of tobacco suspension cells (*Nicotiana tabacum* CV. KY 14) with a purified endoxylanase from *Trichoderma viride* caused a 13-fold increase in the levels of acylated sterol glycosides and elicited the synthesis of phytoalexins.

α-L-Arabinofuranosidase and β-D-glucopyranosidase have been employed in food processing for aromatizing musts, wines, and fruit juice.

Some xylanases may be used to improve cell wall maceration for the production of plant protoplasts.

A recent application of a truncated bacterial xylanase gene from *Clostridium thermocellum* has been demonstrated in rhizosecretion in transgenic tobacco plants.

Xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc., can be used for the generation of biological fuels, such as ethanol and xylitol, from lignocellulosic biomass. The biological process of ethanol fuel production requires delignification of lignocellulose to liberate cellulose and hemicellulose from their complex with lignin, followed by depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and finally fermentation of mixed pentose and hexose sugars to produce ethanol.

A potential application of the xylanolytic enzyme system in conjunction with the pectinolytic enzyme system is in the degumming of bast fibers such as flax, hemp, jute and ramie. A xylanase-pectinase combination is also used in the debarking process, which is the first step in wood processing. The fiber liberation from plants is affected by retting, i.e., the removal of binding material present in plant tissues using enzymes produced in situ by microorganisms. Pectinases are believed to play a major role in this process, but xylanases may also be involved. Replacement of slow natural retting by treatment with artificial mixtures of enzymes could become a new fiber liberation technology in the near future.

Xylanase are used concurrently with cellulase and pectinase for clarifying must and juices, and for liquefying fruits and vegetables, and in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting.

Alkyl glycosides are one of the most promising candidates for new surfactants. Commercially, they are produced from monomeric sugars such as D-glucose and a fatty alcohol. But the direct glycosylation using polysaccharide is more feasible for their industrial production, because hydrolysis of polysaccharide and subsequent steps can be omitted. Thus, use of xylanase in this process provides a challenging opportunity. Recently, xylanase from *Aureobasidium pullulans* has been used for direct transglycosylation of xylan, 1-octanal and 2-ethyl hexanol into octyl-β-D-

xylobioside, xyloside, and 2-ethylhexyl-β-D-xylobioside, respectively (Beg *et al*., 2001).

The use of xylanases in biotechnological applications has stimulated the search for enzymes with high temperature optima and/or alkaline pH optima. Fungal xylanases are generally less thermostable than bacterial xylanases. Most xylanases from fungi have pH optima between 4.5 and 5.5. Xylanases from actinobacteria are active at pH 6.0-7.0. However, xylanases with alkaline pH optima have been described from *Bacillus* sp. and *Streptomyces viridosporus* (Sa-pereira *et al*., 2002). The recent works on isolation of xylanase-producing bacteria and actinobacteria, their xylanase properties, and optimal conditions for the xylanase-production are listed below.

Hurlbert and Preston (2001) reported functional characteristics of novel xylanase (Xylanase A) produced by *Erwinia chrysanthemi* D1 isolated from corn. The xylanase A was found to have molecular mass of 42 kDa, isoelectric point of 8.8, and optimal pH and temperature of 6 and 35 °C. The enzyme was still active at temperature higher than 40 ºC and pH of up to 9.0. It was most active on xylan substrates with low ratio of xylose to 4-*O*-methyl-D-glucuronic acid. Mode of action was unique with no internal cleavages of the xylan backbone between substituted xylose residues.

Sa-pereira *et al*. (2002) isolated xylanolytic *Bacillus subtilis* from hot-spring. Oat spelt xylan was used as xylanase inducer in culture medium. Optimal xylanase production of about 12 units/ml was achieved at pH 6.0, 50 °C within 18 h fermentation. Xylanase production decreased as function of time when xylan was used as substrate. But with trehalose as carbon source, xylanase production maintained constant for at least 80 h. Optimal xylanolytic activity was reached at 60 °C in phosphate buffer pH 6.0. The xylanase was completely stable at 60 °C for 3 h. Under optimized fermentation conditions, no cellulolytic activity was detected. Protein disulfide reducing agents, *e. g.* DTT, enhanced xylanolytic activity about 2.5 fold.

Techapun *et al*. (2003) isolated cellulase-free xylanase producing actinobacteria, *Streptomyces* sp., from agricultural wastes. Their xylanase produced from cane bagasse was active and stable at temperature of 50-80 ºC, active at alkaline pH (pH 7-9), and half-life at 70 °C, pH 9.0 was 5 h.

Roy and Uddin (2004) isolated xylanase producing bacteria, *Paenibacillus* sp. from soil in Bangladesh. The molecular weight of the purified xylanase was 48 kDa. The optimum temperature and pH of the purified enzyme were 55 °C and pH 7.0, respectively.

Roy (2004) isolated xylanase-producing *Bacillus* sp. from soil in Bangladesh. The *Bacillus* sp., grown in xylan medium at pH 7.0, produced xylanase at 55 units/ml. Maximal enzyme activity was obtained by cultivation in oat spelt xylan, but high enzyme production was also obtained on wheat bran. The pH optimum and temperature optimum of the xylanase were between pH 6 and 7, and at 50 °C (pH 7.0), respectively. The enzyme could not hydrolyse cellulose, carboxymethyl-cellulose and starch.

Rawashdeh *et al*. (2005) isolated xylanase producing actinobacteria, *Streptomyces* sp., from soil in Jordan, and studied the effect of some cultural conditions on the xylanase production. Maximal xylanase production was obtained when oat spelt xylan was used as a carbon source. When tomato pomace was used as carbon and nitrogen source, the maximal xylanase production was 1,447 units/ml. The crude enzyme was maximally active at pH 6.5 and 60 °C.

Virupakshi *et al*. (2005) isolated thermostable alkaline xylanase producing *Bacillus* from sugarcane molasses. Xylanase production from various agricultural wastes (wheat bran, rice bran, sugarcane bagasse, ragi husk, gram bran, corn cob) by solid-state fermentation was studied. Maximal xylanase was produced in rice bran moistened with mineral salt solution at 50 °C for 72 h. Yeast extract, beef extract and xylan enhanced enzyme production, while glucose, lactose and fructose strongly repressed the enzyme production.

Characterization of xylanolytic bacteria

Characteristics of *Bacillus*

Bacillus strains in *B. firmus* (Tseng *et al*., 2002), *B. pumilus* (Duarte *et al*., 2000), *B. subtilis* (Yuan *et al*., 2005), *B. polymyxa* (Sandhu and Kennedy, 1984), *B. coagulans* (Wong *et al*., 1988), *B. thermantarcticus* (Lama *et al*., 2004), *B. circulans* (Kyu *et al*., 1994), *B. licheniformis* (Archana and Satyanarayana, 1997) and *B. stearothermophilus* (Khasin *et al*., 1993) were reported to produce xylanase. They were rod-shaped and straight, 0.5-2.5 × 1.2-10 μm, and arranged in pairs or chains,

with rounded or squared ends. Cells were Gram-positive and were motile by peritrichous flagella. Endospores were oval or sometimes round or cylindrical and were very resistant to many adverse conditions. They were aerobic or facultatively anaerobic, chemoorganotrophs, with a fermentative or respiratory metabolisms. Usually, catalase was positive. Found in a wide range of habitats; a few species were pathogenic to vertebrates or invertebrates (Holt *et al*., 1994). They contained DAP in the cell wall and a major menaquinone (MK-7). DNA G+C contents ranged from 32-69 mol% (Claus and Berkeley, 1986)**.** Characteristics of some xylanolytic *Bacillus* species were shown in Table 2.3.

Table 2.3 Characteristics of *Bacillus* species (Archana and Satyanarayana, 1997; Duarte *et al*., 2000; Kyu *et al*., 1994; Tseng *et al*., 2002; Yuan *et al*., 2005.)

+, positive; -, negative.

Characteristics	***B. licheniformis*** ATCC 14580^T	***B. subtilis*** IAM 1026^T	***B. pumilus*** ATCC 7061^T	***B. circulans*** ATCC 4513^T	***B. firmus*** ATCC 14575^T
Enzyme production:					
β-Galactosidase	+	-	-	+	+
Arginine dihydrolase	+	-	-	-	-
Oxidase	+	+	+	-	-
Acetoin production	-	-	+	-	-
Galatin liquefication	-	+	-	-	-
Utilization of:					
Mannitol	-	-	-	+	+
Amygdalin	-	-	+	+	-
Fermentation of:					
Ribose	+	+	+	-	-
D-Xylose	-	-	+	+	-
Adonitol	-	+	-	-	-
Galactose	-	-	+	+	-
Inositol	+	+	+	+	-
Sorbitol	+	+	-	+	-
N-Acetylglucosamine	+	-	+	+	+
Lactose	-	-	+	-	-
Melibiose	-	+	+	+	-
Melezitose	-	-	-	+	-
Raffinose	-	+	-	-	-
Starch	-	+	-	+	-
Glycogen	-	+	-	+	-
Gentiobiose	-	+	+	+	-
D-Turanose	-	+	+	+	+
D-Lyxose	-	-	-	+	-
D-Tagatose	+	-	+	-	-
Gluconate	+	-	-	+	-

Characteristics of *Paenibacillus*

Paenibacillus species that produced xylanase, such as *P. barcinonensis*, *P. cellulosilyticus*, *P. favisporus*, *P. montaniterrae*, *P. panacisoli*, *P. phyllosphaerae*, *P. septentrionalis*, *P. siamensis*, *P. soli*, *P. tundrae*, *P. woosongensis*, *P. xylanexedens* and *P. xylanilyticus* (Velázquez *et al*., 2004; Rivas *et al*., 2005; 2006; Sánchez *et al*., 2005; Ten *et al*., 2006; Park *et al*., 2007; Lee and Yoon, 2008; Khianngam *et al.*, 2009; Nelson *et al*., 2009). They were Gram-variable, rod-shaped and motile with peritrichous flagella. They produced ellipsoidal spores in swollen sporangia. Colonies formed circular, flat, convex, smooth. They are facultatively anaerobic or strictly aerobic rod shaped, and have G+C contents ranging from 45 to 54 mol%. Some of these organisms excrete diverse assortments of polysaccharide-hydrolysing enzymes and produce antibacterial compounds such as polymyxin, octopytin baciphelacin and an antifungal compounds. The major isoprenoid quinone was menaquinone MK-7 and major cellular fatty acid was 12-methyltetradecanoic acid. Cell-wall peptidoglycan contained *meso*-diaminopimelic acid. (Berge *et al*., 2002; Lee *et al.,* 2002; Takeda *et al*., 2002). Characteristics of *Paenibacillus* were shown in Table 2.4.

Table 2.4 Characteristics of *Paenibacillus* species. Strain: *P.barcinonensis* BP-23^T (Sánchez *et al*., 2005), *P. xylanilyticus* LMG 21957^T (Rivas *et al*., 2005), *P.cellulosilyticus* PALXIL08^T (Rivas *et al*., 2006), *P.phyllosphaerae* PALXIL04^T (Rivas *et al*., 2005), *P.siamensis* S5-3^T and *P. septentrionalis* X13-1^T (Khianngam *et al*, 2009)

+, positive; -, negative; w, weakly positive; nd, not determine

Characteristic	**BP-23^T**	**LMG21957^T**	**PALXIL08^T**	**PALXIL04^T**	**S5-3^T**	**X13-1^T**
Growth in 5%NaCl	+	+	-	-	+	+
Catalase	+	+	+	+	+	+
Oxidase	-	-	+	+	+	-
Citrate utilization	-	-	-	-	-	-
Voges-Proskauer	nd	-	+	-	+	+
Nitrate reduction	-	-	-	-	-	-
Urease	-	-	-	-	+	+
Hydrolysis of:						
Gelatin	+	+	-	nd	-	-
Starch	-	+	+	+	+	+
Acid production from:						
N-acetylglucosamine	+	+	-	-	-	-
Amygdaline	+	nd	-	+	+	-
L-Arabinose	+	+	+	+	+	-
Gluconate	+	nd	+	+	-	-
Glucose	+	+	+	+	-	+
D-Maltose	+	+	+	+	+	-
D-Mannitol	+	+	-	+	+	-
D-Mannose	+	+	+	w	-	-
D-Melibiose	+	+	+	+	+	-
D-Raffinose	+	nd	+	+	+	-
D-Rhamnose	-	+	+	w	+	-
D-Sorbitol	-	-	-	-	-	-
D-Sucrose	+	+	+	+	+	-
D-Xylose	-	+	+	+	+	-
DNA G +C %mol	45	50.5	51	50.7	45.8	47.3

Characteristics of *Cohnella*

Cohnella fontinalis was reported to produce xylanase (Shiratori *et al*., 2010). Cells were Gram-positive, aerobic, endospore-forming rods measuring 0.5-0.7 × 1.5-6.5 μm. Motile by means of peritrichous flagella. Grew occurs at 25-55 °C and pH 5·5-8·5. Grew occurs at NaCl concentrations of up to 2·0 % (w/v). Positive for catalase, oxidase, nitrate reduction, hydrolysis of xylan and aesculin while that of DNA, starch, agar, chitin, cellulose, casein, gelatin and tyrosine was negative. Negative for indole production, hydrogen sulfide production, urease and arginine dihydrolase. The major isoprenoid quinone was MK-7. The major fatty acids of were anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Predominant polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The genomic G+C content was 58·6 mol%.

Characteristics of *Cellulomonas*

The strains of *Cellulomonas xylanticus* (Rivas *et al*., 2004), *C. persica* (Elberson *et al*., 2000), *C. terrae* (An *et al*., 2005) and *C. uda* were reported to produce xylanase (Rapp and Wagner, 1986). Cells were Gram positive, slender, irregular rods, 0.5-0.6 × 2.0-5.0 μm, straight or slightly curved in young cultures; some rods were in pairs at an angle to each other giving V formation; rod occasionally showed branching, but no mycelium was formed. Motile by one or a few flagella, non-spore forming, non-acid-fast, facultative anaerobes, but some grew very poorly anaerobically. Growth on peptone-yeast extract agar gives usually convex, yellow colonies. They were chemoorganotroph, respiratory and also fermentative, giving acid from glucose and various other carbohydrates, both aerobic and anaerobic. Catalase and cellulolytic were positive. Nitrate was reduced to nitrite. The optimum temperature was 30 °C. Widely distributed in soils and decaying vegetable matters (Holt *et al*., 1994). They contained L- Orn-D-Glu in the cell wall and had major menaquinones, (MK-8(H_4) and (MK-9(H_4). The DNA G+C contents of were 72-76 mol% (Rivas *et al*., 2004).

Characteristics of *Clostridium*

Clostridium algidixylanolyticum (Broda *et al*., 2000), *C. xylanovorans* (Mechichi *et al*., 1999), *C. xylanolyticum* (Chamkha *et al*., 2001), *C. acetobutylicum*

and *C. stercorarium* strains (Wong *et al*., 1988) were reported to produce xylanase. They were rod-shaped, 0.3-2.0 × 1.5-20.0 μm, and were often arranged in pairs or short chains, with rounded or sometimes pointed end, commonly pleomorphic. They were Gram positive in young cultures, usually motile by peritrichous flagella, form oval or spherical endospores usually distend the cell. Most species were chemoorganotrophic; some were chemoautotrophic or chemolithotrophic as well. May be saccharolytic, proteolytic, neither, or both. Usually they produced mixtures of organic acids and alcohols from carbohydrates or peptones. Did not carry out a dissimilatory sulfate reduction. Usually, catalase was negative and obligately anaerobic; if growth occurred in air, it was scanty and sporulation was inhibited. Metabolically they were very diverse, with optimum temperatures of 10-65 °C (Holt *et al*., 1994). The organisms of these genera were characterized by the presence of the *meso*-diaminopimelic acid in the cell wall, and by G+C contents of 39-43 mol% (Fardeau *et al*., 2001).

Characteristics of *Microbacterium*

Microbacterium species that produced xylanase, such as the strains of *M. ulmi* sp. nov., *M. xylanilyticum* sp. nov. and *M. paludicola* sp. nov had reported (Rivas *et al*., 2004; Kim *et al*., 2005; Park *et al*., 2006). They were slender, irregular rods in young cultures, 0.4-0.8 x 1.0-4.0 μm, arranged singly or in pair, when some were arranged at an angle to give V formation. Primary branching was uncommon, and mycelia were not produced. In old cultures, rods were shorter and cocci, but there was no marked rod-coccus cycle, Gram-positive, non-acid-fast, non-spore forming, non-motile or motile by one to three flagella. Aerobic; weak anaerobic growth may occur. On yeast extract-peptone-glucose agar, colonies were opaque glistening, often with yellowish pigmentation. Chemoorganotrophic, metabolisms primarily respiratory but might be weakly fermentative. Acid was produced from glucose and some other carbohydrates. Nutritional requirements were complex. Catalase was positive. The optimum growth temperature was 30 ºC (Holt *et al*., 1994). The organisms of these genera were characterized by the presence of *N*-glycolyl residues in the cell wall, by having major isoprenoid quinones MK-11, MK-12 and MK-13 and/ or MK-14, and by G+C contents of 65-76 mol% (Takeuchi and Hatano, 1998). Characteristic of *Microbacterium* sp. were shown in Table 2.5.

Table 2.5 Characteristics of *Microbacterium* (Rivas *et al*., 2004; Kim *et al*., 2005; Park *et al*., 2006)

Characteristics	***M. ulmi* LMG 20991^T**	***M. xylanilyticum* DSM 16914^T**	***M. paludicola* DSM16915^T**
Colony colour	White	Yellow	Lemon-yellow
Catalase	-	+	+
Oxidase	-	+	+
Nitrate reduction	-	+	-
Hydrolysis of :			
Casein	+	-	-
Urea	-	-	+
Chemotaxonomic:			
Whole cell sugar	Gal, Fuc, Xyl, Rha	Gal, Gal	Gal, Gal, Man, Rha, Fuc
Major fatty acids	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$	ai-$C_{15:0}$, i-$C_{16:0}$, ai-$C_{17:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$
Major menaquinones	MK-12, MK-13, MK-11, M-14, MK-10	MK-11, MK-12, MK-13	MK-11, MK-12
% mol G+C	69	69.7	66.5

Characteristics of *Bacteriodes*

Bacteroides strains were rod-shaped organisms of variable size with pleomorphic and showed terminal or central swellings, vacuoles, or filaments, non-motile, anaerobic, chemoorganotrophic, metabolizing carbohydrates, peptone, or metabolic intermediates. Especially with strongly saccharolytic species, fermentation products include acetate, succinate, lactate, formate, or propionate. Butyrate was not usually a major product, but when it was formed it was accompanied by isobutyrate and isovalerate. Many species contain high level of branched chain fatty acids, generally anteiso-$C_{15:0}$ acids, and also sphingolipids. Hemin and Vitamin K were highly stimulatory for the growth of many species and were generally added to media for growth of habitats: gingival crevice, intestinal tract (cecum and rumen), sewage sludge, and infective and purulent conditions in human and animal**s** (Holt *et al*., 1994). The organisms of this genus were characterized by having major menaquinone (MK-10) and (MK-11), and by G+C contents of 39-42 mol% (Miyamoto and Itoh, 2000). *B. xylanolyticus* were reported to produce xylanase (Scholten-Koerselman *et al*., 1988).

Characteristics of *Thermotoga*

Thermotoga maritime strain was reported to produce xylanase (Beg *et al*., 2001). This bacterium was rod-shaped and had a characteristic outer sheath-like structure which could be observed under in situ conditions. Members of the recently described genera *Geotoga* and *Petrotoga* also possessed this morphological feature and, as determined by a 16S rRNA sequence analysis, were distantly related to members of the *Thermotogales*. Collectively, the five genera mentioned above represented one of the deepest phylogenetic branches in the domain *Bacteria*. These taxa could be differentiated on the basis of their optimum temperatures for growth; *Thermotoga* species were extreme thermophiles that have optimum temperatures for growth of around 80 °C, *Thermosipho* and *Fervidobacterium* species had optimum temperatures for growth of 65 to 75 °C and were regarded as thermophiles, and *Geotoga* and *Petrotoga* species were moderate thermophiles having optimum temperatures for growth of less than 60 °C. Until recently, members of the three genera belonging to the order *Thermotogales* (*Thermotoga*, *Thermosipho*, and *Fervidobacterium*) had been isolated only from volcanic aquatic environments. Different species had different sodium chloride requirements and optimum temperatures for growth. These differences reflected the restricted ecological habitats (hydrothermal marine environments, hydrothermal terrestrial environments) from which the organisms were isolated. Round colonies (diameter, 1 mm) were present after 7 days of incubation at 60 °C. Cells were rods (0.5 to 1 by 2 to 3 mm), and each cell had an outer sheath-like structure (toga). The cells occurred singly or in pairs and had peritrichous flagella. The cell wall was Gram negative, as determined by electron microscopy or Gram staining. Chemoorganotrophic and obligately anaerobic members of the domain *Bacteria*. The G+C contents of the DNA was 29-46 mol% (Ravot *et al*, 1995).

Characteristics of *Ruminococcus*

Ruminococus albus and *R. flavefaciens* were reported to produce xylanase (Cotta and Zeltwanger, 1995). They were spherical or slightly elongated, might have pointed ends 0.3-1.5 x 0.7-1.8 μm, and arranged in pairs and chain. Might motile with 1-3 flagella per cell, non spore-forming, stain weakly, Gram-positive or Gram-negative, though cell wall structure is of the Gram-positive type. Strict

anaerobes requiring special methods for study. Chemoorganotrophs with a fermentative metabolisms, utilizing carbohydrates with the production of mixed acids, ethanol, CO_2 , and H_2. Catalase negative; nitrate was not reduced, and ammonia was not produced from amino acids. Growth occurred at a temperature 20-45 °C (optimum 40 °C). In habit the rumen, large bowel, and cecum of mammals (Holt *et al*., 1994).

Characteristics of *Thermobacillus*

Thermobacillus xylanilyticus, a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil situated underneath a manure heap in northern France was reported. Cells were Gram-negative, aerobic, thermophilic, xylanolytic and spore-forming short rods, occurred sometimes in chains. Spores were ellipsoidal, central to subterminal and occurred in swollen sporangia. It grew at temperatures up to 63 °C and in the pH range 6.5–8.5. When grown on glucose in optimal conditions, its doubling time was found to be 33 min. CO_2 was observed to have a growth-stimulating effect at the start of the culture. In addition to glucose, the isolate utilized xylose, arabinose, mannose, cellubiose, galactose, maltose, sucrose, xylan and starch. Growth was inhibited by 5% NaCl. The DNA G+C content of strain was 57.5 mol% (Touzel *et al*., 2000).

Characteristics of *Xylanibacter*

The strain, *Xylanibacter oryzae* KB3^T is strictly anaerobic, xylanolytic bacterium. Cells were Gram-negative, non-motile, non-spore-forming, short to filamentous rods. Growth of the strain was remarkably stimulated by the addition of haemin to the medium. The strain utilized various sugars including xylan, xylose, pectin and carboxymethylcellulose and produced acetate, propionate and succinate with a small amount of malate. Propionate production was stimulated by the addition of a B-vitamin mixture or cobalamin to the medium. The strain was slightly acidophilic with an optimum pH 5–6 and the optimum growth temperature was 30 ℃. Oxidase, catalase and nitrate reduction were negative. Aesculin was hydrolysed. The major cellular fatty acids were anteiso-$C_{15:0}$ and iso-3-OH $_{C17:0}$. The major respiratory quinones were menaquinones MK-12(H_2) and MK-13(H_2). The DNA G+C content was 43.6 mol% (Ueki *et al*., 2006).

In addition, *Cotta and Zeltwanger* (1995) reported the prodeominant species of xylanolytic ruminal bacteria included *Butyrivibrio fibrisolvens, Fibrobacter succinogenes, Eubacterium ruminantium* and *Prevotella ruminicola.* Furthermore, Beg *et al*. (2001) reported that *Acidobacterium capsulatum*, *Micrococcus* sp, AR-135, *Staphylococcus* SG-13 and *Thermoanaerobacterium* JW/SL-YS485 produced xylanases.

Characteristics for bacterial identification

In principle, all genotypic, phenotypic, and phylogenetic information may be incorporated in polyphasic taxonomy. Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell, whereas phenotypic information is derived from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features. The number of different molecules which have been applied in taxonomic studies is large, and their applications as markers are manifold. The taxonomic information level of some of these techniques is illustrated in Figure. 2.7 Obviously, typing methods such as restriction enzyme patterning, multilocus enzyme electrophoresis, and serological analyses are not useful for phylogenetic studies, whereas rRNA or protein sequencing is, in general, not adequate to type large numbers of strains. Chemotaxonomic methods such as fatty acid analysis are fast methods, which allow us to compare and group large numbers of strains in a minimal period, whereas DNA-DNA hybridization studies, for example, will be restricted to a minimal but representative set of strains (Vandamme *et al*., 1996).

Phenotypic characteristics

The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (color, dimensions, form) characteristics. The physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, growth in the presence of various substances such as antimicrobial agents, and data on the presence or activity of various enzymes, metabolization of compounds, etc (Vandamme *et al*., 1996).

In addition, standardization of methodologies for many phenotypic tests is inherentl impossible between those organisms whose conditions for growth do not overlap. Miniaturized versions of traditional biochemical tests (e.g. API kits, VITEK cards and Biolog plates) offer standardized methods for a range of biochemical characters (Logan *et al*., 2009).

Chemotaxonomic characteristics

Cell wall composition Determination of the cell wall composition has traditionally been important in gram-positive bacteria. The peptidoglycan type of gram-negative bacteria is rather uniform and provides little information. Cell walls of gram-positive bacteria, in contrast, contain various peptidoglycan types, which may be genus or species specific (Schleifer and Kandler, 1972). For determination of the diagnostic diamino acid is essential, and determination of murein structure is essential for description of new genera and strongly recommended for all novel species (Logan *et al*., 2009).

Whole cell sugar Bacterial cell walls contain some kinds of sugar, in addition to the glucosamine and muramic acid of peptidoglycan. The sugar composition often presents valuable information on the classification and identification of some bacteria, especially some Gram positive bacteria and actinomycetes (Lechevalier and Lechevalier, 1970).

Cellular fatty acids Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. More than 300 different chemical structures of fatty acids have been identified. The variability in chain length, double-bond position, and substituent groups has proven to be very useful for the characterization of bacterial taxa. Bacteria can be divided into three distinct groups on the basis of their membrane lipids. The first group consists of bacteria possessing cell membranes composed of straight-chain acyl esters. Most bacteria are members of this group. The second group has cell membranes composed of branched-chain and alicyclic acyl esters. This includes a significant portion (about 10%) of bacterial species. The third group has cell membranes composed of isoprenoid ethers. This includes a small portion of bacterial species, all of which are archaebacteria (Kaneda, 1991).

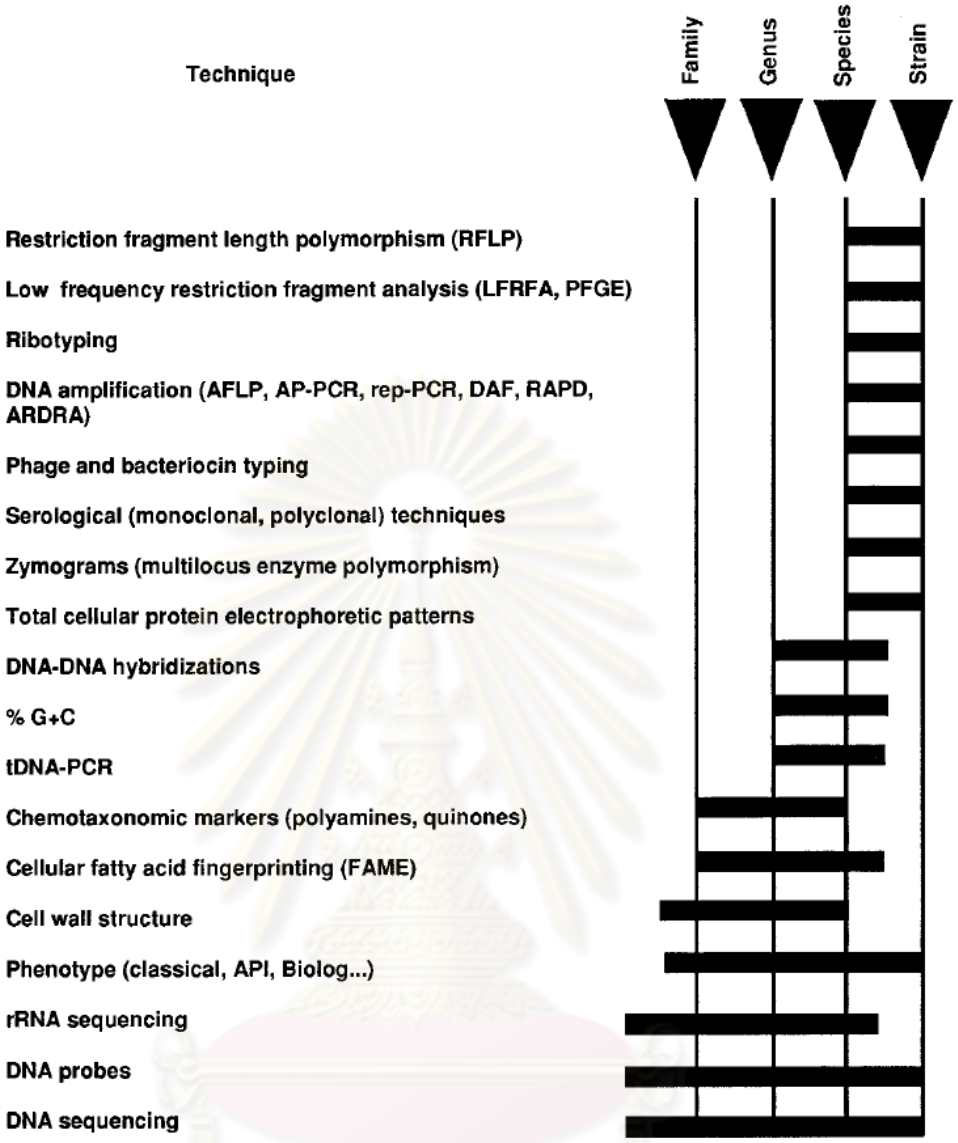

Figure 2.7 Taxonomic resolution of some of the currently used techniques (Vandamme *et al*., 1996).

Polar lipids Polar lipids are the major constituents of the lipid bilayer of bacterial membranes and have been studied frequently for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to be valuable within these groups. Polar lipid method of characterization is essential for description of new genera and recommended for all novel species. Although many polar lipids detected have not yet been structurally characterized, this disadvantage does not necessarily reduce the value of this analysis if, for an unknown lipid, a recognizable designation is used and

the chromatographic behaviour is presented in an image of the two-dimensional thin-layer plate that shows all lipids (Logan *et al*., 2009).

Isoprenoid quinones Isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport. Two major structural groups, the naphthoquinones and the benzoquinones, are distinguished. Naphthoquinones can be divided further into two main types on the basis of structural considerations; these are the phylloquinones and the menaquinones. While, benzoquinones, are two main types, the plastoquinones and the ubiquinones (Collins and Jones, 1981). Menaquinones and ubiquinones have so far been reported for representatives each of genus and hence this method of characterization is essential for description of new genera and recommended for all novel species (Logan *et al*., 2009).

DNA base composition The mol% G+C of the type strain, at least, should be determined and included in the general description, with an indication of the method used. The G+C content of the DNA is strongly recommended for the description of novel species, and essential for the description of new genera (Logan *et al*., 2009).

Genotypic characteristics

16S rRNA gene sequences 16S rRNA gene sequences form the phylogenetic basis for modern bacterial taxonomy and so it is essential that the sequence of the type strain, at least, of each novel species must be deposited in a database with public access, and its sequence similarity with related species represented in that database should be determined. A sequence similarity of 97% or more between an almost complete sequence (>1400 nt, <0.5% ambiguity) of the type strain of the novel species and any sequence of a species with a validly published name available from the database (EMBL/GenBank/DDBJ) should lead to further genotypic (and phenotypic) analysis (Logan *et al*., 2009).

Repitative PCR Genomic fingerprints are the procedures of analyzing the whole genome of the targeted organisms. Rep-PCR is one of the welle stablished genomic fingerprint methods applied for bacterial identification and characterization. The rep-PCR technique is simple, can differentiate between closely related strains of

bacteria, and can assign bacteria potentially up to the strain level based on the presence of repeated elements within the genome examined (Adiguzel *et al*., 2009).

DNA-DNA hybridization DNA-DNA hybridization is one method that provides more resolution than 16S rRNA gene sequencing. DNA–DNA hybridizations are essential in cases of species descriptions when 16S rRNA gene sequences of the novel strains show 97% or more similarity with existing taxa. Several widely used methods do not allow the determination of thermal stability (expressed as ΔT_m) of the hybrid, but differences in ΔT_m between the hybrid and the homologous duplex are important and can be decisive for taxonomic conclusions. It should be borne in mind that, although 70% or more DNA relatedness is recommended to delineate taxa at the species level (Wayne *et al*., 1987), some strains of a species may show less than 70% relatedness with the type strain or other strains of the same species. This threshold should not be rigidly applied less than 70% relatedness between two strains should not be taken automatically to mean that they belong to different species (Cho and Tiedje, 2001; Logan *et al*., 2009).

CHAPTER III

EXPERIMENTAL

3.1 Sample collection and isolation of xylanolytic bacteria

A total of 45 soil samples were collected from various provinces in Thailand (Table 4.1). Xylanase producing bacteria were isolated from the soil samples not later than 24 hours after collection by an enrichment culture method. The soil sample (0.1 g) was put into a 10 ml of xylan-basal medium (XB medium) (Appendix A-1) in 25x250 mm test tube and incubated at 37 °C for 2 days on a rotary shaker at 200 rpm to screening xylanase-producing bacteria. One milliliter of the culture was transferred to fresh XB medium and incubated at the same above condition for 2 more times. The enriched cultures or their dilutions (0.1 ml) were dropped and spreaded on the XB agar medium and incubated at 37 °C for 2 days. Xylanase producing capability of the cultures was screened qualitatively by method described by Teather and Wood (1982). The colonies grown on XB agar medium at 37 °C were flooded with 0.1% (w/v) Congo red solution for 10 minutes and then washed by 0.1 M NaCl. Colonies surrounded by clear zone were selected as xylanase producing isolates and then they were purified by streak plate method. Colony diameter and clear zone diameter of single colony grown on the XB agar medium were measured. Hydrolysis capacity (HC) value was calculated from clear zone diameter divided by colony diameter.

3.2 Identification methods

3.2.1 Phenotypic characteristics

Cell morphology, Gram staining, cell motility, colonial appearance, pigmentation, and spore formation were examined on the cells grown on C agar medium (Appendix A-2) at 37 °C for 1-2 days as described by Barrow and Feltham (1993); Forbes (1981) (Appendix A-24). Catalase, oxidase, citrate utilization, indole production, methyl red (MR) and voges-Proskauer (VP), nitrate reduction, hydrogen sulfide (H_2S) production, hydrolysis of aesculin, L-arginine, casein, gelatin, starch, tyrosine, tween 80, DNA, urea and acid production from carbohydrates were determined as described by

Barrow and Feltham (1993) (Appendix A-2-25). Growth under anaerobic condition on C agar plate was investigated using a Gaspak (BBL) anaerobic jar. Growth at different pH (5, 6, 7, 8 and 9), in 3 and 5% (w/v) NaCl and at different temperature (10, 15, 20, 30, 37, 45, 50, 55 and 60 °C) were tested by using C agar medium. All tests were carried out by incubating the cultures at 37 °C, except for the investigation of the effect of temperature. Furthermore, the strains of novel species and the type strains were tested by using API 50 CH strips, API 20 NE, API 20E and API zym (bioMérieux). The results were recorded after 2 days incubation.

3.2.2 Chemotaxonomic characteristics

3.2.2.1 Diaminopimelic acid analysis Dried cells (5 mg) of the representative isolates in each of group were hydrolyzed with 1 ml of 6N HCl in a screw-capped tube at 100 °C for 18 h. After cooling, the hydrolysate solution was filtered, the resultant filtrate was mixed with 1 ml of distilled water, then concentrated to dryness at 65 °C by a rotary evaporator. The dried material was dissolved in 1 ml of distilled water and repeated drying. Finally, residual was dry material dissolved in 300 µl of distilled water and spotted (3 µl) at base line of a cellulose TLC plate (20 x 20 cm, E. Merck No. 1.05716.0001). One µl of 0.01 M DL-diaminopimelic acid (DAP) was applied as reference. TLC was developed with methanol: pyridine: 4N HCl: water (80: 10: 4: 26, v/v) system which last 3 hours or more. TLC chromatogram was visualized by spraying with 0.2% (w/v) ninhydrin in ethanol followed by heating at 100 °C for 5 min. DAP isomers appeared as dark-green spots and the developed spot gradually disappeared in a few hours (Komagata and Suzuki, 1987).

3.2.2.2 Whole-cell sugar Approximately 50 mg of dried cells of selected isolates was hydrolysed with 1 ml of 1N H_2SO_4 at 100 °C for 2 hours in a screw-capped tube. After cooling, it was mixed with saturated solution of $Ba(OH)_2$ and pH was adjusted to 5.2-5.5. Removal of precipitate by centrifugation and the resultant supernatant was dried by vacuum freeze-drying. The dried residue was dissolved in 0.3 ml of distilled water and it was spotted (10 µl) at the base line of a cellulose TLC plate (20 x 20 cm, E. Merck No. 1.05716.0001). Two standard sugar solutions were used. One consisted of

0.1% (w/v) each of galactose, arabinose and xylose. The other consisted of 0.1% (w/v) each of rhamnose, mannose, glucose and ribose. One microlitre of each standard sugar solution was applied as reference. TLC was developed with *n*-butanol: water: pyridine: toluene (10: 6: 6: 1, v/v) system which last approximately 4 hours. TLC chromatogram was visualized by spraying with acid aniline phthalate (Appendix B-3) followed by heating at 100 °C for 4 min. Hexose sugars appeared as yellowish-brown spots and pentose sugars appeared as maroon coloured spots (Staneck and Roberts, 1974).

3.2.2.3 Cellular fatty acids Two to four loops of the selected cell isolates in exponential phase were put into a 13 mm x 100 mm screw cap test tube containing 1 ml of reagent 1 (Appendix B-1.1) and mixing well. The resultant suspension was heated at 100 °C for 5 min, repeated mixing, reheated at 100 °C for 25 min, then cooled to room temperature in water bath. Two millilitre of reagent 2 (Appendix B-1.2) was added into the suspension, mixed for 5 to 10 sec with vortex mixer, heated at 80±1 °C for 10±1 min and cooled rapidly in ice water. Then, 1.25 ml of reagent 3 (Appendix B-1.3) was added, mixed for 10 min and the upper layer was transferred to new tube. The resultant suspension was mixed with 3 ml of reagent 4 (Appendix B-1.4) for 5 min. Addition of saturation NaCl may required if the suspension became emulsifing. The upper layer was analysed for cellular fatty acid by gas chromatography method (Sasser, 1990; Kämpfer and Kroppenstedt, 1996).

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3.2.2.4 Polar lipids The selected isolates of novel species were analysed. Two ml of aqueous methanol (added 10 ml of 0.3% aqueous NaCl to 100 ml methanol): petroleum benzine was added to the dried cells (100 mg). The solution was mixed for 15 min and centrifuged for 5 min. The lower layer was added with 1 ml of petroleum benzine and mixed for 15 min. The solution was heated at 100 °C for 5 min and cooled immediately at 37 °C for 5 min. The suspension was added with 2.3 ml of chloroform: methanol: 0.3%NaCl (90: 100: 30), mixed for 1 h and centrifuged for 5 min and then transferred the solvent into another tube. Pellets were extracted again with 0.75 ml of chloroform: methanol: 0.3%NaCl (50: 100: 40) and mixed for 30 min. The supernatant after centrifuged was kept and combined. The combined supernatants were extracted with

1.3 ml of chloroform and 1.3 ml of 0.3% NaCl and mixed. The upper layer was removed with Pasteur pipettes while, the lower layer was concentrated with dryness on a rotary evaporator (40 °C). The residues were dissolved with 0.4 ml chloroform: methanol (2: 1) and applied to two-dimensional silica HPTLC (10 x 10 cm, E. Merck No. 5553) and was developed with the following solvent systems. The first solvent system: chloroform: methanol: water (65: 25: 4). The second solvent system: chloroform: acetic acid: methanol: water (80: 15: 12: 4). Subsequently, the first plate was sprayed with Ninhydrin reagent (Appendix B-2.1) and then heated at 100 °C for 5 min. Dittmer and Lester reagent (Appendix B-2.2) was sprayed onto the same plate and then blue spots were detected on the plates containing all phospholipids. The second plate was sprayed with anisaldehyde reagent (Appendix B-2.3) and heated at 100 °C for 10 min after spraying. Green-yellow spots, dark brown spots and violet spots were detected on plates containing mannose-containing substances, glycolipids and other lipids, respectively (Minnikin *et al*., 1977).

3.2.2.5 Quinone analysis The isoprenoid quinone were extracted from dried cells (100-300 mg) of the selected isolates in each of group by using chloroform: methanol (2: 1, v/v) in flask and shaked for 3 h. The residual cells were separated by filtration. The combined filtrate was concentrated to dryness under a reduced pressure on the rotary evaporator. Crude quinone was dissolved in a small amout of acetone. Acetone solution was applied to thin-layer chromatography on a silica-gel plate (20 x 20 cm, E. Merck, Silica gel 60 F_{254}, Art.1.05548.0001) and developed with a solvent system of petroleum and diethyl ether (85: 15, v/v). Standard quinones should also be included. The quinone spots can be visualized by UV light at 254 nm. The R_f of menaquinone was 0.4. The band of menaquinone was scraped off and extracted with acetone. The purified quinones were examined by HPLC (Shimadza model LC-3A). μ-Bondapak C18 column (water Associates, Milford, Mass., USA) was employed and eluted by methanol: diisopropyl ether (3: 1, v/v) with flow rate 1.0 ml/min. The abbreviation (e.q. MK-7, MK-6, etc.) used for menaquinone indicated the number of isoprene unit in the side chain (Shin *et al*., 1996).

3.2.2.6 DNA base composition

Isolation of DNA from representative isolates in each of group was done according to the method described by Saito and Miura (1963). Briefly, log phase cells grown in the C agar medium at 37 °C for 2 day were harvested by scraping and washed in 2-3 ml of saline-EDTA buffer pH 8.0 (Appendix C-1.1). Bacterial cell lysis was done by using 20 mg/ml of lysozyme in 0.1 M Tris buffer pH 9.0 and 10% (w/v) and sodium dodecyl sulfate (SDS) (Appendix C-1.2) at 60 °C for 10 min. After the cell was lysed, the cell suspension was changed from turbid to opalescent and became very viscous. Following, the addition of 2 ml of phenol-chloroform (1: 1 v/v) (Appendix C-1.3), the mixture was mixed for at least 30 sec. Then, it was centrifuged at 12,000 g for 10 min. The supernatant was transferred into a small beaker. After adding of cold 95% (v/v) ethanol into supernatant to precipitate DNA, DNA was spooled with a grass-rod, rinsed with 70% (v/v) ethanol, 95% (v/v) ethanol and air dried. DNA was dissolved in 500 µl of 0.1xSSC (Appendix C-1.4). RNase A solution (Appendix C-1.5) (0.3 ml) was added and the DNA solution was incubated at 37 °C about 20 min for the purification. After adding 0.5 ml of 10xSSC, 1 ml of phenol-chloroform were mixed by vortex for 1 min and centrifuged at 12,000 g for 10 min. The upper layer was transferred to another tube. The DNA was precipitated by adding cold 95% ethanol and DNA was spooled with a grass-rod then rinsed with 70% (v/v) ethanol and 95% (v/v) ethanol, respectively. After air dried, DNA was dissolved in 500 µl of 0.1xSSC. The purity and quality of DNA solution were determined from the ratio between absorbance value at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur and Doty (1962).

DNA was hydrolysed into nucleosides using nuclease P1 (EC 3.1.3.30) and alkaline phosphatase (EC 3.1.3.1) as described by Tamaoka and Komagata (1984). Sample of DNA solution was prepared about 0.5-1.0 g of DNA/liter of distilled water. Heat the DNA solution in boiling water for 5 min and cool in ice water. Took 10 µl of the DNA solution to an eppendorf tube and incubated with adding 10 µl of nuclease P1 solution (Appendix C-1.9) in water bath at 50 ºC for 1 h. After incubation, 10 µl of alkaline phosphatase solution (Appendix C-1.10) was added and kept at 37 ºC for 1 h. After DNA hydrolysis, the sample was analyzed by HPLC with condition show in Table 3.1.

Table 3.1 Conditions for high-performance liquid chromatography

Detector wave length	270 nm
Column	Nakarai Cosmosil packed column $5C_{18}$ (150x4.6 mm)
Column temperature	Room temperature
Eluent	0.2 M $NH_4H_2PO_4$: acetonitrile (20: 1, v/v)
Flow rate	1 ml/min

3.2.3 Genotypic characteristics

3.2.3.1 16S rRNA gene sequence analysis

3.2.3.1.1 16S rRNA gene amplification by PCR The PCR was performed in a total volume of 50 µl containing 1 µl of DNA sample, 0.25 µl of *Taq* DNA polymerase, 5 µl of 10x polymerase buffer, 4 µl of dNTP mixture, 2.5 µl of 10 µM forward and reverse primers (Appendix C-4) and 34.75 µl of Milliq water. A DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 5 min at 94 °C followed by 30 cycles of 30 sec at 94 °C (denaturing of DNA), 30 min at 55 °C (primer annealimg) and 2 min at 72 °C (polymerization) and a final extension for 10 min at 72 °C. The PCR amplified products were analyzed by running 5 µl of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rRNA gene band.

3.2.3.1.2 16S rRNA gene sequencing The amplified 16S rRNA gene was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96 °C followed by 25 cycles of 10 sec at 96 °C (denaturing of DNA), 5 sec at 50 °C (primer annealimg), and 4 min at 60 °C (polymerization). Sequencing for each sample was carried out in both forward and reverse directions.

3.2.3.1.3 Phylogenetic analysis Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequences at the GenBank/EMBL/DDBJ databases. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (NJ) method (Saitou and Nei, 1987) in MEGA4 software (Tamura *et al.*, 2007). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program. Gaps and ambiguous nucleotides were eliminated from the calculations.

3.2.3.2 Repetitive sequence based polymerase chain reaction (rep-PCR) fingerprinting

PCR was carried out as described by Versalovic *et al* (1994) and Gevers *et al* (2001). The PCR was performed in a total volume of 50 µl containing 1 µl of DNA template, 0.5 µl of DNA Taq polymerase, 5 µl of 10x polymerase buffer, 5 µl of dNTP mixture, 5 µl of 10 pmol (GTG)5 primer (5'GTGGTGGTGGTGGTG'3) and 33.5 µl of MilliQ water. Amplifications were performed in a DNA Engine DYAD ALD 1244 thermocycler (MJ Research, Waltham, MA), using the following temperature profile: 94 ºC for 5 min, 30 cycles at 94 ºC for 30 sec, at 45 ºC for 1 min and at 65 ºC for 8 min, followed by a final extension of 16 min at 65 ºC. The PCR products were electrophorised for 4 h at 140 V on a 1.5% (w/v) agarose gel (Appendix C-3.1) in TBE buffer (Appendix C-3.2). The profiles were visualised after staining with ethidium bromide (0.5 mg/ml) under ultraviolet light, followed by digital capturing using the Gel Doc 2000 system (Biorad, Hercules, CA, USA). The resulting fingerprints were analysed by the BioNumerics 4.0 software package (Applied Maths Inc., St Martens Latem, Belgium). Similarities were calculated using Pearson correlation and an average linkage dendrogram was obtained (UPGMA-unweighted pair group method arithmetic averages).

The different dendrograms were visullay interpreted to set the delineation level separately for each species.

3.2.3.3 DNA-DNA hybridization

DNA labeling probe with photobiotin was started by mixing 10 µl of purified DNA solution (1 mg/ml) and 15 µl of photobiotin solution (1 mg/ml) in an eppendorf tube and then the mixture was irradiated with sunlamp for 30 min on ice water. After irradiation, the excess photobiotins were removed by the addition of 100 µl of 0.1 M Tris-HCl buffer pH 9.0 and 100 µl of *n*-butanol. The upper layer was removed. A 100 µl of *n*-butanol was added and mixed well and removed the upper layer. The biotinylated DNA solution was boiled for 15 min and immediately cooled in ice water. The solution was sonicated for 3 min and dissolved with hybridization solution (Appendix C-2.6). DNA-DNA hybridization solution was performed by Ezaki *et al*. (1989). 100 µl of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) and fixed by incubation at 37 °C for 2 h. After incubation, the DNA solution was removed. 100 µl of a hybridization mixture containing biotinylated DNA was added to microdilution wells. The microdilution plate was incubated at hybridization temperature of each Group for 15 h. After hybridization, the microdilution wells were washed three times with 200 µl of 0.2xSSC buffer. Then 200 µl of solution I (Appendix C-2.7) was added to microdilution wells and incubated at 30 °C for 10 min. Solution I was removed from the wells and replaced with 100 µl of solution II (Appendix C-2.8). The microdilution plate was incubated at 37 °C for 30 min. After incubation, the microdilution plate was washed for three times with 200 µl of PBS. 100 µl of solution III (Appendix C-2.9) was added and the plate was incubated at 37 °C for 10 min. The enzyme reaction was stopped with 100 µl of 2M H_2SO_4 (Verlander, 1992). The absorbance was measured at 450 nm with Microplate Reader (Microplate ManagerR 4.0 Bio-Rad Laboratories, Inc) and calculated for the value of percentage DNA homology (Tanasupawat *et al*., 2000). In practice, a DNA-homology above 70% indicates a relationship in the species level as reported by Wayne *et al*. (1987).

3.3 Primary screening and quantitative xylanase activity assay

A loopful of selected xylanase producing isolates was inoculated into 125-ml Erlenmeyer flask containing 20 ml of the XC medium and incubated at 37 °C with shaking (200 rpm) for 2 days. Cell-free supernatant recovered by centrifugation at 4 °C, 10,000 g for 15 min was used for extracellular xylanase activity assay as described by Mandels *et al*. (1976). Oat spelt xylan (Sigma X-0627) was used as a substrate in mixture reaction (Appendix D-2.1). A 1.0 ml of reaction mixture contained 0.1 ml of appropriate diluted enzyme solution and 0.9 ml of 1% oat spelt xylan in 0.1 M sodium phosphate buffer, pH 7.0. The reaction mixture was incubated at 37 °C for 10 min, then the reaction was stopped by the addition of 1.5 ml dinitrosalicylic acid (DNS) (Miller, 1959) (Appendix D-2.2). After mixing on a vortex mixer, the reaction mixtures were boiled in boiling water bath for 15 min and immediately cooled in ice cold water. Then the reaction mixtures were centrifuged with 2,500 g for 10 min. A reaction blank was done in the same manner except the enzyme was added after an addition of dinitrosalicylic acid solution. Yellowish-orange color produced at 540 nm by the reaction mixtures were measured against reagent blank. An absorbance at 540 nm plotted against concentration of standard xylose solution were used as standard curve. One unit (U) of xylanase is defined as the amount of enzyme that releases 1 μmol of xylose per min under the assay condition. Protein content in the supernatant fraction was analysed by Lowry method (1951) (Appendix D-1) with bovine serum albumin (BSA) as standard. Enzyme assays were performed in triplicate. Average values and standard errors were calculated.

3.4 Optimization of xylanase production

3.4.1 Inoculum preparation

Bacterial cells were maintained in 20% glycerol and stored at -80 °C. The inoculum was prepared by inoculating -80 °C maintenance culture into XC medium at 1% (v/v) and incubated at 37 °C on a rotary shaker (200 rpm) for 24 h. The resultant seed cultures (8.5-9.0 CFU/ml) were analysed for xylanase production and growth. To determine growth, the seed cultures were diluted and spreaded on XC agar medium. The colony was counted and expressed as CFU/ml of the spreaded seed culture.

3.4.2 Optimization procedure and experimental design

3.4.2.1 Screening for optimal substrate for cultivation and condition

To investigate the effect of substrate on xylanase production, Oat spelt xylan in the original medium were replaced with different substrate at the 1% (w/v) equal. Substrates were as follows; Beech wood xylan, Birch wood xylan, soy bean and corn cob. For xylanase production, a 1% (v/v) inoculum from the culture was added to 25 ml of XC medium in 125 ml Erlenmeyer flask. After incubation for 24 h at 37 °C under shaking condition 200 rpm and then, the cultivation was harvested by centrifugation at 10,000 g for 15 min at 4°C. The cell-free extract was used as crude enzyme to measure xylanase activity. Each of crude enzyme from various substrate was assayed with three substrates (Oat spelt xylan, Beech wood xylan, Birch wood xylan) at optimal condition. The optimized incubation condition for analysis was at 55 °C for 5 minute.

3.4.2.2 Screening of essential medium compositions and initial pH

Placket-Burman design (PBD) was initially followed to identify important medium components and pH affecting enzyme production. Total of 8 components (variable k = 8, Table 3.2) were selected for the study with each variable being represented at 2 levels, high (+) and low (-) in 12 trials (Table 4.28). Each row represents a trial and each column represents an independent variable. The effect of each variable was determined by the following equation:

$$E(X_i) = 2(\Sigma M_i^{+} - \Sigma M_i^{-})/N$$

where $E(X_i)$ is the concentration effect of the tested variable. M_i^{+} and M_i^{-} represent xylanase production from the trials where the variable (X_i) measured was present at high and low concentrations, respectively. N is the total number of trials that is equal to 12.

A statistical procedure was used to calculate the limit to which the effects of important independent variables were assigned. The significant level (*P*-value) of each main effect was determined using *F*-test.

Table 3.2 Concentration of variables at different levels of the Plackett-Burman design

Variables	Variable code	Lower level (-)	Base level (0)	Higher level (+)
1. Corn (g/l)	X_1	0	5	10
2. Peptone (g/l)	X_2	0	2.5	5
3. Yeast extract (g/l)	X_3	0	0.5	1
4. K_2HPO_4 (g/l)	X_4	2	4	6
5. KCl (g/l)	X_5	0.1	0.2	0.3
6. $MgSO_4.7H_2O$ (g/l)	X_6	0.25	0.5	0.75
7. $FeSO_4.7H_2O$ (g/l)	X_7	0.01	0.02	0.03
8. pH	X_8	6	7	8

3.4.2.3 Optimization of screening of medium compositions and initial pH

Based on the results from PBD experiments, three factors that significantly affected the xylanase production were identified and optimized further, using the response surface methodology (RSM). The CCD with three factors and five levels was used to fit the second order response surface. Each independent variable was studied at five different coded levels ($-\alpha$, 1, -1, 0, +1 and $+\alpha$). The corresponding actual values of five coded levels were shown in Table 3.3. A series of 17 experiments was performed and their coded forms of independent variables investigated and the full experimental plan are listed in Table 4.30. This methodology allowed the modeling of a second-order equation that describes the process. Xylanase production was analyzed by multiple regressions through the least squares method to fit the following equation:

$$Y = a_0 + \sum a_i x_i + \sum a_{ii} X_i^2 + \sum a_{ij} x_i x_j,$$

where Y represents response variable, a_0 is the interception coefficient, a_i is the coefficient of the linear effect, a_{ii} is the coefficient of quadratic effect and a_{ij} is the coefficient of interaction effect. x_i and x_j denote the coded levels of variable X_i and X_j in experiments. The variable X_i was coded as x_i according to the following transformation equation:

$$x_i = (X_i - X_0)/\Delta X_i,$$

where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point and ΔX_i is the step change. Cultures were incubated at 37 °C, 200 rpm for 24 h and xylanase yield was determined at optimal condition.

Table 3.3 Concentration of variables at different levels of the central composite design

Variables	**Variable code**	**Code level (x_i)**				
		-α	**-1**	**0**	**+1**	**+α**
1. Corn (g/l)	X_1	1.34	5	10	15	18.66
2. Peptone (g/l)	X_2	0.67	2.5	5	7.5	9.33
3. pH	X_3	4.27	5	6	7	7.73

3.4.2.4 Statistical analysis

Quantification of enzyme activity was carried out in triplicate experiments and the mean values were given. The significance of each variable in the PBD experiment was determined by applying the *F*-test using Design-Expert 7 P statistical software. For the CCD experiment the working parameters were calculated and generated response surface graphs using Design-Expert 7 P statistical software. The accuracy and general ability of the above polynomial model could be evaluated by the determination coefficient (R^2).

3.4.3 Time course of growth and xylanase production

Xylanase production and microbial growth were compared in the initial and optimized media. Cultivations were conducted at 37 °C in a rotatory shaker at 200 rpm to start at 0-48 h. Samples taken at 6 h interval were assayed for xylanase activity and monitored for growth by spreading.

3. 5 Partial purification of xylanase

3.5.1 Enzyme preparation

The selected strain was cultivated in optimal medium which omitted yeast extract but contained corn 15 g/l, peptone 7.5 g/l, K_2HPO_4 6 g/l, KCl 0.1 g/l, $MgSO_4.7H_2O$ 0.75 g/l and $FeSO_4.7H_2O$ 0.01 g/l, pH 6.0 at 37 °C. Culture broth at 24 h cultivation was centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was used for enzyme purification.

3.5.2 Purification procedure

All purification steps were carried out at 4 °C. The corn cob 20M (appendix D-2.4), suspended in 0.05 M sodium phosphate buffer (pH 6.0) overnight, was packed in a column (3 cm x 23 cm) and equilibrated with the same buffer. 131.50 mg protein of the crude enzyme was applied on the column. After washing the enzyme–corn cob 20M complex four times with a large amount of the same buffer, the xylanase was eluted by 1.5% triethylamine. 1.0 ml fractions were collected and assayed for xylanase activity. The active xylanase fractions were collected and dialyzed against 0.05 M sodium phosphate buffer pH 6.0 at 4 °C.

The active enzyme was concentrated with 10% (w/v) polyethylene glycol (PEG) MW 6000 prior to size exclusion chromatography. The sample was applied to a Superdex 200 10/300 GL column (1.0 x 30.0 cm) (GE healthcare) previously equilibrated with approximately two bed volumes of 0.05 M sodium phosphate buffer pH 6.0. The sample was loaded onto the column and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions (1.0 ml) were collected and the xylanase active fraction were pooled and used for further studies. The purity of the partially purified enzyme was analyzed by polyacrylamide gel electrophoresis.

3.6 Characterization of partially purified xylanase

3.6.1 Molecular weight determination

3.6.1.1 Size exclusion FPLC

Size exclusion chromatography (SEC) was performed on FPLC using a Superdex 200 10/30 GL column (GE Healthcare Bio-Sciences, Uppsala, Sweden) connected with FPLC pump and UV detector. Partially purified enzyme was injected into the column after centrifuge at 10,000 g for 10 minute. The proteins were eluted with isocratically with 0.05 M sodium phosphate pH 6, at a flow rate of 0.5 ml/min. Eluted proteins were monitored by absorbance at 280 nm. The protein separated on SEC-FPLC was estimated for its molecular weight by plotting relative elution volume (V_e/V_o) against the logarithm of M_r of the protein standards. The elution volume (V_e) was measured for each protein standard and the partially purified enzyme, and the void volume (V_o) was estimated by the elution volume of blue dextran (M_r = 2,000,000). The standards used included thyroglobulin (bovine) (M_r 670,000), γ-globulin (bovine) (M_r 158,000), ovalbumin (chicken) (M_r 44,000), myoglobin (horse) (M_r 17,000) and vitamin B12 (M_r 1,350).

3.6.1.2 SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) (Appendix C-3). Polyacrylamide was prepared for 12.5% running gel with 4% stacking gel. Partially purified protein solutions were mixed with 1:1 (v/v) ratio with sample treatment buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenol blue). The sample (15 μg of protein) was loaded on the gel. The proteins were subjected to electrophoresis at a constant current of 20 mA per gel by the ATTO AE-6530 Dual mini-slab system. After electrophoresis, gel was fixed and stained by silver staining (Heukeshoven and Dernick, 1985). PageRulerTM unstained protein ladder (Fermentas, Ontario, Canada) was used as the standard protein marker. The molecular weight of a protein under investigation was estimated by standard curve correlating log molecular weight and migration distance of known molecular weight proteins.

3.6.1.3 Zymogram analysis

The zymogram analysis was a modification of the published method of Nakamura *et al*. (1993). The partially purified in the sample application buffer was subjected to electrophoresis on an SDS-12.5% polyacrylamide gel containing 0.1% Oat spelt xylan. After electrophoresis, the gel was soaked in 2% (v/v) Triton-X100 with gentle shaking to remove the SDS and renature the proteins in the gel. The gel was incubated for 5 minute at 55 °C in 0.1 M sodium phosphate buffer (pH 6.0). After further the gel was soaked in 0.1% Congo red solution for 10 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After the gel was submerged in 0.5% acetic acid, the background turned dark blue and the activity bands were observed as clear colorless areas.

3.6.2 Optimal temperature and thermal stability

Activity of the partially purified enzyme was assayed at different temperatures in the range of 30-80 °C at pH 6.0 as previously described for the enzyme assays. To study the effect of temperature on enzyme stability, the partially purified enzyme previously was incubated at various temperatures ranging from 30-80 °C for 30 minute after that the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions.

3.6.3 Optimal pH and pH stability

The activity of the partially purified enzyme was assayed at 60°C (optimal temperature**)** over the pH range of 2.0–11.0 by using universal buffer which consists of 0.05 M of 0.05 M citric acid, 0.05 M NaH_2PO4, 0.05 M sodium carbonate, the different volume of 0.2 NaOH. The pH stability experiment was conducted by incubating the enzyme without substrate in universal buffer with pH values ranging from 2.0-11.0 at 37 °C for 30 minute and measuring residual activity under the standard assay conditions.

3.6.4 Metal ions, reducing agents and inhibitors studies

Partially purified xylanase in 0.05M sodium phosphate buffer (pH 6.0) with various metal ions (Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+}), reducing (or thiol) agents

(dithiothreitol (DTT), and β-mercaptoethanol (β-Me)) and other reagents or inhibitors such as ethylene diamine tetraacetic acid (EDTA), phenylmethyl sulfonyl fluoride (PMSF) and sodium dodecyl sulfate were incubated at 37 °C for 30 min. The residual activity was assayed under the standard assay conditions.

3.6.5 Substrate specificity

The hydrolytic abilities against 1% Oat spelt xylan, Beech wood xylan, Birch wood xylan, β-glucan, carboxymethylcellulose (CMC) and pectin in 0.1 M sodium phosphate buffer (pH 6.0) were determined to evaluate the substrate specificity of partially purified xylanase. The xylanase activity was measured under the standard assay conditions.

3.7 Statistical analysis

All statistical experimental design and the results were analyzed by ANOVA and means were separated by Duncan's multiple-range test using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All experiments were carried out in triplicates. The average values of the result were presented, in which the standard deviation.Statistical significance was assigned at 95% of confidence level.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Bacterial isolation and source of samples

The xylanolytic bacteria were isolated from 45 samples of soil collected in Thailand (Table 4.1). Seventy isolates showed clear zone surrounded colonies grown on xylan basal agar medium (XB).

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

Location of samples	Sample	Isolate no.	No. of isolate
Chiangrai	Hot spring sediment	CR1-2, CR5-1, CR7-1	3
Mahasarakham	Soil	MS1-1, MS1-2, MS1-4, MS1-5	4
Chaiyaphum	Soil	CP1-1, CP1-2, CP2-1	3
Nan	Soil	MX2-3, MX15-2, MX21-2, P2-3, S1-3, S3-4A, X11-1	7
Nakhonnayok	Cow faeces	FCN3-3, FCN3-4, FXN1-1B, FXN2-3, FXN3-1	5
Samutsongkhram	Soil	SK1-3	1
Kanchanaburi	Soil	K1-4, K1-5, K1-6A, K1-6B, K3-1, K3-2, K3-5B, K3-5S, K3-6	9
Phetchaburi	Wood chip	CE3-4, CE4-1	2
Phetchaburi	Soil	P2-2, P2-3A, P2-5	3
Phetchaburi	Muddy shore sediment	PHC3-3, PHC3-4, PL1-3, PL2-1, PHX1-5, PHX2-5, PHX2-7, PHX3-1	8
Prachuapkhirikhan	Muddy shore sediment	PJ1-1A, PJ1-1B, PJ1-2	3
Trat	Soil	TH2-1A, TH2-2, TH4-1	3
Suratthani	Soil	SRC1-1, SRC2-3, SRC3-3, SRX1-1, SRX1-2, SRX1-4, SRX2-1, SRX2-2, SRX2-3, SRX3-4, SRX4-1, SRX4-2, SRXT1-1, SRXT1-2, SRXT2-1	15
Nakhonsithammarat	Soil	CXT1-1, CXT1-2, CXT3-2, NS1-1	4
Total			**70**

4.2 Identification and characterization of isolates

Seventy isolates were divided into sixteen groups and were identified based on their phenotypic characteristics and the 16S rRNA gene sequence analyses of the representative isolate in each of group. Sixty-one isolates in Group I to VII were Gram-positive rod-shaped bacteria. Nine isolates in Group VIII to XVI were Gram-

negative rod-shaped bacteria. The results of chemotaxonomic characteristics, $(GTG)_5$-PCR patterns and DNA-DNA relatedness were supported their identification.

4.2.1 Group I

Group I contained 25 isolates, CXT1-2, CP1-1, CP1-2, CP2-1, CR7-1, FCN3-4, K1-6A, K1-6B, K3-6, MS1-1, MS1-2, MS1-4, MS1-5, NS1-1, P2-2, P2-3, PHC3-3, PHX1-5, PHX2-2A, PJ1-2, SK1-3, SRC2-3, SRXT1-2, TH2-1A, TH2-2. They were Gram positive, motile rod-shaped (approximate 0.5-2.0 x 1.8-6.0 μm). Central or subterminal ellipsoidal endospores were observed in swollen sporangia. Colonies were 3-12 mm in diameter, irregular or round, entire or lobate or curled, smooth or wrinkled or concentric, raise or flat, opaque, creamy, yellow or white-coloured after 2 days of incubation at 37 ºC on C medium. They contained *meso*-diaminopimelic in cell wall peptidoglycan. Predominant menaquinone of the representative isolates P2-3 and PHX1-5 in this group was MK-7.

Twenty five isolates were divided into Group I(a) to I(g) based on the phenotypic properties (Table 4.2). The representative isolates were identified with 16S rRNA gene sequence and phylogenetic analyses that the representative isolate were clustered within a clade of the genus *Bacillus* (Figure 4.1).

Group I (a) contained 3 isolates, K3-6, PHX2-2A and SRC2-3. Colonies were 3-6 mm in diameter, round, smooth, raise, opaque and creamy or white-coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5% NaCl, at pH 7-9 and at 25-50 °C. Hydrolysis of aesculin, DNA, gelatin and starch were positive but negative for methyl red, Voges-proskaner, H_2S production, indole production, hydrolysis of L-tyrosine and tween 80. No acid production from L-arabinose, D-galactose, gluconate, inositol, lactose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, sorbitol, sorbose and D-xylose as shown in Table 4.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolates K3-6 and SRC2-3 were the representative of this group. Isolates K3-6 (926 nt) and SRC2-3 (963 nt) were closely related to *B. subtilis* subsp. *subtilis* KCTC 3135^T (Figure 4.1) with 100% 16S rRNA gene sequence similarity. Therefore, based on the results mentioned above and phenotypic properties indicated that isolates K3-6 and SRC2-3 were identified as *B. subtilis* subsp. *subtilis* (Cohn, 1872).

Table 4.2. Differential characteristics of *Bacillus* Group I (a) to (g)

Characteristics	a	b	c	d	e	f	g
Growth at pH: 5	+(-1)	+(-1)	-	w	+	+	-
6	+(-1)	+	-	w	+	+	-
Growth at : 10 °C	-(w1)	-	-	-	+	-	-
15 °C	+(-1)	-(+1)	-	+	+	-	-
50 °C	+	+(-1)	+	+	-(1w)	+	-
55 °C	+(-1)	+(-1)	-	w	-	-	-
Oxidase	+(-1)	+(-2)	+(-1)	-	-	+	+
Citrate utilization	-(+1)	+(-6, w1)	-	-	-	-	-
Methyl red	-	-	-	+	-	-	-
Nitrate redcution	-(+1)	(+5)	-	-	+	+	-
Hydrolysis:							
Aesculin	+	+	+	+	+	+	-
L-Arginine	+(-1)	+	-	w	+	+	-
Casein	+(-1)	+	+(-1)	+	+	+	-
DNA	+	+(w1)	+	+	+	+	-
Gelatin	+	+	-(+1)	w	-(1+)	+	-
Starch	+	+(-1)	-	-	-(1+)	+	-
L-Tyrosine	-	-(+1)	-	-	-(1+)	-	-
Urea	+(-1)	+	-(+1)	+	+	+	-
Acid production:							
L-Arabinose	-	+(-1)	+	+	-	+	w
D-Cellobiose	+(-1)	+(-3)	+(-1)	+	-	+	+
D-Fructose	+(-1)	+(-4)	-	+	-	+	+
D-Galactose	-	-(+2,w1)	-	+	-	+	+
D-Glucose	+(-1)	+(-7)	-	+	+	+	+
Gluconate	-	-(+2,w1)	-	+	-(1+)	-	-
Glycerol	+(-1)	+(-3,w2)	-(+1)	+	+	+	-
Inositol	-	-(+2)	-	+	-	+	+
Inulin	(+1)	-(+2)	-	-	-	+	-
Lactose	-	-(+2)	-	+	-	+	+
D-Maltose	+(-1)	+(-2)	-	+	+	+	+
D-Mannitol	+(-1)	+(-3,w1)	-	+	-	+	+
D-Mannose	+(-1)	+(-3)	-	+	+(-1)	+	w
D-Melibiose	-	-(+2)	-	+	-	+	+
D-Melezitose	-	-(+2)	-	+	-	-	+
α-Glucopyranoside	-	+(-6,w1)	-	+	-	+	-
Raffinose	-	-(+2)	-	+	-	+	+
L-Rhamnose	-	-(+2)	-	+	-	-	+
D-Ribose	-	+(-5,w1)	-	+	+	+	-
Salicin	-(+1)	+(5)	-	+	+	+	+
Sorbitol	-	-(+2)	+(-1)	+	-	+	-
Sucrose	-(+1)	+(-7)	-	+	-(+1)	+	+
D-Trehalose	-(+1)	+(-5)	-	+	+	-	+
D-Xylose	-	-(+2,w1)	+(-1)	+	-	+	+

+, positive; –, negative; w, weakly positive

Group I (b) contained 15 isolated, CXT1-2, CP1-1, CP1-2, CP2-1, CR7-1, MS1-1, MS1-2, MS1-4, MS1-5, P2-2, PJ1-2, SK1-3, SRXT1-2, TH2-1A, TH2-2. Colonies were 3-12 mm in diameter, irregular or round, lobate or entire, wrinkled, raise or flat, opaque and creamy or yellow coloured after 2 days of incubation at 37 ºC on C medium. All isolates grew in 3-5% NaCl, at pH 6-9 and at 25-45 °C but no growth at 10 and 60 °C. Hydrolysis of aesculin, L-arginine, casein, gelatin and urea and acid production from D-amygdalin were positive. But negative for methyl red, Voges-proskaner, H_2S production and indole production as shown in Table 4.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolates TH2-2, P2-2, SK1-3 and PJ1-2 were representative of this group and isolates TH2-2 (1,488 nt), P2-2 (971 nt), SK1-3 (927 nt) and PJ1-2 (947 nt) were closely related to *B. licheniformis* KCTC 1918^T (Figure 4.1) with 99.7, 99.2, 99.1 and 99.6% 16S rRNA gene sequence similarity, respectively. This result showed high the similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that isolates TH2-2, P2-2, SK1-3 and PJ1-2 were representative of Group I(b) and were identified as *B. licheniformis* (Chester, 1901).

Group I(c) contained 2 isolates, K1-6A and K1-6B. Colonies were 3-5 mm in diameter, irregular, curled, concentric, flat, opaque and creamy or white-coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5% NaCl, at pH 7-9 and at 25-50 °C, but did not grow at pH 5-6, at 10-15, 55 and 60 °C. Hydrolysis of aesculin and DNA and acid production from L-arabinose were positive but negative for methyl red, Voges-proskaner, citrate utilization, H_2S production, indole production, nitrate reduction, hydrolysis of L-arginine, starch, L-tyrosine and tween 80. No acid producetion from D-fructose, D-galactose, D-glucose, gluconate, inositol, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbose, sucrose and D-trehalose as shown in Table 4.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolates K1-6A (981 nt) and K1-6B (944 nt) were closely related to *B. niabensis* $4T19^T$ (Figure 4.1) with 99.9 and 100% 16S rRNA gene sequence similarity, respectively. This result showed high the similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the

results mentioned above and phenotypic properties indicated that isolates K1-6A and K1-6B were identified as *B. niabensis* (Kwon *et al*., 2007).

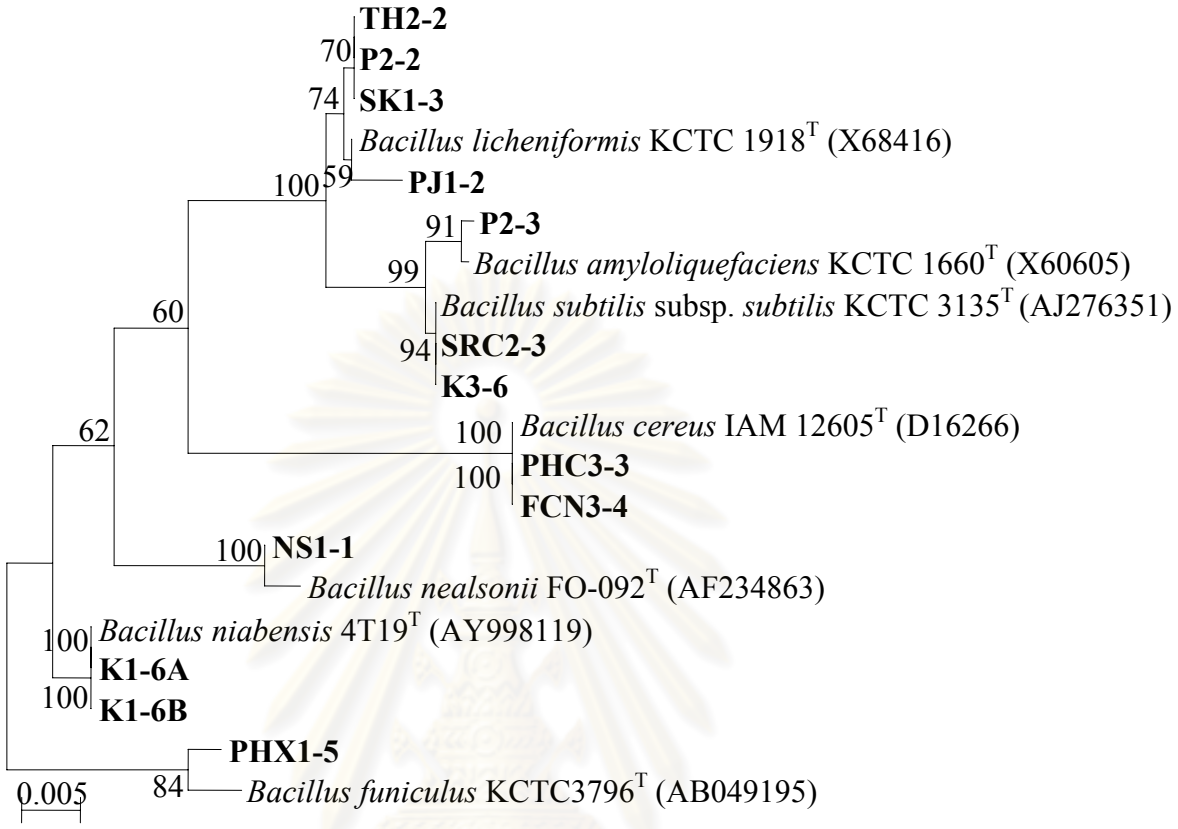

Figure 4.1 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between representative isolates in each of group a to g and known *Bacillus*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position

Group I (d) contained NS1-1. Colonies were 2-3 mm in diameter, round, concentric, flat, opaque and white-coloured after 2 days of incubation at 37 ºC on C medium. The isolate grew in 3- 5% NaCl, at pH 5-7 and at 15-55 °C but did not grow at pH 8-9 and at 10, 60 °C. Methyl red, hydrolysis of aesculin, L-arginine, casein, DNA, gelatin and urea were positive but negative for oxidase, Voges-proskaner, citrate utilization, H_2S production, indole production and nitrate reduction, hydrolysis of starch, L-tyrosine and tween 80. Acid was produced from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inositol, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-

glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sucrose, D-trehalose and D-xylose, but not from inulin and sorbose as shown in Table 4.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate NS1-1 (1,053 nt) was closely related to *B. nealsonii* FO-092^T (Figure 4.1) with 99.7 % 16S rRNA gene sequence similarity. This result showed high similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that NS1-1 was identified as *B. nealsonii* (Venkateswaran *et al*., 2003).

Group I(e) contained 2 isolates, FCN3-4 and PHC3-3. Colonies were 2.5-6 mm in diameter, round, smooth or curled, flat, opaque and yellow or white-coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5% NaCl, at pH 5-9 and at 10-45 °C, but did not grow at 55-60 °C. Positive for nitrate reduction, hydrolysis of aesculin, L-arginine, casein, DNA and urea, but negative for oxidase, methyl red, Voges-proskaner, citrate utilization, H_2S production and hydrolysis of tween 80. Acids were produced from D-glucose, glycerol, D-maltose, D-ribose, salicin and D-trehalose, but not from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, inositol, inulin, lactose, D-mannitol, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, sorbitol, sorbose and D-xylose as shown in Table 4.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolates FCN3-4 (854 nt) and PHC3-3 (895 nt) were closely related to *B. cereus* IAM 12605^T (Figure 4.1) with 100 and 99.8% 16S rRNA gene sequence similarity. This result showed high the similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that FCN3-4 and PHC3-3 were identified as *B. cereus* (Frankland and Frankland, 1887).

Group I(f) contained P2-3. Colonies were 3-8 mm in diameter, irregular or round, lobate, wrinkled, flat, opaque and creamy and yellow coloured after 2 days of incubation at 37 ºC on C medium. The isolate grew in 3-5% NaCl, at pH 5-9 and at 25-50 °C. Catalase, oxidase, H_2S production, nitrate reduction, hydrolysis of aesculin, L-arginine, casein, DNA, gelatin, starch and urea and assimilation of *N*-acetyl-glucosamine, D-glucose, malic acid, D-maltose, D-mannitol, and D-mannose. Able to produce acid from aesculin, D-amygdalin, L-arabinose, arbutine, D-cellobiose, D-fructose, D-galactose, glucose, glycerol, inositol, inulin,

lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, α-glucopyranoside, raffinose, D-ribose, salicin, sorbitol, sucrose, D-turanose, xylitol and D-xylose. Not able to produce acid from *N*-acetyl-glucosamine, D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, gluconate, glycogene, 2-ketogluconate, 5-ketogluconate, D-lyxose, α-mannopyranoside, D-melezitose, L-rhamnose, L-sorbose, starch, D-tagatose, D-trehalose, L-xylose and β-xylopyranoside as shown in Table 4.2 and 4.3. On the basis of 16S rRNA gene sequence and phylogenetic analyses, P2-3 (1,045 nt) was closely related to *B. amyloliquefaciens* KCTC 1660^T (Figure 4.1) with 96.4% 16S rRNA gene sequence similarity. This isolate showed low 16S rRNA gene sequence similarity when compared with type strain. As well as, the isolate P2-3 had differential characteristics with *B. amyloliquefaciens* KCTC 1660^T as shown in Table 4.3. DNA G+C content of isolate P2-3 was 46.3 mol%, which was in the range observed for members of the genus *Bacillus* (32-69 mol%) (Claus and Berkeley, 1986). Therefore, based on the phenotypic properties, chemotaxonomic characteristic and 16S rRNA gene sequence, P2-3 represents as the novel species of the genus *Bacillus* (Cohn, 1872).

Group I (g) contained PHX1-5. Colonies were 2-3 mm in diameter, round, entire, smooth or concentric, raise, opaque and white-coloured after 2 days of incubation at 37 ºC on C medium. The isolate grew in 3-5% NaCl, at pH 7-8 and at 25-45 °C, but did not grow at pH 5-6, 9 and at 10-15, 50-60 °C. Oxidase, hydrolysis of tween 80 and assimilation of D-maltose were positive, but negative for citrate utilization, fermentation glucose, indole production H_2S production, methyl red, Voges-Proskauer, nitrate reduction and urease, hydrolysis of aesculin, arginine, casein, DNA, gelatin, starch and tyrosine and assimilation of *N*-acetyl-glucosamine, adipic acid, L-arabinose, capric acid, D-glucose, malic acid, D-mannitol, D-mannose, phenylacetic acid and potassium gluconate. Acids were produced from *N*-acetyl-glucosamine, aesculin, L-arabinose, D-arabitol, arbutine, D-cellobiose, erythritol, D-fructose, D-galactose, gentiobiose, glucose, α-glucopyranoside, inositol, 5-ketogluconate, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, raffinose, rhamnose, salicine, D-sucrose, D-trehalose, D-turanose, D-xylose and β-xylopyranoside. No acid was produced from D-adonitol, amygdalin, D-arabinose, L-arabitol, dulcitol, D-fucose, L-fucose, gluconate, glycerol, glycogene, inuline, 2-ketogluconate, D-lyxose, α-mannopyranoside, D-ribose, D-sorbitol, L-

sorbose, starch, D-tagatose, xylitol and L-xylose as shown in Table 4.2 and 4.3. On the basis of 16S rRNA gene sequence and phylogenetic analyses, PHX1-5 (1,261 nt) was closely related to *B. funiculus* KCTC 3796^T (Figure 4.1) with 98.4% 16S rRNA gene sequence similarity. The isolate showed low 16S rRNA gene sequence similarity with the type strain. As well as, the isolate PHX1-5 had differential characteristics with *B. funiculus* KCTC 3796^T as shown in Table 4.3. DNA G+C content of isolate PHX1-5 was 42.4 mol%, which was in the range observed for members of the genus *Bacillus* (32-69 mol%) (Claus and Berkeley, 1986). The predominant fatty acids were iso-$C_{15:0}$ (32.2%), anteiso-$C_{15:0}$ (13.8%) and $C_{16:0}$ (13.2%). The PHX1-5 and *B. funiculus* KCTC 3796^T showed the similar cellular fatty acid profiles, but significant quantitative differences were also found as shown in (Table 4.4). Major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The unknown phospholipids was detected (Appendix E-1). Therefore, based on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, PHX1-5 represents as the novel species of the genus *Bacillus* (Cohn, 1872). However, isolate PHX1-5 should be confirmed with DNA-DNA hybridization for the proposal of the new species.

Table 4.3 Differential characteristics of P2-3, *B. amyloliquefaciens* KCTC 1660^T, PHX1-5 and *B. funiculus* KCTC 3796^T

Characteristic	P2-3	KCTC 1660^T	PHX1-5	KCTC 3796^T
Growth in 5%NaCl	+	+	+	-
Voges-Proskauer	-	+	-	+
Nitrate reduction	+	-	-	+
PNPG	-	+	+	+
Urease	+	+	-	+
Hydrolysis of:				
Aesculin	+	+	-	+
Arginine	+	-	-	-
Starch	+	+	-	+
Tween 80	-	+	+	-
Assimilation:				
N-Acetyl-glucosamine	+	+	-	+
D-Glucose	+	+	-	+
Malic acid	+	-	-	-
D-Mannitol	+	-	-	+
D-Mannose	+	+	-	-
Potassium gluconate	-	+	-	+
Acid from:				
Amygdaline	+	+	-	+
D-Arabinose	-	+	-	-
L-Arabinose	+	-	w	-
D-Arabitol	-	-	+	-
Dulcitol	-	-	-	+
α-Glucopyranoside	+	-	+	+
Inositol	+	-	+	+
Inulin	+	-	-	-
D-Lactose	+	-	+	-
D-Mannitol	+	-	+	+
D-Melibiose	+	-	+	+
D-Raffinose	+	-	+	+
L-Rhamnose	-	+	+	+
D-Sorbitol	+	-	-	+
D-Trehalose	-	+	+	+
D-Turanose	+	-	+	+
Xylitol	+	-	-	-
D-Xylose	+	-	+	+
β-Xylopyranoside	-	-	+	-
DNA G+C (mol%)	46.3	44.6*	42.4	37.2†

Data were obtained in this study unless indicated. +, positive; –, negative

*Data were obtained from Priest *et al.* (1987).

†Data were obtained from Ajithkumar *et al*. (2002).

Table 4.4 Cellular fatty acid compositions of PHX1-5 and *B. funiculus* KCTC 3796^T Data were obtained in this study.

Values are percentages of total fatty acids. tr, trace < 1 %; ND, Not detected

Fatty acids	**PHX1-5**	**KCTC 3796^T**
Straight-chain saturated		
$C_{12:0}$	ND	tr
$C_{14:0}$	4.5	4.1
$C_{15:0}$	1.3	ND
$C_{16:0}$	13.2	13.1
$C_{17:0}$	tr	ND
$C_{18:0}$	tr	ND
Branched saturated		
iso-$C_{13:0}$	tr	0.8
iso-$C_{14:0}$	1.9	5.2
iso-$C_{15:0}$	32.2	33.0
iso-$C_{16:0}$	2.2	3.3
iso-$C_{17:0}$	3.4	2.0
anteiso-$C_{15:0}$	13.8	28.5
anteiso-$C_{17:0}$	1.1	tr
Monounsaturated		
$C_{16:1}$ ω11c	ND	1.0

4.2.2 Group II

Group II contained 24 isolates, CXT1-1, CXT3-2, FXN2-3, K1-4, K1-5, K3-1, K3-2, K3-5B, K3-5S, MX2-3, P2-3A, P2-5, PL1-3, PHC3-4, PJ1-1A, PJ1-1B, PL2-1, S3-4A, SRX1-4, SRX4-1, SRX4-2, SRXT1-1, SRXT2-1 and X11-1. They were Gram positive, rod-shaped (approximate 0.4-1.2 x 1.5-12.0 μm). Motile by means of peritrichous flagella. Central or subterminal ellipsoidal endospores were observed in swollen sporangia (Figure 4.2). Colonies were 0.5-20 mm in diameter, circular, curled or entire, concentric or smooth, convex or raise or flat and yellow or white-coloured after 2 days of incubation at 37 ºC on C medium. They contained *meso*-diaminopimelic in cell wall peptidoglycan as in the genus *Bacillus* (Shida *et al.,* 1997). Predominant menaquinone was MK-7.

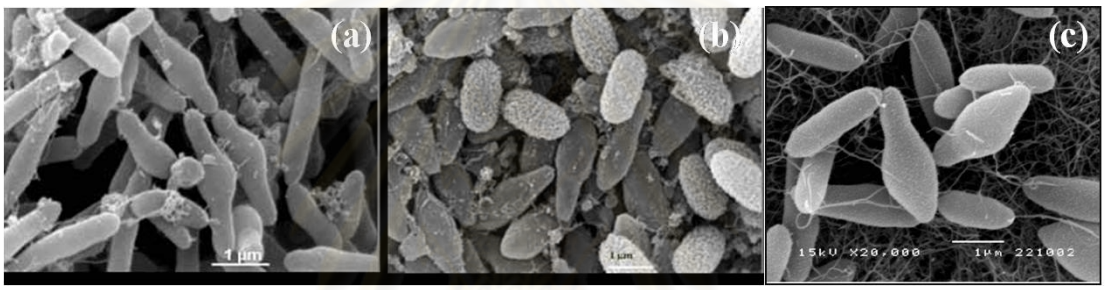

Figure 4.2 Scanning electron micrographs of S3-4A (a), MX2-3 (b), X11-1 (c) grown on C agar for 48 h. Bar 1 μm

Twenty four isolates were divided into 9 (A) to (I) group based on the phenotypic properties (Table 4.5). The representative isolates in each of group II (A to I) were identified with 16S rRNA gene sequence and phylogenetic analyses that the representative isolate was clustered within a clade of the genus *Paenibacillus* (Figure 4.3).

Group II (A) contained K1-4. Colonies were 1.5-4 mm in diameter, round, curled, concentric, flat and white-coloured after 2 days of incubation at 37 ºC on C medium. The isolate grew in 3-5% NaCl, at pH 7-9 and at 25-50 °C. Positive for catalase, oxidase and hydrolysis of aesculin, L-arginine, casein, gelatin and starch, but negative for methylred, Voges-proskaner, citrate utilization, H_2S production, indole production and nitrate reduction. No acid produced from inositol, sorbitol and sorbose as shown in Table 4.5. On the basis of 16S rRNA gene sequence and phylogenetic analyses, K1-4 (1,432 nt) was closely related to *P. macerans* IAM 12467^T (Figure 4.3) with 99.6% 16S rRNA gene sequence similarity. The isolate showed high the

similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that isolate K1-4 was identified as *P. macerans* (Ash *et al*., 1994).

Group II(B) contained 2 isolates, PJ1-1A and PJ1-1B. Colonies were 1-5 mm in diameter, circular, entire or curled, smooth or concentric, flat, opaque and yellow coloured after 2 days of incubation at 37 ºC on C medium. All isolates grew in 3% NaCl, at pH 7-9 and 25-45 °C, but did not grow in 5% NaCl, pH 5-6 and at 15 and 50-60 °C. Positive for catalase, oxidase and hydrolysis of aesculin, starch and urea, but negative for methyl red, Voges-proskaner, citrate utilization, H_2S production, indole production, nitrate reduction and hydrolysis of L-arginine, casein, gelatin, L-tyrosine and tween 80. Acids were produced from D-cellobiose, D-galactose, D-glucose, gluconate, L-rhamnose, D-trehalose and D-xylose. Not able to produce acid from inositol, D-mannose, D-melezitose, α-glucopyranoside, raffinose, salicin, sorbitol, sorbose and sucrose as shown in Table 4.5. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate PJ1-1B was representative of this group, that PJ1-1B (919 nt) was closely related to *P. montaniterrae* MXC2-2^T and *P. siamensis* S5-3^T (Figure 4.3) with 99.7 and 99.6% 16S rRNA gene sequence similarity, respectively. The isolate showed high the similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that isolate PJ1-1B was identified as *P. montaniterrae* (Khianngam *et al*., 2009).

Group II (C) contained PHC3-4. Colonies were 1-5 mm in diameter, round, entire, smooth, flat, opaque and creamy-white coloured after 2 days of incubation at 37 ºC on C medium. The isolate grew at pH 5-9 and at 25-50 °C. Did not grow in 3% and 5% NaCl and at 15, 55-60 °C. Positive for oxidase, indole production and hydrolysis of aesculin, L-arginine, casein, DNA and urea. Able to produce acid from D-amygdalin, sorbitol and D-trehalose as shown in Table 4.5. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate PHC3-4 (904 nt) was closely related to *P. dendritiformis* 105967^T (Figure 4.3) with 99.7% 16S rRNA gene sequence similarity. The result showed high similarity of 16S rRNA gene sequence when compared with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that isolates PHC3-4 was identified as *P. dendritiformis* (Tcherpakov *et al*., 1999).

Table 4.5. Differential characteristics of *Paenibacillus* Group (A) to (I)

Characteristics	A	B	C	D	E	F	G	H	I
Growth in: 3% NaCl	+	+	-	-	-	w	+	w	+(w2)
Growth at pH 6	-	-	+	-	-	-	-	+	+(w2/-1)
9	+	+	+	-	+	-	+	+	+(w2/-1)
Growth at: 15 ºC	-	-	-	-	+	+	-	-	-
45 ºC	+	+	+	-	-	-	+	+	+
50 ºC	w	-	+	-	-	-	-/+	+	+(-3)
55 ºC	-	-	-	-	-	-	-/+	-	+(-4)
Facultative anaerobic	+	-	+	-	-	+	+	+	+
Voges-proskaner	-	-	-	-/+	-	-	+	-	-(w1)
Citrate utilization	-	-	-	-	-	-	-	-	-(+1)
Nitrate reduction	-	-	-	-	+	+	+/-	+	-(+6)
Hydrolysis:									
L-Arginine	+	-	+	-	-	-	-	-	-(+2)
Casein	+	-	+	+/-	-	-	-	+	-(+2)
DNA	-	-/+	+	-	-	+	-/+	-	+(-6)
Gelatin	+	-	-	+/-	-	-	-	-	-(+4)
Starch	+	+	-	+	+	+	+	-	+(-2)
Tween 80	-	-	-	+	-	+	+	+	-(+1)
Urea	-	+	+	+	+	-	+/-	+	-
Acid production:									
D-Amygdalin	+	+/-	+	-	-	+	+	-	-
L-Arabinose	+	-/+	-	-	+	+	+	-	-(+3)
D-Cellobiose	+	+	-	-	-	+	+	-	-(+4)
D-Fructose	+	w/-	-	-	-	+	+	+	-(+6)
D-Galactose	+	+	-	-	-	+	+	-	-(+6)
D-Glucose	+	w	-	-	-	+	+	+	-(+6)
Gluconate	+	+	-	-	-	-	+	-	-(+4)
Glycerol	+	w/-	-	-	+	+	+	+	-
Inulin	+	w/-	-	-	-	+	+	-	-(+1)
Lactose	+	+/-	-	-	+	+	+	-	-(+4)
D-Maltose	+	+/-	-	-	+	+	-/+	+	+(-5)
D-Mannitol	+	+/-	-	-	-	-	-/+	+	-(+2)
D-Mannose	+	-	-	-	-	+	-/+	+	-(+5)
D-Melibiose	+	+/-	-	-	+	+	+	-	-(+4)
D-Melezitose	+	-	-	-	-	+	+	-	-(+1)
α-Glucopyranoside	+	-	-	-	-	+	+	+	-(+3)
Raffinose	+	-	-	-	+	+	+	-	-(+3)
L-Rhamnose	+	+	-	-	-	+	-/+	-	-
D-Ribose	+	+/-	-	-	+	+	+	+	-(w1/+4)
Sucrose	+	-	-	-	+	+	-	+	-(+3)
D-Trehalose	+	+	+	-	-	+	-/+	+	-(+5)
D-Xylose	+	w	-	-/w	-	+	+	-	-(+6)

+, positive; –, negative ; w, weakly positive

Group II (D) contained SRX4-1 and SRX4-2. Colonies were 0.5-3.5 mm in diameter, circular, entire, smooth, convex, translucent and yellow coloured after 2 days of incubation at 37 ºC on C medium. They grew at pH 7-8 and at 25-37 °C but did not grow in 3-5% NaCl, at pH 5-6, 9 and at 15 and 40 °C. Positive for catalase, oxidase and hydrolysis of aesculin, starch, tween 80 and urea, but negative for methyl red, citrate utilization, H_2S production, indole production, nitrate reduction and hydrolysis of L-arginine and DNA. They did not acid production (Table 4.5 and 4.6). On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate SRX4-1 (1,425 nt) and SRX4-2 (1,436 nt) were closely related to each other with 99.9% 16S rRNA gene sequence similarity and to *P.phyllosphaerae* PALXIL04^T (Figure 4.3) with 98.6 and 98.7% 16S rRNA gene sequence similarity, respectively. This result showed low the similarity of 16S rRNA gene sequence when compared with type strain. As the same time, two isolates had differential characteristics with *P.phyllosphaerae* PALXIL04^T as shown in Table 4.6. Therefore, base on the phenotypic properties and 16S rRNA gene sequence, these two isolates represent novel species of the genus *Paenibacillus* (Ash *et al*., 1994). However, isolate SRX4-1 and SRX4-2 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Group (E) contained FXN2-3. Colonies were 4-6 mm in diameter, circular, entire, smooth, convex, opaque and yellow coloured after 2 days of incubation at 37 ºC on C medium. Isolate FXN2-3 grew at pH 7-9 and at 15-37 °C. Did not grow in 3-5% NaCl, at pH 5-6 and 40 °C. Positive for catalase, oxidase, nitrate reduction, hydrolysis of aesculin, starch and urea, but negative for methyl red, Voges-proskaner, citrate utilization, H_2S production, indole production and hydrolysis of L-arginine, casein, DNA, gelatin, L-tyrosine and tween 80. Able to produce acid from L-arabinose, glycerol, lactose, D-maltose, D-melibiose, raffinose, D-ribose and sucrose as shown in Table 4.5 and 4.6. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate FXN2-3 (1,434 nt) was closely related to *P.cellulosilyticus* PALXIL08^T (Figure 4.3) with 98.2% 16S rRNA gene sequence similarity. This result showed low similarity of 16S rRNA gene sequence with type strain. At the same time, FXN2-3 had differential characteristics with *P. cellulosilyticus* PALXIL08^T as shown in Table 4.6. DNA G+C content was 51.6 mol%., which was in the range observed for members of the genus *Paenibacillus*

(Shida *et al*., 1997). As well as, the predominant fatty acids were anteiso-$C_{15:0}$ (42.3%) and iso-$C_{16:0}$ (17.9) (Table 4.7), which were the dominant cellular fatty acid in all members of the genus *Paenibacillus* (Shida *et al*., 1997). Therefore, base on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, FXN2-3 represents novel species of the genus *Paenibacillus* (Ash *et al*., 1994). However, isolate FXN2-3 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Group II (F) contained SRX1-4. Colonies were 3-7 mm in diameter, circular, entire, smooth, convex, translucent and white coloured after 2 days of incubation at 37 ºC on C medium. SRX1-4 grew in 3% NaCl, at pH 7-8 and at 15-37 °C. Did not grow in 5% NaCl, at pH 5-6, 9 and at 40 °C. Positive for catalase, nitrate reduction and hydrolysis of aesculin, DNA, starch and tween 80, but negative for oxidase, methyl red, Voges-proskaner, citrate utilization, H_2S production, indole production and hydrolysis of L-arginine, casein, gelatin, L-tyrosine and urea. Able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, D-maltose, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sucrose, D-trehalose and D-xylose as shown in Table 4.5 and 4.6. On the basis of 16S rRNA gene sequence and phylogenetic analyses, SRX1-4 (1,384 nt) was closely related to *P. edaphicus* KCTC 3995^T (Figure 4.3) with 98.3% 16S rRNA gene sequence similarity. This result showed low the similarity of 16S rRNA gene sequence with type strain. As well as, SRX1-4 had differential characteristics with *P. edaphicus* KCTC 3995^T as shown in Table 4.6. DNA G+C content was 56.8 mol%, which was in the range observed for members of the genus *Paenibacillus* (Shida *et al*., 1997). The predominant fatty acids were anteiso-$C_{15:0}$ (39.0%), $C_{16:0}$ (13.7%) and iso-$C_{15:0}$ (6.2%). The SRX1-4 and *P. edaphicus* KCTC 3995^T showed the similar cellular fatty acid profiles, but significant quantitative differences were also found as shown in (Table 4.7). Major polar lipids were diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine and phosphatidylglycerol (Appendix E-2a). Therefore, base on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, SRX1-4 represents novel species of the genus *Paenibacillus* (Ash *et al*., 1994). However, isolate SRX1-4 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Table 4.6 Differential characteristics of SRX4-1, SRX4-2, FXN2-3, SRX1-4, S3-4A, MX2-3, X11-1 and closely related *Paenibacillus* species

Strains: **1**, SRX4-1; **2**, SRX4-2; **3**, *P. phyllosphaerae* PALXIL04^T; **4**, FXN2-3; **5**, *P. cellulosilyticus* PALXIL08^T; **6**, SRX1-4; **7**, *P. edaphicus* KCTC 3995^T; **8**, S3-4A; **9**, MX2-3; **10**, *P. agaridevorans* KCTC 3849^T; **11**, X11-1; **12**, *P. naphthalenovorans* KACC11505^T; **13**, *P. validus* CCM 3894^T

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Growth in:3% NaCl	-	-	nd	-	+	w	-	+	+	+	+	-	-
Growth at : 10 ºC	-	-	+	-	nd	w	+	-	-	+	-	-	-
15 ºC	-	-	+	+	nd	+	+	-	-	+	+	-	w
Oxidase	+	+	+	+	+	-	+	+	+	+	+	+	+
Voges-proskaner	-	+	-	-	+	-	nd	+	+	-	-	+	+
Nitrate redcution	-	-	-	+	-	+	+	-	+	-	+	-	+
Hydrolysis:													
Casein	+	-	-	-	-	-	-	-	-	-	+	-	-
DNA	-	-	nd	-	nd	+	-	+	-	-	-	nd	nd
Starch	+	+	+	+	nd	+	+	+	+	+	-	-	+
Urea	+	+	-	+	-	-	-	-	+	-	+	+	+
Acid production:													
D-Amgdalin	-	-	+	-	-	+	+	+	+	+	-	-	-
L-Arabinose	-	-	+	+	+	+	+	+	+	-	-	-	-
D-Cellobiose	-	-	+	-	nd	+	+	+	+	+	-	-	-
D-Fructose	-	-	+	-	nd	+	+	+	+	-	+	+	+
D-Galactose	-	-	+	-	nd	+	+	+	+	+	-	+	+
D-Glucose	-	-	+	-	+	+	+	+	+	+	+	+	+
Gluconate	-	-		-	+	-	-	+	+	-	-	-	-
Glycerol	-	-	+	+	nd	+	+	+	+	-	+	-	+
Inulin	-	-	-	-	nd	+	+	+	+	-	-	-	-
D-Maltose	-	-	+	+	+	+	+	+	-	+	+	+	+
D-Mannitol	-	-	+	-	-	-	-	+	-	-	+	+	+
D-Mannose	-	-	w	-	+	+	+	+	-	-	+	+	+
D-Melibiose	-	-	+	+	+	+	+	+	+	+	-	+	-
Raffinose	-	-	w	+	+	+	+	+	+	+	-	+	+
L-Rhamnose	-	-	nd	-	+	+	+	+	-	-	-	-	-
D-Ribose	-	-	-	+	nd	+	+	+	+	-	+	+	+
Salicin	-	-	+	-	nd	+	+	+	+	+	-	-	-
Sorbitol	-	-	nd	-	-	-	+	+	+	-	-	-	-
Sucrose	-	-	+	+	+	+	+	-	-	+	+	+	+
D-Trehalose	-	-	+	-	nd	+	+	+	-	+	+	+	+
D-Xylose	-	w	+	-	+	+	+	+	+	-	-	+	+

+, positive; –, negative; w, weakly positive ; nd, not determined

Group II (G) contained 2 isolates, S3-4A and MX2-3. Colonies were 2-3 mm in diameter, circular, entire, smooth, convex and yellowish-white coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3% NaCl, at pH 7-9 and at 25-45 °C. Did not grow in 5% NaCl, at pH 5-6 and at 15 and 60 °C. Positive for catalase, oxidase, Voges-proskaner and hydrolysis of aesculin, starch and tween 80, but negative for methyl red, citrate utilization, H_2S production, indole production and hydrolysis of L-arginine, casein, gelatin and L-tyrosine. Able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inositol, inulin, lactose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, D-ribose, salicin, sorbitol, sorbose and D-xylose. Not able to produce acid from sucrose (Table 4.5 and 4.6.).

On the basis of 16S rRNA gene sequence and phylogenetic analyses, S3-4A (1,485 nt) and MX2-3 (1,460 nt) were closely related to each other with 97.0% 16S rRNA gene sequence similarity and to *P. agaridevorans* KCTC 3849^T (Figure 4.3) with 97.0 and 97.3% 16S rRNA gene sequence similarity, respectively. Two isolates showed low DNA–DNA relatedness to *P. agaridevorans* KCTC 3849^T (6.0–30.3%) and S3-4A showed low DNA–DNA relatedness to MX2-3 (20.5%). The DNA G+C contents of S3-4A and MX2-3 were 52.9 and 52.7, respectively. The predominant fatty acids of S3-4A were anteiso-$C_{15:0}$ (40.5%), iso-$C_{16:0}$ (18.6%) and anteiso-$C_{17:0}$ (18.3%) as shown in Table 4.7. The predominant fatty acids of MX2-3 were anteiso-$C_{15:0}$ (34.9%), iso-$C_{16:0}$ (19.6%) and $C_{16:0}$ (14.3%) as shown in Table 4.7. The isolates could be clearly distinguished from each other and from known *Paenibacillus* species based on their physiological and biochemical characteristics as well as their phylogenetic positions and DNA-DNA hybridization data. Therefore, these two strains represent novel species of the genus *Paenibacillus* (Ash *et al*., 1994), for which the names *Paenibacillus thailandensis* sp. nov., and *Paenibacillus nanensis* sp. nov., were proposed.

Group (H) contained X11-1. Colonies were 1-5 mm in diameter, circular, lobate, smooth, flat and white coloured after 2 days of incubation at 37 ºC on C medium. The isolate X11-1 grew in 3% NaCl, at pH6-9 and at 25-50 °C. Did not grow in 5% NaCl, at pH 5 and at 15, 55 °C. Positive for catalase, oxidase, nitrate reduction and hydrolysis of aesculin, casein, tween 80 and urea, but negative for methyl red, Voges-proskaner, citrate utilization, H_2S production, indole production

and hydrolysis of L-arginine, DNA, gelatin, starch and L-tyrosine. Able to produce acid from D-fructose, D-glucose, glycerol, D-maltose, D-mannitol, D-mannose, α-glucopyranoside, D-ribose, sucrose and D-trehalose. Not able to produce acid D-amygdalin, L-arabinose, D-cellobiose, D-galactose, gluconate, inositol, inulin, lactose, D-melibiose, D-melezitose, raffinose, L-rhamnose, salicin, sorbitol, sorbose and D-xylose as shown in Table 4.5 and 4.6.

In the 16S rRNA gene-based phylogenetic tree according to the neighbour-joining method, X11-1 was placed in a monophyletic cluster consisting of the closely related *Paenibacillus* species as shown in Figure 4.3. The X11-1 was closely related *P. naphthalenovorans* KACC11505^T and *P. validus* CCM 3894^T with 96.5% 16S rRNA gene sequence similarity. The result of 16S rRNA gene sequence similarity showed less than 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994). DNA G+C content was 51.6 mol%. The predominant fatty acids were anteiso-$C_{15:0}$ (56.6%) and $C_{16:0}$ (14.0%) (Table 4.7). Major polar lipids were diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine and phosphatidylglycerol (Appendix E-2b). The X11-1 could be clearly distinguished from known *Paenibacillus* species based on their physiological and biochemical characteristics as well as their phylogenetic positions. Therefore, X11-1 represent novel species of the genus *Paenibacillus* (Ash *et al*., 1994), for which the name *Paenibacillus xylanisolvens* sp. nov. was proposed.

Table 4.7 Cellular fatty acids compositions of FXN2-3, SRX1-4, S3-4A, MX2-3, X11-1, CXT1-1, P2-3A, P2-5, K3-2, PL1-3 and related taxa Isolates: **1**, FXN2-3; **2**, *P. cellulosilyticus* PALXIL08^T; **3**, SRX1-4; **4**, *P. edaphicus* KCTC 3995^T; **5**, S3-4A; **6**, MX2-3; **7**, *P. agaridevorans* KCTC 3849^T; **8**, X11-1; **9**, *P. naphthalenovorans* KACC11505^T; **10**, *P. validus* CCM 3894^T; **11**, CXT1-1; **12**, P2-3A; **13**, P2-5; **14**, K3-2; **15**, PL1-3. Values are percentages of total fatty acids. tr, trace < 1 %; ND, Not detected

Fatty acids	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Straight -chain saturated															
$C_{14:0}$	1.1	1.3	6.1	ND	tr	tr	tr	2.4	2.2	2.0	tr	11.3	2.3	5.8	6.0
$C_{15:0}$	2.3	ND	2.1	6.7	tr	tr	1.2	3.1	tr	tr	2.9	tr	tr	tr	tr
$C_{16:0}$	8.1	7.4	13.7	3.3	5.6	14.3	13.1	14.0	17.2	15.2	8	36.3	17.4	34.7	20.5
Branched saturated															
iso-$C_{14:0}$	2.2	3	2.2	1.1	1.3	1.3	2.7	2.4	1.2	1.3	1.2	1	tr	1.1	1.8
iso-$C_{15:0}$	8.4	3.3	6.2	4.2	7.4	4.1	5.4	5.1	6.5	9.9	9.6	4.7	10	4.2	7.8
iso-$C_{16:0}$	17.9	28.8	3.3	3.4	18.6	19.6	17.7	7.1	8.8	6.9	15.6	8.2	8.3	12	12.6
iso-$C_{17:0}$	4.4	1.6	1.8	2.2	6.1	7.8	5.6	1.7	4.5	7.7	6.2	2.4	5.6	3.7	4.8
anteiso-$C_{15:0}$	42.3	44.1	39.0	48.1	40.5	34.9	42.4	56.6	47.2	44.6	37.3	25.0	39.7	20.2	30.7
anteiso-$C_{17:0}$	7.9	8.3	2.7	6.4	18.3	12.2	6.5	5.2	9.3	9.7	12.8	6.0	10.2	8.8	9.7

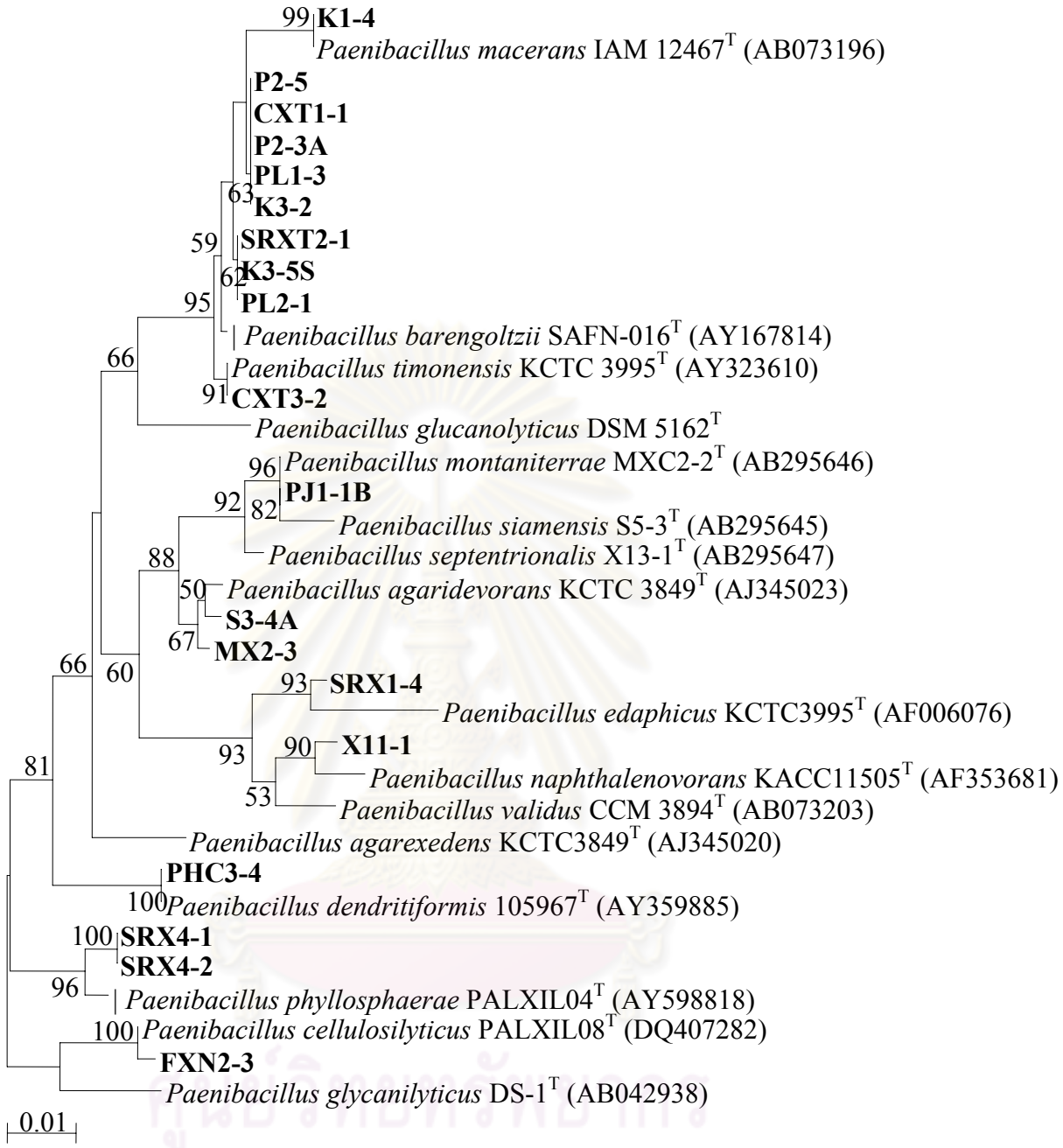

Figure 4.3 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between representative isolates in each of group A-I and known *Paenibacillus* species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

Group (I) contained 13 isolated (K1-5, K3-1, K3-2, K3-5B, PL2-1, SRXT1-1, K3-5S, PL1-3, CXT1-1, P2-3A, CXT3-2, P2-5 and SRXT2-1). Colonies

were 2-20 mm in diameter, circular, entire, smooth, raise or flat and yellow coloured after 2 days of incubation at 37 ºC on C medium. All isolates grew at pH 7-8 and at 25-45 °C. Did not grow in 5% NaCl and at 15 °C. Positive for catalase and oxidase, but negative for H_2S production, indole production and hydrolysis of L-tyrosine and urea. Not able to produce acid from D-amygdalin, glycerol, inositol, L-rhamnose, sorbitol and sorbose as shown in Table 4.5 and 4.9.

On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolates P2-5, CXT1-1, P2-3A, PL1-3, K3-2, SRXT2-1, K3-5S, PL2-1 and CXT3-2 were representative of this group, that isolates P2-5 (1,441 nt), CXT1-1 (1,440 nt), P2-3A (1,441 nt), PL1-3 (1,435 nt), K3-2 (1,446 nt), SRXT2-1 (1,026 nt), K3-5S (1,016 nt), PL2-1 (981 nt) and CXT3-2 were closely related to *P. barengoltzii* SAFN-016^T (Figure 4.3) with 98.5%, 98.8%, 98.7%, 97.9%, 98.1%, 99.8%, 99.8%, 99.7% and 98.2% 16S rRNA gene sequence similarity, respectively as shown in Table 4.8. As the same time, these isolates were closely related *P. timonensis* KCTC 3995^T with 97.6%, 97.7%, 97.6%, 97.0%, 97.3%, 98.1%, 98.1%, 98.0% and 100% 16S rRNA gene sequence similarity, respectively as shown in Table 4.8. This result from SRXT2-1, K3-5S and PL2-1 showed high the similarity of 16S rRNA gene sequence when compared with *P. barengoltzii* SAFN-016^T while isolates P2-5, CXT1-1, P2-3A, PL1-3 and K3-2 showed low the similarity of 16S rRNA gene sequence. The isolate CXT3-2 showed 100% the similarity of 16S rRNA gene sequence when compared with *P. timonensis* KCTC 3995^T.

Table 4.8 Percentage similarities of CXT1-1, K3-2, K3-5S, P2-3A, P2-5, PL1-3, PL2-1, SRXT2-1, CXT3-2 and related taxa

Accession no.		%Similarity										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	**K3-2**	100										
2	**PL1-3**	99.7	100									
3	**P2-3A**	99.1	99.1	100								
4	**CXT1-1**	99	99	99.8	100							
5	**P2-5**	99.2	99.2	99.8	99.7	100						
6	**PL2-1**	98.1	97.9	98.7	98.8	98.5	100					
7	**K3-5S**	98.2	98	98.8	98.9	98.7	99.8	100				
8	**SRXT2-1**	98.2	98	98.8	98.9	98.7	99.8	100	100			
9	**CXT3-2**	97.3	97	97.6	97.7	97.6	98	98.1	98.1	100		
10	AY323610	97.3	97	97.6	97.7	97.6	98	98.1	98.1	100	100	
11	AY167814	98.1	97.9	98.7	98.8	98.5	99.7	99.8	99.8	98.2	98.2	100

Table 4.9 Differential characteristics of CXT1-1, P2-3A, P2-5, K3-2, PL1-3, *P. timonensis* KCTC 3995^T and *P. barengoltzii* SAFN-016^T (Osman *et al*., 2006)

Characteristics	**CXT1-1**	**P2-3A**	**P2-5**	**K3-2**	**PL1-3**	**SAFN-016^T**	**KCTC 3995^T**
Growth at pH: 5	w	-	w	-	w	+	-
9	+	+	+	-	w	+	+
Growth at 10 ºC	-	-	-	-	-	+	-
15 ºC	-	-	-	-	-	+	-
50 ºC	+	+	+	-	+	+	+
55 ºC	+	+	+	-	+	-	+
Voges-Proskauer	-	-	w	-	-	+	-
Nitrate reduction	+	-	-	-	+	+	-
Hydrolysis of:							
Arginine	-	+	-	-	-	-	-
Gelatin	-	+	+	-	+	-	-
Assimilation:							
N-Acetyl-glucosamine	w	+	+	-	-	-	+
L-Arabinose	w	w	w	-	-	-	w
D-Glucose	-	w	+	+	-	-	w
D-Maltose	w	w	w	+	-	-	w
D-Mannitol	w	+	w	-	-	-	-
D-Mannose	w	+	w	-	-	-	+
Potassium gluconate	+	+	+	-	-	+	+
Acid from:							
L-Arabinose	+	+	+	-	-	-	+
D-Fructose	+	+	+	-	-	nd	+
Gluconate	-	+	+	-	+	nd	+
Glucose	+	+	+	-	+	-	+
α-Glucopyranoside	+	+	+	-	-	nd	+
Inuline	+	+	+	-	-	nd	-
D-Mannitol	+	+	+	-	+	-	-
D-Mannose	+	+	+	-	-	nd	+
α-Mannosepyranoside	+	+	+	-	-	nd	+
D-Melibiose	+	+	+	-	+	-	+
D-Melezitose	+	+	+	-	-	nd	-
D-Raffinose	+	+	+	-	-	nd	+
L-Rhamnose	-	-	-	-	-	-	+
D-Ribose	+	+	+	-	+	nd	-
Salicine	+	+	+	-	-	nd	+
D-Sucrose	+	+	+	-	-	-	-
Starch	+	+	+	-	+	-	+
D-Trehalose	+	+	+	-	-	nd	+
D-Turanose	+	+	+	-	+	nd	-
Xylitol	+	+	+	-	-	nd	+
D-Xylose	+	+	+	-	-	nd	+
DNA G+C (mol%)	53.6	52.9	54.4	52.9	59.2	nd	nd

+, positive; –, negative; w, weakly positive ; nd, not determined

The member of Group II (I) were assessed differential between closely related isolates and type strain with $(GTG)_5$-PCR fingerprints. From a cluster analysis based on the $(GTG)_5$-PCR patterns, the banding patterns, two clusters (designated A and B in Figure 4.4 could be delineated of a 52.7% Pearson's correlation coefficient. Clusters (A) was represented by seven isolates and *P. timonensis* KCTC 3995^T as the reference strain. The isolate CXT3-2 showed 83.9% similarity with *P. timonensis* KCTC 3995^T. Furthermore, the isolates SRXT2-1, K3-5S, PL2-1, K1-5, K3-5B and SRXT1-1 were classified of 58.3% similarity with *P. timonensis* KCTC 3995^T. Clusters (B) was represented by six isolates, which resulted in delineation showed 60.3 and 71.4% similarity. The CXT1-1, P2-3A and P2-5 showed the same patterns together as well as K3-1 showed virtually identical with banding of K3-2 with 100% Pearson's correlation coefficient as shown in Figure 4.4.

Based on the phenotypic properties, 16S rRNA gene sequence and $(GTG)_5$-PCR patterns, the isolate CXT3-2 showed 100% the similarity of 16S rRNA gene sequence and had the similarity on patterns more than 80% with *P. timonensis* KCTC 3995^T. Thus, CXT3-2 was identified *P. timonensis* KCTC 3995^T (Roux and Raoult, 2004). While the SRXT2-1, K3-5S, PL2-1 showed high the similarity of 16S rRNA gene sequence with *P. barengoltzii* SAFN-016^T and can distinguish from *P. timonensis* KCTC 3995^T on $(GTG)_5$-PCR patterns. Therefore SRXT2-1, K3-5S and PL2-1 were identified *P. barengoltzii* SAFN-016^T (Osman *et al*., 2006).

On the basis of 16S rRNA gene sequence and phylogenetic analyses, the results of CXT1-1, P2-3A, P2-5, PL1-3 and K3-2 showed low the 16S rRNA similarity with *P. barengoltzii* SAFN-016^T. At the same time, the banding of $(GTG)_5$-PCR patterns were distinguished with *P. timonensis* KCTC 3995^T. DNA G+C contents of CXT1-1, P2-3A, P2-5, PL1-3 and K3-2 were 50.8, 52.9, 52.9, 54.4, 53.6 mol%, which were in the range observed for members of the genus *Paenibacillus* (Shida *et al*., 1997). Therefore, base on the phenotypic properties, chemotaxonomic characteristics, 16S rRNA gene sequence and $(GTG)_5$-PCR patterns, CXT1-1, P2-3A, P2-5, PL1-3 and K3-2 represent novel species of the genus *Paenibacillus* (Ash *et al*., 1994). However, they should be confirmed for DNA-DNA hybridization for the proposal of the new species.

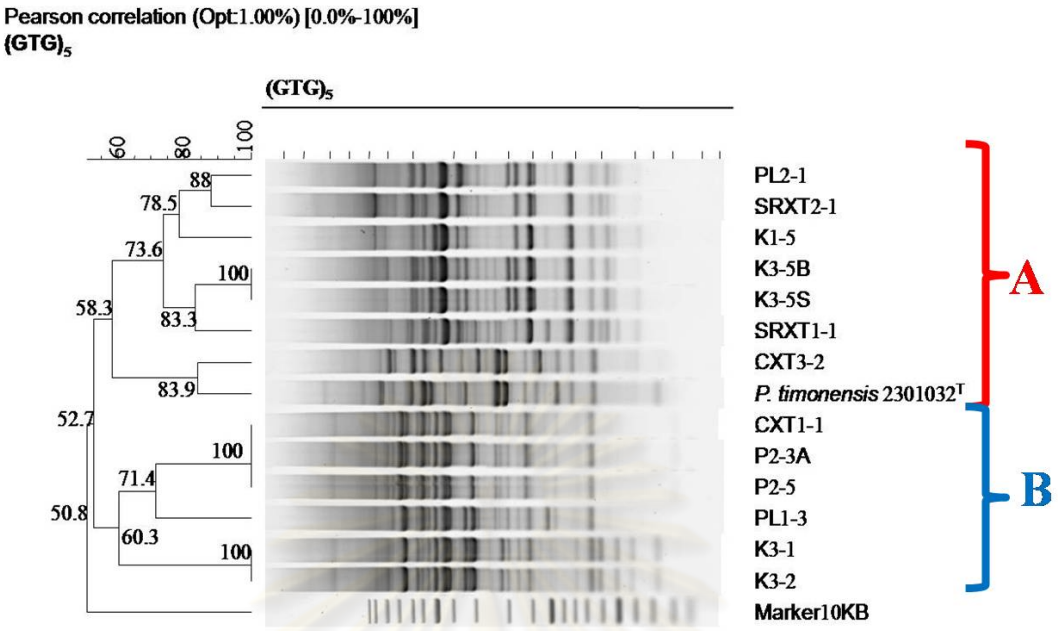

Figure 4.4 Cluster analysis of rep-PCR of the isolates Group II (I) and *P. timonensis* KCTC 3995^{T} (2301032^{T})

4.2.3 Group III

Group III contained 4 isolates, FCN3-3, S1-3, MX15-2 and MX21-2. Cell were Gram positive, rod-shaped (approximate 0.2-0.5 x 1.2-4.0 µm) and motile by means of peritrichous flagella (Figure 4.5). Central and subterminal ellipsoidal endospores were observed in swollen sporangia (Figure 4.6). Colonies were 0.5-3.5 mm in diameter, circular, flat and white-coloured after 2 days of incubation at 37 ºC on C medium. They contained *meso*-diaminopimelic in cell wall peptidoglycan as in the genera *Bacillus* and *Paenibacillus* (Shida *et al.,* 1997). Prodominant menaquinone was MK-7.

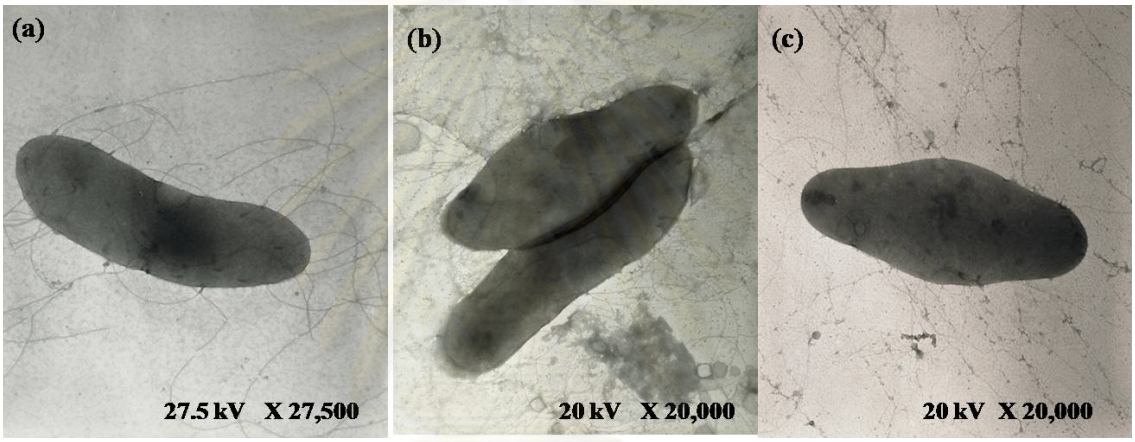

Figure 4.5 Transmission electron micrographs of isolates S1-3 (a), MX15-2 (b) and MX21-2 (c) grown on C agar for 48 h

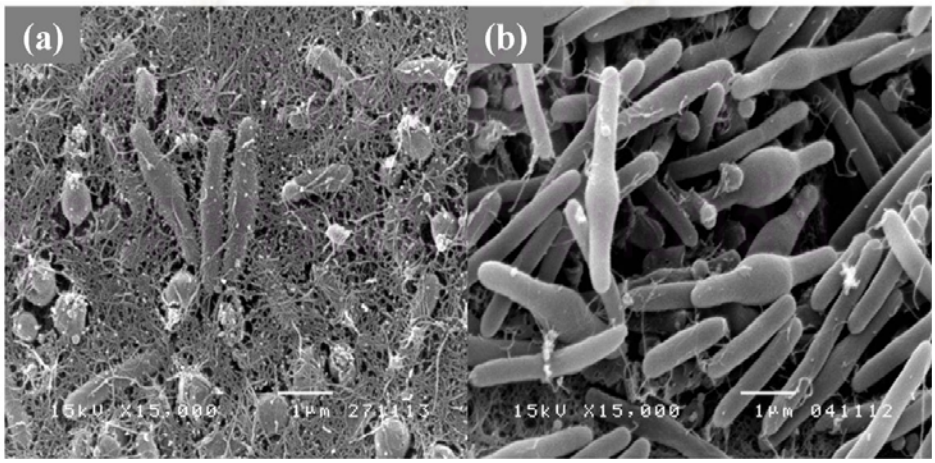

Figure 4.6 Scanning electron micrographs of isolates MX15-2 (a), MX21-2 (b) grown on C agar for 48 h. Bar 1 µm

The isolate FCN3-3 grew in 3% NaCl, at pH 7-9 and at 15-37 °C. Did not grow in 5% NaCl, at pH 5-6 and at 10, 40 °C. Positive for oxidase, nitrate reduction, PNPG, hydrolysis of aesculin and assimilation of L-arabinose, D-glucose, D-maltose, D-mannitol, D-mannose and potassium gluconate. Able to produce acid from aesculin, amygdaline, D-arabinose, L-arabinose, D-arabitol, arbutine, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, glucose, α-glucopyranoside, inositol, D-lactose, D-lyxose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, salicine, D-sucrose, D-trehalose, D-turanose, D-xylose, L-xylose and β-xylopyranoside as shown in Table 4.10.

In the 16S rRNA gene-based phylogenetic tree according to the NJ method, FCN3-3 was placed in a monophyletic cluster consisting of all known *Cohnella* and closely related species as shown in Figure 4.7. The isolate FCN3-3 (1,388 nt) was closely related to *Cohnella phaseoli* KCTC 13070^T with 96.9% 16S rRNA gene sequence similarity. The result of 16S rRNA gene sequence similarity showed less than 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994). The predominant fatty acids of FCN3-3 were anteiso-$C_{15:0}$ (40.7%), iso-$C_{16:0}$ (17.3%), iso-$C_{15:0}$ (14.1%) and $C_{16:0}$ (11.7%) as shown in Table 4.11. Major polar lipids were diphosphatidylglycerol, phosphstidylglycerol, phosphstidylethanolamine and lysyl-phospatidylglycerol. The unknown phospholipids and aminophospholipids were detected (Appendix E-3A). The DNA G+C content was 58.0 mol%, which was close to the values observed for other members of the genus *Cohnella* (Kämpfer *et al*., 2006). Therefore, base on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, FCN3-3 represents novel species of the genus *Cohnella* (Kämpfer *et al*., 2006).

The isolate S1-3 grew at pH 7-9, 20-45 °C and in anaerobic condition, but not in 3-5% NaCl, pH 5-6 and 10 °C, 50-60°C. Positive for oxidase, hydrolysis of aesculin and gelatin, but negative for catalase, methyl red, Voges-Proskauer, nitrate reduction, indole production, citrate, H_2S production, urease, and hydrolysis of L-arginine, casein, starch, tween 80 and L-tyrosine. Able to produce acid from amygdaline, L-arabinose, arbutine, D-cellobiose, aesculin, D-fructose, D-galactose, gentiobiose, glucose, glycogen, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, methyl-α-D-glucoside, methyl-β-D-xyloside, D-raffinose, L-rhamnose, salicine, starch, sucrose, D-trehalose, D-turanose and D-xylose as shown in Table 4.10.

On the basis of 16S rRNA gene sequence and phylogenetic analyses, S1-3 (1,531 nt) was closely related to *C. ginsengisoli* GR21-5^T (Figure 4.7) with 96.0% 16S rRNA gene sequence similarity. The result of 16S rRNA gene sequence similarity showed less than 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994). The predominant fatty acid were iso-$C_{16:0}$ (39.5%) and anteiso-$C_{15:0}$ (26.8%) as shown in Table 4.11. Major polar lipids were diphosphatidylglycerol, phosphstidylglycerol, phosphstidylethanolamine and lysyl-phospatidylglycerol. The unknown phospholipids and aminophospholipids were detected (Appendix E-3B). The DNA G+C content was 53.3 mol%, which was close to the values observed for other members of the genus *Cohnella* (Kämpfer *et al*., 2006). The S1-3 could be clearly distinguished from known *Cohnella* species based on their physiological and biochemical characteristics as well as their phylogenetic positions. Therefore, S1-3 represents novel species of the genus *Cohnella* (Kämpfer *et al*., 2006), for which the name *Cohnella thailandensis* sp. nov. was proposed.

The isolates MX15-2 and MX21-2 grew at pH 7-9 and 20-45 °C, but not in 5% NaCl, at pH 5-6 and at 10-15 and 55 °C. Positive for catalase, oxidase, hydrolysis of aesculin, gelatin, PNPG, starch, tween 80 and assimilation of potassium gluconate, but negative for fermentation of glucose, citrate utilization, indole production, H_2S production, methyl red, Voges-Proskauer, nitrate reduction, urease, hydrolysis of L-arginine, casein, L-tyrosine, assimilation of *N*-acetyl-glucosamine, adipic acid, capric acid and phenylacetic acid. Able to produce acid from aesculin, amygdaline, D-arabinose, L-arabinose, D-arabitol, arbutine, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-glucopyranoside, glucose, glycogene, D-lactose, D-lyxose, D-maltose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, salicine, D-sorbitol, starch, sucrose, D-trehalose, D-turanose, β-xylopyranoside, xylitol and D-xylose. Not able to acid production from *N*-acetylglucosamine, D-adonitol, dulcitol, erythritol, D-fucose, gluconate, glycerol, inositol, inuline, 2-ketogluconate, L-sorbose and L-xylose as shown in Table 4.10.

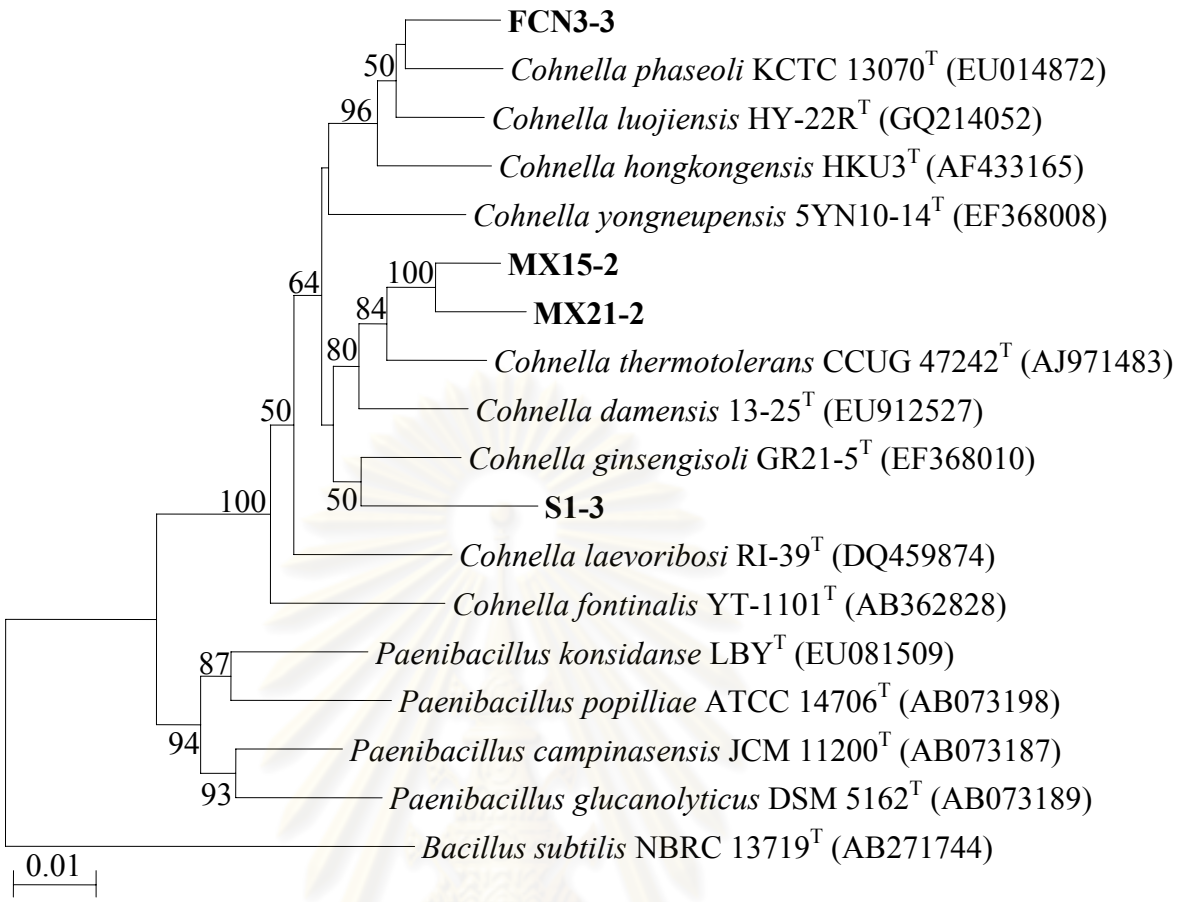

Figure 4.7 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between FCN3-3, S1-3, MX15-2 and MX21-2, all known *Cohnella* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

On the basis of 16S rRNA gene sequence and phylogenetic analyses, MX15-2 (1,507 nt) and MX21-2 (1,553 nt) were closely related to each other with 97.6% 16S rRNA gene sequence similarity and to *C. thermotolerans* CCUG 47242^T (Figure 4.7) with 96.7% and 96.3% sequence similarity, respectively. The DNA–DNA relatedness between MX15-2 and MX21-2 was 52.9% and both isolates showed low DNA–DNA relatedness to *C. thermotolerans* CCUG 47242^T (2.1-5.5%). Predominant fatty acids of MX15-2 and MX21-2 were iso-$C_{16:0}$ and anteiso-$C_{15:0}$ as shown in Table 4.11. Major polar lipids were diphosphatidylglycerol, phosphstidylglycerol, phosphstidylethanolamine and lysyl-phospatidylglycerol. The

unknown phospholipids and aminophospholipids were detected (Appendix E-3D and 3E). DNA G+C contents of MX15-2 and MX21-2 were 63.0 and 65.1 mol%, respectively, which was close to the values observed for other members of the genus *Cohnella* (Kämpfer *et al*., 2006). The MX15-2 and MX21-2 could be clearly distinguished from known *Cohnella* species based on their physiological, biochemical characteristics and their phylogenetic positions as well as DNA-DNA relatedness less than 70% (Wayne *et al*., 1987). Therefore, MX15-2 and MX21-2 represent novel species of the genus *Cohnella* (Kämpfer *et al*., 2006), for which the name *Cohnella xylanilytica* sp. nov. and *Cohnella terrae* were proposed, respectively.

Table 4.10 Differential characteristics of FCN3-3, *C. phaseoli* KCTC 13070^T, S1-3, *C. ginsengisoil* GR21-5^T, MX15-2^T, MX21-2^T and *C. thermotolerans* CCUG 47242^T

Characteristic	FCN3-3	KCTC 13070^T	S1-3	GR21-5^T	MX15-2	MX21-2	CCUG 47242^T
Growth in 3%NaCl	+	-	+	-	w	-	w
Growth at pH 6	-	+	+	+	-	-	+
Growth at : 10 °C	-	+	-	+	-	-	-
45 °C	-	+	+	-	+	+	+
50 °C	-	-	+	-	w	-	+
Catalase test	-	+	w	+	+	+	+
Nitrate reduction	+	-	-	+	-	-	-
Hydrolysis of:							
DNA	-	-	-	-	+	-	-
Gelatin	-	-	+	-	w	+	+
Starch	-	+	+	+	+	+	+
Tween 80	-	-	-	-	+	+	+
Assimilation :							
N-Acetyl-glucosamine	-	+	-	-	-	-	-
L-Arabinose	+	+	+	-	+	-	+
D-Glucose	+	+	+	+	+	-	+
Malic acid	-	-	-	w	+	-	-
D-Maltose	+	+	-	-	+	-	-
D-Mannitol	+	-	-	-	+	-	-
D-Mannose	+	+	+	-	+	-	+
Potassium gluconate	+	-	-	-	+	+	-
Acid from:							
N-acetylglucosamine	-	+	-	-	-	-	-
L-Arabinose	+	+	+	-	+	+	-
D-Arabitol	+	+	-	-	+	+	-
L-Arabitol	-	-	-	-	-	+	-
Arbutine	+	+	+	+	+	+	-
L-Fucose	+	+	+	-	+	+	-
α-Glucopyranoside	+	+	+	-	+	+	-
Glycerol	-	+	-	+	-	-	-
D-Lactose	+	+	+	+	+	+	-
α-mannopyranoside	-	+	-	-	-	+	+
D-Mannitol	+	-	+	+	+	-	-
D-Melezitose	-	+	+	-	-	+	-
L-Rhamnose	+	+	+	-	+	+	-
D-Sorbitol	-	-	-	-	+	+	-
D-Tagatose	-	-	-	-	-	+	-
L-Xylose	+	+	-	-	-	-	+
DNA G+C (mol%)	58.0	60.3*	53.3	61.3†	63.0	65.1	59‡

+, positive; –, negative; w, weakly positive

*Data were obtained from García-Fraile *et al* (2008).

†Data were obtained from Kim *et al* (2010).

‡Data were obtained from Kämpfer *et al* (2006).

Table 4.11 Cellular fatty acids compositions of FCN3-3, S1-3, MX15-2, MX21-2 and related *Cohnella* species

Strains: **1**, FCN3-3; **2**, *C. phaseoli* KCTC 13070^T; **3**, S1-3; **4**, *C. ginsengisoli* GR21-5^T; **5**, MX15-2; **6**, MX21-2; **7**, *C. thermotolerans* CCUG 47242^T

Values are percentages of total fatty acids. tr, trace < 1 %; ND, Not detected

Fatty acids	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Straight -chain saturated							
$C_{14:0}$	2.1	1.8	1.8	5.5	tr	tr	1.5
$C_{15:0}$	2.9	5.3	5.2	1.5	2	4.5	1.1
$C_{16:0}$	11.7	8.9	7.7	16.2	5.4	5.4	6.8
Branched saturated							
iso-$C_{14:0}$	3.0	2.6	4.4	3.8	3.0	3.2	3.4
iso-$C_{15:0}$	14.1	14.3	7.5	9.2	7.5	8.3	4.4
iso-$C_{16:0}$	17.3	14.1	39.5	18.9	39.2	36.1	48.3
iso-$C_{17:0}$	3.1	3.1	1.9	1.2	2.1	2.1	1.1
anteiso-$C_{13:0}$	tr	1.4	ND	ND	ND	ND	ND
anteiso-$C_{15:0}$	40.7	44.5	26.8	40.8	31.6	31.6	26.9
anteiso-$C_{17:0}$	2.7	2.3	3.8	2.9	6.4	5.6	4.8
Monounsaturated							
$C_{16:1}\omega 7c$	ND	1.9	ND	ND	ND	ND	ND
$C_{16:1}\omega 11c$	ND	1.2	ND	ND	ND	ND	tr

4.2.4 Group IV

Group IV contained 2 isolates, CR1-2 and CR5-1. Cell were Gram positive, rod or coccoid shaped (approximate 0.8-1.0 x 0.8-4.5 μm), non-spore forming, facultative anaerobic and non-motile. Colonies were 0.5-2.0 mm in diameter, circular, convex, smooth, opaque and yellow or white-coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5% NaCl, at pH 7-9 (optimally at 7) and 25-45 ºC (optimally at 37 ºC). No growth at pH 5 and at 10-15 and 50-60 ºC. Positive for catalase, DNase, hydrolysis of aesculin, L-arginine, casein, gelatin, starch and urea, but negative for methyl-red and Voges-Proskauer, indole production, citrate utilization, H_2S production. Not able to produce acid from amygdalin, L-arabinose, D-galactose, D-glucose, gluconate, glycerol, inositol, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose and D-xylose as shown in Table 4.12.

Table 4.12 Phenotypic characteristics of CR1-2 and CR5-1 and *Isoptericola variabilis* $MX5^T$

Characteristic	**CR1-2**	**CR5-1**	**$MX5^T$***
Growth at 10 °C	-	-	-
Catalase test	+	+	+
Oxidase test	w	-	-
Hydrolysis:			
Aesculin	+	+	+
Ccasein	+	+	+
Gelatin	+	+	+
Starch	+	+	+
Tyrosine	+	-	+
Tween 80	-	+	-
Urea	+	+	+
Acid production:			
D-cellobiose	w	-	+
D-fructose	+	-	+
Inositol	-	-	-
Inulin	-	-	-
sorbitol	-	-	-
sucrose	+	-	+

+, positive; –, negative; w, weakly positive
*Data were obtained from Wu *et al*. (2010).

In the 16S rRNA gene-based phylogenetic tree according to the NJ method, CR1-2 and CR5-1 were placed in a monophyletic cluster consisting of all known *Isoptericola* and closely related species as shown in Figure 4.8. The isolates

CR1-2 (1,006 nt) and CR5-1 (930 nt) were closely related to each other with 100% 16S rRNA gene sequence similarity and to *Is. variabilis* MX5^T with 99.6% sequence similarity. The isolate CR1-2 contained major menaquinone, MK-9(H_4). The DNA G+C content was 70.0 mol%. Based on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, isolates CR1-2 and CR5-1 were identified as *Isoptericola variabilis* (Stackebrandt *et al*., 2004).

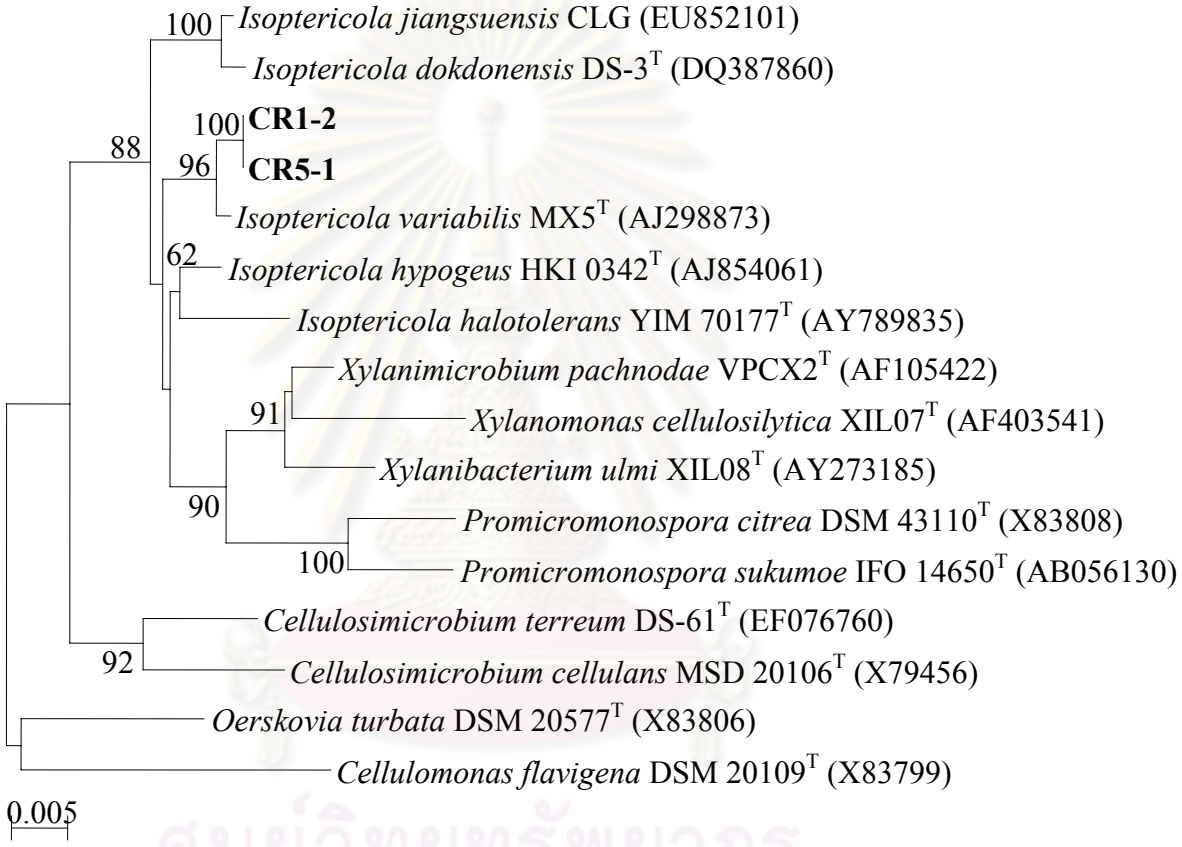

Figure 4.8 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between CR1-2 and CR5-1, all known *Isoptericola* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position

4.2.5 Group V

Group V contained 2 isolates, FXN1-1B and PHX2-5. Cell were Gram positive, rod shaped (approximate 0.5-1.0 x 1.5-2.0 μm), facultative anaerobic and non-motile. Colonies were 0.5-1.0 mm in diameter, circular, convex, smooth, translucent and yellowish coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5% NaCl, at pH 7-9 (optimally at 7) and 15-37 ºC (optimally at 30 ºC). No growth at pH 5 and at 50-60 ºC. Positive for catalase, methyl-red, nitrate reduction, DNase, hydrolysis of aesculin and starch, but negative for Voges-Proskauer, indole production, citrate utilization, H_2S production, urease and hydrolysis of L-arginine, L-tyrosine and tween 80. Able to produce acid from fructose, glucose, D-mannose, salicin and D-xylose, but not amygdalin, gluconate, glycerol, inositol, inulin, D-mannitol, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, sorbitol, sorbose and D-trehalose as shown in Table 4.13.

Table 4.13 Phenotypic characteristics of FXN1-1B, PHX2-5 and *Jonesia denitrificans* ATCC 14870^T

Characteristic	FXN1-1B	PHX2-5	ATCC 14870^T*
Temperature range (°C)	15-37	15-37	30-37
Growth in 5% NaCl	+	+	+
Catalase test	+	+	+
Oxidase test	w	-	-
DNase	+	-	+
Hydrolysis:			
Aesculin	+	+	+
Starch	+	+	+
Acid production:			
Amygdalin	-	-	-
Gluconate	-	-	-
D-Fructose	+	+	+
D-Glucose	+	+	+
α-Glucopyranoside	+	+	+
D-Mannose	+	+	+
Rhamnose	-	-	-
Salicin	+	+	+
Sorbose	-	-	-
D-Xylose	+	+	+

+, positive; –, negative; w, weakly positive
*Data were obtained from Rocourt *et al*. (1987).

On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method, FXN1-1B and PHX2-5 were placed in a monophyletic cluster consisting of all known *Jonesia* and closely related species as shown in Figure 4.9. The FXN1-1B (922 nt) and PHX2-5 (983 nt) were closely related to each other with 99.8% 16S rRNA gene sequence similarity and to *J. denitrificans* ATCC 14870^T with 99.2 and 99.1% sequence similarity, respectively. The isolate PHX2-5 contained major menaquinone, MK-9. DNA G+C content was 58.4 mol%. Based on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, FXN1-1B and PHX2-5 were identified as *J. denitrificans* (Rocourt *et al*., 1987).

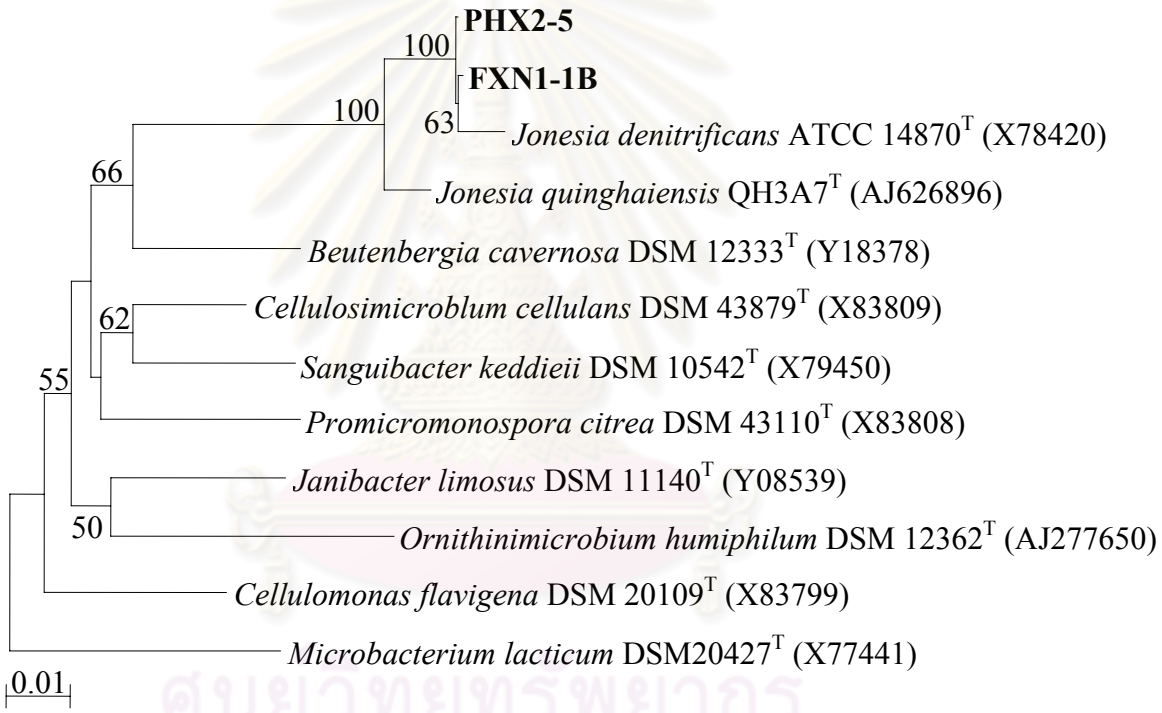

Figure 4.9 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between FXN1-1B and PHX2-5, all known *Jonesia* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.6 Group VI

Group VI contained 3 isolates, CE3-4, SRC1-1 and SRC3-3. Cell were Gram positive, rod shaped (approximate 0.5-1.0 x 0.6-1.5 μm), non-spore-forming and non-motile. Three isolates were classified 2 groups based on the phenotypic properties (Table 4.14 and 4.15). They were identified with 16S rRNA gene sequence and phylogenetic analyses that 3 isolates were clustered within a clade of the genus *Micobacterium* (Figure 4.10).

Group VI (1) contained 2 isolates, SRC1-1 and SRC3-3. Colonies were 0.5-2.5 mm in diameter, circular, convex, smooth, translucent and white coloured after 2 days of incubation at 37 ºC on C medium. They grew in 3-5%NaCl, at pH 5-9 and 30-45 °C, no growth at 10-25 and 50-60 °C. Positive for catalase, urease, hydrolysis of aesculin, L-arginine, DNA, gelatin, starch and L-tyrosine, but negative for oxidase, methyl red, Voges-Proskaner, indole production, nitrate reduction, H_2S production and hydrolysis of tween 80. Able to produce acid from D-cellobiose, D-fructose, D-galactose, D-mannose, D-melibiose and sucrose. Not able to produce acid from D-amygdalin, L-arabinose, gluconate, glycerol, inositol, inulin, lactose, D-mannitol, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, D-trehalose (Table 4.14). Major menaquinone were MK-11 and MK12. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRC1-1 and SRC3-3 were placed in a monophyletic cluster consisting of known *Microbacterium* as shown in Figure 4.10. The SRC1-1 (1,401 nt) and SRC3-3 (1,397 nt) were closely related to each other with 100% 16S rRNA gene sequence similarity and to *M. natoriense* TNJL143-2^T with 99.0% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type strain. Therefore, based on the results mentioned above and phenotypic properties indicated that SRC1-1 and SRC3-3 were identified as *M. natoriense* (Liu *et al*., 2005).

Table 4.14 Phenotypic characteristics of SRC1-1, SRC3-3 and *M. natoriense* TNJL143-2^T

Characteristic	SRC1-1	SRC3-3	TNJL143-2^{T*}
Growth in 3% and 5%NaCl	+	+	+
Growth at pH: 5-9	+	+	+
Growth at:			
30°C	+	+	+
40°C	+	+	+
45°C	w	+	-
Catalase test	+	+	+
Oxidase test	-	-	-
Nitrate reduction	-	-	-
Urease	+	+	-
Hydrolysis of:			
Aesculin	+	+	+
Casein	w	-	-
Gelatin	w	w	+
Starch	w	+	+
Acid production:			
D-Cellobiose	+	+	+
D-Fructose	+	+	+
D-Galactose	+	w	+
Gluconate	-	-	-
Glucose	+	-	+
α-Glucopyranoside	+	-	+
Inositol	-	-	-
D-Lactose	-	-	-
D-Maltose	-	+	+
D-Mannitol	-	-	-
D-Mannose	+	+	+
D-Melibiose	+	+	+
D-Melezitose	-	w	+
L-Rhamnose	-	-	-
D-Ribose	-	-	-
D-Sorbitol	-	-	-
L-Sorbose	-	-	-
D-Sucrose	+	+	+
D-Xylose	-	w	+

+, positive; –, negative; w, weakly positive
*Data were obtained from Liu *et al*. (2005).

Group VI (2) contained CE3-4. Colonies were 0.5-2.5 mm in diameter, circular, convex, smooth, translucent and yellow coloured after 2 days of incubation at 37 ºC on C medium. CE3-4 grew in 3% NaCl, at pH 5-8 (optimally at 7) and 25-37 ºC (optimally at 30 ºC). Did not grow in 5% NaCl, at pH 9 and at 10-15 and 45-60 °C.

Positive for catalase, urease, hydrolysis of aesculin, L-arginine, DNase, gelatin, assimilation of *N*-acetyl-glucosamine, D-glucose, D-maltose, D-mannitol, D-mannose, but negative for oxidase, methyl red, Voges-Proskaner, indole production, nitrate reduction, citrate utilization, H_2S production, hydrolysis of casein, starch, L-tyrosine and tween 80 and assimilation of adipic acid, L-arabinose, capric acid, malic acid, phenylacetic acid and potassium gluconate. Able to produce acid from D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutine, D-cellobiose, aesculin, D-fructose, D-fucose, L-fucose, D-galactose, glucose, glycerol, D-lyxose, D-maltose, D-mannitol, D-mannose, D-melibiose, L-rhamnose, D-sorbitol, D-sucrose, D-trehalose, D-turanose, xylitol, D-xylose and β-xylopyranoside. Gave positive results for the production of *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α-galactosidase, α-glucosidase, leucine arylamidase and trypsin (Table 4.15).

Whole-cell sugars of CE3-4 was galactose, glucose, mannose, xylose, ribose and rhamnose (Appendix E-7). Main menaquinone was MK-13 (51.1%), MK-14 (45.7%) and MK-12 (3.1%). The predominant fatty acids were anteiso-$C_{17:0}$ (60%) and iso-$C_{16:0}$ (16.3%) and anteiso-$C_{15:0}$ (15.3%) as shown in Table 4.16. Major polar lipids were diphosphatidylglycerol, phosphstidylglycerol and glycolipid (Appendix E-4). DNA G+C content was 71.5 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method CE3-4 was placed in a monophyletic cluster consisting of known *Microbacterium* as shown in Figure 4.10. The isolate CE3-4 (1,411 nt) was closely related to *M. imperiale* DSM 20530^T with 98.4% sequence similarity. The result showed low the similarity of 16S rRNA gene sequence with type strain. Therefore, base on the phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence, CE3-4 represents novel species of the genus *Microbacterium*. (Takeuchi *et al*., 1998). However, CE3-4 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Table 4.15 Differential characteristics of CE3-4 and *M. imperiale* DSM 20530^T.

Characteristic	**CE3-4**	**DSM 20530^T***
Catalase test	-	+
Citrate utilization	w	-
Hydrogen sulfide production	-	+
Urease	+	-
Hydrolysis of:		
L-Arginine	+	-
Gelatin	w	-
Starch	-	+
Enzyme assay for:		
Alkaline phosphatase	-	+
α-Chymotrypsin	-	+
Esterase (C4)	+	-
Esterase lipase (C8)	+	-
α-Glucosidase	+	-
α-Mannosidase	-	+
Naphthol-AS-BI-phosphohydrolase	-	w
Trypsin	w	+
Assimilation:		
N-Acetylglucosamine	w	+
α-Glucopyranoside	-	+
Glycerol	+	-
D-Lactose	w	+
D-Melezitose	+	-
D-Raffinose	-	+
D-Ribose	w	-
L-Xylose	-	-
β-Xylopyranoside	+	-
Cell wall sugar	Gal, Glu, Man, Xyl, Rib, Rha	Rha, Man, Gal
Major menaquinones	12, 13, 14	11, 12
DNA G+C (mol%)	71.5	71.2*

+, positive; –, negative; w, weakly positive; Gal, galactose; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose

*Data were obtained from Kageyama *et al*. (2007); Park *et al*. (2006).

Table 4.16 Cellular fatty acids compositions of CE3-4 and *M. imperiale* DSM 20530^T. Values are percentages of total fatty acids. ND, Not detected

Fatty acids	CE3-4	DSM 20530^T*
Straight -chain saturated		
$C_{16:0}$	2.4	5.9
Branched saturated		
iso-$C_{15:0}$	1.9	ND
iso-$C_{16:0}$	16.3	14.0
iso-$C_{17:0}$	3.6	ND
anteiso-$C_{15:0}$	15.3	50.8
anteiso-$C_{17:0}$	60.0	28.2

* Data were obtained from Park *et al*. (2006).

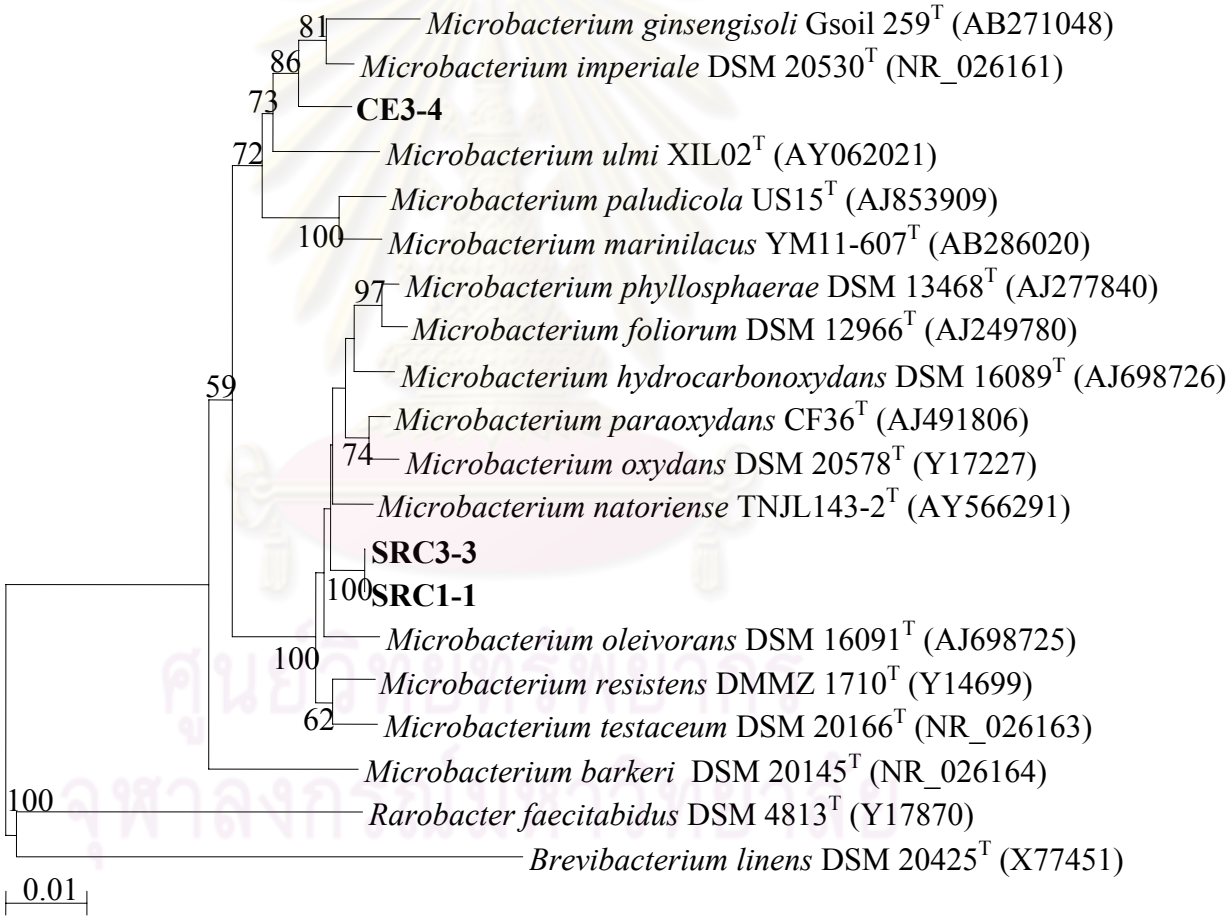

Figure 4.10 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between CE3-4, SRC1-1, and SRC3-3 and known *Microbacterium*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.7 Group VII

Group VII contained SRX2-3. Cell were Gram positive, rod or coccoid shaped (approximate 1.0-1.2 x 1.5-6.0 μm), motile, non-spore-forming and strictly aerobic. Colonies were 0.7-1.0 mm in diameter, irregular, flat, smooth, glistening, yellowish-white coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew at pH 6-9 (optimally at 7) and 25-37 ºC (optimally at 30 ºC), but not growth in 3-5% NaCl, at pH 5 and at 10-15, 45-60 ºC. Positive for catalase, DNase, urease, hydrolysis of aesculin (weakly), L-arginine, casein, gelatin and tween 80, but negative for oxidase, methyl red, Voges-Proskauer, indole production, nitrate reduction, citrate utilization, H_2S production and hydrolysis of starch and L-tyrosine. Able to produce acid from L-arabinose, Not able to produce acid from D-amygdalin, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inositiol, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, *α*-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, sucrose, D-trehalose and D-xylose (Table 4.17).

SRX2-3 contained *meso*-diaminopimelic in cell wall peptidoglycan. MK-8(H_4) was the predominant menaquinone. DNA G+C content was 72.0 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRX2-3 was placed in a monophyletic cluster consisting of known *Nocardioides* as shown in Figure 4.11. The isolate SRX2-3 (900 nt) was closely related to *N. simplex* DSM 20130^T with 99.3% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type strain. Based on the results mentioned above and phenotypic properties indicated that isolate SRX2-3 was identified as *N. simplex* (Yoon *et al*., 1997).

Table 4.17 Phenotypic characteristics of SRX2-3 and *N. simplex* DSM 20130^T (Yoon *et al*., 2007)

Characteristic	**SRX2-3**	**DSM 20130^T**
Optimal temp. (ºC)	25-37	26-37
Catalase test	+	+
Oxidase test	-	+
Citrate utilization	-	+
Indole production	-	-
Nitrate reduction	-	-
Urease	+	-
Hydrolysis of:		
Aesculin	w	+
L-Arginine	+	+
Casein	+	+
DNA	+	+
Gelatin	+	+
Starch	-	w
Tween 80	+	+
Tyrosine	-	+
Acid production:		
L-Arabinose	+	-
D-Cellobiose	-	-
D-Fructose	-	-
D-Galactose	-	-
Glucose	-	+
Glycerol	-	-
Inositol	-	-
D-Lactose	-	-
D-Mannitol	-	-
D-Mannose	-	-
D-Raffinose	-	-
L-Rhamnose	-	-
D-Ribose	-	-
Salicine	-	-
D-Sucrose	-	+
D-Xylose	-	-

+, positive; –, negative; w, weakly positive.

*Data were obtained from Liu *et al*. (2005).

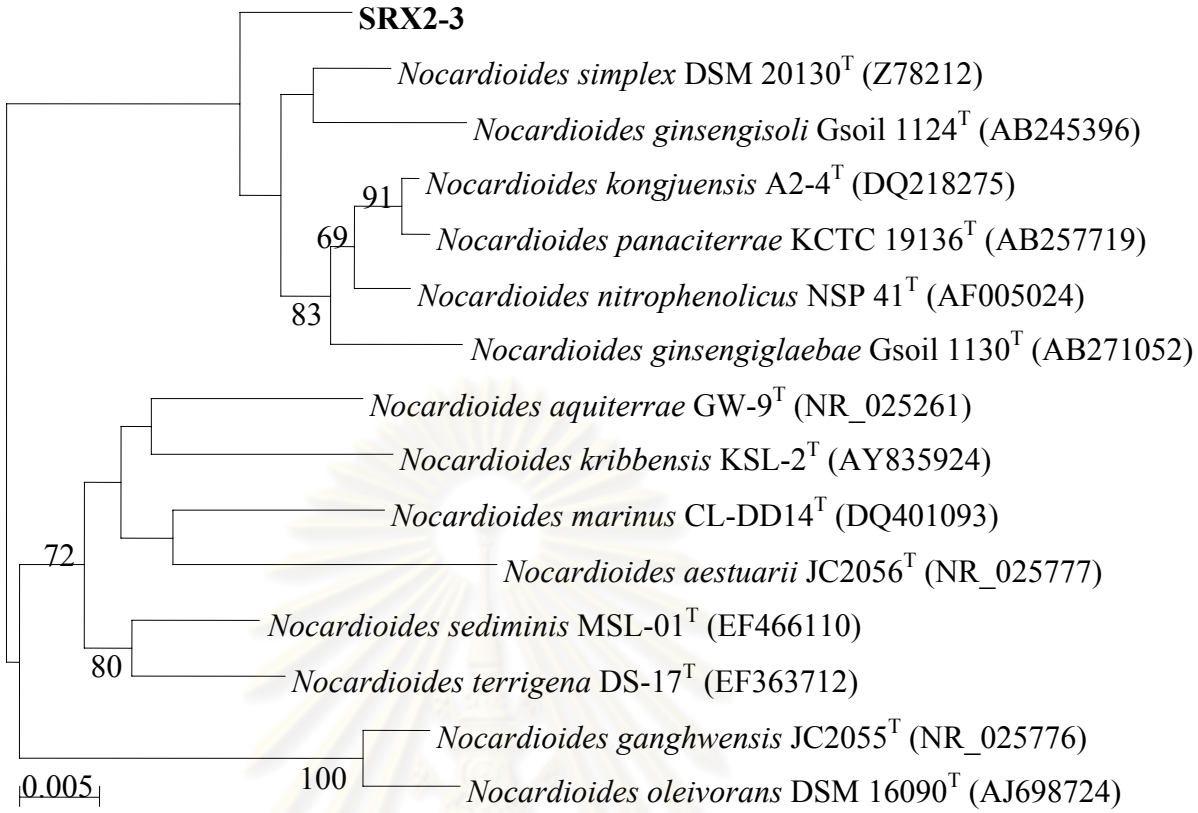

Figure 4.11 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX2-3 and known *Nocardioides*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position

4.2.8 Group VIII

Group VIII contained SRX2-1. Cell were Gram negative, coccobacilli shaped (approximate 0.4-0.7 x 0.5-0.9 μm), facultative anaerobic, non-motile. Colonies were 0.5-1.5 mm in diameter, circular, flat, smooth, yellowish coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3% NaCl, at pH 5-9 (optimally at 7) and 10-50 ºC (optimally at 30 ºC), but not growth in 5% NaCl and at 55-60 ºC. Positive for catalase, citrate utilization, DNase, urease, hydrolysis of L-arginine, casein, gelatin and L-tyrosine, but negative for oxidase, methyl red, Voges-Proskauer, indole production, nitrate reduction, H_2S production and hydrolysis of aesculin, starch and tween 80. Able to produce acid from L-arabinose (weakly), D-fructose, D-galactose, D-glucose, glycerol, D-maltose, D-mannitol, D-mannose, salicin, sucrose and D-trehalose. Not able to produce acid from D-amygdalin, D-cellobiose, gluconate, inositiol, inulin, lactose, D-melibiose, D-melezitose, *α*-glucopyranoside, raffinose, L-rhamnose, D-ribose, sorbitol, sorbose and D-xylose (Table 4.18).

Table 4.18 Phenotypic characteristics of SRX2-1 and *A. junii* LMG 998^T (Bouvet and Grimont, 1986)

Characteristic	**SRX2-1**	**LMG 998^T**
Growth temp. (ºC)	10-50	15-40
Citrate utilization	+	+
Nitrate reduction	-	-
Hydrolysis of:		
L-Arginine	+	+
Gelatin	+	-
Tyrosine	+	+
Acid production:		
L-Arabinose	w	-
D-Cellobiose	-	-
Glucose	+	-
D-Lactose	-	-
D-Melibiose	-	-
D-Mannose	+	-
L-Rhamnose	-	-
D-Xylose	-	-

+, positive; –, negative; w, weakly positive

Predominant ubiquinone of SRX2-1 was Q-9. DNA G+C content was 42.1 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method, SRX2-1 was placed in a monophyletic cluster consisting of known *Acinetobacter* as shown in Figure 4.12. The isolate SRX2-1 (973 nt) was closely related to *A. junii* LMG 998^T with 99.8% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type strain. Based on the results mentioned above and phenotypic properties indicated that isolate SRX2-1 was identified as *A. junii* (Bouvet and Grimont, 1986).

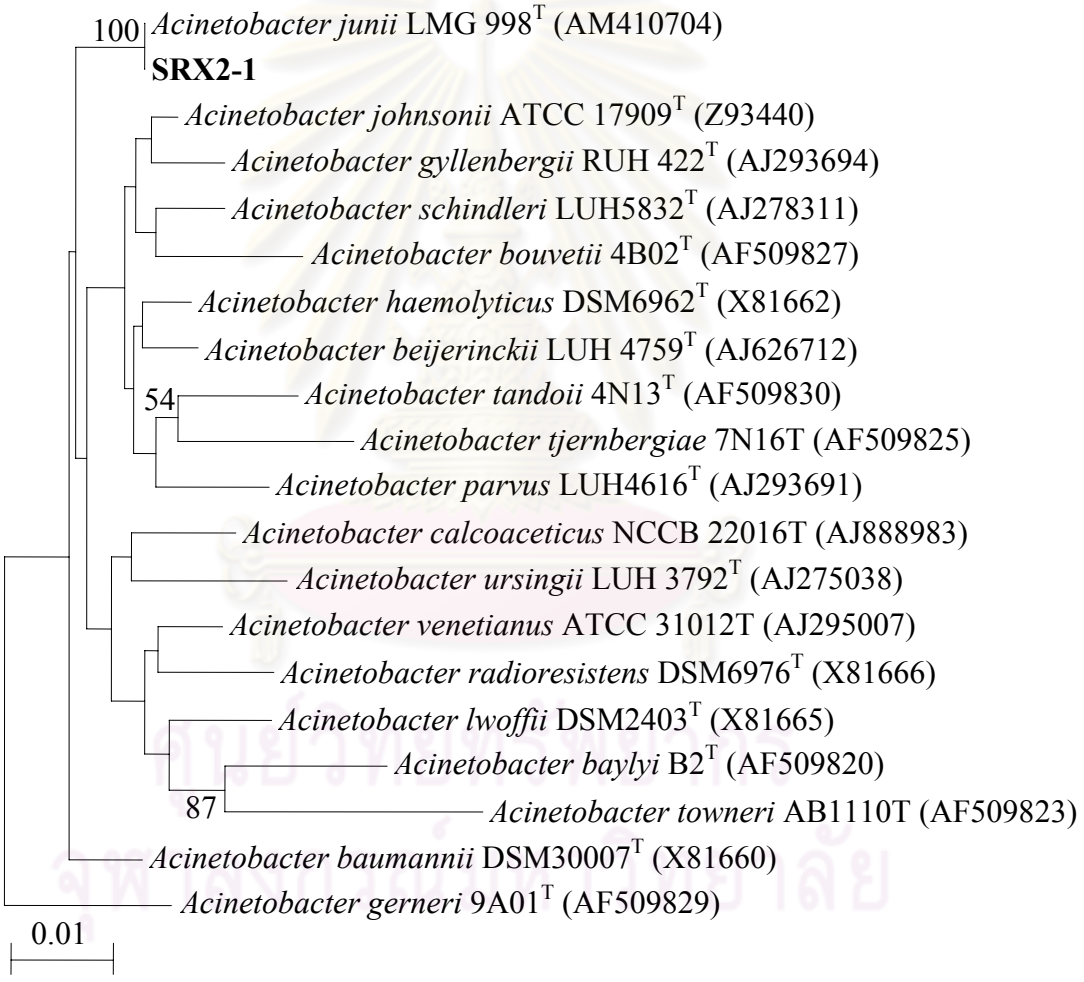

Figure 4.12 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX2-1 and known *Acinetobacter*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.9 Group IX

Group IX contained SRX2-2. Cell were Gram negative, rod (approximate 0.4-1.0 x 1.0-4.0 μm), facultative anaerobic, motile. Colonies were 1-1.6 mm in diameter, irregular, lobate, flat, yellowish coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3% NaCl, at pH 5-9 (optimally at 7) and 30-45 ºC (optimally at 30 ºC), but not growth in 5% NaCl, at 10-25 and 50-60 ºC. Positive for oxidase, methyl red, indole production, DNase, urease, hydrolysis of aesculin, L-arginine, casein, gelatin, starch and L-tyrosine, but negative for catalase, Voges-Proskauer, nitrate reduction, citrate utilization, H_2S production and hydrolysis of tween 80. Able to produce acid from L-arabinose (weakly), D-fructose, D-galactose, D-glucose, glycerol, lactose, D-maltose, D-mannitol, D-mannose, salicin, sucrose and D-trehalose. Not able to produce acid from D-amygdalin, D-cellobiose, gluconate, inositiol, inulin, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, sorbitol, sorbose and D-xylose (Table 4.19).

Predominant ubiquinone of SRX2-2 was Q-8. DNA G+C content was 57.7 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRX2-2 was placed in a monophyletic cluster consisting of known *Aeromonas* as shown in Figure 4.13. The isolate SRX2-2 (1,053 nt) was closely related to *A. enteropelogenes* DSM 6394^T with 99.4% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence.with type strain. Based on the results mentioned above and phenotypic properties indicated that isolate SRX2-2 was identified as *A. enteropelogenes* (Schubert *et al*., 1991).

Table 4.19 Phenotypic characteristics of SRX2-2 and *A. enteropelogenes* DSM 6394^T (Carnahan, *et al*., 1991)

Characteristic	**SRX2-2**	**DSM 6394^T**
Oxidase test	+	+
Citrate utilization	-	+
Facultative anaerobic	+	+
Indole production	+	+
H_2S production	-	-
Nitrate reduction	-	+
Voges-Proskauer	-	-
Hydrolysis of:		
Aesculin	+	-
L-Arginine	+	+
Gelatin	+	+
Starch	+	-
Acid production:		
Amygdalin	-	+
L-Arabinose	w	-
D-Cellobiose	-	+
Gluconate	-	-
Inositol	-	-
D-Mannitol	+	+
D-Mannose	+	+
L-Rhamnose	-	-
Salicin	+	-
Sorbitol	-	-
D-Sucrose	+	-

+, positive; –, negative; w, weakly positive

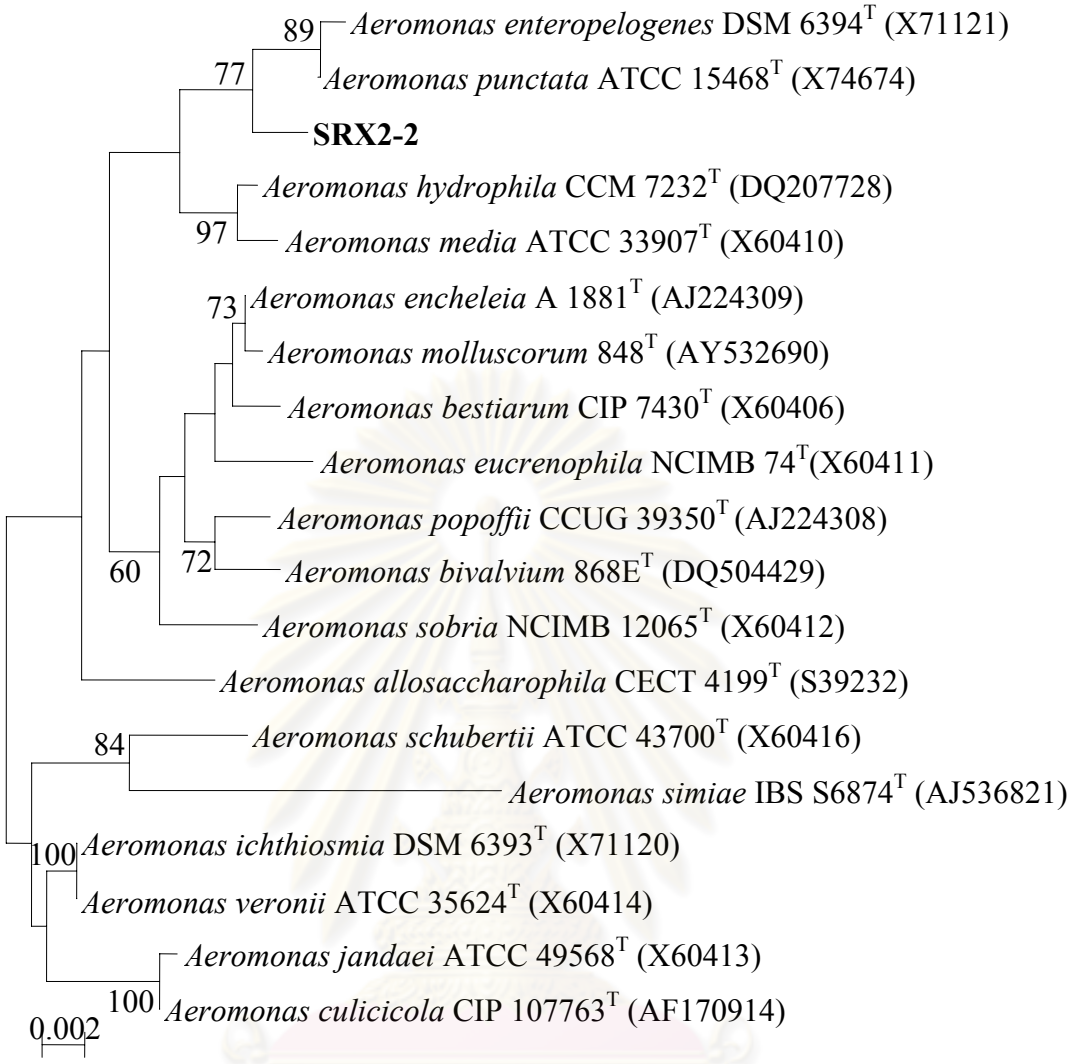

Figure 4.13 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX2-2 and known *Aeromonas*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.002 substitutions per nucleotide position

4.2.10 Group X

Group X contained SRX1-2. Cell were Gram negative, rod shaped (approximate 0.6-0.8 x 1.5-2.5 μm), facultative anaerobic, non-motile. Colonies were 0.5-1.0 mm in diameter, circular, raise, smooth, yellowish-white coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew at pH 7-9 (optimally at 7) and 25-37 ºC (optimally at 30 ºC), but not growth in 3% and 5% NaCl, at pH 5-6 and at 10-15, 45-60 ºC. Positive for catalase, oxidase, urease, hydrolysis of aesculin, L-arginine and PNPG, assimilation of *N*-acetyl-glucosamine, D-glucose, D-maltose, D-mannitol and D-mannose, but negative for methyl red, Voges-Proskauer, citrate utilization, fermentation glucose, indole production, nitrate reduction, H_2S production, DNase, and hydrolysis of casein, gelatin, starch, L-tyrosine and tween 80 and assimilation of adipic acid, L-arabinose, capric acid, malic acid, phenylacetic acid and potassium gluconate. Able to produce acid from D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutine, aesculin, D-cellobiose, D-fructose, D-fucose, L-fucose, D-galactose, D-glucose, glycerol, D-lyxose, D-maltose, D-mannitol, D-mannose, D-melibiose, L-rhamnose, sorbitol, sucrose, D-trehalose, D-turanose, xylitiol, D-xylose and β-xylopyranoside. Not able to produce acid from *N*-acetylglucosamine, D-adonitol, D-amygdalin, dulcitol, erythritol, gentiobiose, gluconate, *α*-glucopyranoside, glycogene, inositol, inuline, 2-ketogluconate, 5-ketogluconate, D-lactose, *α*-mannopyranoside, D-melezitose, D-raffinose, D-ribose, salicine, L-sorbose, starch, D-tagatose and L-xylose. Gave positive results for the production of *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase (weakly), esterase, esterase lipase, *α*-galactosidase, *β*-galactosidase (weakly), *α*-glucosidase, *β*-glucosidase (weakly), leucine arylamidase and trypsin, but negative for the production of *α*-chymotrypsin, *α*-fucosidase, *β*-glucuronidase, lipase, *α*-mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase.

Predominant ubiquinone of SRX1-2 was Q-10. The predominant fatty acids were $C_{18:1}\omega7c$ (83.8%). Major polar lipids were diphosphatidylglycerol, phosphstidylglycerol and phosphstidylethanolamine. The unknown phospholipids were detected (Appendix E-5). DNA G+C content was 63.2 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRX1-2 was placed in a monophyletic cluster consisting of all known *Blastobacter* and closely related species as shown in Figure 4.14. The isolate SRX1-2 (1,371 nt) was closely

related to *Bl. aggregatus* IFAM 1003^T and *Bl. capsulatus* IFAM 1004^T with 98.1% and 95.3% sequence similarity, respectively. SRX1-2 could be clearly distinguished from known *Blastobacter* species based on description of genera *Blastobacter* as well as their phylogenetic positions. The description was as given by Sly (1985) with the following amendment. Cell gram-negative and rod shaped. Cell were usually 0.5-1.0 by 1.0-4.5 μm in size. Colony pigmentation was white or yellow. Positive for catalase, oxidase, acid production from glucose. The G+C content of deoxyribonucleic acid ranges from 59 to 66 mol%. Therefore, SRX1-2 represents novel species of the genus *Blastobacter* (Zavarzin, 1961). However, SRX1-2 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

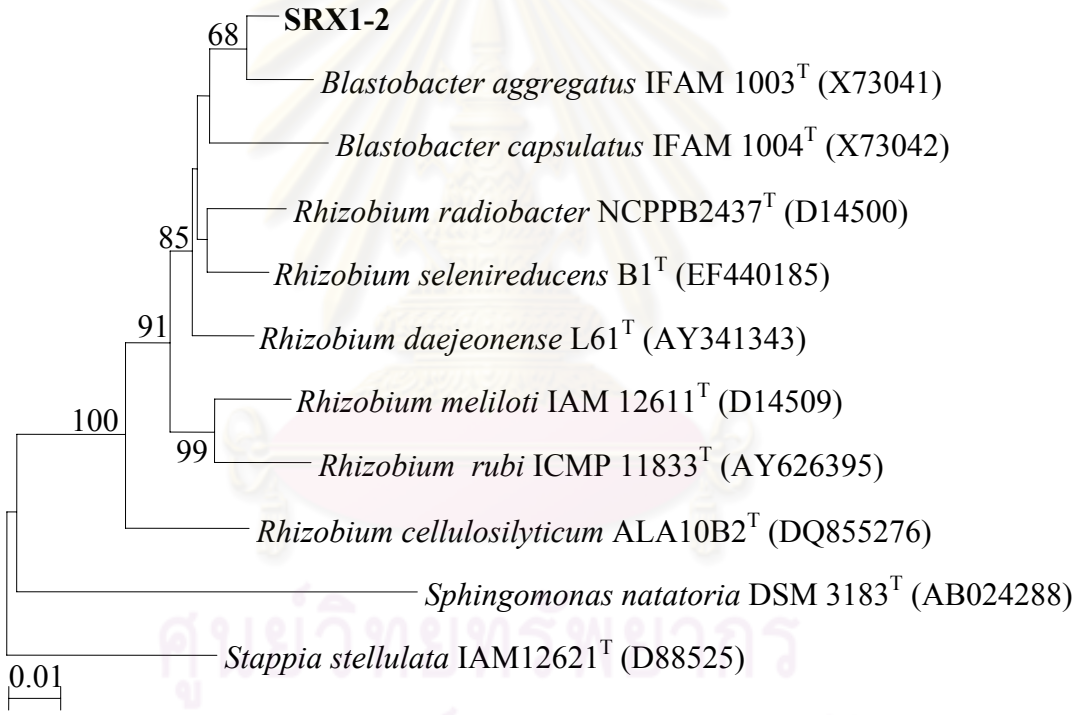

Figure 4.14 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX1-2 and all known *Blastobacter* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.11 Group XI

Group XI contained SRX1-1. Cell were Gram negative, rod shaped (approximate 0.5-1.0 x 1.0-3.0 μm), facultative anaerobic, motile. Colonies were 0.5-1.0 mm in diameter, circular, flat, smooth, shiny, yellowish coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew at pH 5-9 (optimally at 7) and 25-37 ºC (optimally at 30 ºC), but not growth in 3-5% NaCl and at 10-15, 45-60 ºC. Positive for catalase, indole production, citrate utilization, DNase, urease, hydrolysis of aesculin, L-arginine, but negative for oxidase, methy red, Voges-Proskauer, nitrate reduction, H_2S production and hydrolysis of casein, gelatin, starch, L-tyrosine and tween 80. Not able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inositol, inuline, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, sucrose, D-trehalose and D-xylose.

Predominant ubiquinone of SRX1-1 was Q-10. The DNA G+C content was 61.6 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRX1-1 was placed in a monophyletic cluster consisting of known *Ensifer* and closely related species as shown in Figure 4.15. The isolate SRX1-1 (986 nt) was closely related to *E. adhaerens* LMG 20216^T with 99.3% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type strain. Based on the results mentioned above and phenotypic properties as well as their phylogenetic positions indicated that SRX1-1 was identified as *E. adhaerens* (Young, 2003).

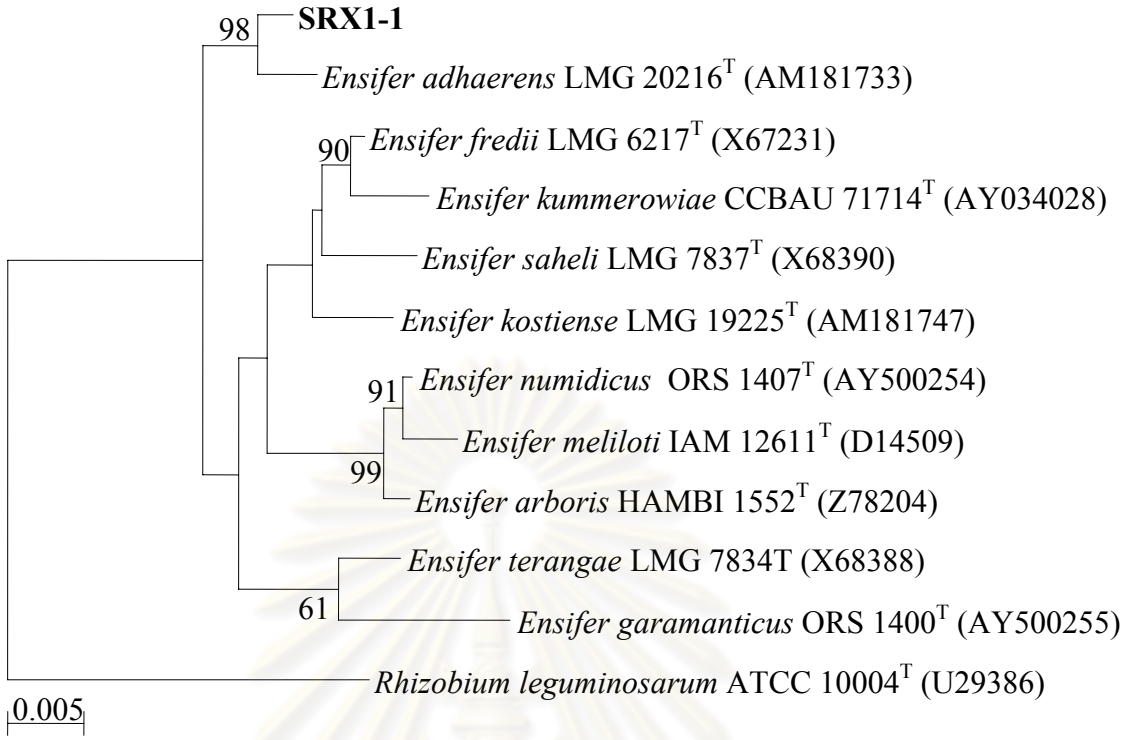

Figure 4.15 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX1-1 and known *Ensifer* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position

4.2.12 Group XII

Group XII contained PHX3-1. Cell were Gram negative, straight rod shaped (approximate 0.3-0.5 x 1.5-2.5 μm), facultative anaerobic, motile. Colonies were 0.5-1.0 mm in diameter, circular, raise, smooth, yellowish-brown coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3-5% NaCl, at pH 6-9 (optimally at 7) and 10-37 ºC (optimally at 30 ºC), but not growth at pH 5 and at 45-60 ºC. Positive for catalase, oxidase, citrate utilization, urease, hydrolysis of L-arginine, starch and L-tyrosine, but negative for methy red, Voges-Proskauer, indole production, nitrate reduction, H_2S production, DNase, and hydrolysis of aesculin, casein, gelatin and tween 80. Able to produce acid from glycerol. Not able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, inositol, inuline, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, sucrose, D-trehalose and D-xylose (Table 4.20).

Table 4.20 Phenotypic characteristics of PHX3-1 and *P. stutzeri* ATCC 17588^T (Nishimori *et al*., 2000)

Characteristic	PHX3-1	ATCC 17588^T
Growth in 3% and 5%NaCl	+	+
Nitrate reduction	-	+
Urease	+	-
Hydrolysis of:		
Aesculin	-	-
L-arginine	+	+
Gelatin	-	-
Starch	+	+
Tween 80	-	+
Acid production:		
L-Arabinose	-	-
D-Fructose	-	+
D-Galactose	-	-
Glycerol	+	+
Inositol	-	-
D-Lyxose	-	-
D-Mannose	-	-
D-Sorbitol	-	-
D-Sucrose	-	-
D-Trehalose	-	-
D-Xylose	-	-

+, positive; –, negative

Predominant ubiquinone of PHX3-1 was Q-9. DNA G+C content was 60.6 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method PHX3-1 was placed in a monophyletic cluster consisting of known *Pseudomonas* as shown in Figure 4.16. The isolate PHX3-1 (962 nt) was closely related to *Ps. stutzeri* ATCC 17588^T with 99.8% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type strain. Based on the results mentioned above and phenotypic properties as well as their phylogenetic positions indicated that PHX3-1 was identified as *P. stutzeri* (Döhler *et al*., 1987).

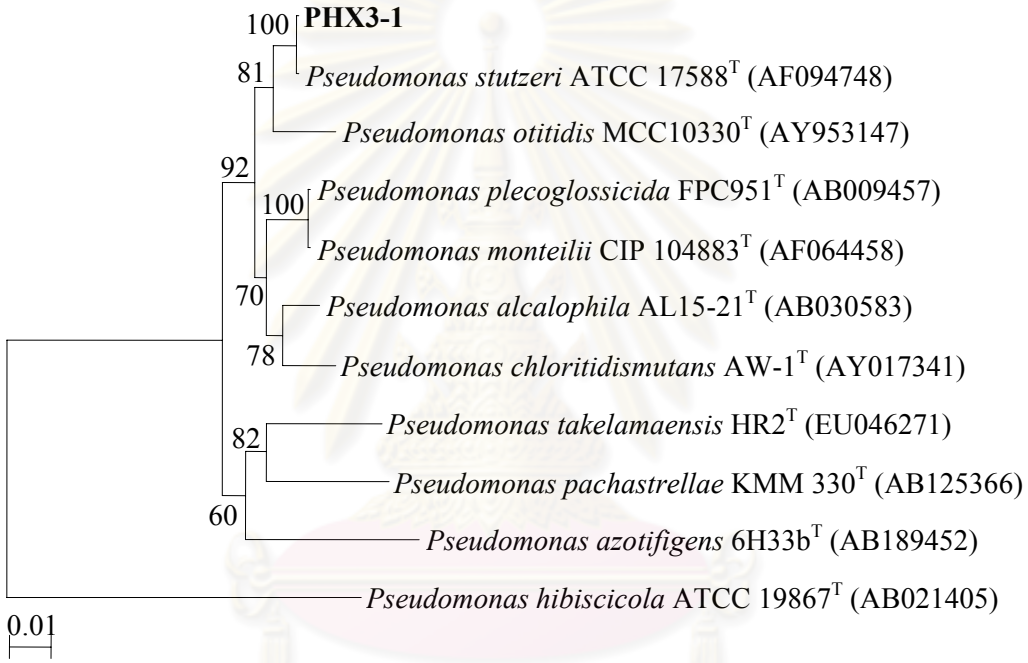

Figure 4.16 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between PHX3-1 and known *Pseudomonas*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.13 Group XIII

Group XIII contained SRX3-4. Cell were Gram negative, rod shaped (approximate 0.3-0.4 x 1.3-1.6 μm), facultative anaerobic, non-motile. Colonies were 2.0-3.0 mm in diameter, circular, convex, smooth, yellowish-brown coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew at pH 5-9 (optimally at 7) and 10 ºC (weakly), 15-37 ºC (optimally at 30 ºC), but not growth in 3-5% NaCl, at 45-60 ºC. Positive for catalase, oxidase, DNase, fermentation glucose, H_2S production, PNPG, urease, hydrolysis of aesculin and L-arginine and assimilation of *N*-acetyl-glucosamine, L-arabinose, D-glucose, D-maltose and D-mannose, but negative for methy red, Voges-Proskauer, citrate utilization, indole production, nitrate reduction and hydrolysis of casein, gelatin, starch, L-tyrosine and tween 80 and assimilation of adipic acid, capric acid, malic acid, D-mannitol, phenylacetic acid and potassium gluconate. Able to produce acid from D-arabinose, L-arabinose, arbutine, aesculin, D-cellobiose, D-fructose, L-fucose, D-galactose, glucose, *α*-glucopyranoside, lactose, D-maltose, D-mannose, *α*-mannopyranoside, D-melibiose, D-melezitose, raffinose, L-rhamnose, sucrose, D-trehalose and D-turanose. Not able to produce acid from *N*-acetylglocosamine, D-adonitol, amygdaline, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, gentiobiose, gluconate, glycerol, glycogene, inositol, inulin, 2-ketogluconate, 5-ketogluconate, D-lyxose, D-mannitol, D-ribose, salicin, D-sorbitol, L-sorbose, starch, D-tagatose, xylitol, D-xylose, L-xylose and *β*-xylopyranoside. Gave positive results for the production of *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase (weakly), esterase, esterase lipase, *α*-fucosidase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, leucine arylamidase, *α*-mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase, but negative for the production of *α*-chymotrypsin, β-glucosidase, *β*-glucuronidase, lipase and trypsin (Table 4.21).

On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method SRX3-4 was placed in a monophyletic cluster consisting of known *Sphingobacterium* as shown in Figure 4.17. The isolate SRX3-4 (1,419 nt) was closely related to *Sp. multivorum* B5533^T, *Sp. canadense* CR11^T, *Sp. siyangense* SY1^T with 98.3, 97.7 and 97.4% sequence similarity. Predominant menaquinone of SRX3-4 was MK-7. The predominant fatty acids were iso-$C_{15:0}$ (17.3%), $C_{16:0}$ (16.9%) and iso-$C_{17:0}$ 3OH (9.2%) as shown in Table 4.22. DNA G+C contents was 44.4 mol%. This

value was almost consistent with the G+C content of members of the genus *Sphingobacterium* (Mehnaz *et al*., 2007). SRX3-4 could be clearly distinguished from known *Sphingobacterium* species based on their physiological and biochemical characteristics as well as their phylogenetic positions. Therefore, SRX3-4 represents novel species of the genus *Sphingobacterium*. (Yabuuchi *et al*., 1983). However, SRX3-4 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Table 4.21 Differential characteristics of SRX3-4 and *Sp. multivorum* B5533^T (Yabuuchi, *et al*., 1983; Yoo *et al*., 2007)

Characteristic	**SRX3-4^T**	**B5533^T**
H_2Sproduction	+	-
Hydrolysis of:		
Starch	-	+
Tyrosine	-	+
Acid from:		
Amygdaline	-	+
Glycerol	-	+
Glycogene	-	+
Inuline	-	+
L-Rhamnose	+	-
Salicine	-	+
L-Sorbose	-	+
D-Trehalose	+	-
D-Xylose	-	+
DNA G+C (mol%)	44.4	39.9-40.5

+, positive; –, negative

Table 4.22 Cellular fatty acids compositions of SRX3-4 and *Sp. multivorum* B5533^T (Yoo *et al*., 2007). Values are percentages of total fatty acids. tr, trace < 1 %; ND, Not detected

Fatty acids	**SRX3-4**	**B5533^T**
$C_{14:0}$	3.9	2.7
$C_{16:0}$	16.9	7.8
Branched saturated		
iso-$C_{15:0}$	17.3	22.2
iso-$C_{15:0}$ 3OH	4.3	3.2
iso-$C_{17:0}$ 3OH	9.2	7.1
Monounsaturated		
$C_{14:0}$ 2OH	1.2	ND
$C_{16:0}$ 2OH	1.5	tr
$C_{16:0}$ 3OH	8.2	5.3

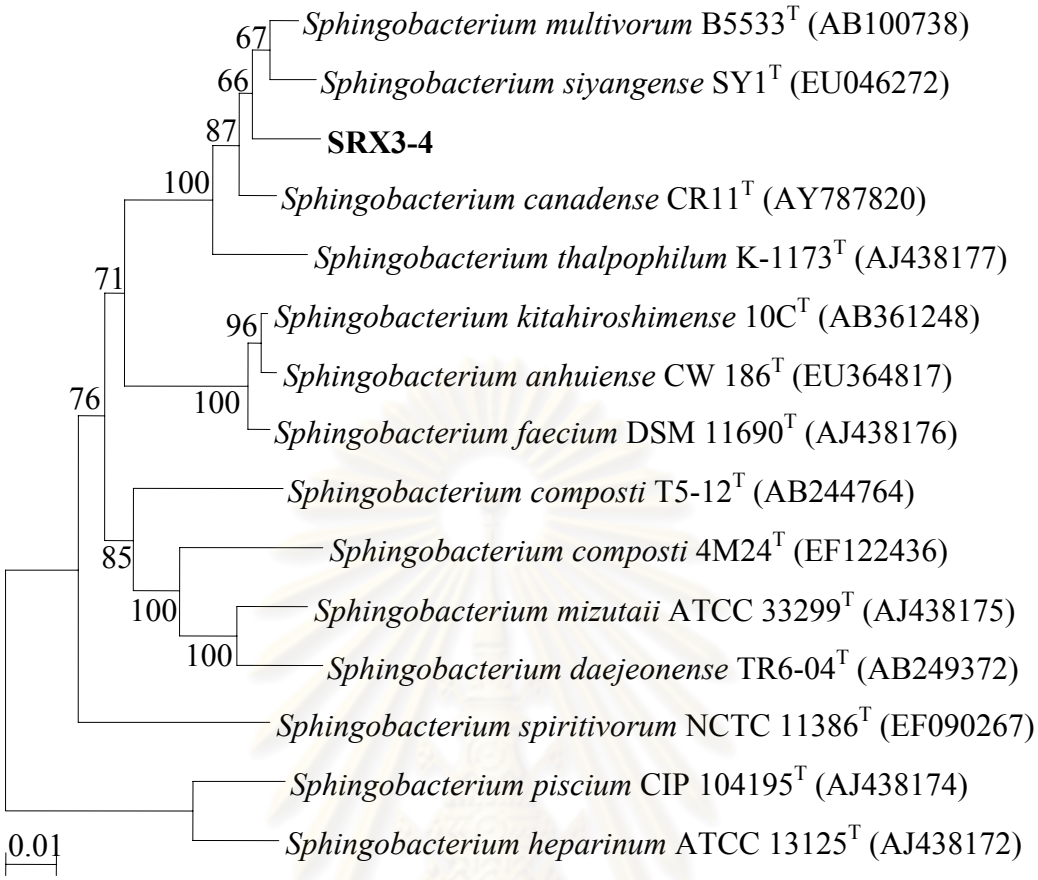

Figure 4.17 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between SRX3-4 and known *Sphingobacterium*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.14 Group XIV

Group XIV contained CE4-1. Cell were Gram negative, rod shaped (approximate 0.5-0.7 x 0.5.0-1.0 μm), facultative anaerobic, non-motile. Colonies were 1.0-1.5 mm in diameter, circular, convex, smooth, yellowish coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3-5% NaCl, at pH 5-9 (optimally at 7) and 25 ºC (weakly), 30-45 ºC (optimally at 30 ºC), but not growth at 10-20 and 50-60 ºC. Positive for catalase, oxidase, citrate utilization, nitrate reduction, DNase, urease, hydrolysis of aesculin, L-arginine, casein, gelatin and starch and assimilation of adipic acid (weakly), L-arabinose, glucose, D-maltose (weakly) and D-mannose, but negative for fermentation glucose, methy red, Voges-Proskauer, indole production, H_2S production, PNPG and hydrolysis of L-tyrosine and tween 80 and assimilation of *N*-acetyl-glucosamine, capric acid, malic acid, D-mannitol, phenylacetic acid and potassium gluconate. Able to produce acid from D-cellobiose, D-fructose, glucose, D-maltose, salicin, sucrose and D-trehalose. Not able to produce acid from amygdaline, L-arabinose, galactose, gluconate, glycerol, inositol, inulin, lactose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, sorbitol, sorbose and D-xylose. Gave positive results for the production of acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase, esterase lipase, α-glucosidase (weakly), β-glucosidase (weakly), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase, but negative for the production of *N*-acetyl-β-glucosaminidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase and α-mannosidase (Table 4.23).

On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method CE4-1 was placed in a monophyletic cluster consisting of known *Sphingomonas* as shown in Figure 4.18. The isolate CE4-1 (1,375 nt) was closely related to *Sp. mucosissima* CP173-2^T with 98.3% sequence similarity. Predominant ubiquinone.of CE4-1 was Q-10. The predominant fatty acids were $C_{18:1}\omega7c$ (64.0%), $C_{17:1}\omega6c$ (8.4%), $C_{16:0}$ (7.2%) and $C_{14:0}$ 2OH (7.1%) as in the genera *Sphingomonas* (Yabuuchi *et al*., 1990) (Table 4.24). Major polar lipid were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, glycolipids and sphingoglycolipid (Appendix E-6). DNA G+C content was 66.5 mol%. This value was almost consistent with the G+C content of members of the genus *Sphingomonas*, which ranges between

59.0 and 67 mol%. (Yabuuchi *et al*., 1990). The CE4-1 could be clearly distinguished from known *Sphingomonas* species based on their physiological and biochemical characteristics as well as their phylogenetic positions. Therefore, CE4-1 represents novel species of the genus *Sphingomonas*. (Yabuuchi *et al*., 1990). However, CE4-1 should be confirmed for DNA-DNA hybridization for the proposal of the new species.

Table 4.23 Differential characteristics of CE4-1 and *Sp. mucosissima* CP173-2^T (Reddy and Garcia-Pichel, 2007)

Characteristic	**CE4-1**	**CP173-2^T**
Colony colour	yellow	orange
Growth in 3%NaCl	+	-
Growth in 5%NaCl	+	-
15°C	-	+
20°C	-	+
25°C	w	+
40°C	+	-
45°C	+	-
Citrate utilization	+	-
Nitrate reduction	+	-
Urease	+	-
Hydrolysis of:		
Aesculin	+	-
Arginine	+	-
Gelatin	+	-
Assimilation:		
D-Maltose	w	-
D-Mannose	+	-
Phenylacetic acid	-	+

+, positive; –, negative ; w, weakly positive

Table 4.24 Cellular fatty acids compositions of CE4-1 and *Sp. mucosissima* CP173-2^T (Reddy and Garcia-Pichel, 2007)

Values are percentages of total fatty acids. tr, trace < 1 %; ND, Not detected

Fatty acids	**CE4-1**	**CP173-2^T**
$C_{14:0}$	1.6	10.3
$C_{15:0}$	tr	1.4
$C_{16:0}$	7.2	7.1
$C_{17:0}$	1.0	ND
$C_{18:0}$	tr	4.1
Monounsaturated		
$C_{14:0}$ 2OH	7.1	22.3
$C_{15:0}$ 2OH	1.6	tr
$C_{16:0}$ 2OH	ND	4.1
$C_{16:1}\omega5C$	1.7	1.0
$C_{17:1}\omega6c$	8.4	2.7
$C_{17:1}\omega8c$	1.2	ND
$C_{18:1}\omega5c$	1.2	tr
$C_{18:1}\omega7c$	64.0	33.9
11 methyl $C_{18:1}$ $\omega7c$	2.4	ND

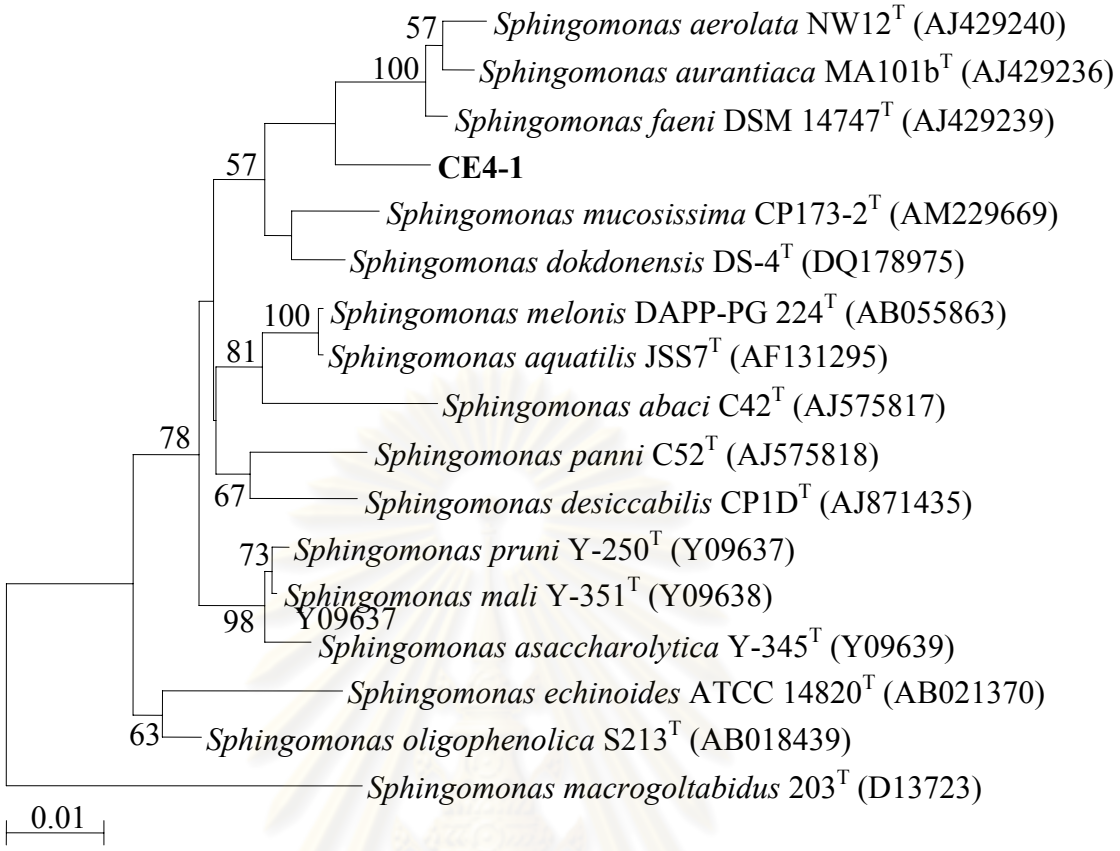

Figure 4.18 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between CE4-1 and known *Sphingomonas*. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.2.15 Group XV

Group XV contained FXN3-1. Cell were Gram negative, straight rod shaped (approximate 0.4-0.5 x 1.5-2.0 μm), facultative anaerobic, motile. Colonies were 0.5-1.0 mm in diameter, circular, flat, smooth, yellowish-brown coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3-5% NaCl, at pH 5-9 (optimally at 7) and 10-37 ºC (optimally at 30 ºC), but not growth at 45-60 ºC. Positive for catalase, citrate utilization, DNase, urease, hydrolysis of aesculin, L-arginine, caseins and L-tyrosine, but negative for oxidase, methy red, Voges-Proskauer, indole production, nitrate reduction, H_2S production and hydrolysis of gelatin, starch and tween 80. Not able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inositol, inuline, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, sucrose, D-trehalose and D-xylose (Table 4.25).

Table 4.25 Phenotypic characteristics of FXN3-1 and *S. maltophilia* IAM 12423^T (Heylen *et al*., 2007; Yoon *et al*., 2006)

Characteristic	**FXN3-1**	**IAM 12423^T**
Growth in 3% and 5%NaCl	+	+
Catalase test	+	+
Oxidase test	-	-
Indole production	-	-
Nitrate reduction	-	+
Urease	+	+
Hydrolysis of:		
Aesculin	+	+
L-Arginine	+	+
Casein	+	+
DNA	+	+
Gelatin	-	+
Starch	-	w
Tween 80	-	+

+, positive; –, negative ; w, weakly positive

Predominant ubiquinone of FXN3-1 was Q-8. DNA G+C content was 65.4 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method FXN3-1 was placed in a monophyletic cluster consisting of all known *Stenotrophomonas* and relate taxa as shown in Figure 4.19. The isolate FXN3-1 (923 nt) was closely related to *St. maltophilia* IAM 12423^T with 99.4% sequence similarity. The result showed high the similarity of 16S rRNA gene sequence with type starin. Based on the results mentioned above and phenotypic properties as well as their phylogenetic positions indicated that isolate FXN3-1 was identified as *St. maltophilia* (Palleroni and Bradbury, 1993).

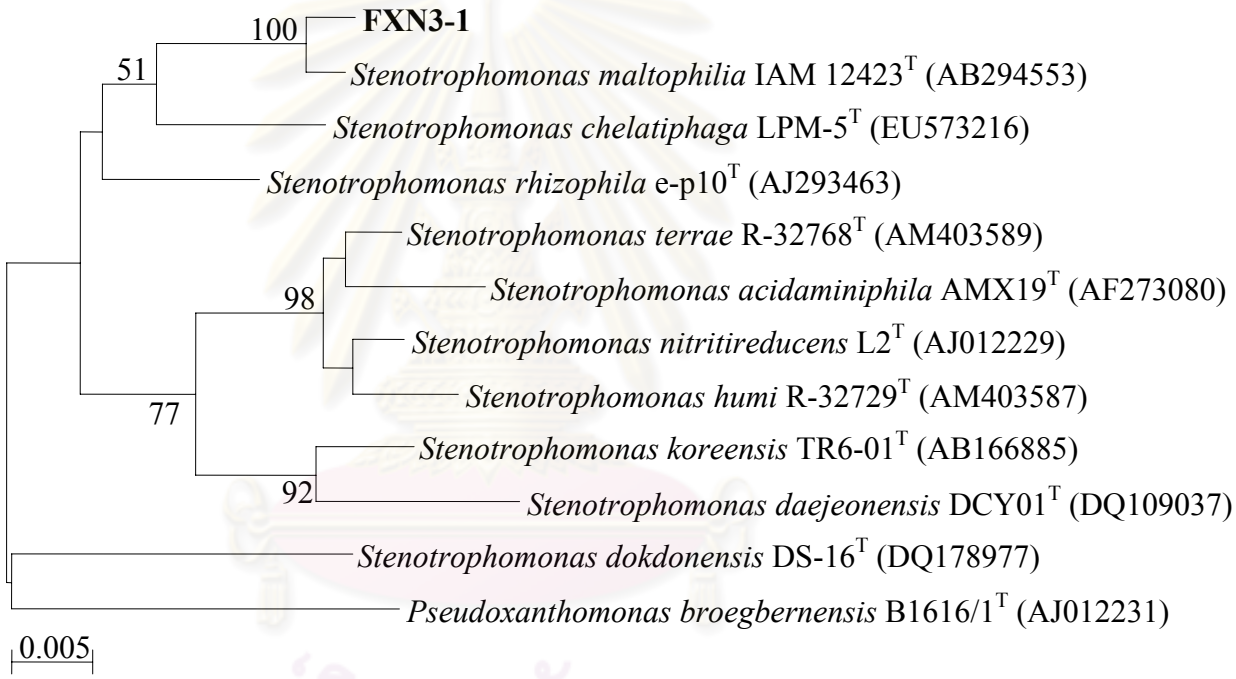

Figure 4.19 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between FXN3-1 and all known *Stenotrophomonas* and related taxa. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position

4.2.16 Group XVI

Group XVI contained PHX2-7. Cells were Gram negative, rod shaped (approximate 0.6-0.7 x 1.5-2.5 μm), facultative anaerobic, motile. Colonies were 1.0-4.0 mm in diameter, circular, raise, smooth, yellowish coloured and opaque after 2 days of incubation at 37 ºC on C medium. Grew in 3-5% NaCl, at pH 5-9 (optimally at 7) and 25-45 ºC (optimally at 30 ºC), but not growth at 10-15 and 50-60 ºC. Positive for oxidase, methyl red, citrate utilization, urease, hydrolysis of L-arginine, starch (weakly) and L-tyrosine, but negative for catalase, Voges-Proskauer, indole production, nitrate reduction, H_2S production, DNase and hydrolysis of aesculin, casein, gelatin and tween 80. Able to produce acid from D-fructose, D-galactose, D-glucose, glycerol, D-maltose, D-mannitol, D-melibiose, D-melezito, α-glucopyranoside, raffinose, sucrose and D-trehalose. Not able to produce acid from D-amygdalin, L-arabinose, D-cellobiose, gluconate, inositol, inuline, lactose, D-mannose, L-rhamnose, D-ribose, salicin, sorbitol, sorbose, and D-xylose (Table 4.26).

Predominant ubiquinone was Q-8. DNA G+C content was 61.9 mol%. On the basis of 16S rRNA gene-based phylogenetic tree according to the NJ method PHX2-7 was placed in a monophyletic cluster consisting of all known *Zobellella* and closely related species as shown in Figure 4.20. The isolate PHX2-7 (911 nt) was closely related to *Z. denitrificans* ZD1^T and *Z. taiwanensis* ZT1^T with 99.2% and 98.6% sequence similarity, respectively. The result showed high the similarity of 16S rRNA gene sequence with *Z. denitrificans* ZD1^T. Based on the results mentioned above and phenotypic properties indicated that isolate PHX2-7 was identified as *Z. denitrificans* (Lin and Shieh, 2006).

Table 4.26 Phenotypic characteristics of PHX2-7, *Z. denitrificans* ZD1^T and *Z. taiwanensis* ZT1^T (Lin and Shieh, 2006)

Characteristic	**PHX2-7**	**ZD1^T**	**ZT1^T**
Growth in 3 and 5%NaCl	+	+	+
Growth at pH 6-9	+	+	+
Growth at 20-45 °C	+	+	+
Growth at 50 °C	-	-	-
Catalase test	-	+	+
Oxidase test	+	+	+
Facultative anaerobic	+	+	+
Indole production	-	-	-
Hydrogen sulfide production	-	-	-
Nitrate reduction	-	+	+
Urease	+	w	+
Hydrolysis of:			
L-Arginine	+	-	-
DNA	-	-	-
Gelatin	-	-	-
Acid production:			
L-Arabinose	-	-	-
D-Cellobiose	-	+	+
D-Galactose	+	+	-
Glucose	+	+	+
Inositol	-	+	+
D-Lactose	-	-	-
D-Maltose	+	+	+
D-Mannitol	+	+	+
D-Mannose	-	+	+
D-Melibiose	+	+	-
D-Melezitose	+	+	+
D-Ribose	-	+	+
D-Sorbitol	-	+	+
D-Sucrose	+	+	+
Starch	w	+	+
D-Trehalose	+	+	-
D-Xylose	-	-	-

+, positive; –, negative; w, weakly positive

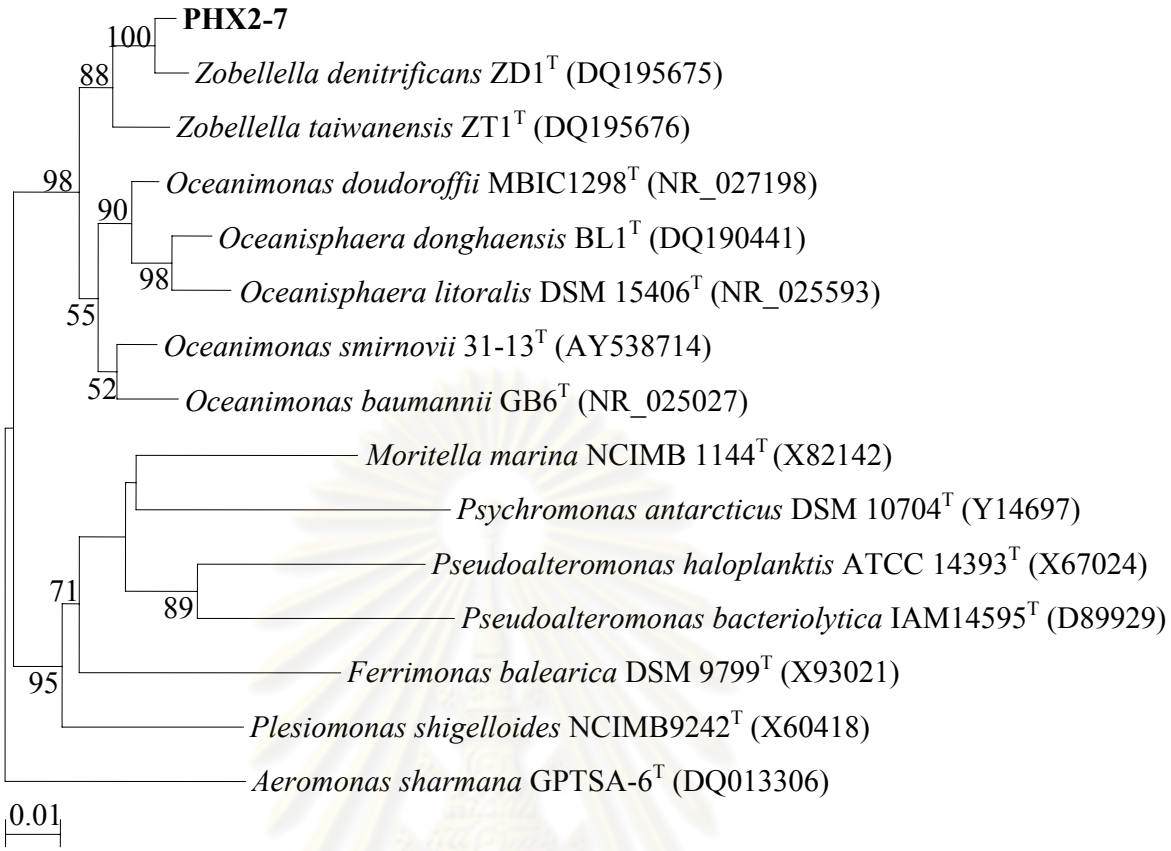

Figure 4.20 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between PHX2-7 and all known *Zobellella* and closely related species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

4.3 Primary screening and quantitative xylanase activity assay

Initially, xylanolytic bacteria were isolated from 45 samples of soil using XB agar medium containing 1% (w/v) Oat spelt xylan and incubated at 37 ºC for 2 days. Seventy isolates showed xylanase clear zone (diameter 1.0-15.0 mm) surrounded their colonies. The 70 isolates were assayed for xylanase activity by DNS method using 1% Oat spelt xylan as substrate (Miller, 1959). The reaction mixtures were incubated at 37 ºC for 10 min. The xylanase producing bacteria of Group I showed clear zone (diameter 1.1-15.0 mm) and produced xylanase activity (1.03-20.81 unit/ml) while Group II showed clear zone (diameter 1.5-9.5 mm) and produced xylanase activity (1.07-4.27 unit/ml). Group III to Group VII were Gram positive bacteria which showed clear zone (radius 1.7-12.0 mm) and produced xylanase activity (1.10-3.93 unit/ml). Group VIII to Group XVI were Gram negative bacteria which showed clear zone (radius 1.0-2.0 mm) and produced xylanase activity (1.12-9.27 unit/ml). It was found that, isolate P2-3 was produced biggest clear zone (radius 15.0 mm) and had highest xylanase activity (20.81 unit/ml) (Table 4.27). Therefore, the isolate P2-3 was selected for further study.

Table 4.27 Clear zone radius and xylanase activity of the isolates

No. isolate	Clear zone (mm)	Xylanase activity (unit/ml)*	No. isolate	Clear zone (mm)	Xylanase activity (unit/ml)*
Group I			**Group II**		
CXT1-2	2.5	2.41±0.20	CXT1-1	4.5	1.91±0.07
CP1-1	6.0	6.01±0.15	CXT3-2	1.9	1.07±0.15
CP1-2	5.4	4.92±0.12	FXN2-3	2.5	1.71±0.12
CP2-1	4.0	3.81±0.02	K1-4	6.5	3.20±0.07
CR7-1	1.1	2.19±0.24	K1-5	6.5	1.10±0.15
FCN3-4	4.0	1.03±0.03	K3-1	5.5	1.75±0.05
K1-6A	5.0	1.49±0.17	K3-2	5.0	2.53±0.18
K1-6B	3.5	1.22±0.24	K3-5B	5.5	1.26±0.20
K3-6	1.7	2.22±0.35	K3-5S	2.0	1.86±0.20
MS1-1	2.0	2.73±0.05	MX2-3	6.5	1.44±0.20
MS1-2	3.7	2.91±0.21	P2-3A	3.0	1.62±0.12
MS1-4	2.0	5.90±0.02	P2-5	2.8	1.68±0.07
MS1-5	2.0	1.94±0.25	PL1-3	3.0	1.63±0.18
NS1-1	3.0	1.07±0.03	PHC3-4	1.5	1.32±0.17
P2-2	3.0	2.89±0.02	PJ1-1A	1.75	1.46±0.12
P2-3	15.0	20.81±0.02	PJ1-1B	1.5	1.31±0.13
PHC3-3	5.0	1.34±0.02	PL2-1	1.95	1.64±0.10
PHX1-5	1.4	1.26±0.23	S3-4A	8.5	4.27±0.13
PHX2-2A	10.0	12.59±0.31	SRX4-1	8.5	1.93±0.21
PJ1-2	2.0	1.21±0.16	SRX4-2	9.5	1.77±0.06
SK1-3	3.0	1.60±0.18	SRXT1-1	4.5	1.55±0.09
SRC2-3	7.5	6.07±0.55	SRXT2-1	5.0	1.16±0.12
SRX1-4	4.5	1.95±0.32	X11-1	1.5	1.95±0.02
SRXT1-2	6.0	5.63±0.22	**Group VIII**		
TH2-1A	4.0	3.23±0.28	SRX2-1	1.0	1.30±0.03
TH2-2	4.0	3.89±0.31	**Group XI**		
Group III			SRX2-2	1.0	1.21±0.10
FCN3-3	2.5	1.84±0.16	**Group X**		
S1-3	5.0	1.94±0.22	SRX1-2	1.2	1.23±0.02
MX15-2	6.0	1.44±0.02	**Group XI**		
MX21-2	6.5	3.93±0.51	SRX1-1	1.0	1.12±0.04
Group IV			**Group XII**		
CR1-2	12.0	17.65±0.25	PHX3-1	1.0	1.28±0.07
CR5-1	11.7	8.10±0.12	**Group XIII**		
Group V			SRX3-4	1.0	1.32±0.02
FXN1-1B	5.0	1.16±0.13	**Group XIV**		
PHX2-5	5.0	1.21±0.16	CE4-1	1.5	1.21±0.09
Group VI			**Group XV**		
CE3-4	1.7	1.10±0.09	FXN3-1	1.0	1.28±0.07
SRC1-1	1.8	1.53±0.03	**Group XVI**		
SRC3-3	1.7	1.18±0.05	PHX2-7	2.0	9.27±0.19
Group VII					
SRX2-3	1.7	1.52±0.02			

*One unit of xylanase activity was defined as 1 μmol of xylose released per min under the condition assayed.

4.4 Optimization of xylanase production

The previously result showed that isolate P2-3 performed a maximal xylanase activity at 20.81 unit/ml. Therefore, P2-3 was selected for further study. Optimization of crude xylanase production of P2-3 was carried out in the XC medium. Firstly, the optimal biomass substrates for cultivation and condition were screened with the one-at-a-time strategy. Subsequently, the medium compositions and initial pH were studied by Placket-Burman design (PBD) and followed by central composite design (CCD).

4.4.1 Screening for optimal substrate for cultivation and condition

The effects of different biomass substrates by P2-3 was investigated in the XC medium. Xylanase production was analysed when Oat spelt xylan in cultural medium was replaced by Beech wood xylan, Birch wood xylan, corn cob and soybean. Xylanase activity of the cultured supernatant was assayed against three substrates including Oat spelt xylan, Beech wood xylan and Birch wood xylan. The maximal xylanase production (167.32 unit/ml) was obtained when corn cob was used as substrate in medium and used Oat spelt xylan as substrate to assay xylanase activity. (Figure 4.21).

4.4.2 Screening of essential medium compositions and initial pH

The effects of medium compositions (corn cob, peptone, yeast extract, K_2HPO_4, KCl, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$) and initial pH on xylanase production were studied using the Placket-Burman design (PBD) (Table 3.2). It can be noted that the xylanase activity varied from 0.02 to 286.58 unit/ml. The maximum activity of these response were attained at the run number 1, employing 10 g/l corn cob, 5 g/l peptone, 6 g/l K_2HPO_4, 0.3 g/l KCl, 0.75 g/l $MgSO_4.7H_2O$, 0.01 g/l $FeSO_4.7H_2O$ and pH 6while yeast extract had no response for xylanase production. The lowest activity was obtained when the isolate was grown in the medium at the run number 12, containing 2 g/l K_2HPO_4, 0.1 KCl, 0.25 g/l $MgSO_4.7H_2O$, 0.01 g/l $FeSO_4.7H_2O$ and pH 6 without corn cob, peptone and yeast extract in the medium (Table 4.28)

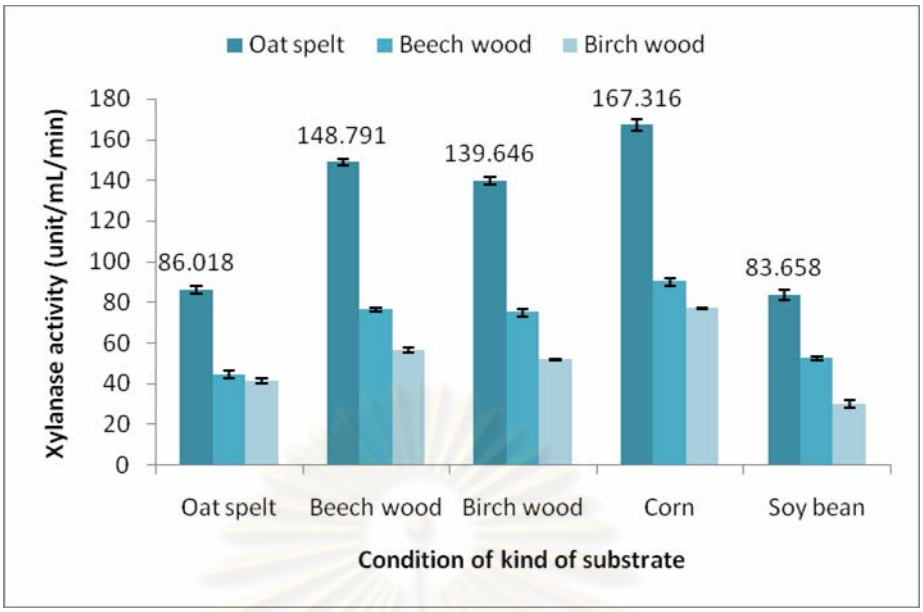

Figure 4.21 Effects of various biomass substrates for cultivation and condition on xylanase production

Table 4.28 Experimental design and results of the Plackett-Burman design

X_1, corn cob; X_2, peptone; X_3, yeast extract; X_4, K_2HPO_4, X_5, KCl; X_6, $MgSO_4.7H_2O$; X_7, $FeSO_4.7H_2O$ and X_8, pH

	Variables								
Run no.	**X_1**	**X_2**	**X_3**	**X_4**	**X_5**	**X_6**	**X_7**	**X_8**	**Xylanase activity (unit/ml)**
1	10	5	0	6	0.3	0.75	0.01	6	286.58±9.39
2	0	5	1	2	0.3	0.75	0.03	6	0.72±0.04
3	10	0	1	6	0.1	0.75	0.03	8	51.21±4.57
4	0	5	0	6	0.3	0.25	0.03	8	1.47±0.37
5	0	0	1	2	0.3	0.75	0.01	8	0.85±0.16
6	0	0	0	6	0.1	0.75	0.03	6	0.45±0.03
7	10	0	0	2	0.3	0.25	0.03	8	12.19±2.46
8	10	5	0	2	0.1	0.75	0.01	8	161.84±5.45
9	10	5	1	2	0.1	0.25	0.03	6	203.33±7.08
10	0	5	1	6	0.1	0.25	0.01	8	0.83±0.13
11	10	0	1	6	0.3	0.25	0.01	6	54.19±2.13
12	0	0	0	2	0.1	0.25	0.01	6	0.02±0.11
13	5	2.5	0.5	4	0.2	0.5	0.02	7	103.17±4.11
14	5	2.5	0.5	4	0.2	0.5	0.02	7	133.59±6.44
15	5	2.5	0.5	4	0.2	0.5	0.02	7	132.81±8.36

Table 4.29 shows the test factors as medium compositions and the rank of significance. The medium compositions were screened at confidence level of 95% based on their effects. The confidence level of corn cob, peptone and pH were 99.39, 98.77 and 96.59%, respectively implying that the effects of these compositions were significant. Rest of the components such as yeast extract, K_2HPO_4, KCl, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ showed confidence level below 95% and hence, were considered insignificant. The K_2HPO_4 and $MgSO_4.7H_2O$ showed positive effect for xylanase production, so they were set at their higher level. While, the *P*-value of the factors with negative effect (yeast extract, KCl and $FeSO_4.7H_2O$) for xylanase production were more than 0.05 and hence, were set at lower level considered for further optimization. Therefore, corn cob, peptone and pH were selected for further optimization using CCD.

Table 4.29 Effect estimates for xylanase production from the results of the PBD

Variables	Medium compositions	Effect	*F*-value	*P*-value	Confidence level (%)
X_1	Corn cob	127.51	162.16	0.0061*	99.39
X_2	Peptone	89.31	79.56	0.0123*	98.77
X_3	Yeast extract	-25.23	6.35	0.1280	87.2
X_4	K_2HPO_4	2.63	0.069	0.8171	18.29
X_5	KCl	-10.27	1.05	0.4128	58.72
X_6	$MgSO_4.7H_2O$	38.28	14.61	0.0621	93.79
X_7	$FeSO_4.7H_2O$	-39.15	15.29	0.0596	94.04
X_8	pH	-52.81	27.82	0.0341*	96.59

* Statistically significant at 95% ($P<0.05$) of confidence level

4.4.3 Optimization of screening of medium compositions and initial pH

The variables used for CCD optimization were corn cob, peptone and pH. The concentrations of these major tested variables were presented in Table 4.30. The centre point in the design was repeated three times for estimation of error. The experimental results of central composite design (CCD) were fitted into a quadratic polynomial equation. The values of the regression coefficients were calculated and an equation for predicting enzyme production can be written:

Xylanase activity (unit/ml) = -2575.52351 +63.22333A +49.28823B +721.41833C +4.69085AB -8.54937AC -4.10845BC -1.10465A^2 -4.20656B^2 -49.18169C^2

Where A, B and C were the coded levels of corn cob, peptone and pH, respectively.

Table 4.30 CCD design and the experimental data

Run no.	Variable level			Xylanase activity (unit/ml)	
	A:Corn cob (g/l)	B:Peptone (g/l)	C:pH	Predicted	Experimental
1	5	7.5	5	31.67	6.483
2	10	0.67	6	37.29	35.82
3	15	7.5	5	367.32	391.26
4	5	7.5	7	147.03	213.61
5	1.34	5	6	45.89	6.45
6	15	2.5	7	67.56	101.26
7	5	2.5	5	19.00	30.54
8	10	5	7.73	139.92	118.77
9	18.66	5	6	276.05	304.14
10	10	9.33	6	292.60	282.72
11	15	2.5	5	82.11	24.04
12	5	2.5	7	137.44	122.01
13	10	5	4.27	52.62	62.42
14	15	7.5	7	311.69	270.66
15	10	5	6	243.82	244.25
16	10	5	6	243.82	242.99
17	10	5	6	243.82	244.21

Figure 4.22 represents the actual values for xylanase production and the predicted values determined by the model equation. The analysis of variance (ANOVA) for the model was performed and was summarized in Table 4.31. The model *F*-value and *P*-value were found to be 9.85 and 0.0032 indicated that the model was significant. The higher value of determination R^2 (0.9269) and adjusted R^2 (0.8328) showed efficacy of the model. Adequate precision measures the signal to noise ratio. A ratio >4 was desirable. The ratio of 10.070 indicated an adequate signal (Table 4.32).

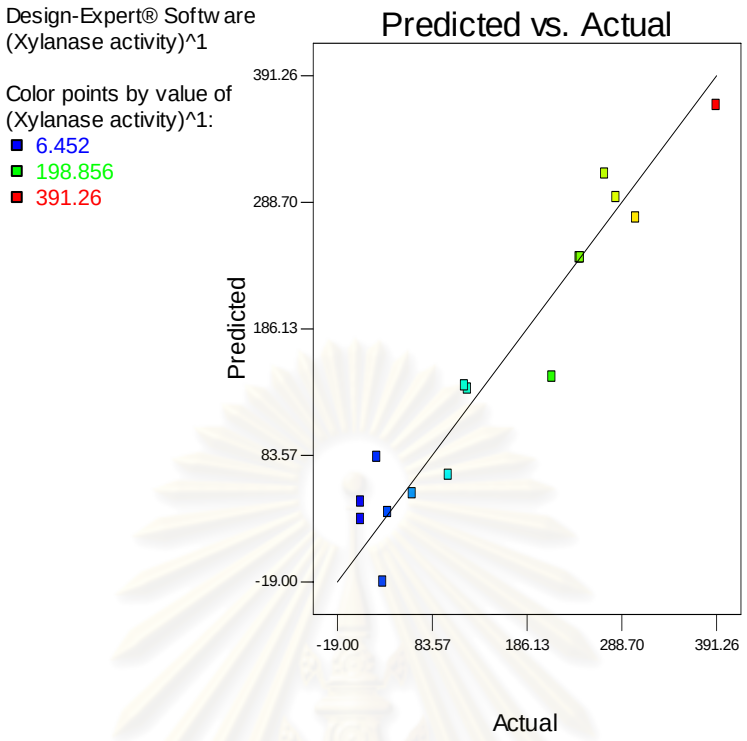

Figure 4.22 Plot of predicted *vs* actual xylanase production for isolate P2-3

Table 4.31 Result of regression analysis for quadratic model

Source	Sum of squares	Degree of freedom	Coefficient	*F*-value	*P*-value
Model	221907.82	9		9.85	0.0032*
A-Corn	61802.06	1	66.44	24.70	0.0016
B-Peptone	76045.69	1	73.70	30.40	0.0009
C-pH	8892.00	1	25.20	3.55	0.1014
AB	27505.09	1	58.64	10.99	0.0128
AC	14618.36	1	-42.75	5.84	0.0463
BC	843.97	1	-10.27	0.34	0.5796
A^2	9299.57	1	-27.62	3.72	0.0952
B^2	8428.40	1	-26.29	3.37	0.1091
C^2	29494.23	1	-49.18	11.79	0.0109
Residual	17512.28	7			
Lack of Fit	17511.25	5		6789.26	0.0001
Error	1.03	2			
Total	239420.10	16			

*Values of *P*-value less than 0.05 indicate model terms were significant.

AB, AC and BC represent the interaction effect of the variables; A^2, B^2 and C^2 were the squared effects of the variables.

Table 4.32 Analysis of variance (ANOVA) for quadratic model for xylanase production

Term	Value	Term	Value
Standard deviation	50.02	R^2	0.93
Mean	158.92	Adjusted R^2	0.83
Coefficient of variation%	31.47	Predicted R^2	0.39
PRESS	146755.77	Adeq Precision	10.07

PRESS Predicted residual sum of squares

The three-dimensional respone surface plots were employed to demonstrate the main effect, interaction effect and squared effect of three factors; corn cob, peptone and pH at different concentrations, which have significant effects on xylanase production. The respone surface plots were shown in Figures 4.23-4.25, which illustrate the relationships between response and the experimental data.

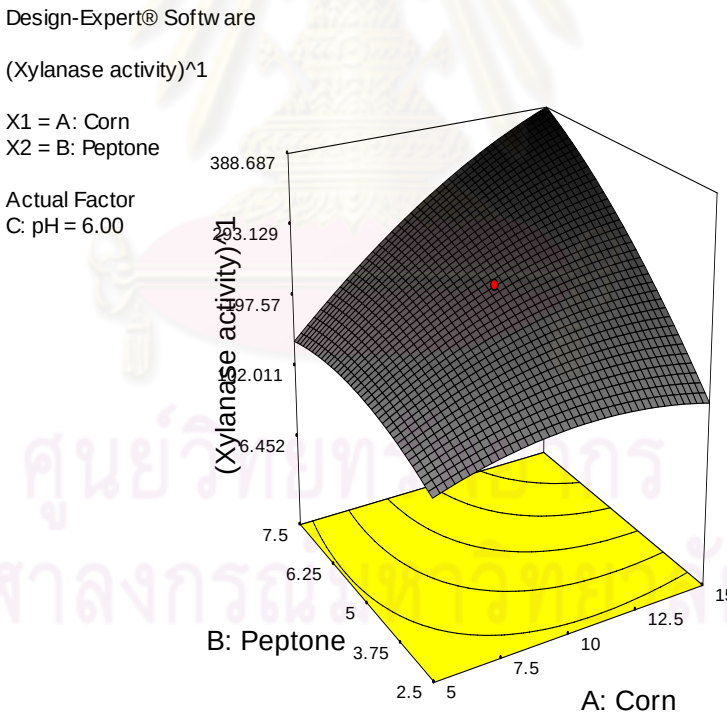

Figure 4.23 Effects of corn cob (A), peptone (B) and their interaction on xylanase production with other variables set at central level

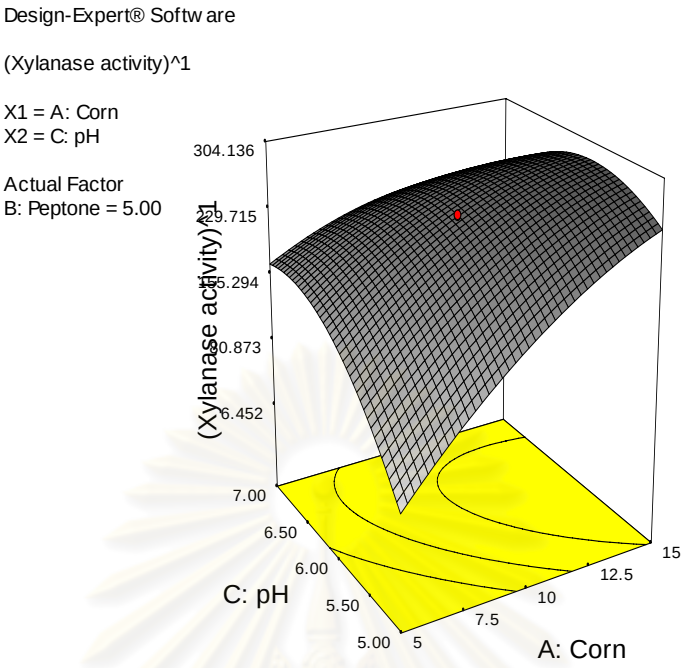

Figure 4.24 Effects of corn cob (A), pH (C) and their interaction on xylanase production with other variables set at central level

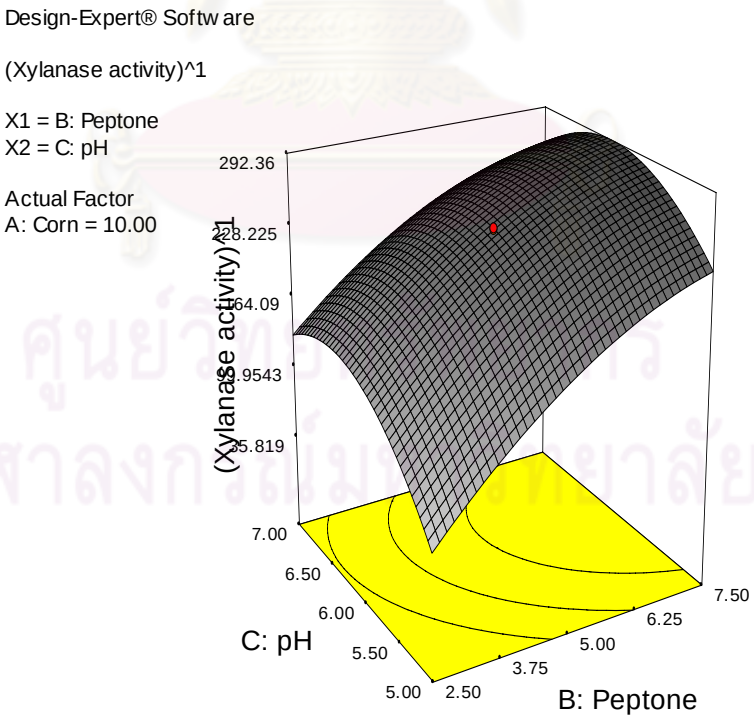

Figure 4.25 Effects of peptone (B), pH (C) and their interaction on xylanase production with other variables set at central level

In Figure 4.23, the interaction effect of corn cob and peptone was found to be highly significant (*P*-value of AB=0.0128); similarly, in Figure 4.24, the interaction effect of corn cob and pH was also found to be highly significant (*P*-value of AC=0.0463); In Figure 4.25, the interaction effect of peptone and pH was found to be not significant (*P*-value of AB=0.5796). It can be seen that when peptone concentration was at a high level, enzyme production increased with the decrease of pH level. According to the results, the optimal medium composed of corn cob 15 g/l, peptone 7.5 g/l, K_2HPO_4 6 g/l, KCl, 0.1 g/l, $MgSO_4.7H_2O$ 0.75 g/l and $FeSO_4.7H_2O$ 0.01 g/l at pH 5.0. Under the optimal medium, the maximum enzyme production at 367.32 unit/ml in predicted model and 391.26 unit/ml in the actual experiment were achieved with an increase of 2.07 times compared to the initial medium.

4.4.5 Time course of growth and xylanase production

Figure 4.26 showed the time course comparison of xylanase production by P2-3 in the initial medium and optimal medium. As the result, the exponential phase of P2-3 was 18 h and after 18 h the bacterial growth reached the stationary phase in initial medium. The extracellular xylanase activity started at 12 h and maximum at 18 h. However, the production decreased during prolonged cultivation. In optimal medium, the late-log phase of P2-3 was 18 h but the maximum produced of xylanase occured at 24 h. The xylanase production was stable upon further incubation. Therefore, incubation time was the significant factor that can lead to a marked increase in xylanase production.

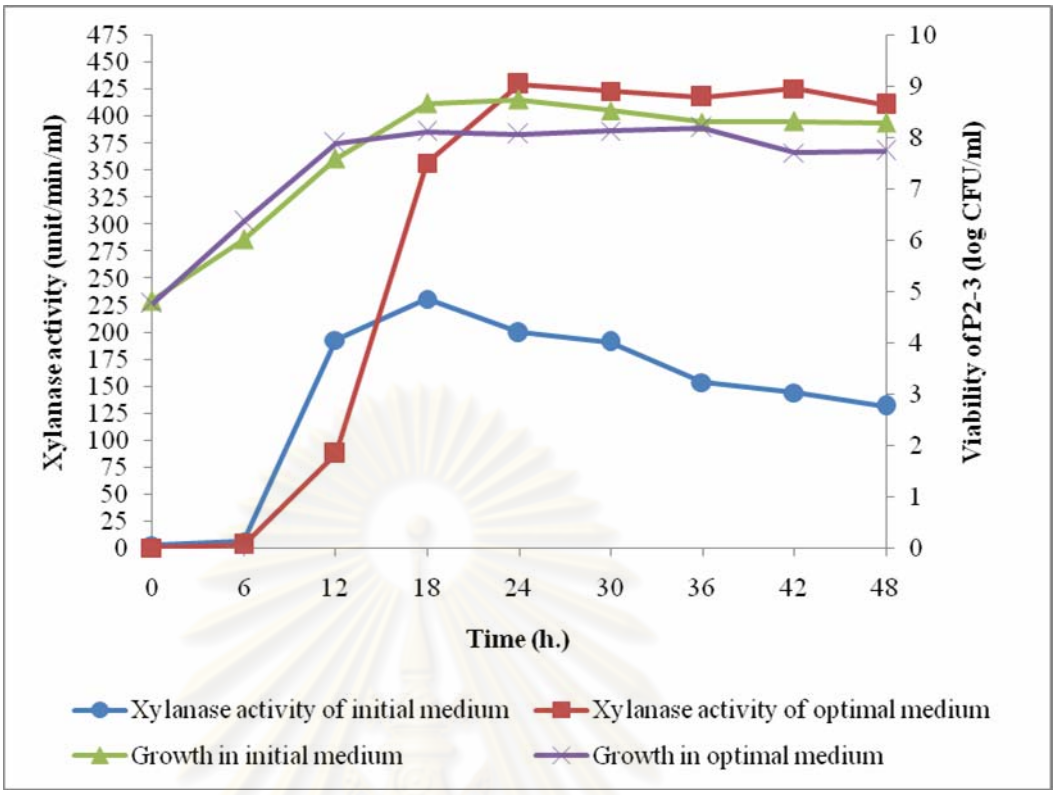

Figure 4.26 Time course of xylanase production by P2-3 in the initial medium and in the optimal medium

4.5 Partial purification of xylanase

Partial purification step of P2-3 xylanase was summarized in Table 4.33. At the first step, crude xylanase was purified by affinity adsorption-desorption chromatography on a corn cob 20M column. Fractions with activity were pooled, total activity (739.50 units) of approximately 27.28% remained, while 72.72% of total protein was removed. From this result, purity of 2.6 folds was achieved. The obtained fraction was pervaporated in dialysis bag against concentrated by polyethylene glycol MW 6000, leading to an increase in purity folds of 3.66 folds. Then, the concentrated enzyme fraction was loaded on a size exclusion chromatography using Superdex 200 10/300 GL. Purification fold was increased 10.62 folds with a yield of 3.07% in this step.

The partially purified xylanase from affinity column were further purified by size exclusion chromatography using Superdex 200 10/300 GL. The column separated xylanase from other proteins by molecular size (Figure 4.27). This step effectively separated xylanase from other protein contaminants.

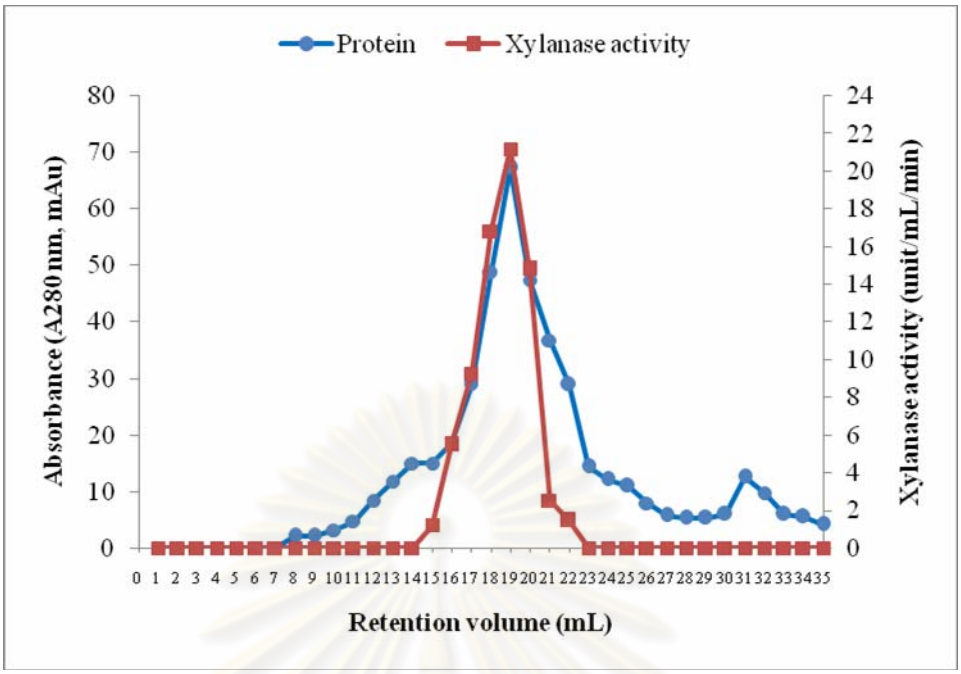

Figure 4.27 Elution profile of P2-3 xylanase on Superdex 200 10/300 GL column

Table 4.33 Partial purification of xylanase from P2-3

Purification step	Total activity (unit*)	Total protein (mg**)	Specific activity (unit/mg)	Purification fold	Yield (%)
Crude extract	2710.60	131.50	20.61	1	100
Corn cob 20M	739.50	13.80	53.59	2.60	27.28
PEG concentration	581.61	7.70	75.53	3.66	21.46
Superdex 200	83.18	0.38	218.89	10.62	3.07

*The unit of enzyme activity was expressed as the μmole of xylose per min.
**Protein concentration was measured by Lowry method.

The partially purified xylanase from P2-3 was evaluated by SDS-PAGE, followed by zymogram (Figure 4.28A and 4.28B). Protein pattern from SDS-PAGE revealed two protein bands (Figure 4.28A). However, the zymogram of xylanase enzyme showed a prominent activity band corresponding to the silver stained band on SDS-PAGE of lower protein band position (Figure 4.28B). Therefore, the lower protein band position was xylanase protein band.

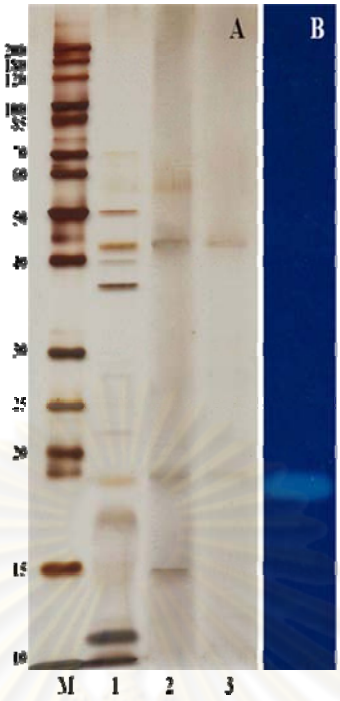

Figure 4.28 Partially purified xylanase from P2-3 visualized on SDS-PAGE (A) and zymogram of xylanase (B). Lane M; standard marker, Lane 1; crude enzyme (20 µg); Lane 2, partially purified passed corn 20M column (8 µg) and Lane 3, partially purified passed superdex 200 10/300 GL (4 µg)

4.6 Characterization of partially purified xylanase

4.6.1 Molecular weight determination

The molecular weight of partially purified xylanase was found to be 17.7 kDa as estimated by size exclusion on Superdex 200 10/300 GL column using AKTA fast FPLC (Figure 4.29A). SDS-PAGE analysis showed that the partially purified xylanase with the molecular weight of 19.8 kDa (Figure 4.29B). This result indicated that partially purified xylanase from P2-3 had similar molecular weights to xylanase from *B. amyloliquefaciens*, 20.7 kDa (SDS-PAGE), but lower than those from *Bacillus* sp. K-1 (23 kDa) and *Bacillus* sp. AR-009 (23 kDa) (Yin *et al.,* 2010).

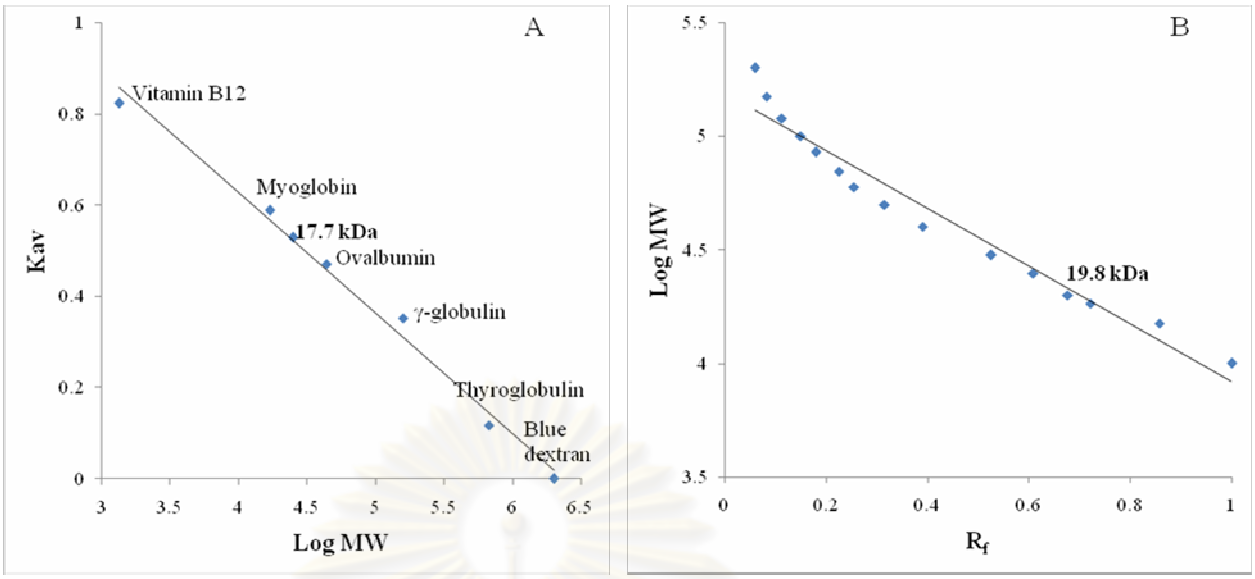

Figure 4.29 Size-exclusion of xylanase from P2-3 by FPLC on Superdex 200 10/300 GL column. The standard marker was eluted through a Superdex 200 10/300 GL column equilibrated with 50 mM sodium phosphate buffer (pH6) at a flow rate of 0.5 ml/min. The elution profiles were monitored by spectrophotometry at 280 nm

4.6.2 Optimal temperature and thermal stability

An effect of optimal temperature and temperature stability on the partially purified P2-3 xylanase activity was presented in Figure 4.30A and B. The xylanase underwent thermal activation at the temperature above 50 °C with a maximum activity at 60 °C but inactivated above 60 °C. In addition, xylanase from P2-3 was stable at 30-40°C after an incubation time of 30 minute which higher than 50% of its maximal was retained. The activity and stability of the enzyme decreased drastically at high temperature, possibly due to the partial unfolding of the enzyme molecule. According to the results, the optimal temperature was similar to some other *Bacillus* (60 °C) (Yin *et al*., 2010).

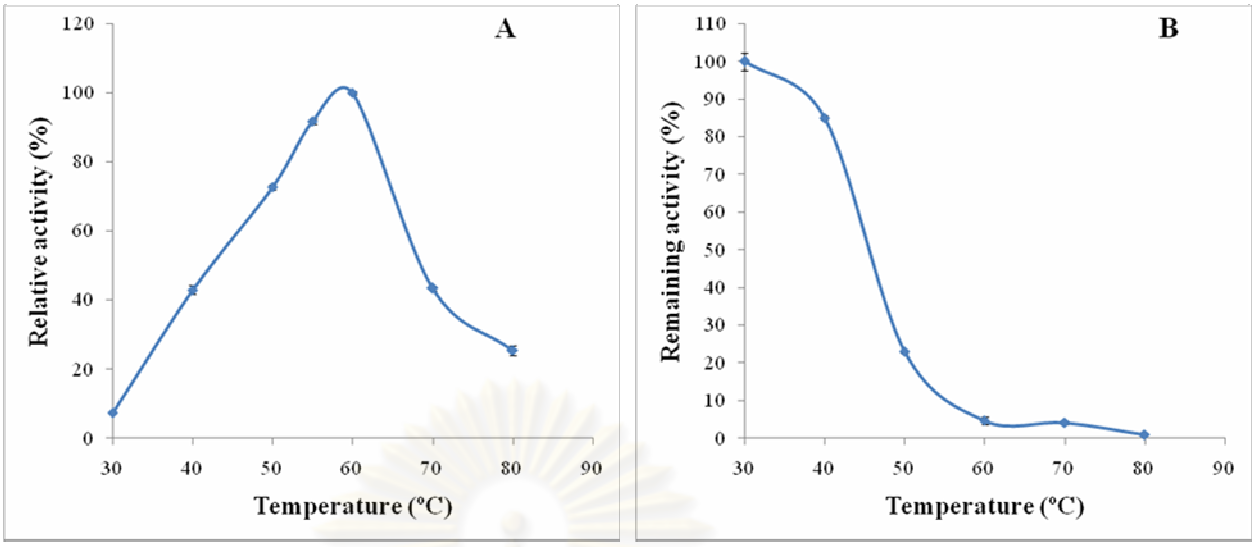

Figure 4.30 The effect of temperature on xylanase activity (A) and on xylanase stability (B) of the partially purified xylanase from P2-3

4.6.3 Optimal pH and pH stability

The effect of pH on the activity of partially purified xylanase was determined with universal buffer at pH 2-11. As shown in Figure 4.31A, the partially purified enzyme was active at pH 5-7, but exhibited maximum activity at pH 6. An effect of pH stability on xylanase activity is shown in Figure 4.31B. The xylanase was stable in broad pH range (pH 5.0-11.0), but the remaining activity was decrease to 60% at pH 3 after incubation for 30 minute. According to previous studies, the optimal pH of most of xylanases from *Bacillus* strains was 6.0, except for those from *Bacillus* sp. K-1 (pH 5.5) and *B. amyloliquefacienats* (pH 6.8-7.0). The pH stability of partially purified xylanase (pH 5.0-11.0) was almost the same as that from *B. circulans* D1 (pH 5.5-10.5) and *Bacillus* sp. GRE7 (pH 5.0-11.0) (Yin *et al*., 2010).

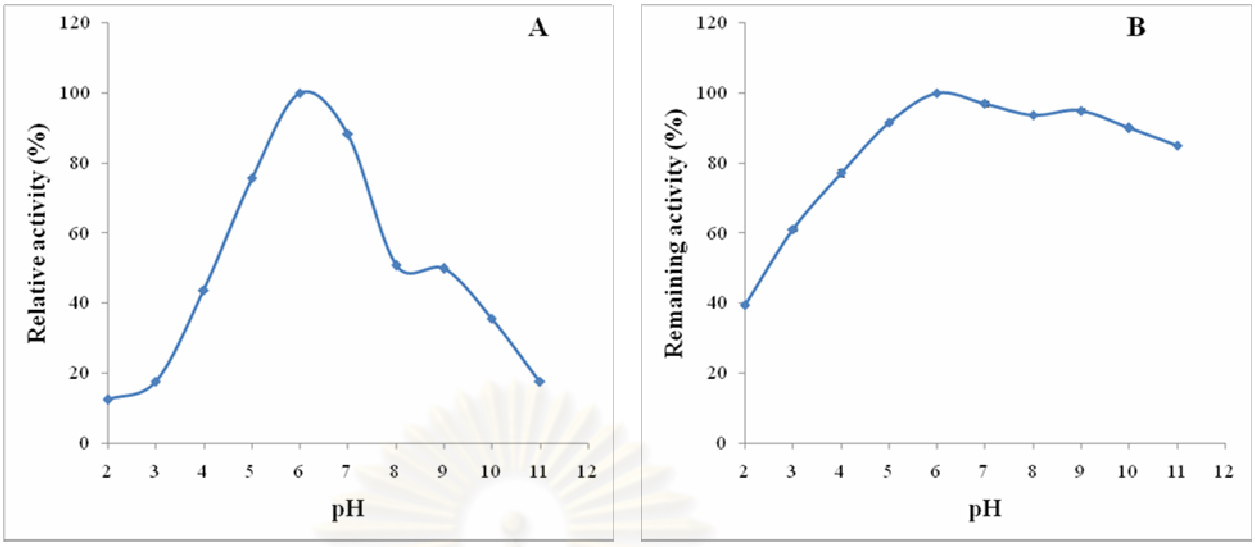

Figure 4.31 The effect of pH on xylanase activity (A) and on xylanase stability (B) of the partially purified xylanase from P2-3

4.6.4 Effects of metal ions, reducing agents and inhibitors

Partially purified xylanase was assayed in the presence and absence of metal ions**,** reducing agents and inhibitors. The xylanase activity was activiated by the addition of 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, dithiothreitol (DTT), and *β*-mercaptoethanol (*β*-Me). In contrast, the xylanase activity was inhibited by Fe^{2+}, Phenylmethyl sulfonyl fluoride (PMSF) and Sodium dodecyl sulfate (SDS) (Table 4.34). Moreover, Na^{+} and Diamine tetraacetic acid (EDTA) were not significantily different (P>0.05) to xylanse production, when compared with control. These results were similar to those xylanase from *B. stearothermophilus* T-6, *B. amyloliquefaciens* and *Bacillus* sp. SPS-0. According to Yin *et al*. (2010), Ca^{2+} and Mg^{2+} could activate the xylanase from *Bacillus* sp. DSNC 101 and xylanases from *B. amyloliquefaciens*, *Bacillus* sp. SPS-0 or *Bacillus* sp. JB-99 were activated by reducing agents such as DTT and β-ME. It was not certain from these studies whether these ions/agents were binding to the enzyme, causing conformational changes that result in increased enzyme activity or whether xylanase requires a metal ion on the active site. Further work using more inhibitors, their analogous and combinations thereof would be necessary to ascertain the mode of action of xylanase.

Table 4.34 Effect of metal ions, reducing agents and other reagents

Chemicals (1 mM)	Relative activity (%)
None	100.0±0.0*
Na^{+}	97.74±0.69
Ca^{2+}	116.52±1.66
Fe^{2+}	94.26±0.97
Mg^{2+}	108.70±4.15
Mn^{2+}	133.91±5.97
Dithiothreitol (DTT)	106.38±0.97
β-mercaptoethanol (*β*-Me)	119.13±2.20
Diamine tetraacetic acid (EDTA)	99.59±0.80
Phenylmethyl sulfonyl fluoride (PMSF)	89.91±1.26
Sodium dodecyl sulfate (SDS)	88.0±1.3

*Xylanase activity in the absence of chemicals mentioned above was set as 100%. All experiments were carried out in triplicates. Statistical significance was assigned at 95% of confidence level.

4.6.5 Substrate specificity

The partially purified xylanase was assayed with various substrates to study the substrate specificity. The xylanase had hydrolytic activity toward Oat spelt xylan, but no activity toward *β*-glucan, carboxymethylcellulose and pectin (Table 4.35). This phenomenon indicated that the substrate binding domain of xylanase had very high affinity for xylans from softwood (Oat spelt xylan). This might be due to the differences in xylan structures, which the binding of xylanase to xylans from Oat spelt xylan might be due to reactive group exposure on the surface that can much more easily binding. Furthermore, the partially purified xylanase from studying was cellulase free xylanase.

Table 4.35 Relative activity of the partial xylanase on polysaccharides

Substrate	**Relative activity (%)**
Oat spelt xylan	100
Beech wood xylan	43.68
Birch wood xylan	42.49
β-glucan	0
Carboxymethylcellulose	0
Pectin	0

CHAPTER V
CONCLUSION

In the course of investigation of xylanolytic bacteria distributed in soils in Thailand, seventy isolates were identified and characterized taxonomically. They were divided into sixteen groups based on their phenotypic, chemotaxonomic characteristics and 16S rRNA gene sequence analyses of the representative isolates. Sixty one isolates were Gram-positive (Group I to VII), rod-shaped bacteria. Nine isolates were Gram-negative rod-shaped bacteria (Group VIII to XVI).

Group I, isolates belonged to the Genus *Bacillus.* All isolates contained *meso*-diaminopimelic in cell wall peptidoglycan. MK-7 was the predominant menaquinone. The DNA G+C contents ranged from 42.4 to 46.3 mol%. On the basis of 16S rRNA gene sequence analyses, the representative of Group I(a), K3-6 and SRC2-3 were closely related to *B. subtilis* subsp. *subtilis* KCTC 3135^T with both 100%. Group I(b), TH2-2, P2-2, SK1-3 and PJ1-2 were closely related to *B. licheniformis* KCTC 1918^T with 99.7, 99.2, 99.1 and 99.6%, respectively. Group I(c), K1-6A and K1-6B were closely related to *B. niabensis* 4T19^T with 99.9 and 100%, respectively. Group (d), NS1-1 was closely related to *B. nealsonii* (99.7%). Group I(e), FCN3-4 and PHC3-3 were closely related to *B. cereus* (100 and 99.8%). The results of the representative of Group I(a) to I(e) showed high the similarity of 16S rRNA gene sequence with the type strains. Therefore, the representative of each group was identified known species of genus *Bacillus.* While Group I(f), P2-3) and I(g), PHX1-5 showed low 16S rRNA gene sequence similarity values with *B. amyloliquefaciens* KCTC 1660^T (96.4%) and *B. funiculus* KCTC 3796^T (98.5%), respectively. Therefore, these two isolates represent novel species of the genus *Bacillus*.

Group II, 24 isolates in this group were divided into nine groups. They had *meso*-diaminopimelic in cell wall peptidoglycan. MK-7 was the predominant menaquinone as in the genera *Bacillus*. The DNA G+C of contents were 50.8-56.8 mol%. On the basis of 16S rRNA gene sequence analyses, the representative of Group (A), K1-4 was closely related to *P. macerans* IAM 12467^T (99.6%). Group II(B), PJ1-1B was closely related to *P. montaniterrae* MXC2-2^T (99.7%). Group II(C), PHC3-4 was closely related to *P. dendritiformis* 105967^T (99.7%). The results of the representative of Group II(A) to II (C) showed high the similarity of 16S rRNA gene

sequence with the reference strains. Therefore, the representative of each group were identified known species of genus *Paenibacillus*. While Group II(D), SRX4-1 and SRX4-2 were closely related to each other with 99.9% 16S rRNA gene sequence similarity and to *P. phyllosphaerae* PALXIL04^T (98.6 and 98.7%). Group II(E), FXN2-3, was closely related to *P. cellulosilyticus* PALXIL08^T (98.2%). Group II(F), SRX1-4, was closely related to *P. edaphicus* KCTC 3995^T (98.3%). The results of Group II(D) to II(F) showed low the similarity of 16S rRNA gene sequence with the reference strains. Therefore, they represent novel species of the genus *Paenibacillus*.

The isolates, S3-4A and MX2-3 are the novel species of genus *Paenibacillus*, for which the names *Paenibacillus thailandensis* sp. nov. and *Paenibacillus nanensis* sp. nov. were proposed. In addition, isolate X11-1 showed low the similarity of 16S rRNA gene sequence with *Paenibacillus naphthalenovorans* KACC 11505^T and *Paenibacillus validus* CCM 3894^T with 96.5% sequence similarity. Thus, X11-1 represent novel species of the genus *Paenibacillus*, for which the name *Paenibacillus xylanisolvens* sp. nov. is proposed.

The representative isolates of Group II (I), P2-5, CXT1-1, P2-3A, PL1-3, K3-2, SRXT2-1, K3-5S, PL2-1 and CXT3-2 were identified with 16S rRNA gene sequence and phylogenetic analyses. Furthermore, the differentiation between strains were supported by the analyses with $(GTG)_5$ patterns. The isolate CXT3-2 was identified as *P. timonensis* KCTC 3995^T with 100% similarity of 16S rRNA gene sequence while SRXT2-1, K3-5S and PL2-1 were identified as *P. barengoltzi* (99.8%, 99.8% and 99.7%, respectively) based on $(GTG)_5$ pattern and 16S rRNA gene sequence. In addition, P2-5, CXT1-1, P2-3A, PL1-3 and K3-2 showed low similarity of 16S rRNA gene sequence with *P. barengoltzii* SAFN-016^T (98.5%, 98.8%, 98.7%, 97.9% and 98.1%, respectively) and showed 52.7% Pearson's correlation coefficient of the banding on $(GTG)_5$ patterns. Therefore, five isolates represent novel species of the genus *Paenibacillus*. However, they should be confirmed by DNA-DNA hybridization for the proposal of the new species.

Group III, isolates FCN3-3, S1-3, MX15-2 and MX21-2 were motile by means of peritrichous flagella. Central and subterminal ellipsoidal endospores were observed in swollen sporangia. They contained *meso*-diaminopimelic in cell wall peptidoglycan and had MK-7 as in the genera *Bacillus* and *Paenibacillus*. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-

phospatidylglycerol. The predominant fatty acids were anteiso-$C_{15:0}$, iso-$C_{16:0,}$ iso-$C_{15:0}$ and $C_{16:0}$. DNA G+C contents were 53.3-65.1 mol%. The similarity of 16S rRNA gene in this group showed low relatedness with the type strain. FCN3-3 was closely related to *C. phaseoli* KCTC 13070^T (96.9%). Therefore, FCN3-3 represents a novel species of the genus *Cohnella.* The phylogenetic analysis using 16S rRNA gene sequences showed that S1-3 was affiliated to the genus *Cohnella*, which was closely related to *C. ginsengisoli* GR21-5^T with 95.7% sequence similarity. Strain S1-3 could be clearly distinguished from related *Cohnella* species by its physiological and biochemical characteristics as well as its phylogenetic position. Therefore, S1-3 represent a novel species of the genus *Cohnella,* for which the name *Cohnella thailandensis* sp. nov. was proposed. The MX15-2 and MX21-2 were closely related to *C. thermotolerans* CCUG 47242^T (96.7% and 96.3%). The DNA–DNA relatedness between MX15-2 and MX21-2 was 52.9% and both strains showed low DNA–DNA relatedness to *C. thermotolerans* CCUG 47242^T (2.1-5.5%). Therefore, MX15-2 and MX21-2 represent novel species of the genus *Cohnella,* for which the names *Cohnella xylanilytica* sp. nov. and *Cohnella terrae* sp. nov.were proposed.

Group IV (CR1-2 and CR5-1) had MK-9(H_4) as major menaquinone. DNA G+C content was 70.0 mol%. The isolates in this Group were closely related to each other (with 100%) and to *Isoptericola variabilis* MX5^T (with 99.6% sequence similarity). Group V isolates (FXN1-1B and PHX2-5) were closely related to each other (with 99.8%) and to *Jonesia denitrificans* ATCC 14870^T with 99.2 and 99.1%. Thus, the isolates were identified as *Is. variabilis* and *J. denitrificans*, respectively.

Group VI (CE3-4, SRC1-1 and SRC3-3) could divided into 2 groups. Main menaquinone of SRC1-1 and SRC3-3 were MK-11 and MK-12, while CE3-4 were MK-13 and MK-14. On the basis of 16S rRNA gene sequence, SRC1-1 and SRC3-3 were closely related to each other (100%) and to *M. natoriense* TNJL143-2^T (99.0%). Therefore, two strains were known *Microbacterium*. The isolate CE3-4 was closely related to *M. imperiale* DSM 20530^T (98.4%). Therefore, CE3-4 represent novel species of the genus *Microbacterium*.

Group VII, SRX2-3 was non-spore-forming and strictly aerobic. The SRX2-3 contained *meso*-diaminopimelic in cell wall peptidoglycan. Major menaquinone was MK-8(H_4). . DNA G+C content was 72.0 mol%. The SRX2-3 was closely related to

N. simplex DSM 20130^T (99.3%). Based on the results mentioned above indicated that isolate SRX2-3 was identified as *N. simplex*.

Group VIII, SRX2-1 was Gram negative, coccobacilli. Q-9 was the predominant ubiquinone. DNA G+C content was 42.1 mol%. The SRX2-1 exhibited sequence similarity values of 99.8% with *Acinetobacter junii* LMG 998^T. In addition, their phenotypic characteristics were similar to *A. junii* LMG 998^T. Group IX (SRX2-2) had predominant ubiquinone as Q-8. DNA G+C content was 57.7 mol%. The sequence of SRX2-2 showed high similarity with *Aeromonas enteropelogenes* DSM 6394^T. (99.4%). Thus, SRX2-1 was identified as *A. junii* and SRX2-2 was identified as *A. enteropelogenes*.

Group X (SRX1-2), had Q-10 as predominant ubiquinone. The predominant fatty acids were $C_{18:1}\omega7c$. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. DNA G+C content was 63.2 mol%. The similarity of 16S rRNA gene sequence showed low similarity with *Blastobacter aggregatus* IFAM 1003^T (98.1%). Therefore, this strain represents novel species of the genus *Blastobacter*.

Group XI (SRX1-1), XII (PHX3-1), XV (FXN3-1) and XVI (PHX2-7) were closely related to *Ensifer adhaerens* LMG 20216^T (99.3%), *Pseudomonas stutzeri* ATCC 17588^T (99.8%), *Stenotrophomonas maltophilia* IAM 12423^T (99.4%) and *Zobellella denitrificans* ZD1^T (99.2%), respectively. The similarity of 16S rRNA gene sequence of their showed high similarity with type strain. Therefore, the four strains were identified as *E. adhaerens, P. stutzeri, S. maltophilia* and *Z. denitrificans*, respectively.

Group XIII, isolate SRX3-4 had MK-7 as predominant menaquinone. The predominant fatty acids were iso-$C_{15:0,}$ $C_{16:0}$ and iso-$C_{17:0}$ 3OH. DNA G+C content was 44.4 mol%. Group XIV, CE4-1 had Q-10 as the predominant ubiquinone. The predominant fatty acids were $C_{18:1}\omega7c$, $C_{17:1}\omega6c$, $C_{16:0}$ and $C_{14:0}$ 2OH. DNA G+C content was 66.5 mol%. Isolates SRX3-4 and CE4-1 were closely related to *Sphingobacterium multivorum* B5533^T (98.3%) and *Sphingomonas mucosissima* CP173-2^T (98.3%), respectively. Therefore, SRX3-4 and CE4-1 represent novel species of the genus *Sphingobacterium* and *Sphingomonas*.

All isolates could produce xylanase activity based on the clear zone on agar plates and xylanase assay. The results revealed that the isolates in Group I to V and

Group XVI had more xylanase activity than Group VI to Group XV. Therefore, Gram-positive isolates in Group I-V were important source of xylanase activity. Among 70 isolates, Group I, (P2-3) was selected for further study due to the novelty of species and high xylanase production. The strain P2-3 produced extracellular xylanase at the middle of exponential phase and the highest xylanase production occurred at 18 h in initial medium, while optimal medium produced highest xylanase at 24 h. The maximum xylanase production was achieved when strain P2-3 was cultivated in corn cob as substrate for culture and used Oat spelt xylan as substrate for condition. The optimal medium, the maximum enzyme produced 367.32 unit/ml with an increase of 2 times compared to the initial medium. The optimal medium which yeast extract was omitted, containing corn 15 g/l, peptone 7.5 g/l, K_2HPO_4 6 g/l, KCl 0.1 g/l, $MgSO_4.7H_2O$ 0.75 g/l and $FeSO_4.7H_2O$ 0.01 g/l, pH 5.0 and incubated at 37 °C with shaking (200 rpm) for 24 h. The xylanase was partially purified with corn cob 20M column as affinity adsorption-desorption chromatography and Superdex 200 10/300 GL as gel filtration chromatography. Partially purified xylanse had molecular weight of 17.7 kDa (calculated by gel filtration method). The enzyme had maximal activity at 60 °C and pH 6. More than 50% of the activity remained when at 30-40 °C and pH 3-11 for 30 minute. The xylanase activity was greatly elevated by the addition of 1 mM of Ca^{2+}, Mg^{2+}, Mn^{2+}, DTT and β-Me which ions/agents were binding to the enzyme, causing conformational changes that result in increased enzyme activity. In contrast, the xylanase activity was inhibited by Fe^{2+}, PMSF and SDS. Furthermore, the substrate binding domain of partial xylanase had very high affinity for xylans from Oat spelt xylan.

In this study, a lot of novel species of xylanolytic bacteria were isolated fromsoil samples collected in Thailand. The 16S rRNA gene sequencing results were useful to indicate their taxonomic position, however the DNA-DNA hybridization of the isolates with the type strains of each species are required for further studies in order to propose them as the new species. As well as, $(GTG)_5$-PCR analysis was supported their identification. From the results mentioned above, the xylanolytic strains, *Bacillus, Paenibacillus* and *Cohnella* including the other genera were distributed in many soil samples in Thailand (Table 5.1). They are the most likely source of enzymes and constitute a heterogeneous group of xylanase producing bacteria belonging to different genera. The isolated bacteria that be able to produce

extracellular enzymes will provide the possibility to have optimal activities at different temperature and pH. The strain P2-3 produced a substantial level of extracellular xylanase activity that was active in extreme conditions and could use the substrate from agriculture waste for the cultivation. Thus, the applications of this strain and the roles of the remained strains should be further study. In addition, the new informations of the xylanolytic bacteria in genera *Isoptericola, Jonesia, Nocardioides, Blastobacter, Ensifer, Sphingobacterium, Sphingomonas, Stenotrophomonas and Zobellella* are reported in this study.

Table 5.1 Biodiversity of xylanase producing bacteria in Thailand

Location of samples	Isolate no.	Closet species	%Similarity	Group	Identification
Chiangrai	CR1-2	*Is. variabilis* MX5^T	99.6	IV	*Is. variabilis*
	CR5-1	*Is. variabilis* MX5^T	99.6	IV	*Is. variabilis*
Nan	MX2-3	*P. agaridevorans* KCTC 3849^T	97.0	II (G)	*P. nanensis*
	MX15-2	*C. thermotolerans* CCUG 47242^T	96.7	III	*C. xylanilytica*
	MX21-2	*C. thermotolerans* CCUG 47242^T	96.3	III	*C. terrae*
	P2-3	*B. amyloliquefaciens* KCTC 1660^T	96.4	I (f)	*Bacillus* sp. nov.
	S1-3	*C. ginsengisoli* GR21-5^T	96.0	III	*C. thailandensis*
	S3-4A	*P. agaridevorans* KCTC 3849^T	97.3	II (G)	*P. thailandensis*
	X11-1	*P. naphthalenovorans* KACC11505^T and *P. validus* CCM 3894^T	96.5, 96.5	II (H)	*P. xylanisolvens*
Nakhonnayok	FCN3-3	*C.phaseoli* KCTC 13070^T	96.9	III	*Cohnella* sp. nov.
	FCN3-4	*B. cereus* IAM 12605^T	100	I (e)	*B. cereus*
	FXN1-1B	*J. denitrificans* ATCC 14870^T	99.2	V	*J. denitrificans*
	FXN2-3	*P.cellulosilyticus* PALXIL08^T	98.2	II (E)	*Paenibacillus* sp. nov
	FXN3-1	*St. maltophilia* IAM 12423^T	99.4	XV	*St. maltophilia*
Samutsongkhram	SK1-3	*B. licheniformis* KCTC 1918^T	99.1	I (b)	*B. licheniformis*
Kanchanaburi	K1-4	*P. macerans* IAM 12467^T	99.6	II (A)	*P. macerans*
	K1-6A	*B. niabensis* 4T19^T	99.9	I (c)	*B. niabensis*
	K1-6B	*B. niabensis* 4T19^T	100	I (c)	*B. niabensis*
	K3-2	*P. barengoltzii* SAFN-016^T	98.1	II (2)	*Paenibacillus* sp. nov.
	K3-5S	*P. barengoltzii* SAFN-016^T	99.8	II (2)	*P. barengoltzii*
	K3-6	*B. subtilis* subsp. *subtilis* KCTC 3135^T	100	I (a)	*B. subtilis* subsp. *subtilis*
Phetchaburi	CE3-4	*M. imperiale* DSM 20530^T	98.4	VI	*Microbacterium* sp. nov.
	CE4-1	*Sp. mucosissima* CP173-2^T	98.3	XIV	*Sphingomonas* sp. nov.
	P2-2	*B. licheniformis* KCTC 1918^T	99.2	I (b)	*B. licheniformis*
	P2-3A	*P. barengoltzii* SAFN-016^T	98.7	II (2)	*Paenibacillus* sp. nov.
	P2-5	*P. barengoltzii* SAFN-016^T	98.5	II (2)	*Paenibacillus* sp. nov.
	PHC3-3	*B. cereus* IAM 12605^T	99.8	I (e)	*B. cereus*
	PHC3-4	*P. dendritiformis* 105967^T	99.7	II (C)	*P. dendritiformis*
	PL1-3	*P. barengoltzii* SAFN-016^T	97.9	II (2)	*Paenibacillus* sp. nov.
	PL2-1	*P. barengoltzii* SAFN-016^T	99.7	II (2)	*P. barengoltzii*
	PHX1-5	*B. funiculus* KCTC 3796^T	98.5	I (g)	*Bacillus* sp. nov.
	PHX2-5	*J. denitrificans* ATCC 14870^T	99.1	V	*J. denitrificans*
	PHX2-7	*Z. denitrificans* ZD1^T	99.2	XVI	*Z. denitrificans*
	PHX3-1	*Ps. stutzeri* ATCC 17588^T	99.8	II	*Ps. stutzeri*
Prachuapkhirikhan	PJ1-1B	*P. montaniterrae* MXC2-2^T	99.7	II (B)	*P. montaniterrae*
	PJ1-2	*B. licheniformis* KCTC 1918^T	99.6	I (b)	*B. licheniformis*
Trat	TH2-2	*B. licheniformis* KCTC 1918^T	99.7	I (b)	*B. licheniformis*
Suratthani	SRC1-1	*M. natoriense* TNJL143-2^T	100	VI	*M. natoriense*
	SRC2-3	*B. subtilis* subsp. *subtilis* KCTC 3135^T	100	I (a)	*B. subtilis* subsp. *subtilis*
	SRC3-3	*M. natoriense* TNJL143-2^T	100	VI	*M. natoriense*
	SRX1-1	*E. adhaerens* LMG 20216^T	99.3	XI	*E. adhaerens*
	SRX1-2	*Bl. capsulatus* IFAM 1004^T	98.1	X	*Blastobacter* sp. nov.
	SRX1-4	*P. edaphicus* KCTC 3995^T	98.3	II (F)	*Paenibacillus* sp. nov.
	SRX2-1	*A. junii* LMG 998^T	99.8	VIII	*A. junii*
	SRX2-2	*A. enteropelogenes* DSM 6394^T	99.4	IX	*A. enteropelogenes*
	SRX2-3	*N.simplex* DSM 20130^T	99.3	VII	*N.simplex*
	SRX3-4	*Sp. multivorum* B5533^T	98.3	XIII	*Sphingobacterium* sp. nov.
	SRX4-1	*P.phyllosphaerae* PALXIL04^T	98.6	II (D)	*Paenibacillus* sp. nov.
	SRX4-2	*P.phyllosphaerae* PALXIL04^T	98.7	II (D)	*Paenibacillus* sp. nov.
	SRXT2-1	*P. barengoltzii* SAFN-016^T	99.8	II (2)	*P. barengoltzii*
Nakhonsrithammarat	CXT1-1	*P. barengoltzii* SAFN-016^T	98.8	II (2)	*Paenibacillus* sp. nov.
	CXT3-2	*P. timonensis* KCTC 3995^T	100	II (2)	*P. timonensis*
	NS1-1	*B. nealsonii* FO-092^T	99.7	I (d)	*B. nealsonii*

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENT FOR IDENTIFICATION

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

1. XB medium

Oat spelt xylan	10	g
$NaNO_3$	2	g
$MgSO_4.7H_2O$	0.5	g
K_2HPO_4	0.05	g
$FeSO_4.7H_2O$	0.01	g
$CaCl_2$	0.02	g
$MnSO_4$	0.002	g
Agar	15	g
Distilled water	1000	ml

Dissolved and adjusted pH 7.0.

2. C medium

Peptone	5	g
Yeast extract	1	g
K_2HPO_4	4	g
$MgSO_4.7H_2O$	0.5	g
KCl	0.2	g
$FeSO_4.7H_2O$	0.02	g
Agar	15	g
Distilled water	1000	ml

Dissolved and adjustd pH 7.0.

3. Simmon Citrate agar

Simmon citrte agar (Difco)	24.2	g
Distilled water	1000	ml

Dissolved the solids in the water, and adjusted pH 6.8±0.2.

4. Tryptone water

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

Adjusted pH 7.2.

5. MR-VP broth

MR-VP medium (Merck)	17	g
Distilled water	1000	ml

Dissolved and adjustd pH 7.2.

6. Nitrate broth

Meat extract	3	g
Peptone	10	g
KNO_3	1	g
Distilled water	1000	ml

Dissolved and adjustd pH 7.2.

7. Triple suger iron agar

Triple suger iron agar (Difco)	60	g
Distilled water	1000	ml

Dissolved and adjustd pH 7.4±0.2.

8. Aesculin broth

Aesculin	1	g
Ferric citrate	0.5	g
C medium	1000	ml

Dissolved and adjusted pH 7.4.

9. L-arginine agar medium

Phenol red, 1.0% aq.solution	1	ml
L(+)arginine monohydrochloride	10	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

10. Casein agar

Skim milk	10	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

11. Gelatin agar

Gelatin	10	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

12. Starch agar

Starch	10	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

13. Tyrosine agar

Tyrosine	5	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

14. Tween 80 agar

Tween 80	2	ml
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

15. Deoxyribonuclease (DNase) media

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

Dissolved and adjusted pH 7.3±0.2.

16. Urea agar

Urea	20	g
Agar	15	g
C medium	1000	ml

Dissolved and adjusted pH 7.2.

17. Acid from carbohydrates

Sugar	5	g
C medium	1000	ml
Phenol red	0.2	g

Dissolved and adjusted pH 7.2.

18. Catalase test

Hydrogen peroxide	3	ml

Dissolved and adjusted volume to 100 ml with distilled water.

19. Oxidase test

Tetramethyl-*p*-phenylenediamine	1	g

Dissolved and adjusted volume to 100 ml with distilled water.

20. Kovacs'reagent

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

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

21. MR-VP solution

21.1 Methy red solution

Methyl red	1	g
95% Ethanol	300	ml
Distilled water	200	ml

Dissolved and mixed thoroughly, then adjust pH to 5.0.

21.2 Potassium hydroxide (KOH) aqueous solution

Potassium hydroxide	40	g
Distilled water	90	ml

Dissolved and adjusted the volume to 100 ml with distilled water.

22. Nitrate test reagent

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

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

23. Lugol' s iodine

Iodine	5	g
Potassium iodide	10	g
Distilled water	100	ml

Dissolved the iodide and potassium iodine in some of the water, and adjusted to 100 ml with distilled water.

24. 10% Trichloroacetic acid (TCA) solution (for gelatin test)

Trichloroacetic acid	10	g

Dissolved and adjusted volume to 100 ml with distilled water.

25. Flagella staining

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

Solvent was composed of a mixture of 2 ml of 95% ethanol, 0.5 ml of glycerol, and 7.5 ml of tris (hydroxymethyl) aminomethane(tris) buffer.

APPENDIX B

REAGENT FOR CHEMOTAXONOMIC CHARACTERISTIC

1. Cellular fatty acid analysis

1.1 Reagent 1 (Saponification reagent)

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

Dissolved NaOH pellets in Mili-Q water and added MeOH.

1.2 Reagent 2 (Methylation reagent)

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

pH must be below 1.5.

1.3 Reagent 3 (Extraction solvent)

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

1.4 Reagent 4 (Base wash reagent)

Sodium hydroxide	1.2	g
Mili-Q water	100	ml

1.5 Reagent 5 (Saturated sodium chloride)

2. Polar lipids

2.1 Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	ml

2.2 Dittmer&Lester reagent

Solution A

MoO_3	11	g
25 N H_2SO_4	100	ml

Dissolved 4.011 g of MoO_3 in 100 ml of 25N H_2SO_4 by heating.

Solution B

Molybdenum powder	0.178	g
Solution A	50	ml

Added 0.178 g of molybdenum powder to 50 ml of solution A, and boiled for 15 minutes. After cooling, removed the precipitate by decantation. Before spraying, mix solution A (50 ml) plus solution B (50 ml) plus water (100 ml). Added 0.178 g of molybdenum powder to 50 ml of solution A and boiled it for 15 minutes. Cooled and removed the precipitate by decantation.

2.3 Anisaldehyde reagent

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

3. Whole cell sugar

Acid aniline phthalate

Phthalic acid	3.25	g
Aniline	2	ml

Dissolved phthalic acid in 100 ml of water-saturated *n*-butanol and 2 ml aniline.

APPENDIX C

REAGENT FOR DNA EXTRACTION AND PURIFICATION DNA BASE COMPOSITION, DNA-DNA HYBRIDIZATION 16S rRNA GENE SEQUENCING AND REP-PCR

1. DNA extraction and DNA base composition

1.1 Saline-EDTA (0.15 M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA	37.22	g

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

1.2 10% (w/v) SDS

Sodium dodecyl sulphate	10	g
Distilled water	90	ml

Dissolved and made up to 100 ml with distilled water.

1.3 Phenol: Chloroform (1:1, v/v)

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

1.4 20 x SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1000	ml

Adjusted pH to 7.0 and steriled by autoclaveing at 121 ºC 15 pounds / inch 2 pressure, for 15 minutes. Note: To prepare 0.1× SSC and 0.2× SSC, the 20× SSC were diluted at 200 and 100 times, respectively before used.

1.5 RNase A solution

RNase A	20	mg
0.15 M NaCl	10	ml

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

1.6 0.1 M Tris-HCl (pH 7.5)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	ml

Dissolved and adjusted to pH 7.5 by adding 0.1 N HCl. Made to 100 ml with distilled water.

1.7 RNase T1 solution

RNase T1	80	µl
0.1 M Tris-HCl (pH 7.5)	10	ml

Mixed 80 µl of RNase T1 in 10 ml of 0.1 M Tris-HCl (pH 7.5) and heated at 95 °C for 5 min. Kept in -20 °C.

1.8 40 mM CH_3COONa + 12 mM $ZnSO_4$ (pH 5.3)

CH_3COONa	3.28	g
$ZnSO_4$	1.94	g
Distilled water	90	ml

Dissolved and adjusted to pH 5.3 by adding 0.1 N HCl or 0.1 N NaOH. Made to 100 ml with distilled water.

1.9 Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH3COONa + 12 mM ZnSO4 (pH 5.3)	1	ml

Dissolved and stored at 4°C.

1.10 Alkaline phosphatase solution

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

2. DNA-DNA hybridization

2.1 Phosphate-buffer saline (PBS)

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.12	g
Na_2HPO_4 (anhydrous)	0.91	g
Distilled water	1	L

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

2.2 20 x SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	L

Adjusted pH to 7.0 and steriled by autoclaveing at 121 ºC 15 pounds /inch2 pressure, for 15 minutes.

2.3 100 x Denhardt solution

Bovine serum albmin (Fraction V)	2	g
Polyvinylpyrrolidone	2	g
Ficoll400	2	ml

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

2.4 Salmon sperm

Salmon sperm DNA 10 mg /ml

Salmon sperm DNA 10 mg was dissolved in 10 mM Tris + EDTA buffer pH 7.6 volume 1 ml, boiled for 10 min and then immediately cooled in ice. Sonicated salmon sperm DNA solution for 3 min and was store at 4 ºC until used.

2.5 Prehybridization solution

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

All of ingredients were dissolved in ultra pure water steriled and kept at 4 ºC.

2.6 Hybridization solution

Prehybridization	100	ml
Dextran sulfate	5	g

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

2.7 Solution I

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

All of ingredients were mixed and keep at 4 ºC.

2.8 Solution II

Strepavidin-POD conjugate	1	µl
Solution1	4	ml

Dissolved Strepavidin-POD conjugate in solution I before used. The solution II was freshly prepared.

2.9 Solution III

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

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

3. Repetitive sequence based polymerase chain reaction (rep-PCR) fingerprinting

3.1 1.5% (w/v) Agarose gel

Agarose	1.5	g
Distilled water	100	ml

Dissolved with distilled water and heating with microwave until agarose gels were dissolved well. After agarose solution cool down to about 50 ºC, pour the solution into the case and leave to solidify at room temperature.

3.2 10X Tris-borate buffer (TBE) buffer

Tris (hydroxymethyl)aminomethane	108	g
Boric acid	55	g
Ethylenediaminetetraacetic acid	7.4	g
Sodium hydroxide	1	g

Dissolved Tris (hydroxymethyl) aminomethane and boric acid with distilled water and adjusted pH to 8.4 with NaOH. Then EDTA was added and brought up the volume to 1000 ml with distilled water. Diluted 10 times before use.

4. 16S rRNA gene sequence analysis

Primers for 16S rRNA gene amplification and Sequencing

Forward primer

27F 5'-AGAGTTTGATC(CT)TGGCTCAG-3’

337F 5'-GCGGTACCTGGAAATTGCTGGGTCCA-3'

Reverse primer

518R 5'-ATTACCGCGGCTGCTGG-3'

1492R 5'-ACGG(CT)TACCTTGTTACGACTT-3'

APPENDIX D

STANDARD ASSAY METHODS

1. Determination of protein

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

1.1 Reagents

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

B: 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium citrate

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

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

1.2 Procedure

1.2.1. Placed 0.1 ml of proper dilution of culture broth (protein determination).

1.2.2. Added 1 ml of Reagent D into the tube and vortexed immediately. Incubated at room temperature for 10 min. After the 10 min incubation, added 0.1 ml of Reagent C to sample and vortexed immediately. Incubated 30 min at room temperature.

1.2.3 Absorbance (OD) of samples was measured at 750 nm.

Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

2. Equipment and Reagent

2.1 Oat spelt xylan substrate

One gram of Oat spelt xylan was homogenized in *ca.* 80 ml of 0.1 M sodium phosphate buffer pH 6 and pH 7 at 60 °C by using a kitchen blender and a heating magnetic stirrer. The solution was cooled by continuing slowly stirring for overnight. The volume was made up to 100 ml with the same buffer. This was then stored at 4 °C for a maximum of one week.

2.2 Dinitrosalicylic acid (DNS)

Dinitrosalicylic acid	1	g
Potassium tartate	300	g
NaOH	16	g

Dissolved NaOH in distilled water and heating at 60 °C. Then, gently put potassium tartate and added DNS. Then made up to 1000 ml with distilled water.

2.3 0.1 M Britton-Robinson universal buffer (Britton and Robinson, 1931)

Sodium citrate	29.41	g
Sodium phosphate, dibasic	14.19	g
Sodium carbonate	10.56	g
Distilled water	950	ml

Dissolved with distilled water and adjusted to the desired pH with 1 N HCl or 1 N NaOH. Then made up to 1000 ml with distilled water.

2.4 Corn cob 20M

Corn cob 20M were washed by 1% triethylamine, to removed other protein. After, that washing several times with warm distilled water, to remove sugars remaining in these residues, the ground corn cob residues were dried at 50 °C.

3. SDS-PAGE and zymogrm

Polyacrylamide gel electrophoresis (PAGE) reagents

3.1 Monomer solution

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

Made up to 100 ml with deionized water.

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

3.2 4× Resolving gel buffer

Tris(hydroxymethyl)aminomethane	18.15	g
Deionized water	90	ml

Dissolved and adjusted the pH to 8.8 by using 0.1 N HCl. Made up to 100 ml with deionized water.

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

3.3 4× Stacking gel buffer

Tris(hydroxymethyl)aminomethane	6	g
Deionized water	90	ml

Dissolved and adjusted the pH to 6.8 by using 0.1 N HCl. Made up to 100 ml with deionized water.

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

3.4 10× Tank buffer for SDS-PAGE

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

Dissolved and made up to 1 litter with distilled water.

Note: Diluted 10 times before use. The solution can be store up to 1 month at room temperature.

3.5 10% Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulfate	10	g
Deionized water	90	ml

Dissolved and made to 100 ml with deionized water.

3.6 2× Sample buffer for SDS-PAGE

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

Dissolved and made up to 10 ml with deionized water.

Note: The reagent should be filtered before use.

3.7 12.5% Running gel for SDS-PAGE

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

3.8 4% Stacking gel for SDS-PAGE

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

3.9 Staining solution

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

Dissolved and made up to 1 litter with distilled water.

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

3.10 Destaining solution

Methanol	300	ml
Acetic acid	100	ml

Dissolved and made up to 1 litter with distilled water.

APPENDIX E

16S rRNA GENE SEQUENCE OF NEW SPECIES

1. The 16S rRNA gene nucleotide sequence of P2-3

CTGGCGGCGTGCCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTG
ATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAG
ACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAAT
AGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGG
CTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCC
TTATGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAACGGTGGAACATGTGGTTTAATT
CAACACCCAAAACCTTACCAGGTCTGACTCCTCTGACATCTAAAAATAGAACGTC
CCCTTCCGGGGCAAATGACGGGGTGCATGTTCCCCCTCCTGTCCGAATGTTGGTAA
TCCCA

2. The 16S rRNA gene nucleotide sequence of PHX1-5

GCTGTCACTTACAGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG
GGCGAAAGCCTGACCGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTA
AAGCTCTGTTGTTAGGGAAGAACACGTACGAGAGTAACTGCTCGTACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCgGGTTTCTT
AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGA
AACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAA

CGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACACCCCTAGAGATAGGGCTTCCCCTT
CGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCGGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAA
AGGGTTGCAAGACCGCGAGGTGGAGCTAATCCCAAAAAACCGTTCTCAGTTCGGA
TTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGCAACACCCGAAGTCGGTGGGGTAACCGTAAGGAGCCAGCCGCCTAAG
GTG

3. The 16S rRNA gene nucleotide sequence of SRX1-4

GTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGAAGGATCGGGAT
AACTACCGGAAACGGTAGCTAAGACCGGATAGCTGGCTCTGGTGCATGCCGGAGT
CATGAAACACGGAGCAATCTGTGGCCTTTGGATGGGCCTGCGGTGCATTAGCTAG
TTGGTGGGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGTTCTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCGTGGGGAGTAAC
TGCCCTGCGAATGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTTCATTAAGTTTGGTGTTTAAGCCCGGGGCTCAACCCCGGTTCGC
ACTGAAAACTGGTGAACTTGAGTGCAGGAGAGGAAAGCGGAATTCCACGTGTAG
CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGG
ACTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCT
TGGTGCCGAAGTAAACACAATAAGCACTCCGCCTGGGGAGTACGCTCGCAAGAG
TGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGAAAGCCCTA
GAGATGGGGTCCTCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAACTTA
GTTGCCAGCATTCAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTAC
TACAATGGCCGGTACAACGGGAAGCGAAGTCGCGAGATGGAGCGAATCCTTACA
AGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTG
CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACA
CCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCCGCA
AGGGAGCCAGCCGCCGAAG

4. The 16S rRNA gene nucleotide sequence of K3-2

GCGGCGTGCCTAATACATGCAAGTCGAGCGGAGTTCATCGGGAGCTTGCTTCGGA
TGAACTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGACTG
GGATAACTACCGGAAACGGTAGCTAATACCGGATACGCAAGTTTCTCGCATGAGG

GGCTTGGGAAAGGCGGAGCAATCTGTCACTTACGGATGGGCCTGCGGCGCATTAG
CTAGTAGGTGGGGTAACGGCCTACCTAGGCGACGATGCGTAGCCGACCTGAGAG
GGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCTTGGAGAG
TAACTGCTCTAAGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAA
GCGCGCGCAGGCGGCTGTTTAAGTCTGGTGTTTAATCCTGGGGCTCAACCCCGGG
TCGCACTGGAAACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCT
CTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATA
CCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGACCGGA
TCAGAGATGATCCTTTCCTACGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACT
TTAGTTGCCAGCAGGTCAGGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTACTACAATGGCCGGTACAACGGGAAGCGAAGCCGCGAGGCGGAGCGAATCT
TAAAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGA
ATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTA
CACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACC
GCAAGGAGCCAGCCGCCGAAGGTG

5. The 16S rRNA gene nucleotide sequence of PL1-3

ATACATGCAAGTCGAGCGGAGTTCATCGGGAGCTTGCTCCTGATGAACTTAGCGG
CGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGACTGGGATAACTACCG
GAAACGGTGGCTAATACCGGATACGCAAGTTTCTCGCATGAGGGGCTTGGGAAAG
GCGGAGCAATCTGTCACTTACGGATGGGCCTGCGGCGCATTAGCTAGTAGGTAGG
GTAACGGCCTACCTAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCTTGGAGAGTAACTGCTCTAAG
AGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CGGCTGTTTAAGTCTGGTGTTTAATCCTGGGGCTCAACCCCGGGTCGCACTGGAA
ACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAA
CTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG

TCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCC
GAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTC
AAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGACCGGATCAGAGATGAT
CCTTTCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACTTTAGTTGCCAG
CAGGTCAGGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAA
TGGCCGGTACAACGGGAAGCGAAGGAGCGATCTGGAGCGAATCCTTGAAAGCCG
GTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCC
CGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACCGCAAGGAG
CCAGCCGCCGAAGGTGG

6. The 16S rRNA gene nucleotide sequence of P2-3A

GGCGTGCCTAATACATGCAAGTCGAGCGGAGTTCATCGGGAGCTTGCTTCGGATG
AACTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGACTGGG
ATAACTACCGGAAACGGTAGCTAATACCGGATACGCAAGTTTCTCGCATGAGGGG
CTTGGGAAAGGCGGAGCAATCTGTCACTTACGGATGGGCCTGCGGCGCATTAGCT
AGTAGGTAGGGTAACGGCCTACCTAGGCGACGATGCGTAGCCGACCTGAGAGGG
TGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCCTGTAGAGTAA
CTGCTCACGGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGCTGTTTAAGTCTGGTGTTTAATCCTGGGGCTCAACCCCGGGTCG
CACTGGAAACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCC
TTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGACCGGTGCA
GAGATGCTCCTTTCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACTTTA
GTTGCCAGCAGGTCAGGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
ACTACAATGGCCGGTACAACGGGAAGCGAAGGAGCGATCTGGAGCGAATCCTTG
AAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT
TGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACA

CACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACCGC
AAGGAGCCAGCCGCCGAAG

7. The 16S rRNA gene nucleotide sequence of CXT1-1

GCGTGCCTAATACATGCAAGTCGAGCGGAGTTCATCGGGAGCTTGCTTCGGATGA
ACTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGACTGGGA
TAACTACCGGAAACGGTAGCTAATACCGGATACGCAAGTTTCTCGCATGAGGGGC
TTGGGAAAGGCGGAGCAATCTGTCACTTACGGATGGGCCTGCGGCGCATTAGCTA
GTAGGTAGGGTAACGGCCTACCTAGGCGACGATGCGTAGCCGACCTGAGAGGGT
GAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCCTGTAGAGTAA
CTGCTAACGGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGCTGTTTAAGTCTGGTGTTTAATCCTGGGGCTCAACCCCGGGTCG
CACTGGAAACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCC
TTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGACCGGTGCA
GAGATGCTCCTTTCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACTTTA
GTTGCCAGCAGGTCAGGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
ACTACAATGGCCGGTACAACGGGAAGCGAAGGAGCGATCTGGAGCGAATCCTTG
AAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT
TGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACCGC
AAGGAGCCAGCCGCCGAAG

8. The 16S rRNA gene nucleotide sequence of P2-5

GGCGTGCCTAATACATGCAAGTCGAGCGGAGTTCATCGGGAGCTTGCTTCGGATG
AACTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGACTGGG
ATAACTACCGGAAACGGTAGCTAATACCGGATACGCAAGTTTCTCGCATGAGGGG
CTTGGGAAAGGCGGAGCAATCTGTCACTTACGGATGGGCCTGCGGCGCATTAGCT
AGTAGGTAGGGTAACGGCCTACCTAGGCGACGATGCGTAGCCGACCTGAGAGGG
TGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG

TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCCTGTAGAGTAA
CTGCTCTCGGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGCTGTTTAAGTCTGGTGTTTAATCCTGGGGCTCAACCCCGGGTCG
CACTGGAAACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCC
TTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCCTGACCGGTGCA
GAGATGCTCCTTTCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACTTTA
GTTGCCAGCAGGTCAGGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
ACTACAATGGCCGGTACAACGGGAAGCGAAGGAGCGATCTGGAGCGAATCCTTG
AAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT
TGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACCGC
AAGGAGCCAGCCGCCGAAG

9. The 16S rRNA gene nucleotide sequence of S3-4A

CCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAGT
TGATGGAGGTGCTTGCACTTCTGANGGTTAGCGGCGGACGGGTGAGTAACACGTA
GGTAACCTGCCCATAAGACCGGGATAACATTCGGAAACGGATGCTAATACCGGAT
ACGCAATTCTCTCGCATGAGGGGATTGGGAAAGGCGGAGCAATCTGTCACTTATG
GATGGACCTGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCA
GGGAAGAACGCTTGGGAGAGTAACTGCTCTCAAGGTGACGGTACCTGAGAAGAA
AGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCATTAAGTCTGGTGTTTA
AGGCTGGGGCTCAACCCCGGTTCGCACTGGAAACTGGTGGACTTGAGTGCAGAAG
AGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA
CCAGTGGCGAAGGCGACTTTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTA
GGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCC
TGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACA

AGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCCCCTGACCGGTCTAGAGATAGGCCTTTCCTTCGGGACAGGGGAGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGATCTTAGTTGCCAGCACTTTGGGTGGGCACTCTAGGATGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTACTACAATGGCCGGTACAACGGGAAGCGAAGGAGC
GATCCGGAGCCAATCCTATAAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTC
GCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAACACCCG
AAGCCGGTGGGGTAACCGCAAGGAGCCAGCCGTCGAAGGTGGGGTAGATGATTG
GGGTG

10. The 16S rRNA gene nucleotide sequence of MX2-3

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAGCTAAGTAGAA
GCTCGCTTTCGCGATGCTTAGCGGCGGACGGGTGAGTAACACGTAGGTAACCTGC
CTGTAAGACTGGGATAACATTCGGAAACGAATGCTAATACCGGATACGCGAGTTG
GTCGCATGGCCGACTCGGGAAAGACGGAGCAATCTGTCGCTTACAGATGGACCTG
CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACG
CTTGGGAGAGTAACTGCTCCCAAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGCGCGCGCAGGCGGTTCATTAAGTCTGGTGTTTAAGGCTGGGGCT
CAACCCCGGTTCGCACTGGAAACTGGTGAACTTGAGTGCAGAAGAGGAAAGTGG
AATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGACTTTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGG
GGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAGT
ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGG
AGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCC
TCTGACCGGTCTAGAGATAGGCCTTTCCTTCGGGACAGAGGAGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTTTTAGTTGCCAGCACTTTGGGTGGGCACTCTAAAGAGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTACTACAATGGCCAGTACAACGGGAAGCGAAGGAGCGATCTGGA
GCCAATCCTATCAAAGCTGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCAT
GAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCG
GGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGT
GGGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTG

11. The 16S rRNA gene nucleotide sequence of FXN2-3

TACATGCAAGTCGAGCGGATCTGATGAGGTGCTTGCACCTCTGATGGTTAGCGGC
GGACGGGTGAGTAACACGTAGGTAACCTGCCTGTAAGACTGGGATAACATTCGGA
AACGAATGCTAATACCAGATACGCGATTTCCTCGCATGGGGGAATCGGGAAAGAC
GGAGCAATCTGTCACTTACAGATGGACCTGCGGCGCATTAGCTAGTTGGTGGGAA
CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGG
TCGTAAAGCTCTGTTGCCAGGGAAGAACACTTGGGAGAGTAACTGCTCTCAAGGT
GACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
CTTTGTAAGTCTGTCGTTTAAGTTCGGGGCTCAACCCCGTATCGCGATGGAAACTG
CAAGGCTTGAGTACAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGGCTGTAACTGAC
GCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCAATACCCTTGGTGCCGAAGT
TAACACATTAAGCATTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGGTCTGGAGACAGGCCTTCC
CTTCGGGGCAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTGCCAGCATTTCG
GATGGGCACTCTAGAATGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCCAGT
ACAACGGGCTGCGAAGGAGCGACCCGGAGCGAATCCTATAAAGCTGGTCTCAGTT
CGGATTGGAGGCTGCAACTCGCCTCCATGAAGTCGGAATTGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACC
ACGAGAGTTTACAACACCCGAAGCCGGTGGGGTAACCGCAAGGAGCCAGCCGTC
GAAGGTGG

12. The 16S rRNA gene nucleotide sequence of SRX4-1

CATGCAAGTCGAGCGGATCTTATCCTTCGGGGTAAGGTTAGCGGCGGACGGGTGA
GTAACACGTAGGTAACCTGCCTGTAAGACCGGGATAACATTCGGAAACGAATGCT
AATACCGGATACACGGCTTGTCCGCATGGACGAGCCGGGAAAGACGGCGCAAGC
TGTCACTTGCAGATGGACCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCTCA
CCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAG
CTCTGTTGCCAGGGAAGAACGAGTGGGAGAGTAACTGCTCCTGCTATGACGGTAC

CTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTTGTAAG
TCAGGTGTTTAAGCTCGGGGCTCAACCCCGATTCGCATcTGAAACTGCAAGACTTG
AGTGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATG
TGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGGCTGTAACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATT
AAGCATTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACG
GGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCCTCTGAATCCTCTAGAGATAGAGGCGGCCCTTCGGG
GACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTGCCAGCACTTTAAGGTG
GGCACTCTAGAATGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCCGTTACAA
CGGGAAGCGAAGTCGCGAGATGGAGCGAATCCTAAAAAGGCGGTCTCAGTTCGG
ATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACG
AGAGTTTACAACACCCGAAGCCGGTGGGGTAACCGCAAGGAGCCAGCCGTCGAA
GGTG

13. The 16S rRNA gene nucleotide sequence of SRX4-2

GTGCCTAATACATGCAAGTCGAGCGGATCTTATCCTTCGGGGTAAGGTTAGCGGC
GGACGGGTGAGTAACACGTAGGTAACCTGCCTGTAAGACCGGGATAACATTCGG
AAACGAATGCTAATACCGGATACACGGCTTGTCCGCATGGACGAGCCGGGAAAG
ACGGCGCAAGCTGTCACTTGCAGATGGACCTGCGGCGCATTAGCTAGTTGGTGGG
GTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGCCAGGGAAGAACGAGTGGGAGAGTAACTGCTCCTGC
TATGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGC
GGTTTTGTAAGTCAGGTGTTTAAGCTCGGGGCTCAACCCCGATTCGCATCTGAAAC
TGCAAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGGCTGTAACTG
ACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAA
GTTAACACATTAAGCATTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAA
GGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCCTCTGAATCCTCTAGAGATAGATGCG
GCCCTTCGGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT

GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTGCCAGCA
CTTTAAGGTGGGCACTCTAGAATGACTGCCGGTGACAAACCGGAGGAAGGCGGG
GATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATG
GCCGTTACAACGGGAAGCGAAGTCGCGAGATGGAGCGAATCCTAAAAAGGCGGT
CTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAA
TCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCG
TCACACCACGAGAGTTTACAACACCCGAAGCCGGTGGGGTAACCGCAAGGAGCC
AGCCGTCGAAGGTGG

14. The 16S rRNA gene nucleotide sequence of X11-1

CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGATTTACCC
TTCGGGGTAAGTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTAA
GATCGGGATAACTACCGGAAACGGTAGCTAAGACCGGATAGGTGGTTTCTTCGCA
TGAAGAGATCCAAGAAACACGGGGCAACCTGTGGCTTACAGATGGGCCTGCGGG
CATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGANCTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAAGGAAGAACGCCTC
GGAGAGTAACTGCTCCGGGGGTGACGGTACTTGAGAAGAAAGCCCCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGG
GCGTAAAGCGCGCGCAGGCGGCCGCTTAAGTTTGGTGTTTAAGCCCGGGGCTCAA
CCCCGGTTCGCACCGAAAACTGGGCGGCTTGAGTGCAGGAGAGGAAAGCGGAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGG
CGGCTTTCTGGACTGTAACTGACGCTGAGGCGCGTAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGG
TTTCGATACCCTTGGTGCCGAAGTAAACACAATAAGCACTCCGCCTGGGGAGTAC
GCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAG
TATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGA
TGAAAGCCATAGAGATATGGCCCCTCTTCGGAGCATTGGAGACAGGTGGTGCATG
GTTGTCGTCAGCTCAAGTGAATNGTGAGATGTTGGGTTAAGTCCNGCAACGAGCN
CAACCCTTGAACTTAGTNNCCAGCATTAAGTTGGGCACTATAAGTTGACTNCCGG
TGACANACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGNCNT
GGGNTACACANGTANNACAATGNCCGGTNCAACGGGAAGCGAANCCGNGAGNCG
GAGNGAATNTTTATAANCCGGTTTCANTTNGGATNNCAGGNTGCAACTNNCCTGC
ATGAAGTNGGAATTGCTAGTNATCGNGGATCCAGCATNCNNGCGGTGAATACGTT
NNCCGNATNTTGTACACACCNCNNGTCACACCANGAGAGTTTNCAACACCCGAAG
TNGGTGGGGTAACCGCAAGGAGCCAGCCGCCGAAGGTGGGGTAGATGAT

15. The 16S rRNA gene nucleotide sequence of FCN3-3

GTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGATCGGGAT
AACATTCGGAAACGGATGCTAAGACCGGATAAACGGTTTGGTCGCATGATCGGAT
CGAGAAACACGGTGCAAGCTGTGACTTGGGGATGGGCCTGCGGCGCATTAGCTAG
TTGGTGGGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTG
AACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAGG
AAGGCTTTCGGGTCGTAAAGCTCTGTTGCCAGGGAAGAATAAGGGTATGTTMACT
GCATATTCGATGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG
CGCAGGCGGTTCTTTAAGTCTGGTGTCTAAGTGCGGGGCTCAACCCCGTGATGCA
CTGGAAACTGGGGGACTGGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGT
CTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGTATCATGCCCTCG
GTGCCGAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGA
AACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGTCCTAGAGA
TAGGGCTTTCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAACTTAGTTG
CCAGCGAGTGAGGTCGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAA
GGCGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACT
ACAATGGCCGGTACAAAGGGCCGCGAAGCCGCGAGGTGGAGCCAATCCCAGCAA
AGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTG
CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACA
CCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCCGCA
AGGGAGCCAGCCGCCGAAGGTG

16. The 16S rRNA gene nucleotide sequence of S1-3

TCCTGGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGA
TCTTTCCTTAAGTAGCTTGCTACTTTAAGAAGGTTAGCGGCGGGACGGGTGAGTA
ACACGTAGGCAACCTGCCCATAAGACCGGGATAACATTCGGAAACGAATGCTAA
GACCGGATACGCAAAAGGAGGGCATCATCCTTTTTGGGAAACACGGTGCAAGCTG
TGGCTTATGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTGTGGAAACT
GAGACACGGCCCAGACTCCTACGGGGAGGCCAGCAGTTAGGGAATTCTTCCACAA
TGGGCGCAAGCCTGATGGAGCAACGCCGCGTGAGTGAGGAAGGCTTTCGGGTCGT
AAAAGCTCTGTTGCCAGGGAAGAATAAGGGCGAGGTAACTACTCGTCCGATGAC
GGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT

AGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCT
TAAGTCTGGTGTTTAAGTGCGGGGCTCAACCCCGTGTCGCATCGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGACTGTAACTGACGCTG
AGGCGCGAAAGCGTGGGGAGCCCAAACAGGGATTAGATACCCTGGTAGTCCACG
CCGTTAAACGATGAGTGTTCTAGGTGTTGGGGGGGTCCACCCCTCGGTGCCGAAG
TTAACACACTTAAGCACTCTCGCCCTGGGGATGTACGGTCGCAAGACTGAAACTC
AAAGGAATTGACGGGGAACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTTACCAGGTCTTGACATCCCTCTGACCGTTCCTAGAGAT
AGGGCTTCCCTTCGGGGCAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAAATGTTGGTTGAANTTCCGGCAACAAGCCCAACCCTGGAATTTATTTCC
CACCACTTTGGGTGGGCACTCTAGATTGACTGCCGGTGACAAACCGGAGGAAGGC
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACA
ATGGCCGGTACAACGGGTTGCGAAGGAGCGATCCGGAGCCAATCCTATAAAGCC
GGTCTCAGTTCGGATTGGAGGCTGCAACTCGCCTCCATGAAGTCGGAATTGCTAG
TAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
CCGTCACACCACGAGAGTTTACAACACCCGAAGCCGGTGGGGTAACCGCAAGGA
GCCAGCCGTCGAAGGTGGGGTAGATGATTGGGGTGAAGTCGTAACAAGGAGCC

17. The 16S rRNA gene nucleotide sequence of MX15-2

CTCCNTGGCTCAGGGACGAAACGCCGGGCGGCGTGCCTAATACATGCAAGTCGAG
CGGATCTTCAAGGGAGCTTGCTCCTGAGAAGGTTAGCGGCGGACGGGTGAGTAAC
ACGTAGGCAACCTGCCCTCAAGACCGGGATAACATTCGGAAACGAATGCTAAGA
CCGGATACGCAAGAAGGAGGCATCTTCTTCTTGGGAAACACGGCGCAAGCTGTGG
CTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGCGGGGTAACGGCCCACCAAG
GCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTTACGGGAGGCAGCAGTTAGGGAATTCTTCCACAATGGGCGC
AAGCCTGATGGAGCAACGCCGCGTGAGTGAGGAAGGCCTTCGGGTCGTAAAGCT
CTGTTGCCAGGGAAGAATAAGAGCCAGTTAACTGCTGGTTCGATGACGGTACCTG
AGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCT
GGTGTTTAAGTGCGGGGCTCAACCCCGTGACGCACTGGAAACTGGGAGACTTGAG
TGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTG
GAGGAACACCAGTGGCGAAGGCGGCTCTCTGGAGCTGTAACTGACCGCTGAGGC
CGCGAAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAGGTGTTGGGGGGGTCCACCCCTCGGTGCCGAAGTTAACACA
TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAA
CCTTACCAGGTCTTGACATCCCTCTGAATCGTCTAGAGATAGGCGCGGCCTTCGGG
ACAGAGGAGACAGGTGGTGCATGGTTGTCGTGCAGCTCGTGTCGTGAGATGTTGG

GTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACTTCGGGTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
ATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCCGGTACAAC
GGGCAGCGAAGGAGCGATCCGGAGCCAATCCTTTAAAGCCGGTCTCAGTTCGGAT
TGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATACGTTACCCGGGTCTTGTACACACCGCCCGTCACACCACGA
GAGTTTACAACACCCGAAGCCGGTGGGGTAACCGCAAGGAGCCAGCCGTCGAAG
GTGGGGTAGATGATTGGGGTGAAATCGTAAT

18. The 16S rRNA gene nucleotide sequence of MX21-2

TTATTGTCCTGGCTCAGGACGAACGCCTGGCCGGCCGTGCCCTTAATGAGCGATG
CAGATGCATTCCTGAGCGGATCTTCCAAGGGAGCTTTGCTCCTGAGAAGGTTTAG
CGGCCGGACCCGGGTTGAGTAACCACGTTAGGCCAACCTTGCCCCTTCCAAGACC
GGGATAACCATTTCGGAAACGAATTGCTAAGACCGGATACGCCAAGGAGGAGGC
ATCTTCTTCTTGGGAAACCACGGCGCAAGCTGTGGCTTGAGGATGGGCCTGCGGC
GCATTAGCTAGTTGGCGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACC
TGAGAGGGTGAACGGCCACACTGGGACTTGAAGACACGGCCCCAGACTCCTTACC
GGGGAGGCCAGCAGTAGGGAATCTTCCACCAATGGGCGCAAGCCTTGATGGAGC
AACGCCCGCGTGAGTGAGGAAGGCCTTCGGGTCGTAAAGCTCTGTTGCCAGGGAA
GAATAAGAGCCAGTTAACTGCTGGTTCGATGACGGTACCTGAGAAAGAAAAGCC
CCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCC
GGAATTATTGGGCGTTAAAGCGCGCGCAGGCGGTTTCTTAAGTTCTGGGTGTTTA
AGTGCGGGGCTCAACCCCGTGACGCACTGGAAACTGGGAGACTTGAGTGCAGAA
GAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGGCTCTCTTGGACTGTAAACTGACGCTGAGGCGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAGGTGTTGGGGGGGTCCACCCCTCGGTGCCGAAGTTAACACATTTAAGCACT
CCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCCTCTGAATGGTCTAGAGATAGCGCAGGCCTTCGGGACAGAGGA
GACAGGTGGTGCATGGTTGTTCGTGCAGCTCGTGTCTTGGGAGATGTTGGGTTAA
GTCCNGCAACGAGCGCAACCCTNGNTNTTAGTNNCCANCATTTNGGGTGGGCACT
TTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT
CATGCCCCTTATNNCCTGGGCTACACACGTANTNCAATGGCCGGTACAACGGGCA
GCGAAGGAGCGATCGGGAGCCAATCCTNTAAAGCCGGTCTCAGTTCGGATTNCAG
GNTGCAACTCNCNTGCATGAAGTCCGGAATTGCTAGTAATCGCGGATCAGCATGC
CCGCGGTGAATACGTTACCCGAATACTTGTACACACCGCCCGTCACACCACGAGA
GTTTNCANCACCNGAAGCCGGTGGGGTAACCGCAAGGAGCCAGCCGTCGAAGGT
GGGGTAGATGNTANGGGTGAAG

19. The 16S rRNA gene nucleotide sequence of CE3-4

GCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAGCCAAGCTTGCTTGGTGGAT
CAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCAGTCTCTGGGATAAC
AGTTGGAAACAGCTGCTAATACCGGATACGAACCGCGATCGCATGGTCAGTGGTT
GGAAAGATTTTTCGGTCTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGA
ATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAA
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTAT
CCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAT
CCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAG
GGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACA
CCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGT
GGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACT
AGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCG
CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCA
CAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG
CTTGACATATAGAGGAAACGGCTGGAAACAGTCGCCCCGCAAGGTCTCTATACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATC
TGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTA
GGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGA
CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATAC
GTTCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCTGA
AGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAA

20. The 16S rRNA gene nucleotide sequence of SRX1-2

GGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGT
AACGCGTGGGAATCTACCGTGCCCTACGGAATAGCTCCGGGAAACTGGAATTAAT
ACCGTATACGCCCTACGGGGGAAAGATTTATCGGGGTATGATGAGCCCGCGTTGG
ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTG
AGTGATGAAGGTCTTAGGATTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGT
AACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG
GGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCATGTAGGCGGATATTTA
AGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAACTGCCTTTGATACTGGGTAT

CTTGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGAT
ATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCAT
TAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAAC
CTTACCAGCTCTTGACATCCGGGTCGCGGACAGTGGAGACATTGTCCTTCAGTTAG
GCTGGACCCAGGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTCAGTTG
GGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTC
AAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACA
GTGGGCAGCGAGACGGTGACGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGA
TTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAC
ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTTGGTTTTACCCGAAGGTAGTGCGCTAACCCGCAAGGGAGGCAGCTAACCACG

21. The 16S rRNA gene nucleotide sequence of SRX3-4

CGGCAGGCCTAATACATGCAAGTCGGACGGGATTTAACTTAAAGCTTGCTTTAAG
TTAATGAGAGTGGCGCACGGGTGCGTAACGCGTGAGCAACCTACCTCTATCAGGG
GGATAGCCTCTCGAAAGAGAGATTAACACCGCATAACATCAACTGTTCGCATGTC
CGGTTGATTAAATATTTATAGGATAGAGATGGGCTCGCGTGACATTAGCTAGTTG
GTAGGGTAACGGCTTACCAAGGCGACGATGTCTAGGGGCTCTGAGAGGAGAATC
CCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTAAGG
AATATTGGTCAATGGGCGGAAGCCTGAACCAGCCATGCCGCGTGCAGGATGACTG
CCCTATGGGTTGTAAACTGCTTTTGTCCAGGAATAAACCCAGATACGTGTACCTGG
CTGAATGTACTGGAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGG
CCTGTTAAGTCAGGGGTGAAATACGGTGGCTCAACCATCGCAGTGCCTTTGATAC
TGACGGGCTTGAATCCATTTGAAGTGGGCGGAATAAGACAAGTAGCGGTGAAAT
GCATAGATATGTCTTAGAACTCCGATTGCGAAGGCAGCTCACTAAGCTGGTATTG
ACGCTGATGCACGAAAGCGTGGGGATCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCCTAAACGATGATAACTCGATGTTGGCGATAGACCGCCAGCGTCCAAGCGAA
AGCGTTAAGTTATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGAGGAGCATGTGGTTTAATTCGATGATACGCGA
GGAACCTTACCCGGGCTTGAAAGTTAGTGAAGGATGCAGAGACGCATCCGTCCTT
CGGGACACGAAACTAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCCTATGTTTAGTTGCCAGCAAGTAATGT
TGGGGACTCTAAACAGACTGCCTGCGCAAGCAGAGAGGAAGGTGGGGACGACGT
CAAGTCATCATGGCCCTTACGTCCGGGGCTACACACGTGCTACAATGGATGGTAC
AGCGGGCAGCTACATAGCAATATGGTGCTAATCTCTAAAAGCCATTCACAGTTCG

GATTGGGGTCTGCAACTCGACCCCATGAAGTTGGATTCGCTAGTAATCGCGTATC
AGCAATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCA
TGAAAGTTGGGGGTACCTAAAGCATGTGACCGCAAGGAGCGTGTTAGG

22. The 16S rRNA gene nucleotide sequence of CE4-1

GGCATGCCTAAAACATGCAAGTCGAACGAAGGCTTCGGCCTTAGTGGCGCACGGG
TGCGTAACGCGTGGGAATCTGCCCCTTGGTTCGGAATAACAGTTAGAAATGACTG
CTAATACCGGATGATGACGTAAGTCCAAAGATTTATCGCCGAGGGATGAGCCCGC
GTAGGATTAGCTAGTTGGTGTGGTAAGAGCGCACCAAGGCGACGATCCTTAGCTG
GTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCC
GCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGATGATAATG
ACAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGAGCTAGCGTTATTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTT
TGTAAGTAAGAGGTGAAAGCCCAGAGCTCAACTCTGGAATTGCCTTTTAGACTGC
ATCGCTTGAATCATGGAGAGGTCAGTGGAATTCCGAGTGTAGAGGTGAAATTCGT
AGATATTCGGAAGAACACCAGTGGCGAAGGCGGCTGACTGGACATGTATTGACG
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGATAACTAGCTGTCCGGGGACTTGGTCCTTGGGTGGCGCAGCTA
ACGCATTAAGTTATCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGC
AGAACCTTACCAGCGTTTGACATGGTAGGACGGCTTCCAGAGATGGATTCCTTCC
CTTCGGGGACCTACACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTACCATCATTCAG
TTGGGTACTCTAAAGGAACCGCCGGTGATAAGCCGGAGGAAGGTGGGGATGACG
TCAAGTCCTCATGGCCCTTACGCGCTGGGCTACACACGTGCTACAATGGCAACTA
CAGTGGGCAGCAATCCCGCGAGGGTGAGCTAATCTCCAAAAGTTGTCTCAGTTCG
GATTGTTCTCTGCAACTCGAGAGCATGAAGGCGGAATCGCTAGTAATCGCGGATC
AGCATGCCGCGGTGAATACGTTCCCAGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTTGGATTCACCCGAAGGCGTCGCGCTAACCCGCAAGGGAGGCAGGCGAC
CACG

APPENDIX F

POLAR LIPID AND WHOLE CELL SUGAR

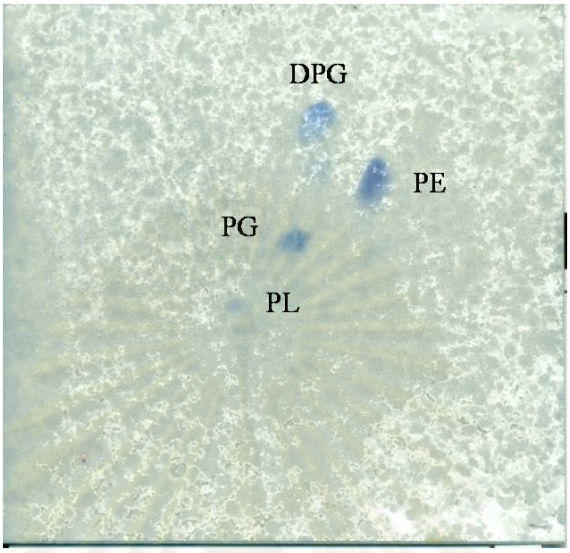

Figure 1 Polar lipid profiles of PHX1-5 after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine;; PL, unknown phospholipids

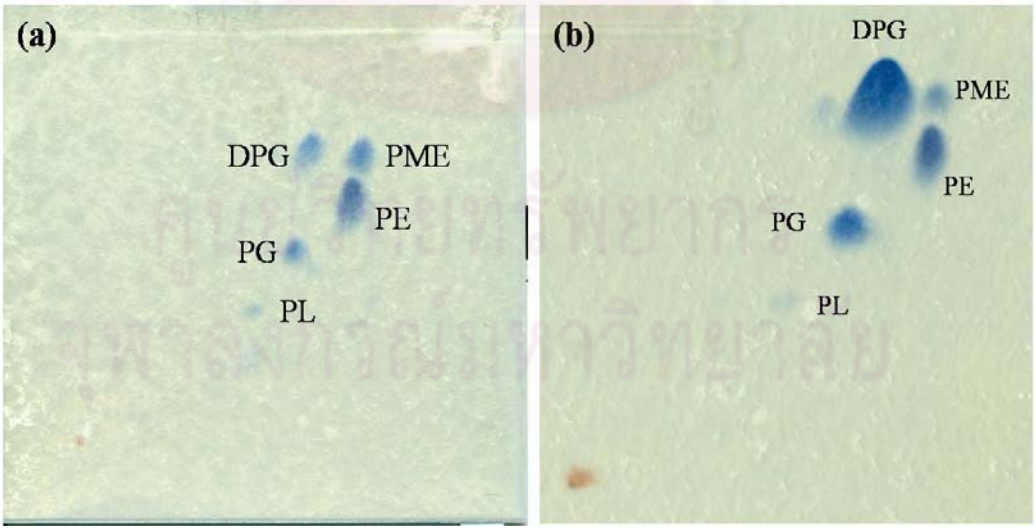

Figure 2 Polar lipid profiles of SRX1-4 (a) and X11-1 (b) after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PME, phosphatidylmonomethylethanolamine; PL, unknown phospholipids

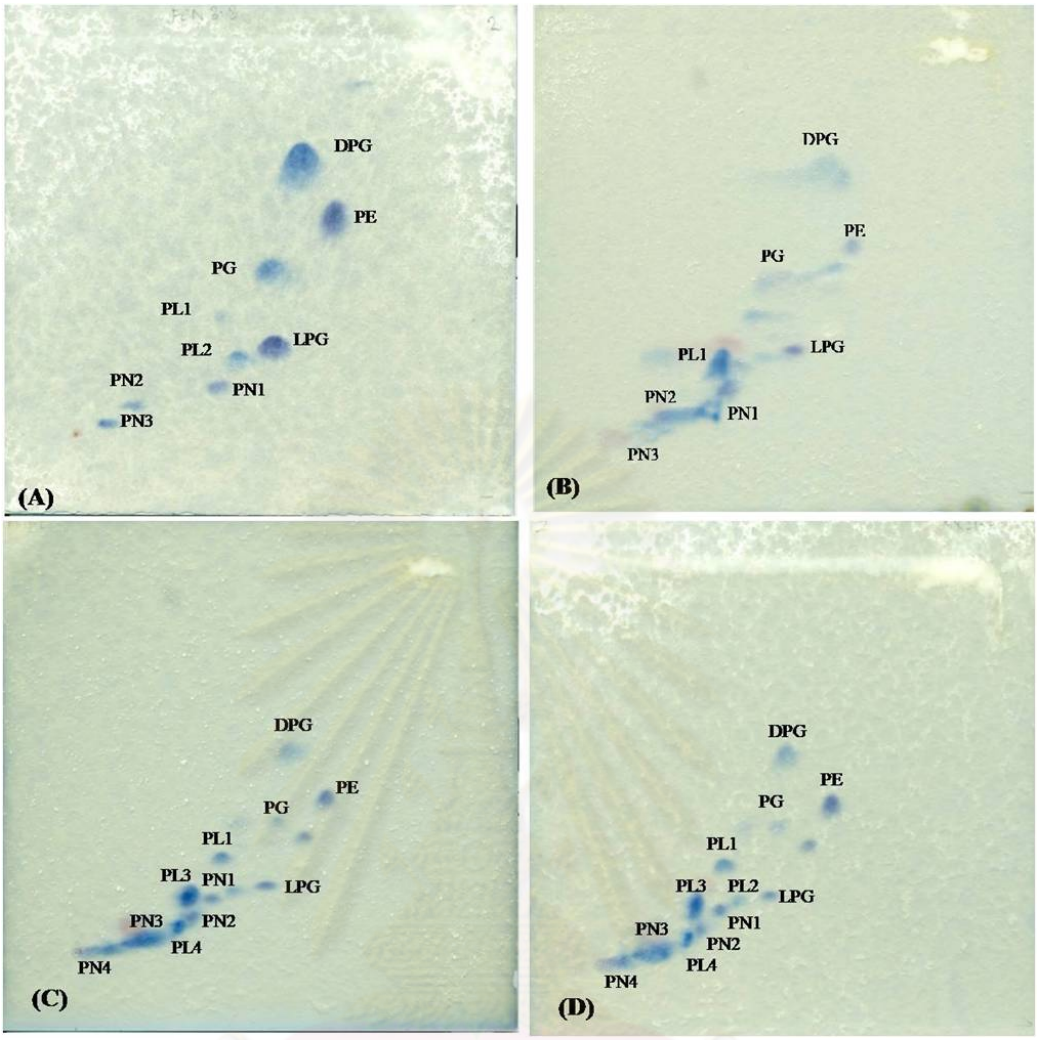

Figure 3 Polar lipid profiles of FCN3-3 (A), S1-3 (B), MX15-2 (C) and MX21-2 (D) after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPG, lysyl-phosphatidylglycerol; PL1-4, unknown phospholipids; PN1–4, unknown aminophospholipids

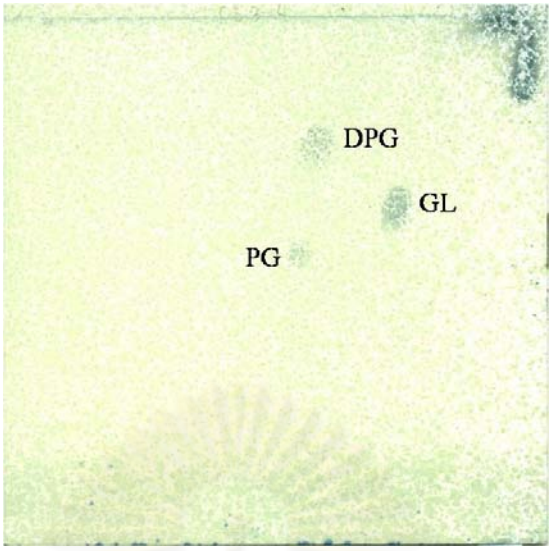

Figure 4 Polar lipid profiles of CE3-4 after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, glycolipid

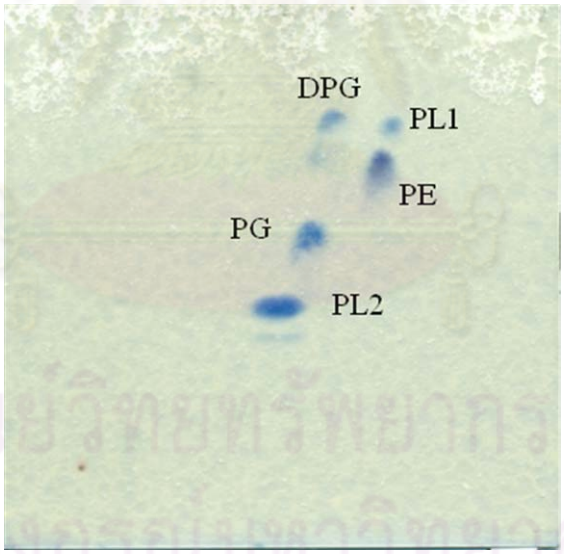

Figure 5 Polar lipid profiles of SRX1-2 after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL1-2, unknown phospholipids

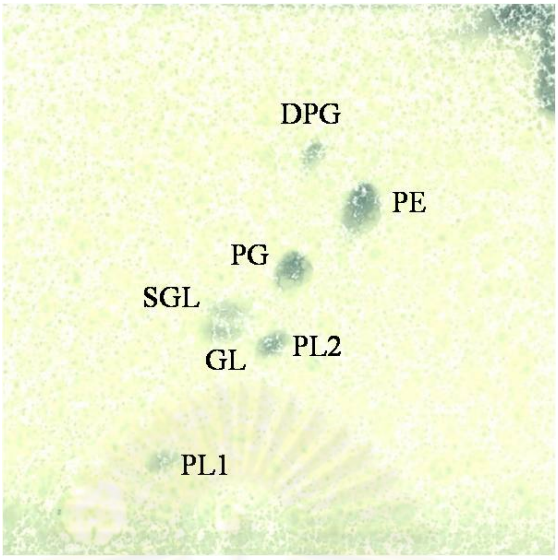

Figure 6 Polar lipid profiles of CE4-1 after separation by two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; GL, glycolipids; SGL, sphingoglycolipid; PL1-2, unknown phospholipids

Figure 7 Whole cell sugar profiles of CE3-4 after separation by thin layer chromatography and spraying with acid aniline phthalate

BIOGRAPHY

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