ไฟบริโนไลทิกโพรทิเอสจากแบคทีเรียที่คัดแยกได้จากอาหารหมักดอง

นางสาว ปุณณาณี สัมภวะผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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FIBRINOLYTIC PROTEASES FROM BACTERIA ISOLATED FROM FERMENTED FOODS

Miss Punnanee Sumpavapol

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Chemistry and Natural Products
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Thesis Title	FIBRINOLYTIC PROTEASES FROM BACTERIA ISOLATED FROM FERMENTED FOODS
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ปุณฑาณี สัมภวะผล : ไฟบริโนไลทิกโพรทิเอสจากแบคทีเรียที่คัดแยกได้จากอาหารหมักดอง. (FIBRINOLYTIC PROTEASES FROM BACTERIA ISOLATED FROM FERMENTED FOODS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ลินนา ทองยงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.สมบูรณ์ ธนาศุภวัฒน์, ดร.วรรณพ วิเศษสงวน 189 หน้า.

ทำการคัดแยกแบคทีเรียที่สามารถผลิตไฟบริโนไลทิกโพรทิเอสจากอาหารหมักดอง 20 ชนิด โดยเบื้องต้นนำแบคทีเรียทั้งหมด 163 สายพันธุ์มาทำการคัดเลือกแบคทีเรียที่สามารถผลิตไฟบริโนไลทิกโพรทิเอสได้โดยวิธีไฟบรินเพลท และคัดเลือกแบคทีเรียจำนวน 21 สายพันธุ์ที่มีกิจกรรมของเอนไซม์สูงมาทำการศึกษา พบเป็นแบคทีเรียสกุล *Bacillus* และทำการแบ่งกลุ่มตาม internal transcribed spacers-polymerase chain reaction (ITS-PCR) fingerprinting ได้ 4 กลุ่ม โดยกลุ่มที่ 1 (12 สายพันธุ์) มีความใกล้เคียงกับ *B. subtilis*, กลุ่มที่ 2 (3 สายพันธุ์) มีความใกล้เคียงกับ *B. vallismortis*, กลุ่มที่ 3 (4 สายพันธุ์) มีความใกล้เคียงกับ *B. amyloliquefaciens* ส่วนกลุ่มที่ 4 (2 สายพันธุ์) พบว่ามีรูปแบบที่แตกต่างจากแบคทีเรียที่เคยมีการรายงาน และไม่สามารถจัดจำแนกได้ ต่อมาทำการจัดกลุ่มแบคทีเรียตามรูปแบบของเอนไซม์โดยใช้ไฟบรินไซโมแกรม แบ่งได้เป็น 6 กลุ่ม แล้วคัดเลือกแบคทีเรียตัวแทนที่มีค่ากิจกรรมของเอนไซม์สูงที่สุดจากแต่ละกลุ่มด้วยวิธีการย่อยสลายก้อนไฟบริน ได้แก่สายพันธุ์ THY-C1, PD-A10, K-A7, K-B16, TJW-A9 และ TISTR 651 ไปทำการศึกษาลำดับเบสบนสาย 16S rRNA gene พบว่าสายพันธุ์ดังกล่าวมีค่าความคล้ายคลึงของ 16S rRNA gene ใกล้เคียงกับสายพันธุ์มาตรฐาน *B. licheniformis* (98.9 – 99.8%), *B. subtilis* (98.9 - 99.5%), *B. vallismortis* (98.9 – 99.4%) และ *B. amyloliquefaciens* (97.6 – 98.34%) นอกจากนี้ยังพบว่าสายพันธุ์ THY-C1, PD-A10 และ K-A7 มีลักษณะทางฟีโนไทป์, มีค่า G+C content, มีรูปแบบกรดไขมัน และรูปแบบ rep-PCR fingerprinting ที่แตกต่างจาก *Bacillus* species และมีค่า DNA-DNA relatedness กับ *Bacillus* species ที่ใกล้เคียงต่ำกว่า 60% ดังนั้นทั้ง 3 สายพันธุ์ จึงเป็นสปีชีส์ใหม่และเสนอตั้งชื่อว่า *B. thailandensis* sp. nov., *B. siamensis* sp. nov. และ *B. kapii* sp. nov. ตามลำดับ

การศึกษาหาสภาวะที่เหมาะสมในการผลิตเอนไซม์ไฟบริโนไลทิกจากสายพันธุ์ THY-C1 พบว่าเชื้อสามารถผลิตเอนไซม์ได้สูงขึ้นสิบเท่า (101.2 unit/ml) เมื่อทำการเพาะเลี้ยงในสภาวะที่มีสารสกัดจากยีสต์ 0.6% น้ำตาลซูโครส 1.5% และมีค่า pH เริ่มต้นที่ 9 รวมทั้งมีการเสริมไฟบรินลงไปในอาหารที่เพาะเลี้ยง โดยใช้กล้าเชื้อเริ่มต้นที่ 5% และบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 30 ชั่วโมง เอนไซม์สามารถทำงานได้ดีที่ pH 7-8 และมีความคงทนที่ pH ในช่วง 6-11 เอนไซม์สามารถทำงานได้ดีที่อุณหภูมิ 50 องศาเซลเซียส และทนความร้อนได้สูงสุด 50 องศาเซลเซียส เอนไซม์ถูกยับยั้งการทำปฏิกิริยาด้วย PMSF ซึ่งเป็นสารยับยั้งเอนไซม์ในกลุ่ม serine protease ทำการศึกษาเปรียบเทียบกิจกรรมของเอนไซม์ที่ได้กับเอนไซม์ทางการค้า 3 ยี่ห้อ พบว่าเอนไซม์ที่ได้มีค่ากิจกรรมสูงกว่าเอนไซม์ทางการค้ายี่ห้อที่ 1 และ 2 แต่มีค่ากิจกรรมต่ำกว่าเอนไซม์ทางการค้ายี่ห้อที่ 3 นอกจากนี้พบว่าเอนไซม์ที่ได้ทนต่อการย่อยในสภาวะของลำไส้เล็ก แต่ไม่ทนต่อการย่อยในสภาวะกระเพาะอาหาร

ทำการเพาะเลี้ยงเชื้อตัวแทนทั้ง 6 สายพันธุ์ในสภาวะที่เหมาะสมต่อการผลิตเอนไซม์ พบว่าสายพันธุ์ PD-A10 สามารถผลิตเอนไซม์ได้สูงสุด นำเอนไซม์ที่ได้มาทำให้บริสุทธิ์ด้วยวิธีทางโครมาโทกราฟฟีโดยใช้คอลัมน์ Resource Q พบว่าเอนไซม์บริสุทธิ์ที่ได้มีค่าความบริสุทธิ์เพิ่มขึ้น 12.2 เท่า และมีผลผลิตเท่ากับ 51.4% เอนไซม์มีน้ำหนักโมเลกุลประมาณ 371.5 kDa และมีความจำเพาะต่อ H-D-Val-Leu-Arg-*p*NA โดยมีค่า V_{max} และ K_m เท่ากับ 0.295 mM/ml/min และ 0.28 mM ตามลำดับ เอนไซม์สามารถทำงานได้ดีที่ pH 7 และที่อุณหภูมิ 50 องศาเซลเซียส เอนไซม์มีความคงทนที่ pH ในช่วง 7-9 และทนความร้อนได้สูงสุด 50 องศาเซลเซียส เอนไซม์ถูกยับยั้งการทำกิจกรรมด้วย chymostatin และ *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) ซึ่งจัดเป็นเอนไซม์ในกลุ่ม chymotrypsin-like serine protease เอนไซม์บริสุทธิ์สามารถย่อยสลายไฟบริน และไฟบริโนเจนในสภาวะหลอดทดลองได้อย่างสมบูรณ์ภายในเวลา 2 และ 1 ชั่วโมง ตามลำดับ นอกจากนี้พบว่าเอนไซม์บริสุทธิ์ที่ได้มีคุณสมบัติเป็น fibrinolytic activity, fibrinogenolytic activity แต่ไม่มีคุณสมบัติของ thrombin-like activity

สาขาวิชา...เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ..... ลายมือชื่อนิสิต ปุณฑาณี สัมภวะผล

ปีการศึกษา...2552..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก ลินนา ทองยงค์

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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KEYWORDS : FIBRINOLYTIC PROTEASE / *BACILLUS* / FERMENTED FOODS / IDENTIFICATION / OPTIMIZATION / PURIFICATION / CHARACTERIZATION

PUNNANEE SUMPAVAPOL: FIBRINOLYTIC PROTEASES FROM BACTERIA ISOLATED FROM FERMENTED FOODS. ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., CO-ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D. AND WONNOP VISESSANGUAN, Ph.D. 189 pp.

Fibrinolytic protease-producing bacteria were isolated from 20 kinds of fermented food. Initially, 163 isolates were screened for their fibrinolytic activities by fibrin plate assay, and 21 isolates with strong activity were selected and subjected to identification. They were identified as *Bacillus* sp. and divided into 4 groups by internal transcribed spacers-polymerase chain reaction (ITS-PCR) fingerprinting. Group I (12 isolates) was closely related to *B. subtilis*, Group II (4 isolates) was related to *B. vallismortis* and Group III (4 isolates) was related to *B. amyloliquefaciens*. However, Group IV (2 isolates) showed different pattern from type strains and unidentified. Next, the enzymatic pattern was performed by fibrin-zymogram technique, which divided the fibrinolytic protease-producing bacteria into 6 groups based on their activity patterns. Then fibrinolytic activity of all isolates was evaluated by the hydrolysis of fibrin clot assay. Six representative isolates that showed the highest activity in each group of enzyme pattern , THY-C1, PD-A10, K-A7, K-B16, TJW-A9 and TISTR 651, were chosen and subjected to 16S rRNA gene sequencing. They were closely related to the type strains of *B. licheniformis* (98.9 - 99.8%), *B. subtilis* (98.9 – 99.5%), *B. vallismortis* (98.9 – 99.4%) and *B. amyloliquefaciens* (97.6 – 98.3%) base on 16S rRNA gene sequence similarity. Moreover, THY-C1, PD-A10 and K-A7 were differentiated from related *Bacillus* species based on the phenotypic characteristics, DNA G + C content, fatty acid profile, rep-PCR fingerprinting and low DNA-DNA relatedness (<60%). Therefore, they presented the novel species of genus *Bacillus*, namely *Bacillus thailandensis* sp. nov., *Bacillus siamensis* sp. nov. and *Bacillus kapii* sp. nov., respectively.

The optimal condition for fibrinolytic protease production by THY-C1 was evaluated. The enzyme production increased about 10 folds (101.2 unit/ml) when the bacterium was inoculated at 5% (v/v) into a medium containing 0.6% (w/v) yeast extract and 1.5% (w/v) sucrose at pH 9.0 with fibrin supplementation and incubated for 30 h at 37°C. The enzyme was optimally active at pH 7-8 and stable over a broad pH range from 6-11. Its optimum temperature was around 50°C. Enzyme was stable up to 50°C and strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), a specific inhibitor of serine protease. Compared to commercial fibrinolytic proteases, the cell-free supernatant of THY-C1 showed stronger activity than the commercial Nattokinase 1 and the commercial Nattokinase 2, but lower than the commercial Nattokinase 3. Moreover, fibrinolytic protease showed it stability to pancreatine and bile salt but lost it activity after incubated with pepsin in *in vitro* digestive model.

Lastly, fibrinolytic protease produced by PD-A10, that showed the highest enzyme production in the optimal condition among 6 representative bacteria, was purified to homogeneity by column chromatography on Resource Q. The enzyme was purified 12.2 fold, with a yield of 51.4%. The molecular weight of the purified enzyme was estimated as 371.5 kDa. The enzyme exhibited a higher affinity toward H-D-Val-Leu-Arg-*p*NA with V_{max} and K_m values of 0.295 mM/ml/min and 0.28 mM, respectively. The enzyme was optimally active at pH 7.0, and its optimum temperature was at 50°C. The enzyme activity was relatively stable at pH 7-9 and maximum temperature at 50°C. The activity was inhibited by chymostatin and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), indicating that the chymotrypsin–like serine protease. The purified enzyme could completely hydrolyze a fibrin and fibrinogen substrate *in vitro* within 2 h and 1 h respectively. Moreover, the purified enzyme possessed its fibrinolytic activity and fibrinogenolytic activity rather than thrombin-like activity.

Field of Study : ...Pharmaceutical Chemistry and Natural Products.....

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LIST OF ABBREVATIONS

α	=	Alpha
AEFB	=	Aerobic endospore-forming bacteria
Asp	=	Asparagine
β	=	Beta
bp	=	Base pair
BCC	=	BIOTEC Culture Collection, Pathumthani, Thailand
BSA	=	Bovine serum albumin
^{o}C	=	Degree Celsius
Ca	=	Calcium
Ca^{2+}	=	Calcium ion
$CaCl_3$	=	Calcium chloride
CCD	=	Central composite design
$CHCl_3$	=	Chloroform
Co	=	Cobalt
Co^{2+}	=	Cobalt ion
cm	=	Centimeter
CVD	=	Cardiovascular diseases
Cu^{2+}	=	Copper ion
DAP	=	Diaminopimelic acid
DDBJ	=	DNA Data Bank of Japan
DFP	=	Diisopropylfluorophosphate
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
DSMZ	=	Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Germanany
E-64	=	Trans-epoxysuccinyl-L-leucyamino(guanidine)–butane
ECLT	=	Euglobulin clot lysis time
EDTA	=	Ethylenendiaminetetraacetic acid
EFPs	=	Extracellular fibrinolytic proteases
EMBL	=	European Molecular Biology Laboratory
EMS	=	Ethyl methane sulfonate

F	=	Forward
FAME	=	Fatty acid methyl ester
FFD	=	Full factorial design
FPLC	=	Fast protein liquid chromatography
g	=	Gram
g	=	Gravitational acceleration
Gal	=	Galactose
GC	=	Gas chromatography
G+C	=	Guanine-plus-cytosine
GenBank	=	National Institute of Health genetic sequence database
Glu	=	Glucose
h	=	Hour
Hb	=	Heamoglobin
HCl	=	Hydrochloric acid
Hib	=	*Haemophilus influenzae* type B
Hg	=	Hemoglobin
His	=	Histidine
H_2O	=	Water
H_2O_2	=	Hydrogen peroxide
HPLC	=	High performance liquid chromatography
HPTLC	=	High performance thin layer chromatography
H_2S	=	Sulphide
H_2SO_4	=	Sulfuric acid
HV	=	Hypervariable
ICSP	=	International Committee on Systematics of Prokaryotes
IEF	=	Isoelectric focusing electrophoresis
IEX	=	Ion exchange chromatography
ITS	=	Intergenic transcribed spacer
K_{cat}	=	Turn over number
KCl	=	Potassium chloride
KCTC	=	Korean Collection for Type Cultures, Korea
kDa	=	Kilo Dalton
K_2HPO_4	=	Potassium phosphate

KOH	=	Potassium hydroxide
l	=	Liter
LB	=	Luria-Bertani
M	=	Molar
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
MLB	=	Modified Luria-Bertani
min	=	Minute
μg	=	Microgram
mg	=	Milligram
Mg^{2+}	=	Magnesium ion
$MgCl_2$	=	Magnesium chloride
μl	=	Microliter
ml	=	Milliliter
μm	=	Micrometer
μM	=	Micromole
mm	=	Millimeter
mM	=	Millimole
Mn^{2+}	=	Magnesium ion
MR	=	Methyl red test
mw	=	Molecular weight
M	=	Molar
mA	=	Milli amp
Mg	=	Magnesium
$MgCl_2$	=	Magnesium chloride
MeOH	=	Methanol
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate
$NaHCO_3$	=	Sodium hydrogencarbonate
NaN_3	=	Sodium azide
NaOH	=	Sodium hydroxide
NB	=	Nutrient broth
NIH	=	National Institute for Health and Clinical Excellence

NH_3	=	Ammonia
$(NH_4)_2SO_4$	=	Ammonium sulfate
NK	=	Nattokinase
nm	=	Nanometer
NO_2	=	Nitrite
NO_3	=	Nitrate
nov.	=	Novel
O_2	=	Oxygen
OD	=	Optical density
ONPG	=	o-Nitrophenyl-β-D-galactopyranoside
%	=	Percent
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
pI	=	Isoelectric point
pmol	=	Picomole
PMSF	=	Phenylmethylsulfonyl fluoride
*p*NA	=	Para-nitroaniline
R	=	Reward
rDNA	=	Ribosomal deoxynucleic acid
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
SBTI	=	Soybean trypsin inhibitor
scu-PA	=	Single chain urokinase plasminogen activator
sec	=	Second
SEX	=	Size exclusion chromatography
Ser	=	Serine
SDS	=	Sodium dodesylsulfate
SIM	=	Sulphid-indole-motility
S-layer	=	Surface layer
SO_4	=	Sulfate
sp.	=	Specie
SSC	=	Saline sodium citrate

SSF	=	Solid state fermentation
ssRNA	=	Single stand RNA
TAE	=	Tris-acetate EDTA
TBE	=	Tris-borate EDTA
TCA	=	Trichloroacetic acid
TEM	=	Transmission electron microscope
TEMED	=	*N,N,N',N'*-tetramethyl ethylene diamine
TISTR	=	Thailand Institute of Scientific and Technological Research, Pathumthani, Thailand
TLC	=	Thin layer chromatography
TLCK	=	N alpha-p-tosyl-L-lysine chloromethyl ketone
t-PA	=	Tissue plasminogen activator or alteplase (trade name)
TPCK	=	N-tosyl-L-phenylalanine chloromethyl ketone
TSA	=	Tryptic soy agar
UK	=	United Kingdom
u-PA	=	Urokinase plasminogen activator
V_{max}	=	Maximal velocity
VP	=	Voges-Proskaüer test
UV	=	Ultraviolet
UV-Vis	=	Ultraviolet-Visible
V_o	=	Void volume
V_e	=	Elution volume
v/v	=	Volume by volume
v/w	=	Volume by weight
w/v	=	Weight by volume
WHO	=	World Health Organization
Zn^{2+}	=	Zinc ion

CHAPTER I

INTRODUCTION

Thrombotic events affect many individuals in a number of ways, all of which cause significant morbidity and mortality. Deep vein thrombosis has a worldwide prevalence of more than 4 million and an incidence of 1 in 1000, approximately 6% of which result in death within a month of diagnosis (Fowkes *et al*., 2003; White, 2003). Ischemic stroke is the third leading cause of death in developed countries, affecting 12 million people each year and causing 5.5 million deaths, and permanent disability in an additional 5 million people (Rosamond *et al*., 2007). Acute coronary syndrome accounts for more than 4 million hospital admissions each year worldwide and is the leading cause of death in the developed world (Rosamond *et al*., 2007). Altogether, 20 million people each year could benefit from effective resolution or reversal of thrombosis (White, 2008).

Fibrinolysis is the process wherein a fibrin clot is broken down by its main enzyme, plasmin. Plasminogen activators are the agents that convert plasminogen to the active plasmin and found to be effective for treating patients with acute myocardial infarction, peripheral arterial embolism and deep vein thrombosis (Verstraete, 1978). However, their use also led to bleeding complications (Marder, 1979), including a 1% risk of intracranial hemorrhage. Although clot dissolution using enzymes from the human fibrinolytic system has long been a goal of physicians and scientist, their expensive prices and undesirable side effects prompt researchers to search for cheaper and safer resources. Over the years, more fibrinolytic proteases from various sources have been discovered. Microorganisms are one of the important resources for thrombolytic agents, especially the genus *Bacillus* from traditional fermented foods (Peng *et al*., 2005).

In 1987, *B. natto* producing Nattokinase was first screened from a traditional Japanese soybean-fermented food named *natto* (Sumi *et al*., 1987). Subsequently, some other bacilli from different fermented foods were discovered to produce fibrinolytic proteases. These finding imply the possibility of consuming fermented foods to prevent cardiovascular diseases. Suzuki *et al*. (2003) reported that dietary supplementation of *natto* could shorten euglobulin clot lysis time, which is used to evaluate the total intrinsic fibrinolytic activity in plasma. At the same time, dietary

natto extract did not prolong bleeding time, indicating the safety of *natto* to be developed as functional food and drug to prevent or cure cardiovascular disease (Peng *et al*., 2005).

Fermented foods play an important role in the Thai diet and in the life of the Thai people. Most Thais eat a fermented product in one form or another every day (Phithakpol *et al*., 1995). In this study, the investigation of microbial fibrinolytic protease from Thai fermented foods deserves special attention to explore the potential food sources of fibrinolytic protease. The study was undertaken with an aim of the screening of fibrinolytic protease producing bacteria from Thai fermented foods and it identification. Furthermore, the condition of fibrinolytic protease production was evaluated. Consequently, characterization and purification of microbial fibrinolytic proteases were investigated. The results provide some information regarding the microbial fibrinolytic proteases from Thai fermented foods.

Experimental objectives

General objective

To investigate the microbial fibrinolytic protease from Thai fermented foods.

Specific objective

1. To screen for fibrinolytic protease-producing bacteria from traditional fermented foods produced in Thailand
2. To identify the selected fibrinolytic protease-producing bacteria
3. To optimize the fibrinolytic protease production condition of the selected isolate
4. To purify and characterize the fibrinolytic protease produced by the selected bacteria

CHAPTER II

LITERATURE REVIEW

2.1 Microbial fibrinolytic protease

Cardiovascular diseases (CVD), including acute myocardial infarction, ischemic heart disease, vascular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke, are the leading causes of death throughout the world. According to data provided by the World Health Organization (WHO) in 2005, heart diseases are responsible for 30% of the total mortality rate in the world (Figure 1). Indeed heart diseases not only affect the elderly, but are the leading cause of premature death in individuals under age 75. Based on the mortality rates by different types of cardiovascular diseases, acute myocardial infarction and ischemic heart disease are the most important heart problems (WHO, 2005).

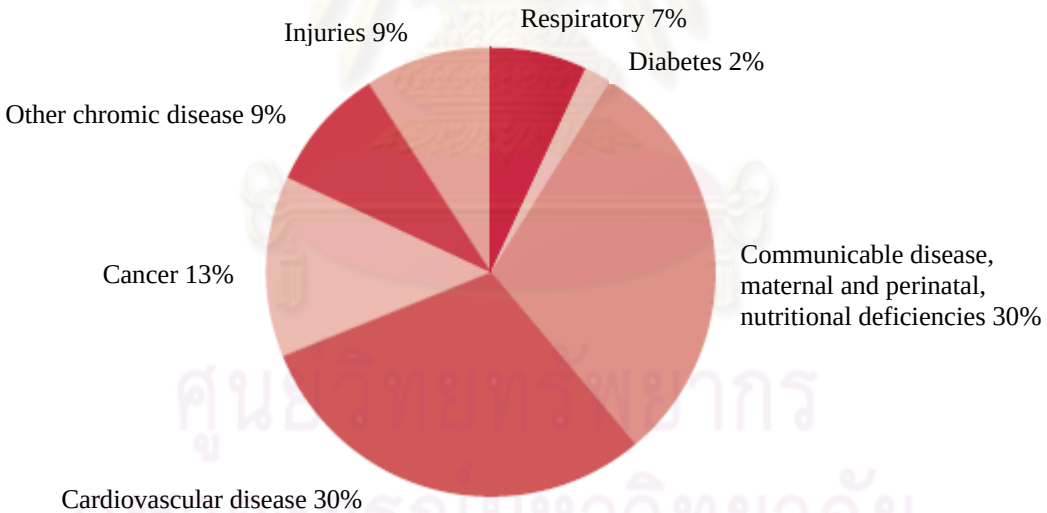


Figure 1. Leading causes of death worldwide (WHO, 2005).

Intravascular thrombosis, the formation of a clot of blood in a blood vessel, is one of the main causes of a variety of CVDs. The major protein component of blood clots, fibrin, is formed from fibrinogen via the proteolysis by thrombin. Meanwhile, the fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels. Under an unbalanced situation due to some disorders, the clots are not hydrolyzed, and thus thrombosis occurs. Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated (Goldhaber and Bounameaux, 2001; Tough 2005).

Based on their different working mechanisms, the thrombolytic agents are classified into two types. One is plasminogen activators, such as urokinase (Duffy, 2002) and tissue-type plasminogen activator (t-PA) (Collen and Lijnen, 2004), which activate plasminogen into active plasmin to degrade fibrin. The other type is plasmin-like proteins,which directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly and completely. Lumbrokinase from earthworm and fibrolase from snake venom are well-known plasmin-like proteins (Chen *et al*., 1991; Mihara *et al*., 1991).

Although t-PA and urokinase are still widely used in thrombolytic therapy today, their expensive prices and undesirable side effects, such as the risk for internal hemorrhage within the intestinal tract when orally administrated, prompt researchers to search for cheaper and safer resources. Therefore, microbial fibrinolytic proteases attracted much more medical interest during these decades.

Sources of microbial fibrinolytic protease

Microorganisms are important resources for thrombolytic agents. Streptokinase from *Streptococcus hemolyticus* and staphylokinase from *Streptococcus aureus* were earlier proved to be effective in thrombolytic therapy (Collen and Lijnen, 1994). Over the years, more fibrinolytic proteases from various microbes have been discovered in succession, such as nattokinase (NK) from *Bacillus natto* and subtilisin DFE and subtilisin DJ-4 from *Bacillus amyloliquefaciens* (Kim and Choi, 2000; Peng and Zhang, 2002a; Sumi *et al*., 1987). The microorganisms producing fibrinolytic proteases, including bacteria, actinomyces, fungi, and algae, are summarized in Table 1. *Streptomyces megasporus* SD5, isolated from the water of a hot spring, can produce a strong thermostable fibrinolytic protease (Chitte and Dey, 2000). Some kinds of fungi have also been found to produce the protease with high fibrinolytic activity, for example, *Aspergillus ochraceus* 513 (Batomunkueva and Egorov, 2001), *Fusarium*

oxysporum (Sun *et al*., 1998), *Penicillum chrysogenum* (El-Aassar *et al*., 1990), *Rhizopus chinensis* 12 (Xiao-Lan *et al*., 2005), and so on. In addition, Matsubara *et al*., (1998, 1999, 2000) found the fibrinolytic proteases from marine algae *Codium latum*, *Codium divaricatum*, and *Codium intricatum*. Lee *et al*., (2005) recently purified the fibrinolytic protease, designated as AMMP, from cultural mycelia of the mushroom *Armillaria mella*.

Table 1. Sources of microbial fibrinolytic proteases

Microorganism	References
Bacilli	
B. subtilis BK-17	Jeong *et al*., 2001
B. subtilis A1	Jeong *et al*., 2004
B. subtilis 168	Kho *et al*., 2005
Actinomyces thermovulgaris	Egorov *et al*., 1976
Streptomyces	
S. spheroids M8-2	Egorov *et al*., 1985
Streptomyces sp. Y405	Wang *et al*., 1999
S. megasporus SD5	Chitte and Dey, 2000 and 2002
Fungi	
Cochliobolus lunatus	Abdel-Fattah and Ismail, 1984
Fusarium pallidoroseum	El-Aassar, 1995
F. oxysporum	Tao *et al*., 1997 and 1998
Pleurotus ostreatus	Choi and Shin, 1998
P. chrysogenum H9	El-Aassar *et al*., 1990
Aspergillus ochraceus 513	Batomunkueva and Egorov, 2001
Rhizopus chinensis 12	Xiao-Lan *et al*., 2005
Algae	
Codium intricatum	Matsubara *et al*., 1998
C. latum	Matsubara *et al*., 1999
C. divaricatum	Matsubara *et al*., 2000

The strains of *Bacillus* from traditional fermented foods are an important one among the microorganisms that have been found to produce the fibrinolytic proteases (Table 2). In 1987, *B. natto* producing NK was first screened from a traditional Japanese soybean-fermented food named *natto* (Sumi *et al*., 1987). Subsequently, some other bacilli from different fermented foods were discovered to produce fibrinolytic proteases. They are *B. amyloliquefaciens* DC-4 from the Chinese soybean-fermented food named *douche* (Peng and Zhang, 2002a), *Bacillus* sp. CK from the Korean fermented-soybean sauce named *chungkook-jang* (*Kim et al*., 1996), *Bacillus* sp. strains DJ-2 and DJ-4 from the Korean *doen-jang* (Kim and Choi, 2000; Choi *et al*., 2005), and *Bacillus* sp. KA38 from the Korean salty fermented fish called *jeot-gal* (Kim *et al*., 1997). Yoon *et al*. (2002) systematically screened the fibrinolytic protease-producing strains from many commercial and homemade fermented foods including *natto*, *chungkook-jang*, *doen-jang*, *jeot-gal*, and the Indonesian fermented food *tempe* and successfully isolated the strain *Enterococcus faecalis* producing higher fibrinolytic activity. These exciting findings imply the possibility of consuming fermented foods to prevent cardiovascular diseases. Suzuki *et al*. (2003a) reported that dietary supplementation of *natto* could shorten euglobulin clot lysis time (ECLT), which is used to evaluate the total intrinsic fibrinolytic activity in plasma. At the same time, dietary *natto* extract did not prolong bleeding time, indicating the safety of *natto* to be developed as a functional food.

Table 2. Bacilli from traditional food

Microorganism	**Food**	**Name of enzyme**	**References**
Bacillus natto	*Natto*, Japan	Nattokinase, NK	Fujita *et al*., 1993
Bacillus sp. CK	Chungkook-jang, Korea	CK	Kim *et al*., 1996
Bacillus sp. KA38	Jeot-gal, Korea	Jeot-gal enzyme	Kim *et al*., 1997
Bacillus sp. DJ-4	Doen-jang, Korea	*Subtilis*in DJ-4	Kim and Choi, 2000
B. subtilis IMR-NK1	*Natto*	-	Chang *et al*., 2000
Bacillus sp. KDO-13	Soybean paste, Korea	-	Lee *et al*., 2001
B. amyloliquefaciens DC-4	Douchi, China	*Subtilis*in DFE	Peng *et al*., 2003
B. subtilis QK02	Fermented soybean	QK-1 and QK-2	Ko *et al*., 2004
B. firmus NA-1	*Natto*	-	Seo and Lee, 2004
Bacillus sp. DJ-2	Doen-jang, Korea	bpDJ-2	Choi *et al*., 2005

Production of microbial fibrinolytic protease

The cost of enzyme production and downstream process is the major obstacle against the successful application of protease in the industry. For fibrinolytic proteases, many attempts in laboratories have been done to improve expression of the fibrinolytic protease, including selection of an ideal culture medium, optimization of environmental conditions, and overexpression by genetically engineered strains.

- **Fermentation conditions** Selection of medium components is usually critical for the fermentative production of fibrinolytic proteases. Since different microbes possess diverse physiological characteristics, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic protease production (Lee *et al*., 1999; Seo and Lee, 2004). For instance, soluble starch or dextrin is the best carbon source for *B. amyloliquefaciens* DC-4 due to the strong amylase activity (Peng and Zhang, 2002b). The optimal temperature of *S. megasporus* SD5 for enzyme synthesis is 55°C because the strain was isolated from a hot spring (Chitte and Dey, 2002). In some cases, fibrin was found to enhance the enzyme production, suggesting that fibrin, as a substrate of fibrinolytic protease, could activate or induce enzyme production during cultivation (Chitte and Dey, 2002; Peng and Zhang, 2002b).

Although the traditional one-at-a-time optimization strategy is simple and easy, it frequently fails to locate the region of optimal response because the comprehensive effect of factors is not taken into consideration. Liu *et al*. (2005) employed the statistical methods fractional factorial design (FFD) and central composite design (CCD) to optimize the fermentation media for production of NK and finally increased the fibrinolytic activity to 1,300±60 unit/ml, about 6.5 times higher than the original one (Fu *et al*., 1997).

Liquid fermentation is usually considered as the first choice for bacteria, whereas solid-state fermentation (SSF) is favored by fungi. SSF has numerous advantages for enzyme production, such as low wastewater output, low operating costs, and large productivity. Tao *et al*., (1997, 1998) systematically studied the production of the fibrinolytic protease from *F. oxysporum* with different SSF methods, largely increasing the production and reducing the cost.

- **Construction of genetically engineered strains** Conventional mutagenesis and current recombinant DNA technology have also been adopted to improve the protease production and simplify the downstream manipulation. For the

former, random mutagenesis is combined with high throughout screening methods to isolate the expected mutant strains. Recently, Lai *et al.* (2004) showed they have successfully doubled the specific activity of fibrinolytic protease through random mutagenesis in vitro by the chemical ethyl methane sulfonate (EMS).

In molecular biology applications, *Bacillus subtilis* has been recognized as a good host for the expression of foreign proteins with pharmacological activities because of its nonpathogenicity and capability of secreting functional extracellular proteins to the culture medium (Wong, 1995). Subtilisin DFE was actively expressed in the protease deficient strain *B. subtilis* WB600 (Peng *et al*., 2004). Furthermore, the native promoter of subtilisin DFE gene was replaced by that of α-amylase gene from *B. amyloliquefaciens* DC-4, resulting in a sharp increase in fibrinolytic activity from 80 to 200 urokinase units milliliter (Xiao *et al*., 2004). Liu and Song (2002) succeeded in the functional expression of NK in *B. subtilis* as well.

When expressed in *E. coli*, NK and subtilisin DFE formed insoluble aggregates without enzymatic activity (Peng and Zhang, 2002c). However, two newly published papers reported the successful expression of active NK and subtilisin DFE in *E. coli* (Chiang *et al*., 2005; Zhang *et al*., 2005). Both papers took advantage of the principle that the extracellular protease subtilisin from the genus *Bacillus* is synthesized as pre-proenzymes, and its propeptide may function as an intramolecular chaperone to facilitate correct folding of the protease domain (Ikemura and Inouye, 1988; Zhu *et al*., 1989). Zhang *et al*., (2005) showed that subtilisin DFE was highly expressed in *E. coli* BL21 (DE3) as fusion protein of Trx-prosubtilisin DFE via the expression vector pET32a and that strong fibrinolytic activity was detected in both soluble fraction and inclusion bodies fraction after *in vitro* renaturation. Moreover, the fusion proteins are easily purified and refolded in a column to active enzyme. Most importantly, Trx-propeptide can be automatically cleaved during *in vitro* refolding to form the mature subtilisin DFE. Chiang *et al*. (2005) indicated that either nattokinase or pronattokinase could be overexpressed in *E. coli* as a recombinant protein fused to the C terminus of olesin, a unique structural protein of seed oil bodies, by a linker polypeptide intein. After reconstitution of artificial oil bodies, active NK was released through self-splicing of intein induced by temperature alteration and spontaneous cleavage of the propeptide (Chiang *et al*., 2005).

Microbial fibrinolytic protease properties

Some microbial fibrinolytic proteases including those from *Streptomyces* (Wang *et al*., 1999), genus *Bacillus* (Jeong *et al*., 2001; Paik *et al*., 2004), *R. chinensis* (Liu *et al*., 2005) and *Armillaria mellea* (Lee *et al*., 2005) have been purified and characterized. Their biochemical properties, such as molecular weight, optimal pH and temperature, stability, and substrate specificity, are summarized in Table 3. Some N-terminal sequences have been determined as well (Table 4). According to their catalytic mechanisms, these enzymes are classified into serine protease (NK, subtilisin DFE, and CK) and metalloprotease (jeot-gal enzyme, AMMP, and Bacillokinase II), except for those from *R. chinensis* 12 and *Streptomyces* sp. Y405, which are both serine and metalloprotease (Wang *et al*., 1999; Liu *et al*., 2005).

Table 3. Properties of microbial fibrinolytic proteases

Protease	Enzyme	Mol. wt (kDa)	Optimum pH and temp.	pH and temp. Stability	Substrate specificity	Reference
Subtilisin-family serine protease	Nattokinase	27.7		7-12, < 50ºC	Synthetic substrate for plasmin and subtilisin	Sumi *et al*., 1987, Fujita *et al.*, 1993
	Subtilisin DFE	28	9, 48ºC	6-10, <50ºC	Synthetic substrate for subtilisin	Peng *et al*., 2003
	Subtilisin QK-2	28	8.5, 55ºC	3-12, 40ºC	Synthetic substrate for subtilisin	Ko *et al*., 2004
Metalloprotease	Jeot-gal enzyme	41	7, 40ºC	7-9, 40ºC	fibrin	Kim *et al*., 1997
	Bacillokinase II (BK II)	31.4	7, 50ºC	4, 50ºC	Synthetic substrate for Kallilrein	Jeong *et al*., 2004
	KDO-13	45	7, 60ºC	7-9, <50ºC	fibrin	Lee *et al*., 2001
Chymotrypsin-like mettalloprotease	AMMP	21	6, 33ºC		fibrinogen	Lee *et al*., 2005
Serine and metalloprotease	SW-1	30	8	4-9, 4-37ºC	fibrin	Wang *et al*., 1999

Table 4. Comparison of N-terminal amino acid sequence of microbial fibrinolytic proteases

Enzyme	N-terminal amino acid sequence	Reference
NK	AQSVPYGISWIKAPALHSQGYTGS	Fujita *et al*., 1993
Subtilisin QK-2	AQSVPYGISQIKAPALHSQG	Ko *et al*., 2004
Subtilisin DFE	AQSVPYGVSQIKAPALHSQGFTGS	Peng *et al*., 2003
Subtilisin DJ-4	AQSVPYGVSQIKAP	Kim and Choi, 2000
31-kDa enzyme	AQSVPYGBSQIKAPAAHN	Jeong *et al*., 2001
CK	AQTVPYGIPLIKAD	Kim *et al*., 1996
Bacillokinase II	ARAGEALRDIYD	Jeong *et al*., 2004
KA38	VYPFPGPIPN	Kim *et al*., 1997
CLP	VVGGDEPP	Matsubara *et al*., 1999
CIP	X-TPLTQVLSGNAVLVEAVLVEAVKA	Matsubara *et al*., 2000
SW-1	R/N/F-P/D-GMTMTAIANQNTQIN	Wang *et al*., 1999
AMMP	MFSLSSRFFLYTLCLSAVAVSAAP	Lee *et al*., 2005
bpDJ-2	TDGVEWNVDQIDAPKAW	Choi *et al*., 2005

The fibrinolytic proteases belonging to serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 8.0 and 10. Their molecular weights range between 27.7 and 44 kDa, and the isoelectric points are about 8.0 (Kim and Choi, 2000, Ko *et al*., 2004), with the exception of bpDJ-2 (pI 3.5–3.7) (Choi *et al*., 2005). The optimal temperature has a wide range, between 30 and 70°C (Kim *et al*., 1996; Kim and Choi, 2000), mostly about 50°C (Peng *et al*., 2003; Paik *et al*., 2004). Almost all serine fibrinolytic proteases belong to subtilisin of *Bacillus* origin. They own the same catalytic triad made up of Ser_{221}, His_{64}, and Asp_{32} and have no intramolecular disulfide bond. Their fibrinolytic activity can be irreversibly inhibited by phenylmethylsulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), or E-64 (Sumi *et al*., 1987; Kim *et al*., 1996; Kim and Choi, 2000; Peng and Zhang, 2002a,b,c).

The fibrinolytic proteases belonging to metalloprotease require divalent metal ions for their activities, for example, Zn^{2+} for jeot-gal (Kim *et al*., 1997), Co^{2+} and Hg^{2+} for enzymes from *Bacillus* sp. KDO-13 (Lee *et al*., 2001), Ca^{2+} and Mg^{2+} for AMMP (Lee *et al*., 2005), so their activities can be inhibited by chelating agents such as EDTA. These enzymes have an optimal pH between 6.0 and 7.0, except one from *R. chinensis* 12, with an optimal pH of 10.5 (Liu *et al*., 2005).

All these fibrinolytic proteases have high substrate specificity to fibrin, different from other proteases with broad substrate specificity. For instance, CK activity degrading fibrin is about eight times higher than that of subtilisin Carlsberg, a common alkaline protease with identical N-terminal sequence (Kim *et al*., 1996). Similar examples also include NK and subtilisin E (Nakamura *et al*., 1992), subtilisin DFE and subtilisin BPN (Peng *et al*., 2004), and subtilisin DJ-4 and subtilisin BPN (Choi *et al*., 2004). Although these common alkaline proteases (subtilisin E, BPN, and Carlsberg) have highly homologous sequence with the corresponding fibrinolytic proteases, why is it that only these fibrinolytic proteases have very high substrate specificity to fibrin? The evolutionary changes of the critical amino acid residues in the substrate binding site probably account for this difference. However, more research should be done to completely elucidate this interesting phenomenon and provide some hints of the structure–function relationship. Furthermore, some microbial fibrinolytic proteases can activate plasminogen and further enhance the fibrinolysis (Kim *et al*., 1996).

Thrombolytic effect of nattokinase *in vivo*

The fibrinolysis mechanism of NK has been explored more extensively than other microbial fibrinolytic proteases. NK not only directly cleaves cross-linked fibrin, but also activates the production of t-PA, resulting in the transformation of inactive plasminogen to active plasmin (Kumada *et al*., 1994; Fujita *et al*., 1995c). Furthermore, NK enhances its fibrinolysis through cleavage and inactivation of PAI-1, which is the primary inhibitor of fibrinolysis and regulates total fibrinolytic activity by its relative ratio with t-PA (Urano *et al*., 2001). NK can be effectively absorbed across the rat intestinal tract after intraduodenal administration and then induces fibrinolysis (Fujita *et al*., 1995a). So far, NK is the only fibrinolytic protease from microorganisms whose thrombolytic effect *in vivo* has been best characterized.

In 1990, Sumi *et al*. reported the effectiveness of NK capsules in dissolving thrombi in dogs. After blood clots were experimentally induced in a major leg vein of male dogs, each dog was orally administered either four capsules of NK or placebo. The results showed that the blood clots in the dogs that received NK capsules completely dissolved within 5 h of treatment, and normal blood circulation was restored. However, as a negative control, blood clots in dogs that have received the placebo did not show any sign of thrombolysis even after 18 h of treatment (Sumi *et al*., 1990). Moreover, dietary supplementation of *natto* can suppress intimal thickening and modulate the lysis of mural thrombi after endothelial injury in rat femoral arteries (Suzuki *et al*., 2003b).

Fujita *et al*. (1995b) investigated the thrombolytic effect of NK on a thrombus in the common carotid of rat where the endothelial cells of the vessel wall have been injured by acetic acid. Animals treated with NK recovered 62% of the arterial blood flow, whereas those treated with plasmin regained just 15.8%, and those treated with elastase did not get any recovery. It was concluded that the *in vivo* thrombolytic activity of NK is stronger than that of plasmin or elastase.

Another human trial involved 12 healthy Japanese volunteers (six men and six women, between 21 and 55 years old). Each participant had 200 g of *natto* daily before breakfast, and their fibrinolytic activity was tracked through a series of blood plasma tests. The results showed that oral administration of *natto* (nattokinase) enhanced the ability of participants to dissolve blood clots. Furthermore, the volunteers retained such enhanced fibrinolytic activity for 2 to 8 h after administration (Sumi *et al*., 1989).

Application and perspectives

The microbial fibrinolytic proteases, especially those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure cardiovascular diseases. NK has already been developed as drugs in the market, including Nattokinase NSKSD, Jarrow NattoMax JR-154, and Natto-K. Development of other microbial fibrinolytic proteases is still ongoing, and much work needs to be done intensively and extensively, especially concerning thrombolytic effects *in vivo*.

The new trend for improving thrombolytic agents is to increase the efficacy and fibrin specificity, focusing on developing effective targeted thrombolytic agents. Several reports have illustrated successful construction of the chimeric proteins, in which a thrombus-specific polypeptide or antibody was attached to the plasminogen activator to enhance the thrombolytic specificity (Tait *et al*., 1995; Ruppert *et al*., 2003). These advances direct the way of future research on microbial fibrinolytic proteases.

2.2 Taxonomy of the genus *Bacillus*

In 1872, Ferdinand Cohn recognized and named the bacterium *Bacillus subtilis*. The organism is Gram-positive, capable of growth in the presence of oxygen, and forms a unique type of resting cell called an endospore (Figure 2). The organism represented what was to become a large and diverse genus of bacteria named *Bacillus*, in the Family *Bacillaceae*. The genus *Bacillus* remained intact until 2004, when it was split into several families and genera of endospore-forming bacteria, justifiable on the basis of ssRNA analysis. In order to accommodate former members of the genus *Bacillus*, its title has been changed to "Gram-positive aerobic or facultative endospore-forming bacteria". The unifying characteristic of these bacteria is that they are Gram-positive, form endospores, and grow in the presence of O_2. The trivial name assigned to them is aerobic sporeformers. The ubiquity and diversity of these bacteria in nature, the unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects, and the pathogen *Bacillus anthracis*, have attracted ongoing interest in these bacteria since and Cohn and Koch's discoveries in the 1870s (Claus and Berkeley, 1986).

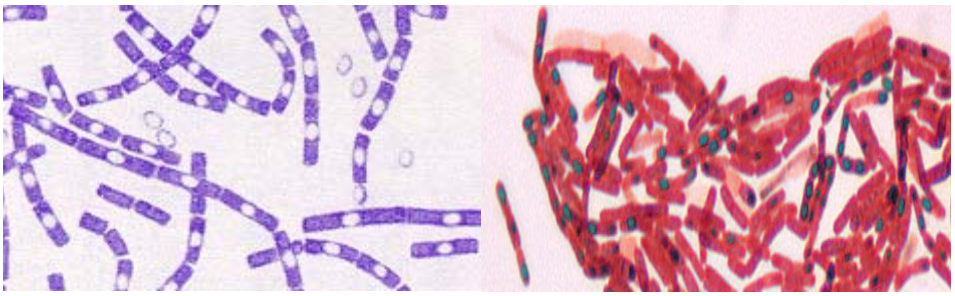


Figure 2. Endospores can be readily recognized microscopically by their intracellular site of formation and their extreme refractility. Left, crystal violet stain viewed by light microscopy. Endospores are highly resistant to application of basic aniline dyes that readily stain vegetative cells. *Bacillus thuringiensis* by phase micrograph.. Right, spore stain of a *Bacillus* species. The staining technique employed is the Schaeffer-Fulton method. A fixed smear is flooded with a solution of malachite green and placed over boiling water for 5 minutes. After rinsing, the smear is counterstained with safranine. Mature spores stain green, whether free or still in the vegetative sporangium; vegetative cells and sporangia stain red.

There is great diversity of physiology among the aerobic sporeformers, not surprising considering their recently-discovered phylogenetic diversity. Their collective features include degradation of most all substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, and others; antibiotic production; nitrification; denitrification; nitrogen fixation; facultative lithotrophy; autotrophy; acidophily; alkaliphily; psychrophily; thermophily; and parasitism. Endospore formation, universally found in the group, is thought to be a strategy for survival in the soil environment, wherein these bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of aerobic spore formers in most habitats examined.

Established by Cohn in 1872, the genus *Bacillus* has undergone considerable taxonomic changes. Starting off with two prominent and truly endospore-forming species, *Bacillus anthracis* and *B. subtilis* (until the early 1900s some taxonomists did not restrict the genus to endospore-forming bacteria), the number of species allocated to this genus increased to an incredible 146 in the 5th edition of Bergey's Manual of Determinative Bacteriology (Bergey *et al*., 1939). Meticulous comparative studies of Smith *et al*. (Cohn, 1872) and Gordon *et al*. (1973) on 1,114 strains of aerobic endospore-forming bacteria (AEFB) helped to reduce this number to 22 well-defined

species in the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). These researchers made a case for being "lumpers" rather than "splitters" in their taxonomic work, thus being able to present morphologically and physiologically well-defined taxa while accepting that these taxa might lather again be taken apart to form better species when better methods became available. The advantage of their approach is that today modern taxonomy still can confidently rely on their results.

A general new starting point for bacterial taxonomy occurred in 1980 with the publication of the Approves Lists of Bacterial Names (Skerman *et al*., 1980) where numerous taxonomic experts had cooperated in establishing a comprehensive and agreed upon list of accepted names of bacterial species. Herein, 38 species of AEFB were listed of which 31 were allocated to the genus *Bacillus* and 7 to other aerobic endospore-forming genera. An amended edition was published in 1989 (Skerman *et al.*, 1989). From this base, a steady increase was seen that lately developed into a virtual explosion of newly and validly described species of AEFB.

Only four of the genera of AEFB (*Bacillus*, *Sporolactobacillus*, *Sporosarcina*, and *Thermoactinomyces*) had already been included in the approved lists and are still valid today. For most of the others, phenotypic differentiation from *Bacillus sensu stricto* is not so clear-cut and only possible through a combination of a number of traits. For some of the genera, the determination of such traits has yet to be completed and differentiation is not satisfactory. All in all, over 88 species of *Bacillus* have been validly published (Fritze, 2004).

Taxonomic developments in *Bacillus subtilis* group

B. subtilis is not only a renowned classical model organism for genetic research but also widely used in traditional and industrial fermentation processes as well as in agriculture. Production of enzymes, antibiotic or probiotic components, antagonistic substances or surfactants or on the other hand, degradation of xenobiotics is only some of the long list of applications of this and related organisms.

Species of the *B. subtilis* group are closely related and thus not easily distinguishable. Cells of these organisms are less than 1 μm wide, sporangia are not swollen, and spores are ellipsoidal. They are in general mesophilic with regard to temperature and neutrophilic with respect to pH for growth, while often being tolerant to higher pH levels. All members of the group are placed in 16S rRNA/DNA group 1. Application of the classical phenotypic tests for the differentiation of *Bacillus* species

(Claus and Berkeley, 1986) indicates that only for some of them have clearly discriminating features been determined. For example, *B. pumilus* is starch negative and hippurate positive, and *B. licheniformis* is propionate positive, grows in temperatures up to 55°C, and is facultatively anaerobic. For others, phenotypic discrimination is weak, such as for *B. atrophaeus*, where pigment formation on tyrosine medium was described to differ from *B. subtilis* from which it is otherwise not distinguishable, and for *B. amyloliquefaciens*, where a faster acid production from lactose and slower gluconate utilization was described in contrast to *B. subtilis*. The two subspecies of *B. subtilis* (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*), *B. mojavensis*, and *B. vallismortis* are not distinguishable phenotypically so far. The same is true for *B. licheniformis* and *B. sonorensis*.

The history of the presently accepted species of the *B. subtilis* group is presented in Figure 3. All species can be differentiated on the genetic level and it is to be expected that when genotypic analyses are applied to a wider range of strains of the classical species mentioned above, additional genospecies will be detected. *B. clausii* is, strictly speaking, not a member of the *B. subtilis* group; however, it is listed here for the sake of completeness, because a number of strains previously classified as *B. subtilis* and used as probioticum have recently been reclassified as *B. clausii* (Senesi *et al*., 2001). Even more loosely attached to this group are the species *B. firmus*, *B. lentus*, and *B. sporothermodurans*, which are clearly distinguishable from the other species and are not further discussed.

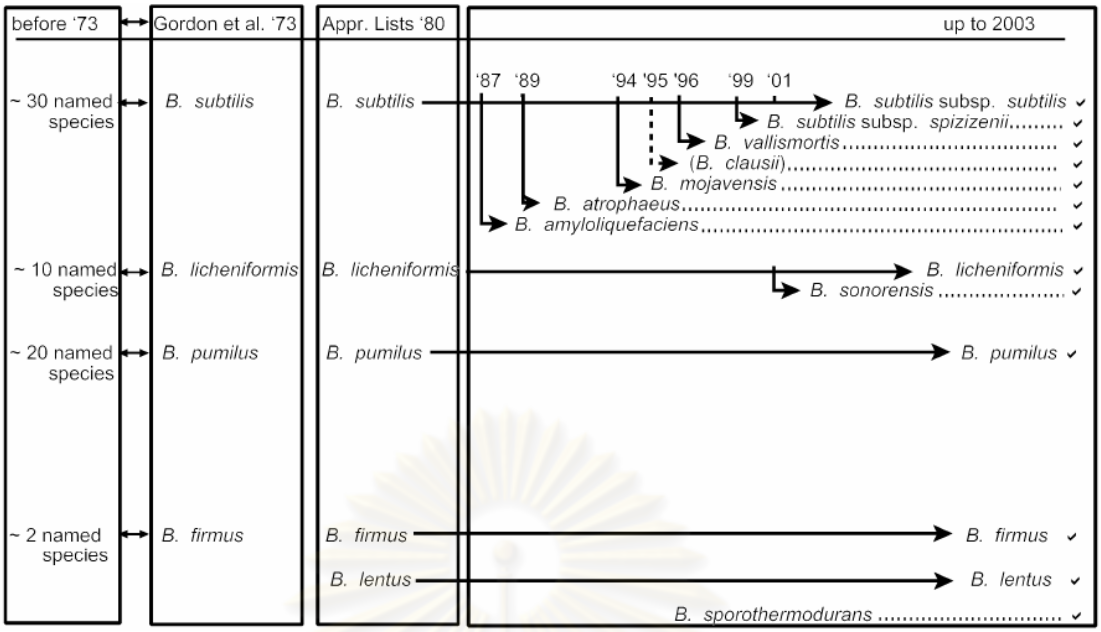


Figure 3. Taxonomic development of the *Bacillus subtilis* group (Fritze, 2004).

Intensive work is going on and, indeed, needs to be done to evaluate the whole group taxonomically in more depth. The decision on redefinition of species in this group will mainly depend on the availability of appropriate methods and the agreement of taxonomists regarding how and where to set border lines between species. At least, the new definitions should be expected to serve the applied areas of sciences, such as regulatory bodies, to support their work. In addition, easy-to-check correlating diagnostic features need to be determined. According to the Ad Hoc Committee for the Re-evaluation of the Species Definition in Bacteriology (Stackebrandt *et al*., 2002), up to now, the final proof for conspecificity of two given bacterial strains is DNA/DNA hybridization, a laborious and time-consuming method. New and quicker methods need to be developed to substitute this method appropriately. However, it is definitely clear that within some of the above-mentioned species, which were isolated and described in the early days of bacteriology and which therefore were and still are mainly phenotypically defined, a broad range of genotypic diversity (be it species or subspecies) is hidden.

Present systematic status

Two factors are chiefly responsible for this rapid increase in named species. One is the application of more diverse and intelligent methods for the enrichment and isolation processes to better account for the true physiological breadth and nutritional and cultural requirements of these ubiquitous organisms. The other is the development

of new and ever more sophisticated methods for the characterization and identification of bacterial strains, in particular on the genetic level, of which especially the 16S rRNA/DNA sequence analysis has exerted major influence.

In particular, sequence analysis has led and continues to lead, on the one hand, to the separation of groups of species from the core genus *Bacillus* to form new genera and, on the other hand, to the definition of novel genera to allocate new isolates. Today, *Bacillus* is only one out of a whole series of genera of AEFB comprising increasing numbers of validly published species. Descriptions of these genera and species are based to a varying extent upon morphological, biochemical, physiological, and chemotaxonomical traits and on the results of various molecular genetic techniques. However, while the basic systematic structure is today determined by the comparison of the sequences of the 16S rRNA/DNA (Stackebrandt and Swidersky, 2002), it is understood, in areas where discrimination through this method is insufficient or unsatisfactory, that classical methods are still useful and newly developed methods need to be applied.

Identification of *Bacillus*

The identification of *Bacillus* species has been performed mainly with morphological and physiological criteria, and this method is widely employed in various fields. However, the process used requires skillful techniques and is very complex and time-consuming. Moreover, it is still difficult to identify and characterize new isolates, mainly because some species share morphological and biochemical characteristics (Priest *et al*., 1988; Ash *et al*., 1991; Harrel *et al*., 1995), which makes it hard to separate them. In addition, species isolated from the environment have considerable diversity with respect to physiology, DNA G + C content and nutritional requirements (Claus and Berkeley, 1872; Priest *et al*., 1988; Ash *et al*., 1991; Dong and Cote, 2003).

Over the years, a sizable database of 16S rRNA gene (rDNA) has been built, and this has been successfully applied in determining phylogenetic relationships or in identifying bacteria (Goto *et al*., 2000). Using 16S rDNA sequence analysis, Ash *et al*. (1991) described the presence of five phylogenetically distinct groups in the genus *Bacillus*, and Nielsen *et al*. (1994) subsequently described a sixth group belonging to the alkaliphilic bacilli. Two of these groups are the *B. cereus* group (*B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides and B. weihenstephanensis*) and the *B. subtilis* group (*B. subtilis, B. pumilus, B. atrophaeus, B. licheniformis and*

B. amyloliquefaciens) (Ash *et al*., 1991). Although the *Bacillus* isolates had identical 16S rRNA gene sequences, they demonstrated considerable genotypic and phenotypic heterogeneity. Therefore, analysis of 16S rRNA gene sequences alone was not sufficient to identify *Bacillus* species (Hammer *et al*., 2007; Pontes *et al*., 2007; Freitas *et al*., 2008). Recently, the fingerprint methods have been developed to characterize and distinguish *Bacillus* isolates (Versalovic *et al*., 1991; Goto *et al*., 2000; Dong and Cote, 2003; Daffonchio *et al*., 2003).

Molecular approaches are increasingly being used for rapid species identification (Goto *et al*., 2000). Various techniques, including tDNA-PCR (tDNA-intergenic spacer length polymorphisms) and ITS-PCR (16S–23S intergenic transcribed spacer region) analyses have been used for identification and differentiation of *Bacillus* species (Daffonchio *et al*., 2003; Cherif *et al*., 2003). Freitas *et al*. (2008) used ITS-PCR and tDNA-PCR to examine phylogenetic relatedness among *Bacillus* isolates. They found that the isolates phylogenetically closest to *B. sphaericus* and *B. pumilus*, and to the *B. subtilis* and *B. cereus* groups were clearly different from the reference strains by these techniques (Figure 4). Although there was sufficient resolution to differentiate among reference strains, it was not possible to associate the *Bacillus* isolates with the reference strains using this technique.

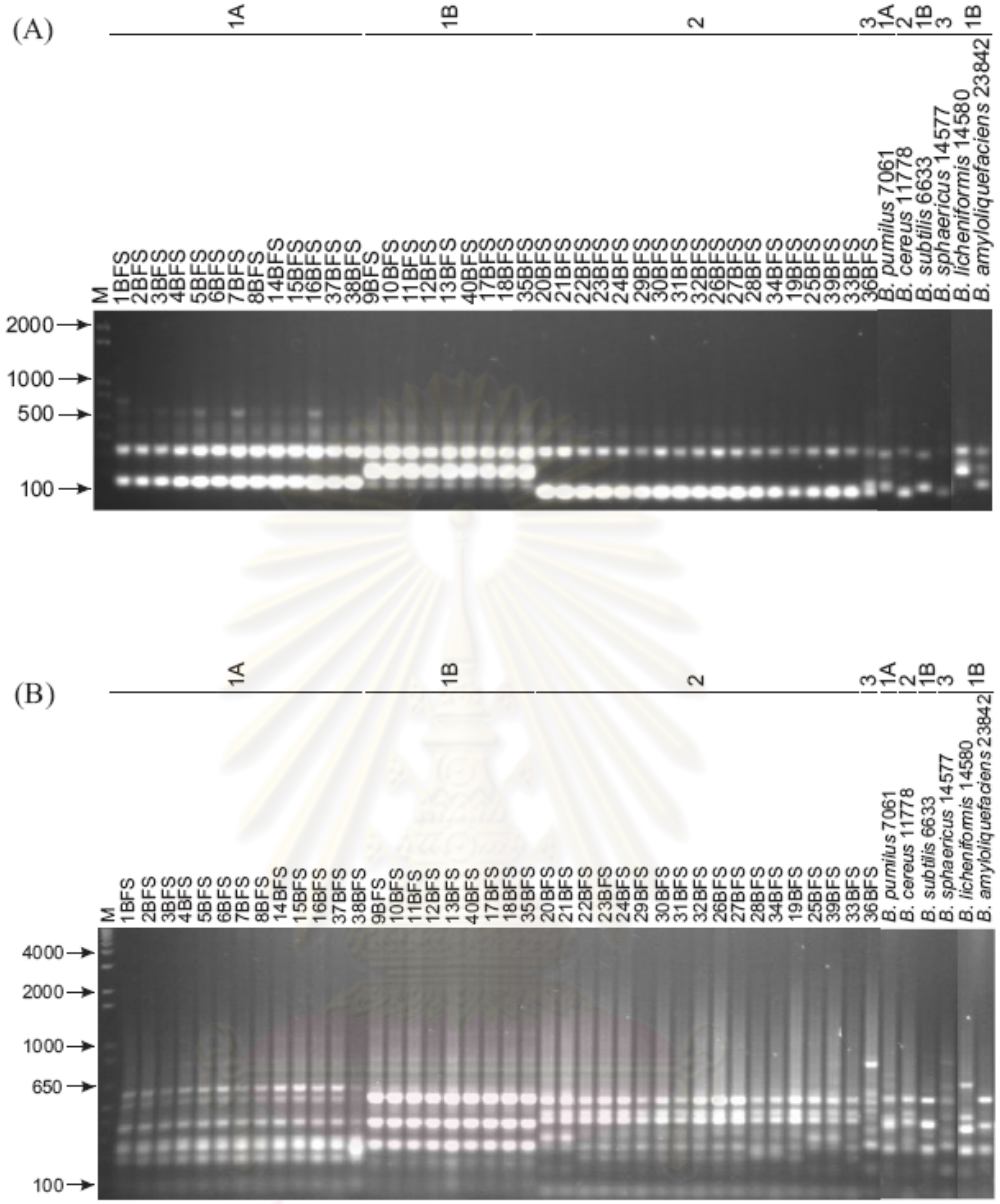


Figure 4. (A) ITS- and (B) tDNA-PCR fingerprinting of isolates and reference *Bacillus* strains. Lane M, Molecular size marker (1 Kb). The numbers above the figure identify the 16S rDNA-sequence-base phylogeny clusters obtained for the isolates (Freitas *et al*., 2008)**.**

Repetitive element sequence-based PCR (rep-PCR) genomic fingerprinting has also been used for discriminating among a wide range of bacterial genera and species, and to compare bacterial genome diversity. This method can be used to generate more accurate information because it is capable of screening several parts of the bacterial genome (Versalovic *et al*., 1991; Versalovic *et al*., 1994). Therefore, the rep-PCR method was applied to determine intraspecies diversity among the different *Bacillus* isolates. reported that isolates with the same partial 16S rDNA sequence often had quite dissimilar rep-PCR patterns. Freitas *et al*. (2008) revealed that the primer specific for $(GTG)_5$, the BOX and ERIC-PCR was efficient for grouping the *Bacillus* sp (Figure 5). Moreover, Freitas *et al*. (2008) found that ERIC-PCR fingerprinting clustered reference and environmental *B. cereus* strains in the same way as the 16S rDNA tree, suggesting the former is a good approach to examining genetic relationships among *Bacillus* isolates (Figure 6).

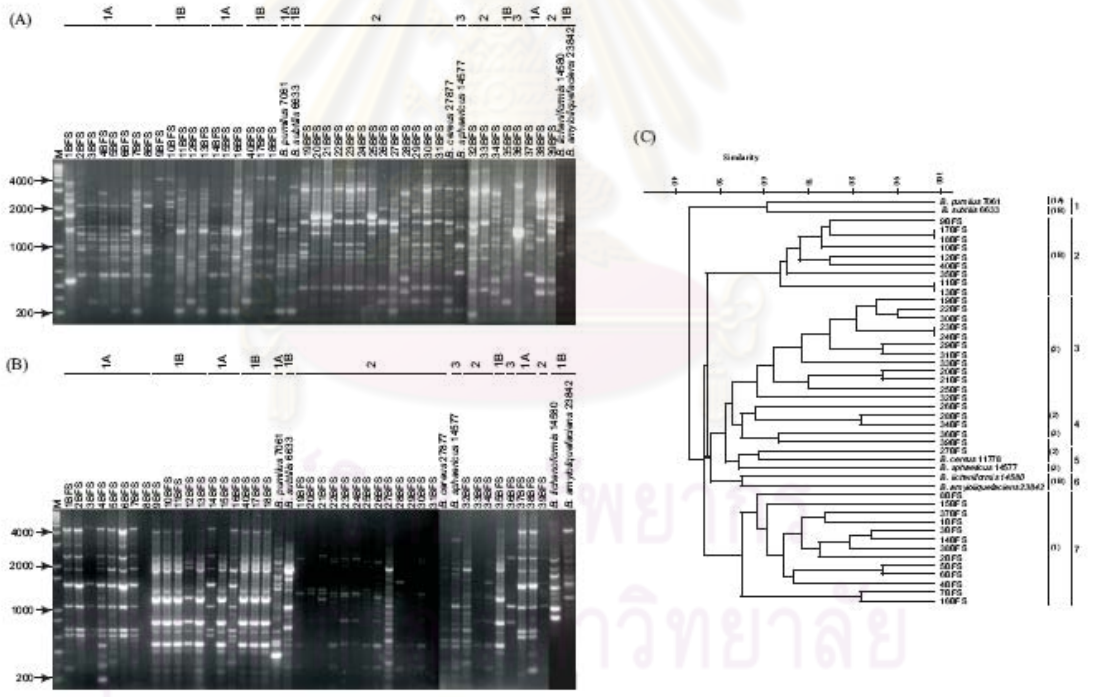


Figure 5. (A) $(GTG)_5$-PCR, (B) BOX-PCR fingerprinting patterns of the isolates and reference *Bacillus* strains. Lane M, Molecular size marker (1 Kb). (C) UPGMA cluster analysis of isolates and reference strains based on multi rep-PCR global matrix of ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR. The numbers above the figure identify the 16S rDNA-sequence-base phylogeny clusters obtained for the isolates (Freitas *et al*., 2008)**.**

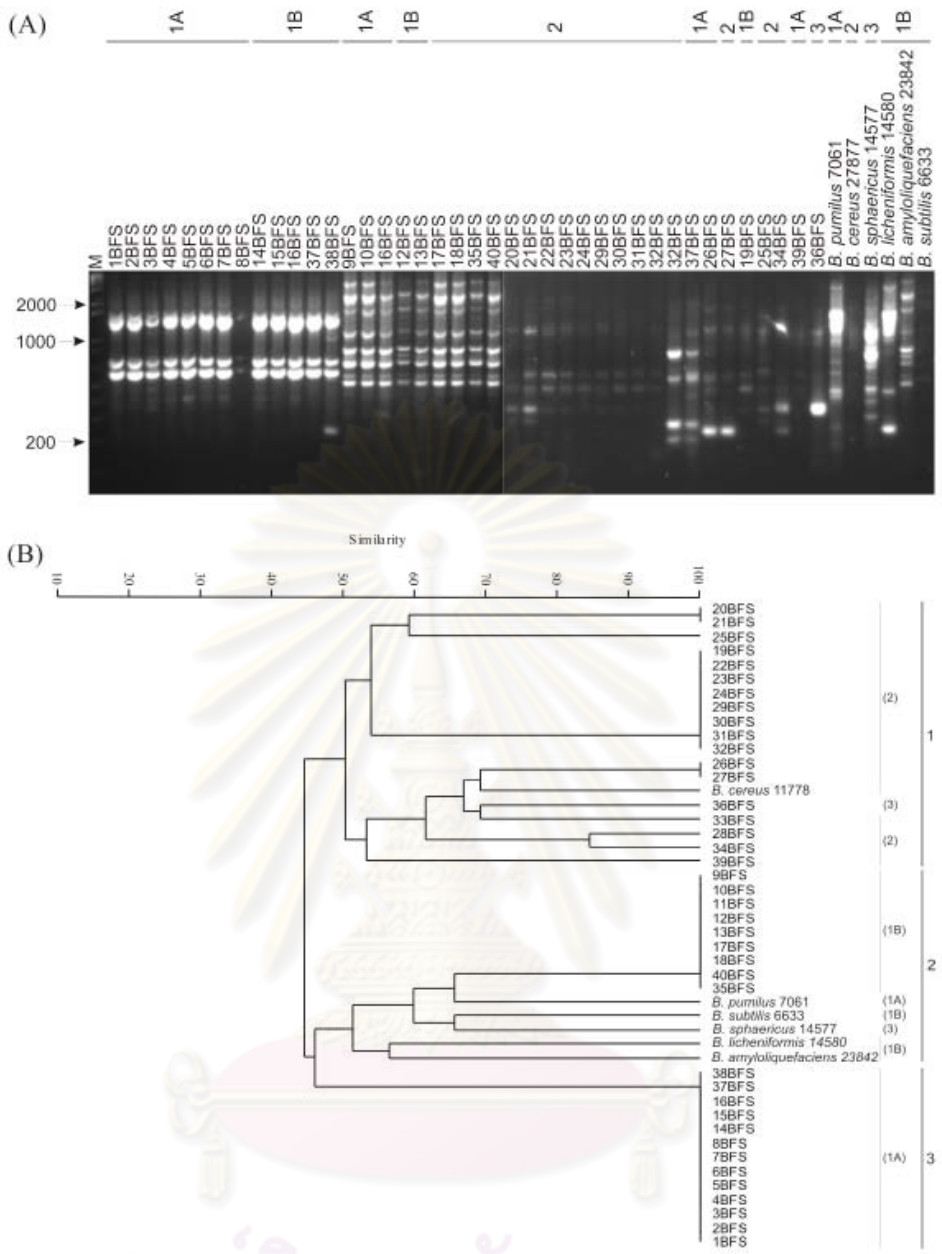


Figure 6. (A) ERIC-PCR fingerprinting patterns of the isolates and reference Bacillus strains. Lane M, Molecular size marker (1 Kb). The numbers above the figure identify the 16S rDNA-sequence-base phylogeny clusters obtained for the isolates. (B) UPGMA cluster analysis of isolates and reference strains based on ERIC-PCR. Numbers in parentheses identify the 16S rDNA-sequence-base phylogeny clusters obtained for the isolates (Freitas *et al*., 2008)**.**

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Traditional fermented food products, for example, *Bu-du* (fish sauce or muslim sauce), *Plaa-chao* (Thai sweetened fermented fish), *Pla-chom* (fermented small fish), *Tai-plaa* (fermented fish viscera), *Ka-pi* (fermented shrimp paste), *Koong-chom* (Thai sweetened fermented shrimp), *Hoi-dong* (pickled sea mussel), *Poo-chem* (fermented crab), *Ma-nao-dorng* (pickled lime), *Khing-dorng* (pickled ginger), *Kra-tiam-dorng* (pickled garlic bulbs), *Nor-mai-dorng* (fermented bamboo shoot), *Pak-kaat-dorng* (pickled mustard greens), *Sa-tor-dorng* (fermented stink bean), *Tooa-ngork-dorng* (pickled mungbean sprouts), *Khao-maak* (fermented glutinous rice), *Tao-hoo-yee* (fermented soybean cheese), *Tao-jieo* (soybean paste), *Tao-jieo-khaw* (white soybean paste), *Tooa-nao* (soybean paste or Thai natto) were purchased from local markets in Thailand.

Human plasmin, bovine fibrinogen, bovine thrombin, bovine pepsin, bovine pancreatine, bovine casein, bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), leupeptin, chymostatin, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and N alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK), soybean trypsin inhibitor (SBTI), phosphoramidon, 1,10-phenanthroline, pepstatin A, trans-epoxysuccinyl-L-leucyamino(guanidine) - butane (E-64), H-D-Val-Leu-Lys-*p*NA (for plasmin), pyro-Glu-Gly-Arg-*p*NA (for urokinase), H-D-Val-Leu-Arg-*p*NA (for thrombin), D-galactose, D-mannose, D-mannitol, D-maltose, D-fructose, D-xylose, L-tyrosine, copper sulphate ($CuSO_4$), calsium sulphate ($CaSO_4$), manganese sulphate ($MnSO_4$), magnesium sulphate ($MgSO_4$), zinc acetate ($(CH_3CO_2)_2Zn$), Ferrous sulphate ($FeSO_4$), and Ferric chloride ($FeCl_3$) were obtained from Sigma Chemical Co. (St. Louis, MO). Skim milk powder, yeast extract, meat extract, D-glucose, D-lactose, sucrose, soluble starch, glycerol, ethylenediaminetetraacetic acid (EDTA) and sodium azide were provided by Merck (Darmstadt, Germany). Agar, tryptone, peptone and gelatin were obtained from Difco (Detroit, USA). Ammonium sulfate, sodium chloride and trichloroacetic acid were purchased from Carlo Erba (Milan, Italy). Photobiotin was obtained from New England Biolabs (Ontario, Canada). PageRuler^{TM} unstained protein ladder was provided by Fermentas (Ontario, Canada).

Resource Q, superpose 12 10/300 GL and PlusOne^{TM} Silver Staining Kit were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). API ZYM, API 50CHB and API 50CH were provided from BioMerieux (Basingstoke, UK). All other chemicals were of analytical grade.

3.2 Experimental designs

In this study, bacteria were isolated from 20 kinds of traditional fermented food and screened for fibrinolytic activity. Consequently, fibrinolytic protease-producing bacteria were subjected to identification. Furthermore, the optimal condition for fibrinolytic protease production was evaluated. Finally, purification and characterization of fibrinolytic protease were carried out. The research procedure of this study was showed in Figure 7.

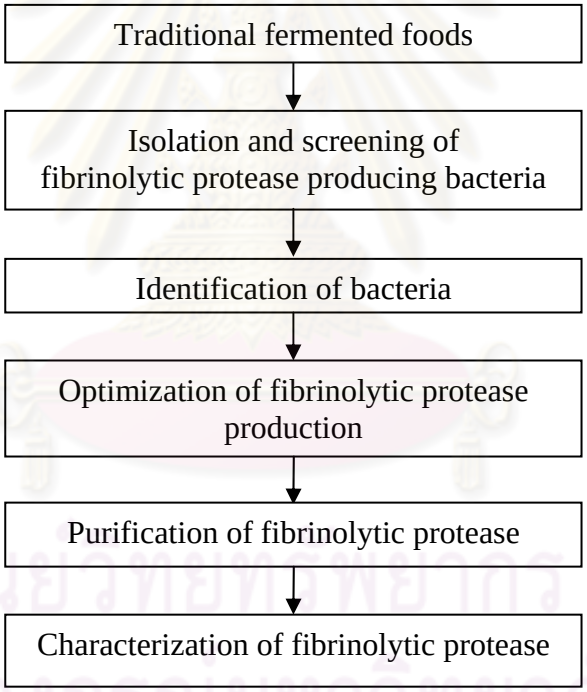


Figure 7. Overall experimental design.

3.3 Isolation of bacterial strains

Bacteria were isolated from 20 kinds of traditional fermented foods produced in Thailand by means of the usual dilution plating technique on modified Luria-Bertani (MLB) medium that composed of 1.0% tryptone, 0.5% yeast extract, 2.5% glucose, 5.0% NaCl, and 1.5% agar (Kim *et al*., 1997). Briefly, a small quantity of fermented food (1 g) was mixed with 9 ml sterile diluent containing 0.85% NaCl. Ten

fold dilutions (10^{-1} to 10^{-8}) were then prepared and 100 μl of each dilution was inoculated on MLB agar plate. The petri dishes were incubated at 37°C for 24 h and colonies were separately isolated on MLB agar plate. The isolates were then reisolated by streaking out on MLB agar plate until a single colony was obtained. The pure cultures were maintained on MLB slant, keep at 4°C and in 20% glycerol, stored at -80°C.

3.4 Screening of fibrinolytic protease-producing bacteria

Inoculum preparation

Bacteria were propagated at 37°C on modified MLB agar plate, composed of 1.0% tryptone, 0.5% yeast extract, 2.5% glucose and 1.5% agar, pH 7.2, for 24 h. The inoculum was prepared by adding a loop full of pure culture from modified MLB agar plate into 5 ml of seed culture medium (modified MLB broth) in 15 ml test tube and incubated at 37°C on a rotary shaker (Model Certomat BS-1, Sartorius, Goettingen, Germany) at 200 rpm for 18 h.

Enzyme production

The production medium was consisted of 1.0% tryptone, 0.5% yeast extract, 2.5% glucose, pH 7.2. The production medium was inoculated with 9% (v/v) inoculum and cultivated at 37°C on a rotatory shaker at 200 rpm for 18 h. Samples of the culture broth were collected and the growth was measure with an absorbance at 600 nm (OD_{600}) by UV-Vis spectrophotometer (Model Helios alpha, Thermo Fisher Scientific, Bremen, Germany). The culture was harvested by centrifugation at 17700 x g for 15 min at 4°C and the cell-free supernatant was used as crude enzyme to measure fibrinolytic activity and protein content.

Primary screening

A fibrin plate assay (Astrup and Mullertz, 1952) was used to screen for fibrinolytic activity of crude enzyme from each isolated bacteria. In brief, 5 ml of 2% (w/v) agarose warmed to 50°C was mixed with 5 ml of 0.6% (w/v) fibrinogen solution in 50 mM phosphate buffer pH 7.4 warmed to 45°C and 20 μl of thrombin solution (10 NIH unit/ml) and poured into a Petri dish. The dish was left at room temperature for 30 min to form a fibrin clot layer. Nine holes (3 mm in diameter) were punched for sample application on the fibrin plate and ten microliters of crude enzyme was placed in each hole. After incubated at 37°C for 18 h, the radius of clear

zone around the hole was measured. Human plasmin was used as the standard fibrinolytic protease.

Secondary screening

SDS-fibrin zymography SDS-fibrin zymography gel was prepared as described by Kim *et al*. (1998). Briefly, separating gel solution (12%, w/v) containing fibrinogen (0.6%, w/v) was prepared in a total volume of 10 ml. Thrombin (1 NIH unit/ml) solution was added to the gel solution at a final concentration of 0.1 μunit/ml. Ammonium persulfate (10%, w/v) and *N,N,N´,N´*,-tetramethylethylenediamine were used to catalyze the polymerization. Crude enzyme was mixed with sample buffer (5X) consisting of 0.5 M Tris-HCl, pH 6.8, 20% (w/v) SDS, 20% (w/v) glycerol and 0.03% (w/v) bromophenolblue at 4:1 (v/v) ratio, and 25 μg of proteins were loaded on to the gel. The proteins were subjected to electrophoresis at a constant current of 20 mA per gel by the Hoefer SE250 mini-gel unit (Amersham pharmacia biotech, Uppsala, Sweden). After electrophoresis, gel was immersed in 100 ml of 2.5% (w/w) Triton X-100 solution for 30 min and then immersed in 100 ml of reaction buffer (30 mM Tris, pH 7.4 containing 200 mM NaCl and 0.02% (w/v) NaN_3) for 18 h at 37°C. The gel was fixed and stained with 0.025% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid for 1 h and then destained in destaining solution (30% (v/v) methanol and 10% (v/v) acetic acid). Development of clear zone on blue background indicated fibrinolytic activity. PageRuler^{TM} unstained protein ladder 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.

Fibrinolytic activity assay Fibrinolytic activity was measured by the hydrolysis of fibrin clot as described by Kim *et al*. (1997) with some modifications. In brief, 500 μl of 0.6% (w/v) fibrinogen solution in 0.1 M borate buffer, pH 7.0 was mixed with 20 μl of thrombin solution (50 NIH unit/ml thrombin in 0.1 M borate buffer, pH 7.0 containing 0.85% NaCl). The fibrin clot was allowed to form for 5 min at room temperature and then mixed with 380 μl of 20 mM sodium phosphate buffer (pH 7.4). To initiate the enzymatic reaction, 100 μl of crude enzyme was added and incubated at 37°C for 20 min. The reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and allowed to stand on ice for 10 min. After centrifugation at 4117 x g for 10 min, the resulting oligopeptides in the supernatant

were measured by Lowry method (Lowry, 1957) and converted to the amount of tyrosine equivalent. One unit of fibrinolytic activity was defined as 1 nmol of tyrosine released per min. A blank was run in the same manner except that the enzyme was added after the addition of TCA solution.

Protein determination Protein concentration was determined by Lowry method (Lowry, 1957) using BSA as a standard (Appendix B-1).

3.5 Identification of isolates

3.5.1 Phenotypic characterization

Prior to the genotyping, the bacteria were phenotyped according to Gordon *et al.* (1973), Claus and Berkeley (1986) and Barrow and Feltham (1993)

Morphological and cultural characteristics Cell arrangement and Gram staining (Hucker and Conn, 1923) (Appendix A-1) were examined after grown on modified Luria-Bertani (MLB) agar plate (Appendix A-2.1) at 37°C for 1-2 days. The shape, position and size of spores were determined by phase-contrast microscopy.

Pigmentation production was examined by inoculated a nutrient agar plate (Appendix A-2.2) with a drop of light suspension of the bacteria, incubated for 24 h at 37°C and then transferred to room temperature and observed for up to 5 days. The color was recorded as red, orange, yellow, green, violet, brown, or negative i.e. produce none of these pigments. Moreover, the pigmentation production on Tryptic soy agar plate (Appendix A-2.3), Luria-Bertani agar plate (Appendix A-2.4), modified Luria-Bertani agar plate (Appendix A-2.1), Tyrosine agar plate (Appendix A-2.5), Potato dextrose agar plate (Appendix A-2.6) and Mannitol yeast-extract agar plate (Appendix A-2.7) were evaluated.

Physiological and biochemical characteristics Sulphide production, indole and motility test were examined by stap-inoculated the culture into the tubes of sulphid-indole-motility (SIM) medium (Appendix A-3.1) and incubated at 30°C for 18-24 h. Diffusion of growth from the line of inoculum indicated motility. Blackening at surface of the medium indicated H_2S production and the development of a red color within one hour after the addition of Kovac's reagent (Appendix A-3.2) indicated indole production.

Catalase test was determined by flooding the colonies grown overnight on Nutrient agar plate with 3% hydrogen peroxide (H_2O_2) (Appendix A-4.1). After 5 min, the appearance of gas bubbles denoted as a positive reaction.

Oxidase test was performed by dropping small amount of 1% tetramethyl-p-phenylenediamine (Appendix A-5.1) on sterile filter paper disc. The test culture (grown on MLB medium free from glucose) then was smeared across the moist paper with a platinum loop. The colonial appearance of dark-purple color on the paper within 30 sec revealed a positive reaction.

For MR-VP test, the culture was inoculated to glucose phosphate (GP) medium (Appendix A-6.1) and incubated at 30ºC for 2 days. After added 2 drops of methyl red solution (Appendix A-6.2) and shake well, the appearance of a red color at the surface indicated positive MR reaction. An orange or yellow color regarded as negative. After reading the MR reaction, the same culture was used for VP test by adding 2 drops of creatine solution (Appendix A-6.3) (about 0.05 ml) and 1 ml of 40% KOH aqueous solution (Appendix A-6.4) and shake well. After 1 and 4 h, positive reaction was indicated by an eosin-pink color.

Citrate utilization was done by a single streak over the surface of a slope of Simmons' citrate medium (Appendix A-7.1). The result was examined daily for up to 7 days for growth, original green color changed to blue color indicated citrate utilized.

Deoxyribonuclease (DNase) activity was performed by inoculated test cultures as streak on the surface of DNase test agar (Difco) (Appendix A-8.1) (replicate plates) and incubated at 30 and 37ºC. After incubation for 36 h, the DNase plates were flooded with 1N HCl, a positive result showed as a clear zone around the growth and a negative result of the test was opaque. Moreover, indole test was performed by inoculated the cultures in peptone water (Appendix A-9.1) and incubated at 37ºC. After 2 days of incubation, 0.5 ml of Kovacs' reagent (Appendix A-9.2) was added and shake well, a red color in the reagent layer indicated indole production.

For nitrate reduction test, the cultures were inoculated into nitrate broth (Appendix A-10.1) and incubated for up to 5 days. After added 1 ml of nitrite reagent A (Appendix A-10.2) followed by 1 ml of reagent B (Appendix A-10.3), a deep red color showed the presence of nitrite and thus the nitrate was reduced, indicated a positive reaction.

Urease activity was tested according to the method of Stuart *et al*. (1945) by using SSR medium (Appendix A-11.1). After heavy inoculated a tube of SSR Urea

medium, they were incubated at 37°C and examined daily up for 7 days. Red color indicated urea hydrolyzed.

Tween 80 hydrolysis was determined. The test culture was streak-inoculated on the surface of Tween 80 agar (Appendix A-12.1) and incubated at 37°C. The result was observed daily up to 7 days for an opaque halo of precipitation around the growth, indicating hydrolysis of the Tween. Starch hydrolysis was examined by streak-inoculating the test culture on the surface of starch agar plate (Appendix A-12.2). The streaked agar was incubated at 37°C for 2 days. The result was observed by flooding the plate with Lugol's iodine solution (Appendix A-12.3). The medium turned blue where starch has not been hydrolysed and clear colorless zone indicated hydrolysis. Gelatin hydrolysis or liquefaction was done. The test culture was streak-inoculated on the surface of gelatin agar plate (Appendix A-12.4) and incubated at 37°C for 3 days. The result was observed by flooding the surface of plate with 5-10 ml of 10% trichloroacetic acid solution (Appendix A-12.5). The clear zone indicated areas of gelatin hydrolysis. Digestion of casein was performed by streak-inoculating the test culture on the surface of casein agar plate (Appendix A-12.6) and intervals were examined for up to 7 days for clearing of the medium around the bacterial growth. Decomposition of L-tyrosine was evaluated by streak-inoculating the test culture on the surface of tyrosine agar plate (Appendix A-12.7) and incubating it at 37°C. The result was examined daily up to 7 days for dissolution of crystals under and around the bacterial growth. Aesculin hydrolysis was tested by inoculating the fresh cell culture suspension into aesculin broth (Appendix A-12.8) and incubating at 37°C. The result was examined daily up to 5 days for blackening, indicating hydrolysis of the aesculin.

Phenylalanine deamination was examined as described by International Committee on Systematics of Prokaryotes (ICSP) (1958). Cell culture suspension was heavily inoculated on a phenylalanine agar slope (Appendix A-13.1). After incubating the agar at 37°C overnight, 0.2 ml of a 10% aqueous solution of $FeCl_3$ (Appendix A-13.2) was flooded over the growth. A positive result gave a green color on the slope and in the free liquid at the base. Decarboxylations of L-arginine, L-lysine and L-ornithine were evaluated using Falkow's medium (Appendix A-14.1) supplemented with 0.5% of each amino acid according to the method of Falkow (1958). Tubes of the four media (the last is negative control without any amino acid) were inoculated

with the fresh cell culture suspension and incubated at 37°C. The results were examined daily up to 4 days and decarboxylation was indicated by a purple color, whereas the control and tubes with negative reactions were shown as yellow color.

Growth in media with increased NaCl concentration (0, 5, 8, 10, 12, 15 and 20% NaCl) was tested using nutrient broth (NB), and pH was adjusted to 7.2 with 0.1 N NaOH and 0.1 N HCl. Temperature range for growth (4, 25, 30, 37, 40, 45, 50 and 55°C) was examined using NB. Growth at various pHs (4.5, 5, 6, 7, 8, 9, and 10) were evaluated using NB and pH was adjusted with sterile 0.1 N NaOH and 0.1 N HCl. The growth was observed by measuring the optical density at 600 nm (OD_{600}) and then optimal condition for growth was evaluated.

Fermentation profiles of carbohydrates were determined by API50CH. Briefly, the fermentation test was inoculated with the fresh culture suspended in API50CHB medium to rehydrate the substrates (Table 5), and incubated at 37ºC. During incubation, fermentation was revealed by a color change in the tubes. The change was caused by the anaerobic production of acid and it was detected by the pH indicator present in the medium. Moreover, the oxidation was revealed by a color change in the capule, caused by the aerobic production of acid. The first tube, which did not contain any active ingredient, was used as a negative control. A positive corresponds to acidification revealed by the phenol red indicator contained in the medium changing to yellow. For the esculin test, a change in color from red to black was observed. If a positive test became negative at the second reading, only the positive result was taken into account. This was caused by an alkalinization due to the production of ammonia from peptone. The biochemical profiles obtained for the strain after the final reading were identified using the identification software with database (V3.0).

Table 5. The composition of the API50CH strip.

Tube	Test	Active ingredients	QTY (mg/cup.)	Tube	Test	Active ingredients	QTY (mg/cup.)
1		Control	-	26	ESC	Esculin*	1.16
2	GLY	Glycerol	1.64	27	SAL	Salicin	1.04
3	ERY	Erythritol	1.44	28	CEL	D-Cellubiose	1.32
4	DARA	D-Arabinose	1.40	29	MAL	D-Maltose	1.40
5	LARA	L-Arabinose	1.40	30	LAC	D-Lactose (bovine origin)	1.40
6	RIB	D-Ribose	1.40	31	MEL	D-Melibiose	1.32
7	DXYL	D-Xylose	1.40	32	SAC	D-Sacch arose (sucrose)	1.32
8	LXYL	L-Xylose	1.40	33	TRE	D-Trehalose	1.32
9	ADO	D-Adonitol	1.36	34	INU	Inulin	1.28
10	MDX	Methyl-βD-xylopyranoside	1.28	35	MLZ	D-Melezitose	1.32
11	GAL	D-Galactose	1.40	36	RAF	D-Raffinose	1.56
12	GLU	D-Glucose	1.56	37	AMD	Aminod (starch)	1.28
13	FRU	D-Fructose	1.40	38	GLYG	Glycogen	1.28
14	MNE	D-Mannose	1.40	39	XLT	Xylitol	1.40
15	SBE	L-Sorbose	1.40	40	GEN	Gentiobiose	0.50
16	RHA	L-Rhamnose	1.36	41	TUR	D-Turanose	1.32
17	DUL	Dulcitol	1.36	42	LYX	D-Lyxose	1.40
18	INO	Inositol	1.40	43	TAG	D-Tagatose	1.40
19	MAN	D-Mannitol	1.36	44	DFUC	D-Fucose	1.28
20	SOR	D-Sorbitol	1.36	45	LFUC	L-Fucose	1.28
21	MDM	Methyl-αD-Mannopyranoside	1.28	46	DARL	D-Arabitol	1.40
22	MDG	Methyl-αD-Glucopyranoside	1.28	47	LARL	L-Arabitol	1.40
23	NAG	N-Acetylglucosamine	1.28	48	GNT	Potassium Gluconate	1.84
24	AMY	Amygdalin	1.08	49	2KG	Potassium 2-Ketogluconate	2.12
25	ARB	Arbutin	1.08	50	5KG	Potassium 5-Ketogluconate	1.80

Enzymatic activity profiles of bacterial strains were evaluated by API ZYM. In brief, 65 μl of heavily bacteria suspension (in normal saline solution with the absorbance of 0.699 measured at 600 nm that equal to a McFarland No.4) was distributed into API ZYM strips and incubated at 37ºC in the dark incubation box with normal atmospheric condition. After 6 h of incubation, reagent A and B were added to each cupule following the manufacturer's direction. The reactions were allowed to develop for 5 min under a powerful light source. After light exposure, the reactions were read and recorded on the result sheets. A value ranging from 0-5 was assigned, corresponding to the color developed : 0 corresponds to a negative reaction, 5 to a reaction of maximum intensity and values 1, 2, 3 or 4 were intermediate reactions depending on the level of intensity (3, 4 or 5 being considered as positive reactions) (Table 6).

Table 6. Reading table of API ZYM

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

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

3.5.2 Genotypic characterization

Preparation of genomic DNA Genomic DNA was isolated according to the method of Saito and Miura (1963). Cells were harvested after 12-16 h cultivation and suspended in 2 ml of saline-EDTA buffer pH 8.0 (Appendix A-15.1). The cell suspension was inoculated with 1 mg of lysozyme and incubated at 37ºC for 1 h. Then 100 µl of 10% SDS (Appendix A-15.2) were added and incubated at 50ºC for 10 min. The phenol extraction was carried out by adding an equal volume of phenol:chloroform (1:1) (Appendix A-15.3) to the sample for removal of protein and other debris. The upper layer of the mixture was collected after centrifugation at 4ºC, 17700 x g for 30 min. Genomic DNA was then precipitated with two volumes of ice cold absolute ethanol and left for air-dryness. Genomic DNA was redissolved in 0.1x SSC (Appendix A-15.4) and treated with RNase A, RNase T_1 and protease K solution (Appendix A-15.5) at 37ºC for 1 h to removal of RNA and protein, respectively. Then phenol extraction and ethanol precipitation were performed and genomic DNA was stored in 0.1x SSC at 4ºC.

16S-23S rRNA internal transcribed spacers-polymerase chain reaction (ITS-PCR) fingerprinting Modified and combined methods of Daffonchio *et al*. (1998), Daffonchio *et al*. (2003) and Ouoba *et al*. (2007) were used. Amplification of the 16S-23S internal transcribed spacer (ITS) region was carried out in 50 µl of reaction mixture containing 1 µl of DNA template, 5 µl of PCR buffer, 5 µl of dNTP, 5 µl of $MgCl_2$, 1 µl of the forward primer S-D-Bact-1494-a-S-20 (10 pmol/µl) (Table 7), 1 µl of the backward primer L-D-Bact-0035-a-A-15 (10 pmol/µl) (Table 8), 1 unit of Taq polymerase and 31 µl of autoclaved high purity water. Amplification consisted of 30 PCR cycles in a DNA Engine DYAD ALD 1244 thermocycler (MJ Research, Waltham, MA). The cycling program was: initial denaturation at 94ºC for 4 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 7 min and elongation at 72ºC for 2 min. The PCR was ended with a final extension at 72ºC for 7 min and amplified product was cooled at 14ºC.

The DNA fragments were separated by applying 3 µl of each PCR product with 2 µl of loading buffer (Appendix A-16.1) to 2.5% agarose gel (Appendix A-16.2). DNA molecular weight marker (Bioexcellent, Bangkok, Thailand) was included as standard for the calculation of the fragments. The gel was run in Tris Acetate-EDTA buffer (Appendix A-16.3) for 55 min at 100 V and then stained for 5

min in a 0.5 mg/ml of ethidium bromide solution (Appendix A-16.4). The profiles were visualized after staining under ultraviolet light, followed by digital capturing using the Gel Doc 2000 system (Biorad, Hercules, CA, USA).

Sequencing of 16S rRNA gene The 16S rRNA gene were PCR amplifed with 27F and 1492R (Table 7). The PCR was performed in a total volume of 50 ul containing 1 μl of DNA template, 0.25 μl of Taq DNA polymerase, 5 μl of 10x polymerase buffer, 5 μl of dNTP mixture, 2.5 μl of 10 μM forward primer (27F), 2.5 μl of 10 μM reverse primer (1492R) and 33.75 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 95ºC followed by 30 cycles of 30 sec of 95ºC (denaturing of DNA), 15 sec at 55ºC (primer annealing), and 1 min at 72ºC (polymerization) and a final extension for 7 min at 72ºC. The PCR amplified products were analyzed by running 2 μl of the reaction mixture on a 0.8% agarose gel in Tris Acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution (0.5 mg/l) and examined under UV-transillumiator (UVP Inc.) to visualize the amplified 16S rDNA band.

The 16S rRNA gene fragments were PCR amplified with 357R, 802R, 1115R and 1539R primer (Table 7). The 16S rDNA fragments were purified and sequenced using BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.0: Applied Biosystems) in the ABI PRISM 310 Genetic analyzer (Applied Biosystems, USA)

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 sequences were multiple aligned with selected sequences obtained from the tree main databases using CLUSTAL X version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods (Kluge and Farris, 1969) in the MEGA program version 2.1 (Kumar *et al*., 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise

Table 7. Sequences and positions of the used primers.

Primer	Target sequence	Position	Sequence 5'---3'	Expected size
S-D-Bact-1494-a-S-20	ITS 16S-23S	1494 rDNA 16S	GTCGTAACAAGGTAGCCGTA	Pattern
L-D-Bact-0035-a-A-15	ITS 16S-23S	35 rDNA 23S	CAAGGCATCCACCGT	Pattern
27F	16S rDNA	27 rDNA 16S	AGAGTTTGATC(CT)TGGCTCAG	1500 bp in length
1492R	16S rDNA	1492 rDNA 16S	ACGG(CT)TACCTTGTTACGACTT	1500 bp in length
357R	16S rDNA	343-357 rDNA 16S	CTGCTGCCTCCCGTAGGAGT	400 bp in length
802R	16S rDNA	785-802 rDNA 16S	TACCAGGGTATCTAATCC	600 bp in length
1115R	16S rDNA	1000-1115 rDNA 16S	AGGGTTGCGCTCGTTG	600 bp in length
1539R	16S rDNA	1518-1539 rDNA 16S	CCAGTGAGCAGAGTGACG	600 bp in length
$(GTG)_5$	16S rDNA		GTGGTGGTGGTGGTG	Pattern

alignments obtained using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

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 (Table 7) 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: 94ºC for 5 min, 30 cycles at 94ºC for 30 sec, at 45ºC for 1 min and at 65ºC for 8 min, followed by a final extension of 16 min at 65ºC. The PCR products were electrophorised for 4 h at 140 V on a 1.5% (w/v) agarose gel (Appendix A-17.1) in TBE buffer (Appendix A-17.2). 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.

DNA-DNA hybridization Only high-molecular-mass DNA with A_{280}/A_{260} absorption (A) ratios of <0.6 was used for DNA-DNA hybridizations. DNA labeling probe with photobiotin was performed 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 the mixture was irradiated with sunlamp for 30 min on ice. Then the excess photobiotins were removed by the addition of 100 µl of 0.1 M Tris-HCl buffer pH 9.0 (Appendix A-18.1) and 100 µl of n-butanol. The upper layer was removed and then 100 µl of n-butanol was added. After removed the upper layer, the biotinylated DNA solution was boiled for 15 min and immediately cooled on ice. The solution was sonicated for 3 min and dissolved with hybridization solution.

DNA-DNA hybridizations were performed according to a modification of the microplate method described by Ezaki *et al*. (1989). Briefly, 10 µl of purified DNA (1 mg/ml) was boiled and immediately cooled on ice. Then 390 µl of steriled distilled

water, 500 µl of 2X PBS (Appendix A-18.2) and 100 µl of 0.1 M $MgCl_2$ (Appendix A-18.3) were added. One hundred microliters of denatured DNA solution was added to microdilution wells (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) and incubated at 37ºC for 2 h. Then DNA solution was removed and 100 µl of hybridization solution containing biotinylated DNA (Appendix A-18.11) was added. The microdilution plate was incubated at hybridization temperature of each isolated for 5 h. The hybridization temperature was 43±1ºC (*B. subtilis* group) or 45±1ºC (*B. velezensis* group). After hybridization, the hybridization solution was removed and the microdilution wells were washed 3 times with 200 µl of 0.2X SSC buffer (Appendix A-18.12). Then 200 µl of solution I (Appendix A-18.13) was added to microdilution wells and incubated at 30ºC. After 10 min of incubation, solution I was removed and 100 µl of solution II (Appendix A-18.14) was added and incubate at 37ºC for 30 min. Then the microdilution plate was washed with 200 µl of PBS for 3 times. One hundred microliters of solution III (Appendix A-18.15) was added and the plate was incubated at 37ºC for 10 min. The enzyme reaction was stopped with 100 µl of 2M H_2SO_4 (Appendix A-18.16) (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 of DNA-DNA homology.

Reference strains *Bacillus subtilis* subsp. *subtilis* KCTC 3135^{T}, *B. amyloliquefaciens* KCTC 1660^{T}, *B. licheniformis* KCTC 1918^{T}, *B. mojavensis* KCTC 3706^{T}, *B. vallismortis* KCTC 3707^{T}and *B. velezensis* KCTC 13012^{T} were used as reference strains.

3.5.3 Chemotaxonomic characterization

Diaminopimelic acid (DAP) analysis Determination of diaminopimelic acid was performed as previously described by Komagata and Suzuki (1987). In brief, approximately 10 mg of dried cells were hydrolyzed with 1 ml of 6 N HCl in a screw-capped tube at 100ºC for 18 h. After cooling, the hydrolysate was filtered and evaporated. Then 400 µl of distilled water was added to the dried filter and the sample was applied on the base line of a cellulose TLC plate no. 5787. As a standard, DL-diaminopimelic acid was also applied. TLC was developed with the solvent system MeOH:H_2O:6 N HCl:Pyridine (80:26:4:10, v/v). The spot was visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol (Appendix A-19.1) followed by heating at 100ºC for 5 min. DAP isomer appeared as dark-green spots

with R_f 0.24 for *meso*- and D-isomer. Spots were circled by pencil as to it gradually disappeared in a few hours.

Quinones Freeze-dried cells (200 mg) were suspened in 20 ml of $CHCl_3$:MeOH (2:1, v/v) and gently shake for 6 h. After filtration, the extract was evaporated and dissolved in a small amount of acetone. Acetone solution was applied to silica gel TLC (Merck no.1.05744) and developed with benzene. Standard for quinones was also applied. The quinine spots were visualized by UV light at 254 nm. The menaquinines were appeared at R_f ca. 0.7, and ubiquinones at R_f ca. 0.4. The band corresponding to menaquinone was scraped off and extracted with acetone. Before concentration, the acetone solution was filtered. The quinine sample was separted by isocratic HPLC (Collins and Jones, 1981, Collins, 1982 and Tamaoka *et al*., 1983). Quantitative data was obtained and was based on the area ratio of UV absorbance to molar composition of cellular quinine (Komagata and Suzuki, 1987).

Polar lipids analysis Polar lipids were extracted using the modified method of Kamekura (1993), Bligh and Dyer (1959) and Card (1973). Briefly, 200 mg of dried cells were suspended in 3 ml of MeOH: 0.3%NaCl aqueous solution (100:10). Three milliliter of petroleum ether was added and the mixture was mixed for 60 min with shaking at 250 rpm in a rotatory shaker. After centrifugation at 4117 x g for 10 min, the lower layer was collected and 1 ml of petroleum ether was added and mixed thoroughly for 15 min. After centrifugation at 4117 x g for 10 min, the upper layer was removed and the lower layer was heated at 100ºC for 5 min. After cooling, 2.3 ml of $CHCl_3$:MeOH:H_2O (90:100:30) was added and mixed thoroughly for 1 h. After centrifugation, the upper layer was transferred into another tube and 2.3 ml of $CHCl_3$:MeOH:H_2O (50:100:40) was added to the debris cell and mixed well for 30 min. The supernatant was transferred to the upper layer tube and the lower layer was extracted again with 1.3 ml of chloroform and water. The final lower layer was mixed with the upper layer and dried with N_2 gas at temperature below 37ºC. The polar lipid fraction was redissolved with 60 µl of $CHCl_3$:MeOH (2:1) and applied to two-dimentional silica HPTLC no. 1.05633 and was developed with the following solvent systems. The first solvent system was $CHCl_3$:MeOH:H_2O (65:25:4). The second solvent system was $CHCl_3$:acetic acid:MeOH: H_2O (40:7.5:6:2). Then HPTLC plate was incubated with iodine crystal until polar lipid appeared. Then the plate was sprayed with Ninhydrin reagent (Appendix A-20.1) and then heated at 110ºC for 10

min. Dittmer and Lester reagent (Appendix A-20.2) was sprayed onto the plate and then blue color were detected on the spot containing phospholipids.

Cellular fatty acid profiles Fatty acid profiles were identified by gas chromatographic (GC) analysis of extracted microbial fatty acid methyl ester (FAMEs) as described by Myron (2006) at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellukulturen GmbH, Germany).

DNA base composition The guanine-plus-cytosine (G+C) content of the DNA was determined by the HPLC method Tamaoka and Komagata (1984). In brief, 10 μl of purified DNA (1 mg/ml) was heated in boiling water for 5 min and immediately cooled on ice. Denatured DNA was then hydrolyzed with 10 μl of nuclease P_1 solution (Appendix A-21.2) at 50ºC for 1 h followed by incubated with 10 μl of alkaline phosphatase solution (Appendix A-21.4) at 37ºC for 1 h. The hydrolyzed DNA was determined using the reversed-phase HPLC on Nakarai Cosmosil packed column $5C_{18}$ (150 X 4.6 mm), eluted with 0.2 M $NH_4P_2PO_4$:acetonitrile (20:1, v/v) at flow rate of 1 ml/min. An equimolar mixture of nucleotides was used as the quantitative standard for analysis of DNA base composition.

3.6 Optimization of the fibrinolytic protease production condition

Inoculum preparation

Bacterium, maintained in 20% glycerol and stored at -80°C, was propagated on modified MLB agar plate and incubated at 37°C. The inoculum was prepared by adding a loop full of pure culture from modified MLB agar plate into 5 ml of seed culture medium (modified MLB broth) in 15 ml test tube and incubated at 37°C on a rotary shaker for 18 h to obtain a seed culture with an absorbance at 600 nm (A_{600}) of 7-8.

Optimization procedure and experimental design

Screening of the optimal nitrogen and carbon sources To investigate the effect of nitrogen sources on fibrinolytic protease production, tryptone and yeast extract in the original medium were replaced with different nitrogen sources at the equivalent nitrogen content. Nitrogen sources and the corresponding concentrations were as follows; tryptone 1.15%, peptone 1.04%, casein 1.13%, skim milk 2.98%, yeast extract 1.52%, meat extract 1.27%, $(NH_4)_2SO_4$ 0.74%, monosodium glutamate

2.15% and gelatin 1.04%. The medium was sterilized by autoclaving at 121°C for 15 min. For protease production, a 9% inoculum from the culture was added to 25 ml medium in 125 ml Erlenmeyer flask. After incubation for 18 h at 37°C under shaking condition, the growth was measured at 600 nm (A_{600}) and the culture was harvested by centrifugation at 17700 x g for 15 min at 4°C. The cell-free extract was used as crude enzyme to measure fibrinolytic activity.

To evaluate the effect of carbon sources, glucose in the original medium was replaced with various carbon sources at the concentration of 2.5%. The carbon sources used were D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, D-sucrose, D-maltose, D-lactose, D-mannitol, glycerol and soluble starch. Erlenmeyer flasks (125 ml) containing 25 ml production medium consisted of the optimal nitrogen source and the evaluated carbon source were inoculated with 9% (v/v) fresh inoculum. Fibrinolytic protease production and microbial growth were determined after 18 h incubation at 37°C.

Optimization of the medium compositions A 3^3 full factorial design for three factors was used to determine the optimal concentrations of nitrogen source, carbon source, and initial medium pH. The dependent variable selected in this study was the enzyme activity, expressed in unit/ml, and growth. Each independent variable was studied at three different coded levels (-1, 0, +1). The corresponding actual values of three coded levels were 0.6, 0.9 and 1.2% for yeast extract, 1.5, 2.0 and 2.5% for sucrose and 5, 7 and 9 for initial medium pH, respectively (Table 8). A series of 27 experiments were performed and their coded forms of independent variables investigated and the full experimental plan are listed in Table 7. Cultures were incubated at 37°C, 200 rpm for 18 h and fibrinolytic protease yield was determined.

Table 8. Values of independent variables at different levels of the 3^3 full factorial design.

Independent variables	Symbol	Levels		
		-1	0	+1
Yeast extract (%)	X_1	0.61	0.91	1.22
Sucrose (%)	X_2	1.5	2.0	2.5
pH	X_3	5	7	9

Optimization of the cultural conditions The effect of inoculum level, incubation temperature and fibrin supplementation were studied using an one-at-a-time strategy. In the investigation of inoculum level, cultivations were carried out with the optimized medium, inoculated with different level of inoculums (1, 5, 10 and 15% (v/v)) and cultured at 37°C, 200 rpm for 18 h. In the screening of optimum incubation temperature, cultivations were carried out with optimized medium inoculated with the optimum level of inoculum and incubated at different temperatures (30, 35, 37, 40, 45 and 50°C) for 18 h at 200 rpm. To investigate the effect of fibrin supplementation, cultivations were carried out with optimized medium supplemented with different level of fibrin (0, 1.24, 2.48 and 3.72% (w/v)) and cultivated at optimum temperature. Fibrinolytic protease production and microbial growth were determined after 18 h incubation.

Time course of fibrinolytic protease production Fibrinolytic protease production and microbial growth were compared in the original and optimized media. Cultivations were conducted at 37°C in a rotatory shaker at 200 rpm up to 54 h. Samples were periodically removed to determine fibrinolytic protease production and microbial growth.

3.7. Purification of fibrinolytic protease

Preparation of crude extract The crude extract was prepared from the cell-free supernatant by adding chilled acetone into the supernatant solution up to a final concentration of 80%. The mixture was allowed to stand at -20°C for 2 h followed by centrifugation, 30 min at 17700 x g. The resultant precipitate was resuspended in a minimal volume of 50 mM Tris-HCl (pH 8.0)

Ion exchange-Fast protein liquid chromatography (FPLC) Ion exchange chromatography (IEX) was performed on FPLC using a Resource Q column (GE Healthcare Bio-Sciences, Uppsala, Sweden) connected with FPLC pump and UV detector. Crude extract was injected into the column after filtration through a 0.22 μM filter to remove debris cells. The proteins were eluted with linear gradient of 0-1 M NaCl at a flow rate of 0.2 ml/min. Eluted proteins were monitored by absorbance at 280 nm and collected in 1 ml aliquots for further analyses. The fibrinolytic activity of each fraction was determined fibrinolytic activity assay and the active fractions were pooled.

3.8. Characterization of fibrinolytic protease

Fibrinolytic activity assay Fibrinolytic activity was measured by the hydrolysis of fibrin clot as described by Kim *et al*. (1997) with some modifications. In brief, 500 µl of 0.6% (w/v) fibrinogen solution in 0.1 M borate buffer, pH 7.0 was mixed with 20 µl of thrombin solution (50 NIH unit/ml thrombin in 0.1 M borate buffer, pH 7.0 containing 0.85% NaCl). The fibrin clot was allowed to form for 5 min at room temperature and then mixed with 380 µl of 20 mM sodium phosphate buffer (pH 7.4). To initiate the enzymatic reaction, 100 µl of enzyme was added and incubated at 37°C for 20 min. The reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and allowed to stand on ice for 10 min. After centrifugation at 4117 x g for 10 min, the resulting oligopeptides in the supernatant were measured by Lowry method (Lowry, 1957) and converted to the amount of tyrosine equivalent. One unit of fibrinolytic activity was defined as 1 nmol of tyrosine released per min. A blank was run in the same manner except that the enzyme was added after the addition of TCA solution.

Proteolytic activity assay Proteolytic activity was measured using various protein substrates such as, bovine fibrinogen, bovine serum albumin, bovine heamoglobin and bovine casein. The reaction mixture, composed of 225 µl of protein substrate (1% (w/v) of protein substrate such as, bovine fibrinogen, bovine serum albumin, bovine hemoglobin, and bovine casein, in 20 mM phosphate buffer, pH 7.4) and 25 µl of fibrinolytic protease, was incubated at 37°C. After 20 min, the reaction was stopped by the addition of 0.25 ml of 10% (w/v) trichloroacetic acid (TCA) and allowed to stand on ice for 10 min. After centrifugation at 4117 x g for 10 min, the resulting polypeptides in the supernatant were measured by Lowry method (1951) and converted to the amount of tyrosine equivalent. One unit of fibrinolytic activity was defined as the amount of enzyme releasing 1 nmol of tyrosine equivalent per min per milliliter.

Amidolytic activity assay Amidolytic activities were measured toward a variety of chromogenic substrates such as, H-D-Val-Leu-Lys-*p*NA (for plasmin), pyro-Glu-Gly-Arg-*p*NA (for urokinase) and H-D-Val-Leu-Arg-*p*NA (for thrombin) according to the method of Peng *et al*. (2005) with slight modification. To initiate the enzymatic reaction, 180 µl of 0.5 mM substrate solution in 20 mM phosphate buffer (pH 7.4) was added to 20 µl of enzyme and then incubated at 37°C for 20 min. The

reaction was stopped by adding 200 μl of 15% acetic acid. The amount of released *p*-nitroaniline was determined at $A_{405\ nm}$ using UV-Vis spectrophotometer. A unit of activity was expressed as 1 nmol of *p*-nitroaniline released/min.

Kinetic studies The activity was assayed with different final concentrations of H-D-Val-Leu-Arg-*p*NA (for thrombin) ranging from 0.1-1 mM. The purified enzyme concentration for the assay was 1 mg/ml. The kinetic constants were graphically determined according to Hanes-Woolf plots using the initial velocity of the reaction. The values of Michaelis-Menten constant (K_m), maximal velocity (V_{max}) and catalysis constant (k_{cat}) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration.

Molecular weight determination

- **Size exclusion FPLC** Size exclusion chromatography (SEC) was performed on FPLC using a Superose 12 10/30 GL column (GE Healthcare Bio-Sciences, Uppsala, Sweden) connected with FPLC pump and UV detector. Purified enzyme was injected into the column after filtration through a 0.22 μM filter. The proteins were eluted with isocratically with 50 mM Tris-HCl containing 0.15 M NaCl, at a flow rate of 0.1 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 purified enzyme, and the void volume (V_o) was estimated by the elution volume of blue dextran (M_r = 2000000). The standard used included bovine serum albumin (M_r = 66000), ovalbumin (M_r = 45000), carbonic anhydrase (M_r = 29000), trypsin inhibitor (M_r = 20100), and α-lactalbumin (M_r = 14200).

- **SDS-Polyacrylamide gel electrophoresis** SDS-PAGE was performed according to the method of Laemmli (1970) (Appendix B-2). Polyacrylamide was prepared for 10% running gel with 4% stacking gel. Purified protein solutions were mixed with 5X sample treatment buffer (0.5 M Tris-HCl, pH 6.8, containing 20% (v/v) glycerol, 20% (v/v) SDS, 0.03% (w/v) bromophenolblue and 10% (w/v) β-mercaptoethanol) at the ratio of 4:1 (v/v), and 20 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 Hoefer SE250 mini-gel unit. After electrophoresis, gel was fixed and stained by silver staining (Heukeshoven and Dernick, 1985). PageRuler^{TM} 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.

Native electrophoresis Native electrophoresis was carried out by leaving out the SDS and reducing agent from the standard Laemmli protocol (1970). Polyacrylamide was prepared for 10% running gel with 4% stacking gel. Purified enzyme was mixed with 5X sample treatment buffer (0.5 M Tris-HCl, pH 6.8, containing 20% (v/v) glycerol) at 4:1 (v/v) ratio, and 20 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 Hoefer SE250 mini-gel unit (Amersham pharmacia biotech, Uppsala, Sweden). After electrophoresis, gel was fixed and stained by silver staining (Heukeshoven and Dernick, 1985).

SDS-fibrin zymography SDS-fibrin zymography gel was prepared as described by Kim *et al*. (1998). Briefly, separating gel solution (10%, w/v) containing fibrinogen (0.6%, w/v) was prepared in a total volume of 10 ml. Thrombin (1 NIH unit/ml) solution was added to the gel solution at a final concentration of 0.1 μunit/ml. Ammonium persulfate (10%, w/v) and *N,N,N´,N´*,-tetramethylethylenediamine were used to catalyze the polymerization. Crude enzyme was mixed with sample buffer (5X) consisting of 0.5 M Tris-HCl, pH 6.8, 20% (w/v) SDS, 20% (w/v) glycerol and 0.03% (w/v) bromophenolblue at 4:1 (v/v) ratio, and 25 μg of proteins were loaded on to the gel. The proteins were subjected to electrophoresis at a constant current of 20 mA per gel by the Hoefer SE250 mini-gel unit (Amersham pharmacia biotech, Uppsala, Sweden). After electrophoresis, gel was immersed in 100 ml of 2.5% (w/w) Triton X-100 solution for 30 min and then immersed in 100 ml of reaction buffer (30 mM Tris, pH 7.4 containing 200 mM NaCl and 0.02% (w/v) NaN_3) for 18 h at 37°C. The gel was fixed and stained with 0.025% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid for 1 h and then destained in destaining solution (30% (v/v) methanol and 10% (v/v) acetic acid). Development of clear zone on blue background indicated fibrinolytic activity. PageRuler^{TM} unstained protein ladder 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.

pH and Temperature profiles Fibrinolytic activity of enzyme was assayed over the pH range of 3-11 in 0.1 M the Britton-Robinson universal buffer (citrate-phosphate-carbonate buffer) (Appendix A-22) (Britton and Robinson, 1931) at 37°C for 20 min. For temperature profile study, the activity was assayed at different temperatures (30, 40, 50, 60 and 70°C) for 20 min under the optimum pH. The relative activity was expressed as a percentage of the highest enzyme activity measured.

pH and temperature stability The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation in 50 mM the Britton-Robinson universal buffer at various pHs (3-11) for 60 min at 37°C. For thermal stability, enzyme was diluted with 0.1 M phosphate buffer, pH 7.4 at a ratio of 1:1 (v/v) and incubated at different temperatures (30, 40, 50, 60 and 70°C) for 60 min in a temperature-controlled water bath. Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed at pH 7.0 and 50°C for 20 min. The relative activity was expressed as a percentage of the activity before incubation.

Inhibition studies The effect of various protease inhibitors was determined by incubating enzyme with an equal volume of inhibitor solution to obtain the final concentration designed (1 mM PMSF, 10 µM leupeptin, 10 µM chymostatin, 10 µM TLCK, 10 µM TPCK, 0.1 mg/ml SBTI, 1 mM EDTA, 10 µM phosphoramidon, 1 µM E-64 and 1 µM Pepstatin A). The mixture was allowed to stand at room temperature for 60 min. Thereafter, the remaining activity was measured by the fibrinolytic activity assay at pH 7.0 and 50°C for 20 min and percent inhibition was calculated. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

The effect of digestive enzymes on fibrinolytic activity

Pepsin solution and the extract of pancreatine and bile were prepared as followed. Pepsin solution was consisted of 2 mg pepsin per milliliter of an electrolyte solution I (53 mM NaCl, 1 mM $CaCl_2$, 14.8 mM KCl and 5.7 mM Na_2CO_3, pH 2.5). The extract of pancreatine and bile was consisted of 0.7 g pancreatine in an electrolyte solution II (21 mM NaCl, 0.5 mM $CaCl_2$ and 2 mM KCl, pH 6.5). The volume of the mixture was adjusted to 10 ml with an electrolyte solution II and subsequently centrifuged at 17700 x g at 4°C for 20 min. To 1 ml of the obtained supernatant, 19

mg of bile was added and the obtained mixture was considered as the extract of pancreatine and bile.

- ***In vitro* stomach model** Freshly prepared pepsin solution was added to 100 unit of fibrinolytic protease and pH was adjusted to 2.5 with 1 N HCl with the final volume of 1 ml. After mixing for 20 s, the reaction mixture was incubated in a shaking water bath at 37°C for 60 min. In the case of the incubation without pepsin, the pepsin solution was replaced by the electrolyte solution I at pH 2.5.

- ***In vitro* intestine model** The extract of pancreatine and bile were added to 100 unit of fibrinolytic protease and the pH was adjusted to 6.5 with 1 M $NaHCO_3$ with the final volume of 1 ml. After mixing for 20 s, the reaction mixture was incubated in a shaking water bath at 37°C for 60 min. In the case of the incubation without pancreatin and bile, the pancreatin and bile solution was replaced by the electrolyte solution II at pH 6.5. After all types of *in vitro* incubations, the reaction mixture was kept on ice and was used to evaluate the remaining fibrinolytic activity.

Analysis of degradation products of fibrin Briefly, 125 µl of 0.6% bovine fibrinogen solution in 20 mM phosphate buffer (pH 7.4) was mixed with 5 µl of thrombin solution (50 NIH unit/ml) in the same buffer. The fibrin clot was allowed to stand for 5 min at room temperature. A total of 25 µl of purified fibrinolytic protease was mixed with the clot and incubated at 37°C for various time intervals. The resulting peptides were analysed using SDS-PAGE on a 12% gel (Leammli, 1970).

Analysis of degradation products of fibrinogen Briefly, 130 µl of 0.6% (w/v) fibrinogen in 20 mM phosphate buffer (pH 7.4) was incubated with 25 µl of purified enzyme at 37°C for various time intervals. The resulting peptides were analyzed using SDS-PAGE on a 12% gel (Leammli, 1970).

Fibrin and Fibrinogen agarose plate assay Fibrin plate assay was performed according to the method of Astrup and Mullertz (1952). In brief, 5 ml of 2% (w/v) agarose warmed to 50°C was mixed with 5 ml of 0.6% (w/v) fibrinogen dissolved in 50 mM phosphate buffer pH 7.4 warmed to 45°C and 20 µl of thrombin solution (10 NIH unit/ml) and poured into a Petri dish. The dish was left at room temperature for 30 min to form a fibrin clot layer. Nine holes (3 mm in diameter) were punched for sample application on the fibrin plate. Ten microliter of enzyme was placed in each hole and incubated at 37°C for 18 h. Following the incubation period, the radius of

clear zone around the hole was measured. Human plasmin was used as the standard fibrinolytic protease.

Fibrinogen-agarose plate assay was performed according to the method of Joo *et al*. (2002) with some modifications. Five milliliter of 2% agarose warmed to 50°C was mix with 5 ml of 0.6% (w/v) fibrinogen dissolved in 20 mM phosphate buffer, pH 7.4, at 45°C. Fibrinogen agarose mixture was poured into a plate, and allowed to solidify for 30 min at room temperature. Nine holes (3 mm in diameter) were punched for sample application on the fibrin plate. Ten microliters of enzyme was placed in each hole and incubated at 37°C for 18 h. Following the incubation period, the diameters of the turbid fibrin rings formed around the hole due to the polymerization of fibrinogen, were measured. Thrombin was used as the positive control.

3.9 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 and repeated twice. The average values of the result were presented, in which the standard deviation was within 10%. Statistical significance was assigned at 95% of confidence level.

CHAPTER IV

RESULTS

4.1 Isolation, screening and identification of fibrinolytic protease producing bacteria

Twenty kinds of traditional fermented food product were collected from local market in Thailand and bacteria were isolated by diluted plating technique on modified Luria-Bertani (MLB) medium (Table 9). From the result, all of the strains exhibited typical characteristics of *Bacillus* sp., namely, Gram-positive rod, facultative aerobic, catalase-positive reaction and grew in 5% NaCl.

Primary screening

Initially, 163 isolates were screened for their fibrinolytic activity using fibrin plate assay. One hundred and forty-one isolates showed fibrinolytic activity by ranging from 0.1 – 9.3 mm in radius of clear zone (Table 9). Overall the result in Table 9, 71 isolates from fermented fishery products and 68 isolates from fermented soybean products showed fibrinolytic activity while, 1 isolate from fermented fruits and 1 isolate from vegetable products showed fibrinolytic activity.

Table 9. Fibrinolytic protease producing bacteria isolated from fermented foods produced in Thailand.

Products	Samples	Province	No. of bacteria
Fishery products			
Fish	Bu-du (B-A)	Pattani	-
	Pla-chao (PG-A)	Ayutthaya	3
	Pla-chom (PJ-A)	Ayutthaya	7
	Tai-plaa (T-A)	Chumphon	3
	Tai-plaa (T-B)	Chumphon	1
	Tai-plaa (T-C)	Chumphon	2
	Tai-plaa (T-D)	Ayutthaya	1
Shrimp	Ka-pi (K-A)	Surat Thani	8
	Ka-pi (K-B)	Samut Prakan	11
	Ka-pi (K-C)	Samut Prakan	13
	Ka-pi (K-D)	Samut Sakhon	6
	Kung-chom (KG-A)	Ayutthaya	5
Others	Hoi-ma-laeng-poo-dorng (HD-A)	Nakhon Pathom	1
	Poo-khem (PD-A)	Nakhon Pathom	10
Soybean products	Tao-hoo-yee (THY-A)	Bangkok	8
	Tao-hoo-yee (THY-B)	Ayutthaya	7
	Tao-hoo-yee (THY-C)	Ayutthaya	3
	Tao-jieo (TJ-A)	Bangkok	9
	Tao-jieo (TJ-B)	Ayutthaya	3
	Tao-jieo (TJ-C)	Ayutthaya	8
	Tao-jieo-khaw (TJW-A)	Ayutthaya	9
	Tooa-nao (BCC, TISTR)	Chiang Mai	2, 19
Vegetable products	Khing-dorng (KD-A)	Nakhon Pathom	-
	Kra-tiam-dorng (KT-A)	Ayutthaya	-
	Nor-mai-dorng (NM-A)	Ayutthaya	1
	Pak-kaat-dorng (PK-A)	Ayutthaya	-
	Sa-tor-dorng (ST-A)	Surat Thani	-
	Tooa-ngork-dorng (TN-A)	Nakhon Pathom	-
Fruit product	Ma-nao-dorng (MN-A)	Ayutthaya	1
Rice product	Khao-maak (KM-A)	Ayutthaya	-
		Total	141

Twenty-one isolates with strong fibrinolytic activity, ranging from 7.0 – 9.3 mm in radius of clear zone on fibrin plate, were selected for the further screening (Figure 8). It was noted that in 21 isolates with strong activity, 11 isolates were from fermented soybean products while the other 10 strains were from fermented fishery products.

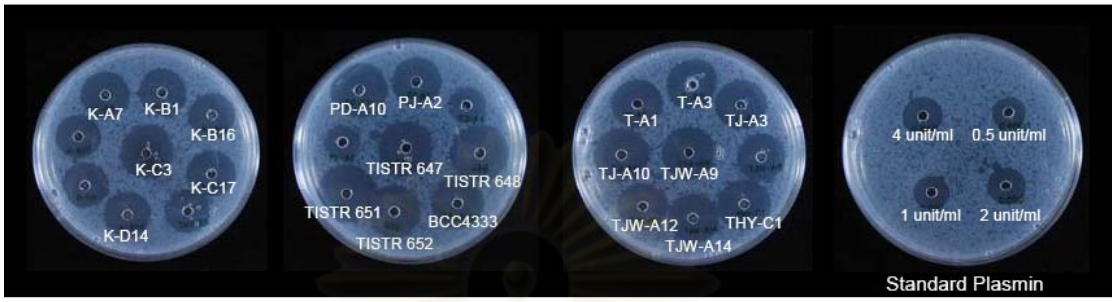


Figure 8. Fibrinolytic activity of 21 isolates that showed the strong fibrinolytic activity on fibrin plate compared with standard plasmin. Various concentrations of standard human plasmin were tested (0.5, 1, 2 and 4 unit/ml).

Identification of isolates

All isolates of fibrinolytic protease producing bacteria were characterized as Gram-positive, spore-forming, rod-shaped bacteria that are the common characteristic of *Bacillus*. However, there were significant variations in colony morphology.

ITS-PCR fingerprinting was used to allow typing mainly at species level and four groups of bacteria were observed (Figure 9 and Table 10). Group I was characterized by three constant DNA bands and comprised the reference isolates of *B. subtilis* subsp *subtilis* KCTC 3135^T, *B. licheniformis* KCTC 1918^T and *B. mojavensis* KCTC 3706^T and 12 isolates. Group II was very similar to Group I and was characterized by four constant bands. This group comprised of the reference isolates of *B. vallismortis* KCTC 3707^T and 3 isolates. Group III was characterized by six constant DNA bands and comprised of the reference isolates of *B. amyloliquefaciens* KCTC 1660^T and *B. velezensis* KCTC 13012^T and 4 isolates. Group IV was characterized by seven constant DNA bands. They showed different pattern from the reference isolates and comprised 2 of isolates.

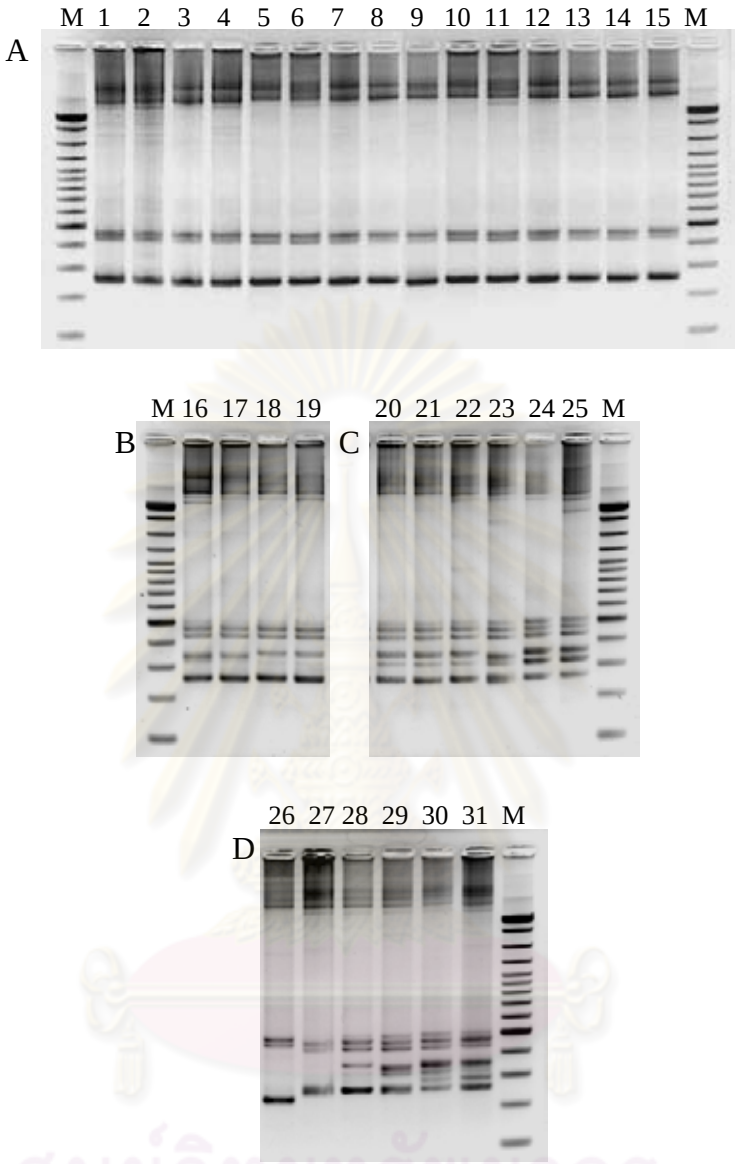


Figure 9. Intergenic transcribed spacer-PCR profiles of 21 fibrinolytic protease-producing bacteria. A: Group I of isolates; B: Group II of isolates; C: Group III of isolates; D: Group IV of isolates. Lane 1 and 27: *B. subtilis* subsp *subtilis* KCTC 3135^{T}; 2: *B. licheniformis* KCTC 1918^{T}; 3: *B. mojavensis* KCTC 3706^{T}; 4: TISTR 647; 5: TISTR 648; 6: TISTR 651; 7: TISTR 652; 8: BCC 4333; 9: K-C3; 10: K-C17; 11: K-D14; 12: T-A1; 13: T-A3; 14: PD-A10; 15: PJ-A2; 16 and 28: *B. vallismortis* KCTC 3707^{T}; 17: TJ-A3; 18: TJW-A9; 19: TJW-A12; 20 and 29: *B. amyloliquefaciens* KCTC 1660^{T}; 21: *B. velezensis* KCTC 13012^{T}; 22: TJ-A10; 23: K-B1; 24: TJW-A14; 25: THY-C1 ; 26: *B. cereus*; 30: K-A7; 31: K-B16.

Table 10. Identification of isolates based on ITS-PCR pattern.

ITS-PCR pattern type	Signature bands (bp)	Isolate no.	Identification by ITS-PCR similarity
1	260, 430, 450	*B. subtilis* KCTC 3135^T	
		B. licheniformis KCTC 1918^T	
		B. mojavensis KCTC 3706^T	
		TISTR 647	*B. subtilis*
		TISTR 648	*B. subtilis*
		TISTR 651	*B. subtilis*
		TISTR 652	*B. subtilis*
		BCC 4333	*B. subtilis*
		K-C3	*B. subtilis*
		K-C17	*B. subtilis*
		K-D14	*B. subtilis*
		T-A1	*B. subtilis*
		T-A3	*B. subtilis*
		PD-A10	*B. subtilis*
		PJ-A2	*B. subtilis*
2	260, 330, 430, 450	*B. vallismortis* KCTC 3707^T	
		TJ-A3	*B. vallismortis*
		TJW-A9	*B. vallismortis*
		TJW-A12	*B. vallismortis*
3	260, 320, 330, 430, 450, 510	*B. amyloliquefaciens* KCTC 1660^T	
		B. velezensis KCTC 13012^T	
		TJ-A10	*B. amyloliquefaciens*
		K-B1	*B. amyloliquefaciens*
		TJW-A14	*B. amyloliquefaciens*
		THY-C1	*B. amyloliquefaciens*
4	260, 280, 320, 330, 430, 450, 510	K-A7	Unidentified
		K-B16	Unidentified

Group I of isolate

Group I comprised of 12 isolates, namely, TISTR 647, TISTR 648, TISTR 651, TISTR 652, BCC 4333, K-C3, K-C17, K-D14, T-A1, T-A3, PD-A10 and PJ-A2 and the reference isolates of *B. subtilis* subsp *subtilis* KCTC 3135^T, *B. licheniformis* KCTC 1918^T and *B. mojavensis* KCTC 3706^T. All strains showed positive reactions to catalase and hydrolyzed casein, starch and gelatin. They showed negative reaction to MR and indole. They were grown in the presence of 0-10% NaCl, at 25-50ºC and pH 5-9. No growth was observed at pH 3.5-4.0 and in the presence of 20% NaCl. Hydrolyzed tween-80 and L-tyrosine were negative in all strains. Acids from glycerol, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. Different characteristics were shown in Table 11. In addition, the enzymatic profile was performed. All isolates in Group I showed positive reaction for alkaline phosphatase, esterase (C4), esterase lipase (C8) and Naphthol-AS-BI-phosphohydrolase. They showed negative reaction for lipase, valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The different enzymatic reactions were shown in Table 11.

Table 11. Characteristics of Group I isolate and type strains.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Nitrate reduction	-	-	-	-	+	+	+	+	+	+	+	+	+
VP	-	-	+	+	+	-	+	-	+	+	+	+	+
Citrate	-	-	-	-	-	+	+	+	+	-	-	-	+
Oxidase	+	-	+	-	+	+	-	-	+	-	-	+	+
Growth in:													
12% NaCl	+	+	+	+	+	-	+	+	+	+	+	+	-
15% NaCl	+	+	+	+	+	-	+	+	+	-	+	+	-
Growth at:													
pH 10	+	+	+	+	+	+	+	+	+	+	-	+	+
55ºC	-	+	+	+	-	-	-	+	+	-	+	-	+
Acid from:													
D-Xylose	+	-	+	-	+	+	+	-	+	+	-	+	+
D-Galactose	+	+	-	+	-	-	-	-	-	-	-	-	-
D-Mannose	+	-	+	-	-	+	+	-	+	+	-	+	+
L-Rhamnose	-	+	-	-	-	-	-	-	-	-	+	-	-

1, TISTR 647 ; 2, TISTR 648; 3, TISTR 651 ; 4, TISTR 652; 5, BCC 4333 ; 6, K-C3 ; 7, K-C17 ; 8, K-D14 ; 9, T-A1 ; 10, T-A3 ; 11, PD-A10 ; 12, PJ-A2 ; 13, *B. subtilis* subsp *subtilis* KCTC 3135^{T}.

+, Positive reaction; -, negative reaction

Table 11 (cont). Characteristics of Group I isolate and type strains.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Inositol	-	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	+	+	+	+	+	+	+	+	+	+
Methyl-αD-glucopyranoside	+	+	-	-	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	+	+	+	+	-	-	+	+	+
Arbutin	-	-	-	-	+	+	+	+	-	-	+	+	+
Salicin	-	-	-	-	+	+	+	+	+	+	+	+	+
D-Cellobiose	-	-	-	+	+	+	+	+	+	+	+	+	+
D-Maltose	-	-	-	+	+	+	+	+	+	+	+	+	+
D-Lactose	-	-	-	-	+	+	-	-	+	+	+	+	-
D-Melibiose	-	+	+	-	-	+	+	+	+	+	-	+	-
D-Trehalose	+	-	+	+	-	-	+	+	+	+	-	+	+
Inulin	+	+	+	+	-	+	-	+	-	-	-	-	+
D-Raffinose	-	+	+	-	-	+	+	+	+	+	+	+	+
Amidon	-	+	+	+	+	+	+	+	+	+	+	+	+

1, TISTR 647 ; 2, TISTR 648; 3, TISTR 651 ; 4, TISTR 652; 5, BCC 4333 ; 6, K-C3 ; 7, K-C17 ; 8, K-D14 ; 9, T-A1 ; 10, T-A3 ; 11, PD-A10 ; 12, PJ-A2 ; 13, *B. subtilis* subsp *subtilis* KCTC 3135^{T}.

+, Positive reaction; -, negative reaction

Table 11 (cont). Characteristics of Group I isolate and type strains.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Glycogen	-	+	+	-	+	+	+	+	+	+	+	+	+
Gentibiose	-	-	+	-	-	+	-	-	-	+	+	+	+
D-Turanose	-	+	-	+	-	+	+	+	+	-	-	-	-
Leucine arylamidase	-	-	-	-	-	-	-	+	+	+	-	-	-
Trypsin	-	-	-	-	+	-	-	-	-	-	-	-	-
α-Chymotrypsin	-	-	-	+	+	-	-	+	-	-	-	-	-
Acid phosphatase	-	-	-	-	+	+	-	+	+	+	-	-	-
α-Glucosidase	+	+	+	+	-	-	+	+	+	+	-	-	-
β-Glucosidase	-	-	-	-	-	-	-	-	+	+	-	-	-

1, TISTR 647 ; 2, TISTR 648; 3, TISTR 651 ; 4, TISTR 652; 5, BCC 4333 ; 6, K-C3 ; 7, K-C17 ; 8, K-D14 ; 9, T-A1 ; 10, T-A3 ; 11, PD-A10 ; 12, PJ-A2 ; 13, *B. subtilis* subsp *subtilis* KCTC 3135^{T}.

+, Positive reaction; -, negative reaction

Group II of isolate

Group II comprised of 3 isolates, namely, TJ-A3, TJW-A9 and TJW-A12 and the reference isolates of *B. vallismortis* KCTC 3707^T. All strains showed positive reactions to nitrate reduction, VP, catalase and hydrolyzed casein, starch and gelatin. They showed negative reaction to MR and citrate. They were growth in the presence of 0-10% NaCl, at 25-50ºC and pH 5-10. No growth was observed at pH 3.5-4.0 and in the presence of 20% NaCl. Hydrolyzed tween-80 and L-tyrosine were negative in all strains. Acids from glycerol, L-arabinose, D-ribose, D-xylose, D-trehalose, D-mannose, inositol, D-sorbitol, methyl-β-D-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, inulin, L-rhamnose, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. Different characteristics were showen in Table 12. In addition, there enzymatic profile was performed. All isolates in Group II showed positive reaction for alkaline phosphatase, esterase (C4), esterase lipase (C8) and Naphthol-AS-BI-phosphohydrolase. They showed negative reaction for lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The different enzymatic reactions were showed in Table 12.

Table 12. Characteristics of Group II isolate and type strains.

Characteristics	1	2	3	4
Indole	+	-	-	+
Oxidase	+	-	+	+
Growth in:				
12% NaCl	+	+	+	-
15% NaCl	+	+	+	-
Growth at:				
55^{o}C	-	-	+	+
Acids from:				
D-Galactose	-	-	+	-
D-Melibiose	+	+	+	-
D-Raffinose	-	+	+	-
Amidon	-	-	-	+
Glycogen	-	+	+	+
Gentibiose	-	+	-	+
D-Turanose	-	-	+	-
D-Lactose	+	+	+	-
Acid phosphatase	+	-	-	ND
α-Glucosidase	+	-	-	ND

1, TJ-A3; 2, TJW-A9; 3, TJW-A12; 4, *B.vallismortis* KCTC 3707^{T}.

+, Positive reaction; -, negative reaction; ND, Not determined.

Group III of isolate

Group III comprised of 4 isolates, namely, TJ-A10, K-B1, TJW-A14 and THY-C1 and the reference isolates of *B. amyloliquefaciens* KCTC 1660^{T} and *B. velezensis* KCTC 13012^{T}. All strains showed positive reactions to nitrate reduction, catalase and hydrolyzed casein, starch and gelatin. They showed negative reaction to MR and indole. They were growth in the presence of 0-10% NaCl, at 25-40ºC and pH 4.5-10. No growth was observed at pH 3.5-4.0 and in the presence of 20% NaCl. Hydrolyzed tween-80 and L-tyrosine were negative in all strains. Acids from glycerol, L-arabinose, D-ribose, D-xylose, D-mannose, inositol, D-sorbitol, methyl-β-D-glucopyranoside, salicin, D-cellobiose, D-maltose, D-melibiose, D-trehalose, D-raffinose, amidon, glycogen, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while L-rhamnose, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. Different characteristics were shown in Table 13. There enzymatic profile was performed by API ZYM. All isolates in Group III showed positive reaction for alkaline phosphatase and Naphthol-AS-BI-phosphohydrolase. They showed negative reaction for lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The different enzymatic reactions were shown in Table 13.

Table 13. Characteristics of Group III isolate and type strains.

Characteristics	1	2	3	4	5
VP	-	-	-	+	+
Citrate	+	+	+	-	+
Oxidase	+	+	+	-	+
Growth in:					
12% (w/v) NaCl	-	+	+	+	-
15% (w/v) NaCl	-	-	-	+	-
Growth at:					
50^{o}C	-	+	-	+	+
55^{o}C	-	-	-	+	-
Acids from:					
L-Rhamnose	+	-	-	+	+
Amygdalin	+	+	-	-	+
Arbutin	+	+	+	-	+
D-Lactose	-	+	+	+	-
Inulin	-	-	-	+	+
Gentibiose	+	-	-	-	-
D-Turanose	+	+	-	+	-
D-Tagatose	+	-	-	-	-
Esterase	-	+	+	+	ND
Esterase lipase	-	+	+	+	ND
Leucine arylamidase	+	-	-	-	ND
α-Chymotrypsin	+	+	-	-	ND
Acid phosphatase	-	+	+	+	ND
α-Glucosidase	-	+	-	+	ND
β-Glucosidase	-	+	-	-	ND

1, TJ-A10; 2, K-B1; 3, TJW-A14; 4, THY-C1; 5, *B.amyloliquifaciens* KCTC 1660^{T}.

+, Positive reaction; -, negative reaction; ND, Not determined.

Group IV of isolate

Group IV comprised of 2 isolates, namely, K-A7 and K-B16. They showed different ITS-PCR pattern from the reference isolates. All strains showed positive reactions to nitrate reduction, VP, citrate, catalase and hydrolyzed casein, starch and gelatin. They showed negative reaction to MR and indole. They were grown in the presence of 0-10% NaCl, at 25-45ºC and pH 4.5-10. No growth was observed at pH 3.5-4.0 and in the presence of 20% NaCl. Hydrolyzed tween-80 and L-tyrosine were negative in all strains. Acids from glycerol, L-arabinose, D-ribose, D-xylose, D-mannose, inositol, D-sorbitol, methyl-β-D-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-trehalose, amidon, glycogen, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while L-rhamnose, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. Different characteristics were shown in Table 14. In addition, there enzymatic profile was performed. Both isolate showed positive reaction for Naphthol-AS-BI-phosphohydrolase. They showed negative reaction for lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The different enzymatic reactions were shown in Table 14.

Table 14. Characteristics of Group IV isolate and type strains.

Biochemical characteristic	1	2	3	4	5	6
Oxidase	+	-	+	+	+	+
Growth in:						
12% NaCl	+	+	-	+	-	-
15% NaCl	+	-	-	-	-	-
Growth at:						
50^{o}C	+	+	+	+	-	+
55^{o}C	-	-	+	+	-	-
Acids from:						
D-Galactose	-	-	-	+	-	-
D-Lactose	-	+	-	-	-	-
D-Melibiose	+	+	-	-	-	-
Inulin	+	-	+	-	+	+
D-Raffinose	+	+	+	-	+	+
Gentibiose	+	-	-	+	+	+
D-Turanose	+	-	+	+	-	-
Alkaline phosphatase	+	-	ND	ND	ND	ND
Esterase	-	+	ND	ND	ND	ND
Esterase lipase	-	+	ND	ND	ND	ND
Leucine arylamidase	+	-	ND	ND	ND	ND
Acid phosphatase	+	-	ND	ND	ND	ND
α-Glucosidase	+	-	ND	ND	ND	ND

1, KA7; 2, K-B16; 3, *B.subtilis* subsp *subtilis* KCTC 3135^{T} ; 4, *B. licheniformis* KCTC 1918T ; 5, *B. amyloliquifaciens* KCTC 1660^{T} ; 6, *B.velezensis* KCTC 13012^{T}.
+, Positive reaction; -, negative reaction; ND, Not determined.

Secondary screening

Later, fibrin zymography was performed to analyze the pattern of extracellular fibrinolytic protease (EFPs) from crude culture of bacteria. According to the results, several EFPs were secreted from the isolates, at least 5 EFPs with approximate molecular sizes of 20 - >200 kDa. Therefore, fibrinolytic protease producing bacteria could be divided into 6 groups based on enzymatic pattern (Figure 10 and Table 15).

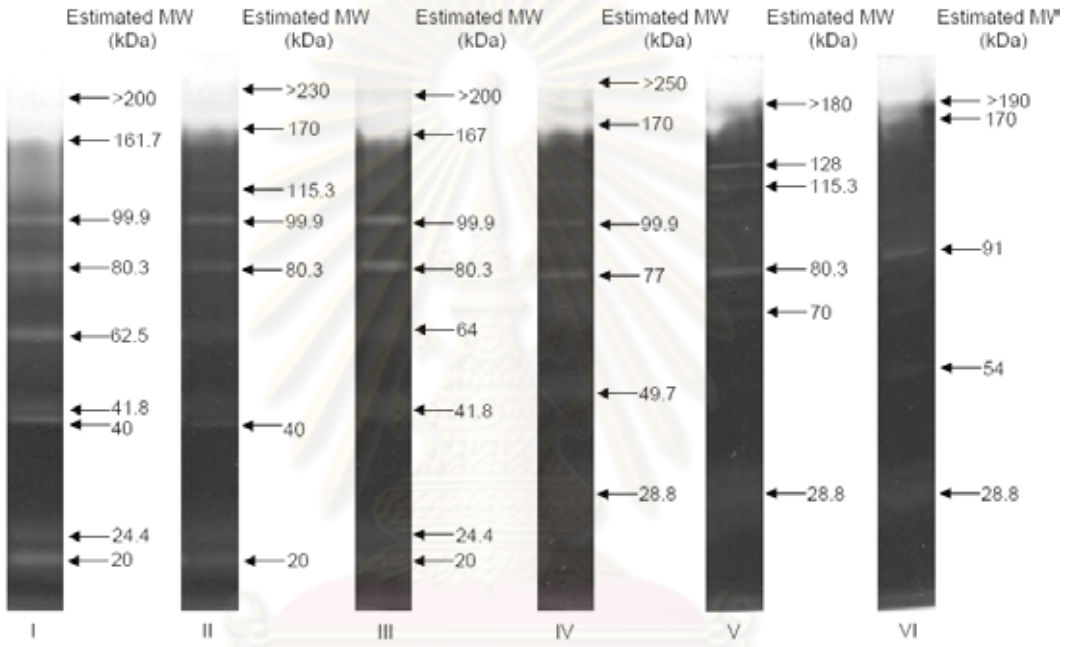


Figure 10. Enzymatic pattern of culture supernatant on fibrin zymogram. White bands on each lane represent hydrolysed fibrin band.

Table 15. Grouping of fibrinolytic protease producing bacteria based on enzymatic pattern on fibrin zymography

Group	No. of band	MW (kDa)	Isolate
I	9	>200, 161.7, 99.9, 80.3, 62.5, 41.8, 40, 24.4, 20	THY-C1, TJW-A14, TJ-A3
II	7	>230, 170, 115.3, 99.9, 80.3, 40, 20	TISTR 651, TISTR 647, TISTR 648, TISTR 652
III	8	>200, 167, 99.9, 80.3, 64, 41.8, 24.4, 20	TJW-A9, TJ-A10, TJW-A12, BCC 4333, K-B1
IV	6	>250, 170, 99.9, 77, 49.7, 28.8	K-B16, T-A3
V	6	>180, 128, 115.3, 80.3, 70, 28.8	PD-A10, K-C17, K-D14, T-A1,
VI	5	>190, 170, 91, 54, 28.8	K-A7, K-C3, PJ-A2

Furthermore, fibrinolytic activity of 21 selected isolates was investigated by the hydrolysis of fibrin clot assay. It was found that fibrinolytic activity of the enzyme assayed by the hydrolysis of fibrin clot, ranging from 4.7-11.1 unit/ml as shown in Table 16, was correlative with the radius of clear zone on fibrin plate. Standard plasmin was also performed and 1 unit/ml of plasmin was corresponded to 3.14 unit/ml of fibrinolytic activity in this study.

Table 16. Fibrinolytic activity of 21 isolates determined by fibrin plate and the hydrolysis of fibrin clot.

Isolate	Radius of clear zone on fibrin plate (mm)	Fibrinolytic activity[a] (unit/ml)
TISTR 651	9.25 ± 0.10	10.34 ± 0.46
TISTR 647	8.75 ± 0.31	9.86 ± 0.23
THY-C1	8.45 ± 0.34	11.09 ± 0.61
TJW-A9	8.43 ± 0.25	9.32 ± 0.00
K-A7	8.35 ± 0.24	7.22 ± 0.08
TJ-A10	8.30 ± 0.28	8.35 ± 0.23
PD-A10	8.08 ± 0.26	7.06 ± 0.61
K-B16	8.05 ± 0.17	8.08 ± 0.46
TISTR 648	8.03 ± 0.33	7.22 ± 0.08
TJ-A3	7.95 ± 0.26	6.41 ± 0.08
TJW-A14	7.78 ± 0.05	6.95 ± 0.46
T-A1	7.60 ± 0.18	5.88 ± 0.46
K-C3	7.48 ± 0.33	5.49 ± 0.31
TISTR 652	7.48 ± 0.13	5.88 ± 0.46
K-C17	7.43 ± 0.13	5.58 ± 0.08
TJW-A12	7.38 ± 0.25	5.98 ± 0.46
BCC 4333	7.38 ± 0.30	5.71 ± 0.00
K-B1	7.35 ± 0.37	5.22 ± 0.08
K-D14	7.20 ± 0.26	5.50 ± 0.08
PJ-A2	7.20 ± 0.22	5.71 ± 0.00
T-A3	7.05 ± 0.37	4.74 ± 0.46

[a]One unit of fibrinolytic activity was defined as 1 nmol of tyrosine released per min.

For further study, six representative strains, THY-C1, TISTR 651, TJW-A9, K-A7, K-B16 and BCC 4333, that showed the highest fibrinolytic activity in each group of enzymatic pattern were selected and subjected to 16S rRNA gene sequencing and phylogenetic analysis (Figure 11). Almost complete 16S rRNA gene sequence of representative bacteria determined in this study contained about 1500 nucleotide positions. Eliminating ambiguous and unalignable bases, 1412 positions were compared in the phylogenetic revealed that the representative isolates were included in a cluster of *Bacillus subtilis* group and distinguish separate from a cluster of *Bacillus cereus* group.

Moreover, 16S rRNA gene sequence similarity of THY-C1, TISTR 651, K-B16, K-A7, PD-A10 and TJW-A9 with the related taxa were evaluated (Table 17). From the result of 16S rRNA gene sequence analysis, isolate THY-C1 was closely related to isolate TISTR 651. In addition, isolate THY-C1 showed levels of 16S rDNA similarities of 98.99% to isolate TISTR 651. The close relationship between isolates THY-C1 and TISTR 651 was supported by high bootstrap values (>96%). However, isolate THY-C1 showed levels of 16S rDNA similarities of 80.6-98.99% to members of the genera *Bacillus*, while isolate TISTR 651 showed levels of 16S rDNA similarities of 81.59-99.50% to members of the genera *Bacillus* (Table 17). Furthermore, the result of 16S rRNA gene sequence analysis found that isolate PD-A10, TJW-A9, K-A7 and K-B16 were closely related to each other and the level of 16S rDNA similarities were 98.99-99.35% (Table 17). The close relationship between isolates K-B16, K-A7, PD-A10 and TJW-A9 were supported by high bootstrap values of more than 96%. Isolate PD-A10, TJW-A9, K-A7 and K-B16 showed levels of 16S rDNA similarities of 80.93-99.28%, 81.49-99.79%, 81.39-99.50% and 81.67-99.64% to members of the genera *Bacillus*, respectively (Table 17).

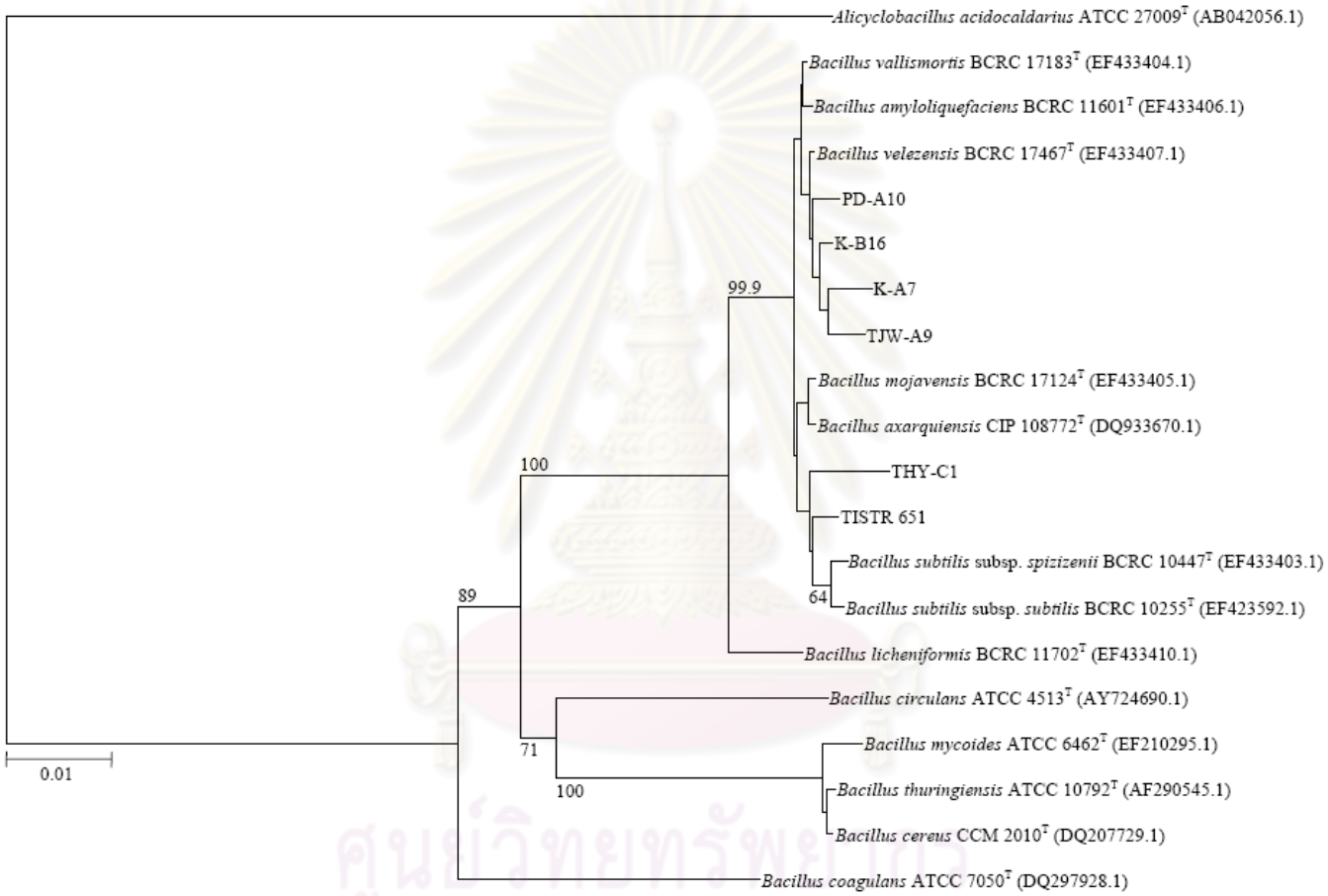


Figure 11. Neighbour-joining-tree showing the phylogenetic position of THY-C1, TISTR 651, PD-A10, K-B16, K-A7 and TJW-A9 and related taxa based on 16S rRNA gene sequences. Bootstrap values are given for each node having 60% or greater support. Bar = 0.01 nucleotide substitution per site.

Table 17. Percentages similarities of THY-C1, TISTR 651, PD-A10, K-B16, K-A7 and TJW-A9 and related taxa

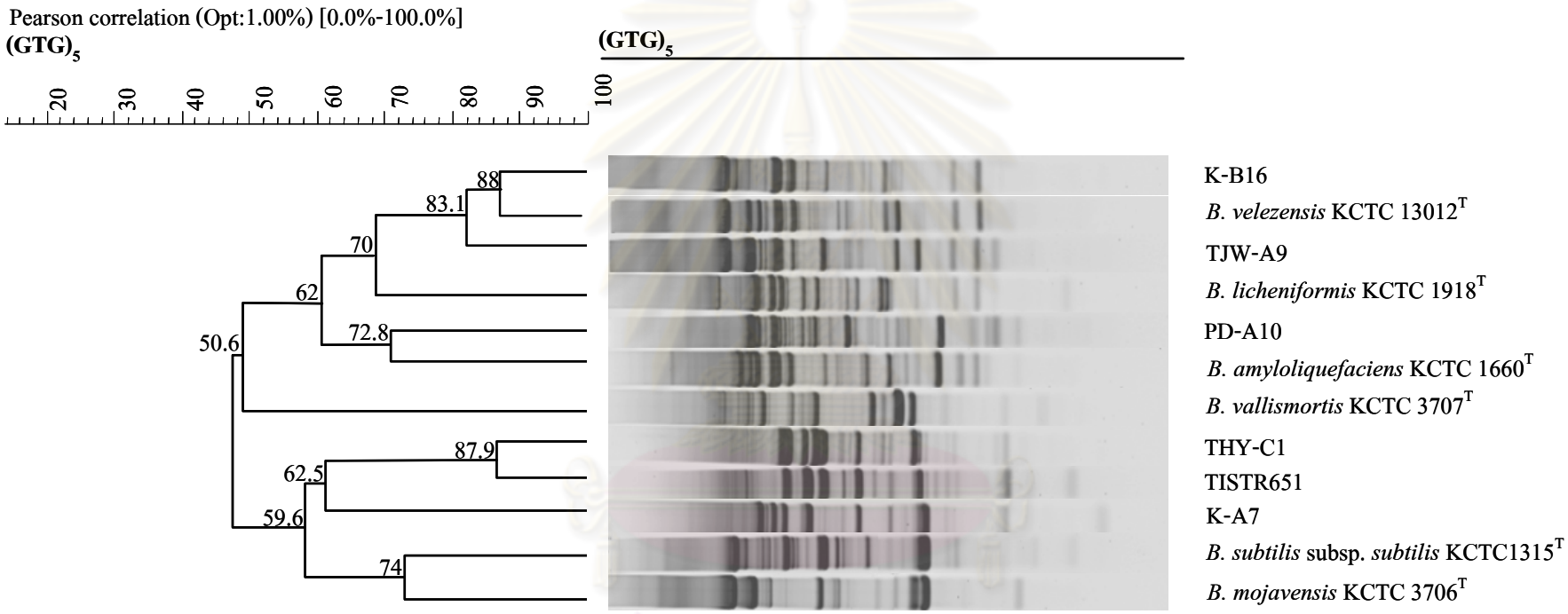
	B. mycoides	*B. cereus*	*B. thuringensis*	*B. circulans*	*B. licheniformis*	*B. axarquiensis*	*B. subtilis subsp. subtilis*	THY-C1	*B. subtilis subsp. spizizen*	TISTR651	*B. mojavensis*	PD-A10	TJW-A9	K-A7	*B. velezensis*	K-B16	*B. vallismortis*	*B. amyloliquefaciens*	*B. coagulans*	*A. acidocadarius*
B. mycoides	100																			
B. cereus	99.50	100																		
B. thuringensis	99.50	99.86	100																	
B. circulans	94.36	94.59	94.43	100																
B. licheniformis	93.59	93.97	93.97	93.94	100															
B. axarquiensis	93.51	93.89	93.89	94.09	99.57	100														
B. subtilis subsp. subtilis	93.35	93.74	93.74	93.78	99.50	99.50	100													
THY-C1	92.63	93.02	93.02	93.14	98.99	98.85	98.92	100												
B. subtilis subsp. spizizen	93.27	93.66	93.66	93.86	99.36	99.79	99.71	98.78	100											
TISTR651	93.43	93.66	93.66	93.86	99.43	99.43	99.50	98.99	99.36	100										
B. mojavensis	93.66	93.89	93.89	94.01	99.71	99.57	99.36	98.85	99.36	99.43	100									
PD-A10	93.35	93.58	93.58	93.70	99.64	99.21	99.14	98.63	98.99	99.36	99.64	100								
TJW-A9	93.12	93.35	93.35	93.47	99.43	99.00	98.92	98.56	98.78	99.14	99.28	99.35	100							
K-A7	93.20	93.58	93.58	93.47	99.21	99.21	99.00	98.49	98.99	99.07	99.07	98.99	99.21	100						
B. velezensis	93.66	94.05	94.05	94.02	99.86	99.71	99.50	98.99	99.50	99.43	99.86	99.50	99.28	99.21	100					
K-B16	93.35	93.74	93.74	93.70	99.79	99.36	99.28	98.92	99.14	99.36	99.50	99.57	99.50	99.28	99.64	100				
B. vallismortis	93.66	94.05	94.05	94.25	99.57	99.86	99.50	98.85	99.64	99.43	99.57	99.21	99.00	99.07	99.71	99.36	100			
B. amyloliquefaciens	93.66	93.89	93.89	94.10	98.34	98.49	98.27	97.60	98.27	98.34	98.63	98.27	97.90	97.97	98.49	98.12	98.49	100		
B. coagulans	92.65	93.04	92.88	93.27	93.48	93.32	93.24	92.52	93.08	93.17	93.32	93.08	92.85	93.08	93.48	93.24	93.32	93.40	100	
A. acidocadarius	81.69	81.59	81.50	81.89	81.78	81.68	81.69	80.60	81.59	81.59	81.77	81.67	81.39	80.93	81.59	81.49	81.68	82.27	82.53	100

$(GTG)_5$-PCR fingerprinting has been found to be a promising genotypic tool for rapid and reliable speciation and typing of lactobacilli (Gevers *et al*., 2001) and enterococci (Svec *et al*., 2005). In this study, $(GTG)_5$-PCR fingerprinting was evaluated for *Bacillus*, using reference strains of *Bacillus* sp. and the isolate strains. The result showed that all isolates belong to the genus *Bacillus* (Figure 12).

The $(GTG)_5$-PCR patterns resulted in the delineation of two $(GTG)_5$-PCR clusters at a 50.6% Pearson's correlation coefficient, comprising seven and five isolates. Within the first cluster, the $(GTG)_5$ patterns resulted in the delineation at 62, 70, 72.8, 83.1 and 88% Pearson's correlation coefficient. While the second cluster, the $(GTG)_5$ patterns resulted in the delineation at 59.6, 62.5, 74 and 87.9% Pearson's correlation coefficient. The results indicated that they were different genotypically and belong to the different species.

Moreover, isolate THY-C1 was closely related to isolate TISTR 651, isolate K-A7 and the type strains of *B. subtilis* subsp. *subtilis* KCTC 3135^T and *B. mojavensis* KCTC 3706^T. While isolate K-B16 was closely related to the type strain of isolate TJW-A9, *B. velezensis* KCTC 13012^T and *B. licheniformis* KCTC 1918^T, isolate PD-A10 was closely related to the type strain of *B. amyloliquefaciens* KCTC 1660^T. In addition, it was noted that cluster analysis result of rep-PCR was similar to the result of 16S rRNA gene sequence analysis.

Figure 12. Cluster analysis of rep-PCR of the representative strains and other *Bacillus* species.



Furthermore, the DNA-DNA relatedness was assessed between isolates and the reference strain of *Bacillus* (Table 18). It was found that isolate THY-C1 exhibited low levels of DNA-DNA relatedness (<70%) to the reference strains of *B. subtilis* and *B. amyloliquefaciens*, while isolate PD-A10 and K-7 exhibited low levels of DNA-DNA relatedness (< 70%) to the reference strains of *B. subtilis, B. amyloliquefaciens* and *B. vallismortis*.

Table 18. DNA-DNA relatedness of strains and related *Bacillus*

Isolate	% DNA-DNA relatedness with labeled type strain		
	KCTC 3135^T	KCTC 1660^T	KCTC 3707^T
THY-C1	46.54	25.17	ND
TISTR 651	78.93	22.87	ND
PD-A10	25.34	59.46	20.22
K-B16	35.13	75.78	37.43
K-A7	22.58	38.76	32.88
TJW-A9	35.79	78.34	43.81
B. subtilis KCTC 3135^T	100	ND	ND
B. amyloliquefaciens KCTC 1660^T	ND	100	ND
B. vallismortis KCTC 3707^T	ND	ND	100

ND, Not determined.

Based on all of these data, isolate THY-C1, PD-A10 and K-A7 would be representing the new species in genus *Bacillus*. The characterization and identification of isolate THY-C1, PD-A10 and K-A7 were then performed.

Identification of isolate THY-C1

The almost complete 16S rRNA gene sequence of THY-C1 were directly determined following PCR amplification and comprised of more than 1500 nucleotides. A phylogenetic tree showed that THY-C1 fall within the radiation of the cluster comprising *Bacillus* species (Figure 13). THY-C1 exhibited more than 98% 16S rDNA similarity to *Bacillus* species whose 16S rRNA gene sequences are known. Levels of 16S rDNA similarity between THY-C1 and the type strains of some phylogenetically related *Bacillus* species are as follows: *Bacillus subtilis* subsp *subtilis*, 98.78; *Bacillus mojavensis*, *Bacillus* axarquiensis and *Bacillus subtilis* subsp. *spizizen*, 98.64; and *Bacillus amyloliquefaciens*, 98.42 (Table 19). Taking into consideration of the 16s rRNA gene sequence similarity value of 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994) many isolates could be assigned to previously described species. At the same time, isolates closely affiliated, for instance, with *Bacillus nematocida*/*Bacillus malacitensis* distincta cluster with sequence similarity values of 99.79% or 99.5% could not be assigned to known species because they were not consistent in their properties with those species of cluster above (Huang *et al*., 2005 and Garcia *et al*., 2005). It was previously reported that similar or almost identical 16S rRNA gene sequence similarity values of >99% did not always imply a close relationship at the species level for bacteria in the genus *Bacillus* (Ash *et al*., 1991). In addition, $(GTG)_5$-PCR fingerprinting of THY-C1 and closely related *Bacillus* sp. was evaluated and the result was shown in Figure 14.

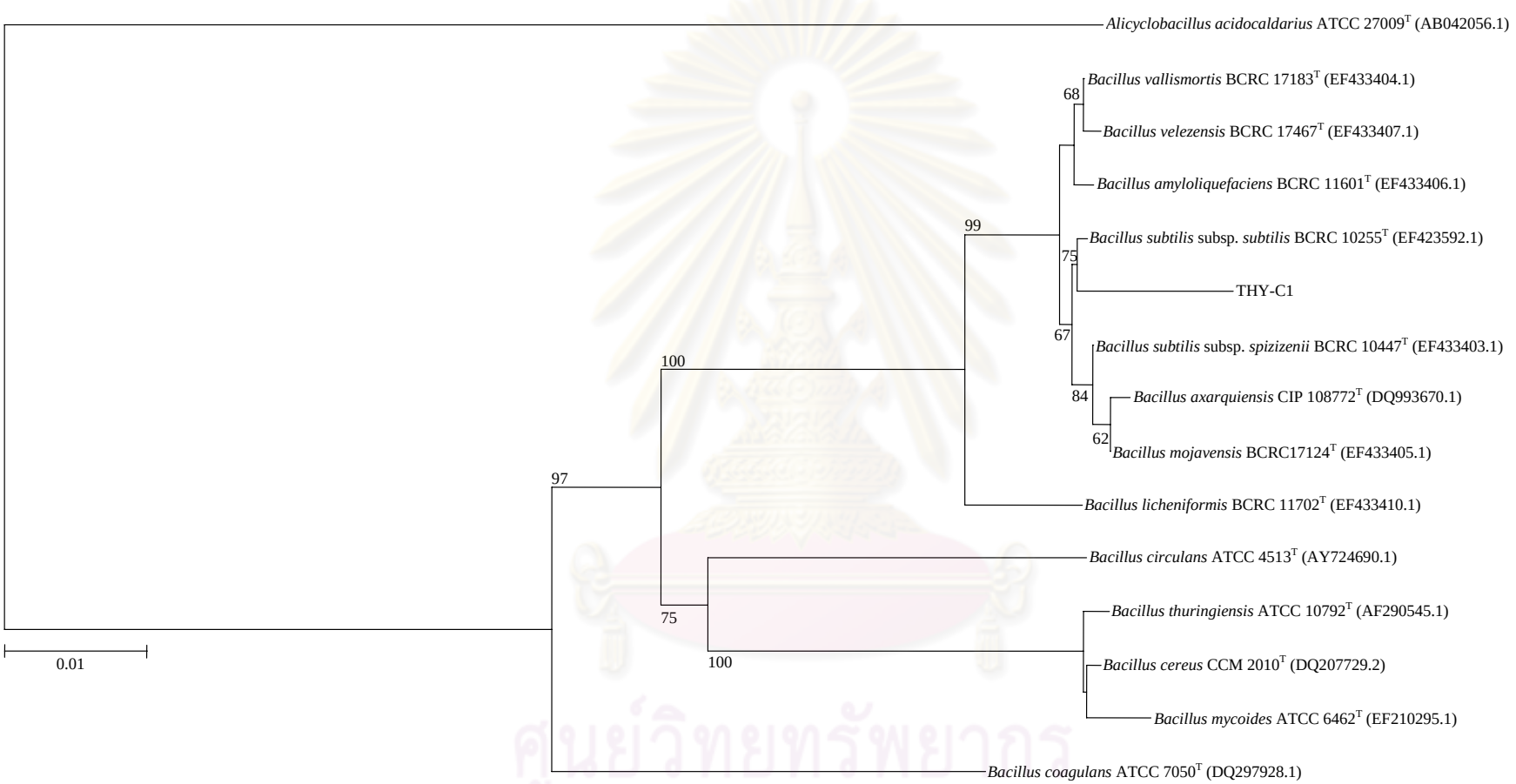
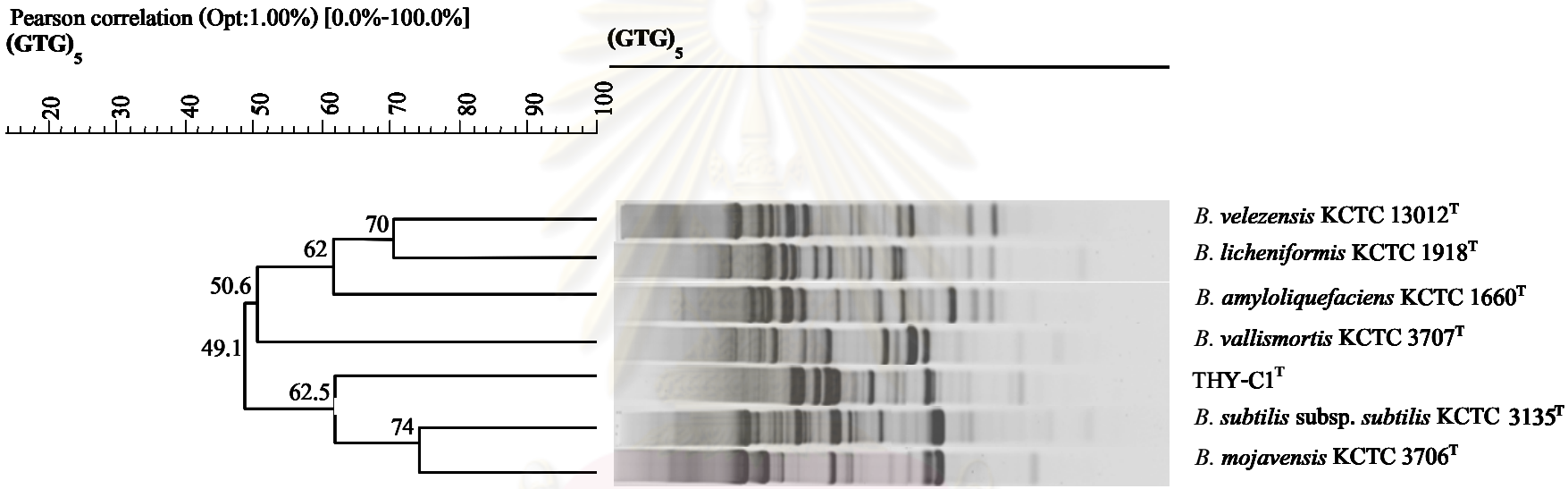


Figure 13. Neighbour-joining-tree showing the phylogenetic position of THY-C1 and related taxa based on 16S rRNA sequences. Bar 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; values greater than 50% were considered significant.

Table 19. Percentage similarities of THY-C1 and related taxa.

	B. mycoides	*B. cereus*	*B. thuringiensis*	*B. circulans*	*Bacillus* sp. THY-C1	*B. subtilis*	*B. mojavensis*	*B. axarquiensis*	*B. subtilis subsp. spizizenii*	*B. velezensis*	*B. vallismortis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. coagulans*	*Alicyclobacillus acidocaldurius*
B. mycoides	100														
B. cereus	99.5	100													
B. thuringiensis	99.5	99.86	100												
B. circulans	94.17	94.39	94.24	100											
Bacillus sp. THY-C1	92.44	92.83	92.83	92.79	100										
B. subtilis	93.64	94.02	94.02	93.98	98.78	100									
B. mojavensis	93.71	94.09	94.09	94.21	98.64	99.72	100								
B. axarquiensis	93.56	93.94	93.94	94.06	98.64	99.72	99.86	100							
B. subtilis subsp. spizizenii	93.56	93.94	93.94	94.06	98.64	99.72	99.86	100	100						
B. velezensis	93.71	94.09	94.09	93.83	98.57	99.65	99.5	99.5	99.5	100					
B. vallismortis	93.79	94.17	94.17	93.9	98.56	99.65	99.65	99.65	99.65	99.86	100				
B. amyloliquefaciens	93.78	94.01	94.01	93.9	98.42	99.5	99.5	99.5	99.5	99.72	99.86	100			
B. licheniformis	93.71	93.94	93.94	94.06	97.39	98.5	98.5	98.5	98.5	98.28	98.42	97.57	100		
B. coagulans	92.69	93.08	92.92	93.07	92.32	93.52	93.36	93.36	93.36	93.6	93.59	93.44	93.44	100	
Alicyclobacillus acidocaldurius	81.88	81.7	81.61	81.9	80.6	81.79	81.89	81.89	81.89	81.87	81.88	82.06	82.46	82.43	100

Figure 14. Cluster analysis of rep-PCR of THY-C1 and other *Bacillus* species.



THY-C1 could be distinguished from *B. subtilis* subsp *subtilis* KCTC3135^T, *B. mojavensis* KCTC33706^T and *B. amyloliquifaciens* KCTC1660^T by morphological and metabolic characteristics as shown in Table 20. Negative oxidase test, negative utilization of citrate, maximum growth temperature (55ºC), maximum growth in the presence of 15% NaCl, and inability to ferment D-xylose were the trait that differentiated THY-C1 from *B. subtilis* subsp *subtilis* KCTC 3135^T, *B. mojavensis* KCTC 3706^T and *B. amyloliquifaciens* KCTC 1660^T. The ability to ferment D-lactose differentiated THY-C1 from *B. subtilis* subsp *subtilis* KCTC 3135^T and *B. mojavensis* KCTC 3706^T while ability to ferment D-melibiose and D-Turanose could separated THY-C1 from *B. amyloliquifaciens* KCTC 1660^T. According to the basis of differences in phenotypic and genetic distinctiveness, THY-C1 should be recognized as a novel species of the genus *Bacillus* and the name *Bacillus thailandensis* sp. nov. was purposed.

Table 20. Differential characteristics of THY-C1 and related *Bacillus* species.

Characteristic	1	2	3	4
Oxidase activity	-	+	+	+
Utilization of citrate	-	+	+	+
Growth in the presence of				
12% NaCl	+	-	-	-
15% NaCl	+	-	-	-
Maximum growth temperature (°C)	55	50	50	50
Acid in API system from:				
D-Lactose	+	-	-	+
D-Xylose	-	+	+	+
D-Melibiose	+	+	-	+
D-Turanose	+	+	-	+
G+C content (%)	40.4	42.9	43.4	45.4

1, THY-C1; 2, *B. subtilis* subsp *subtilis* KCTC 3135^T; 3, *B. mojavensis* KCTC 3706^T; 4, *B. amyloliquifaciens* KCTC 1660^T .

-, negative reaction; +, positive reaction.

Characteristics of *Bacillus thailandensis* sp. nov.

Cells were Gram-positive, rod-shaped and nonmotile. Subterminal ellipsoidal endospores were observed in swollen sporangia. Colonies were white, irregular, and 3-4 mm in diameter after 2 days of incubation at 37ºC on TSA. Optimal growth temperature was 37ºC and growth occurred at 4-55ºC. Optimal pH for growth was 6-7. Growth was observed at pH 4.5. Growth occurred in the presence of 0-15% (w/v) NaCl. Urease reaction was negative. Tyrosine and Tween 80 were not hydrolysed. H_2S and indole were not produced. Voges-Proskauer was positive but methyl-red reaction was negative. When assayed with the API ZYM system, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and α–glucosidase were present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were absent. Acids from glycerol, L-arabinose, D-ribose, D-xylose, D-mannose, inositol, D-sorbitol, methyl-β-D-glucopyranoside, salicin, D-cellobiose, D-maltose, D-melibiose, D-trehalose, D-raffinose, amidon, glycogen, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while L-rhamnose, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. The cell wall peptidoglycan contained *meso*-diaminopimelic acid. The predominant menaquinone was MK-7. The major fatty acids were anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The DNA G+C content was 40.4 mol% (determined by HPLC). The type strain was THY-C1^T (= BCC 22618^T) that isolated from Tao-hoo-yee collected from local market in Ayutthaya Province, Thailand.

Identification of isolate PD-A10

The almost complete 16S rRNA gene sequence of PD-A10 was directly determined following PCR amplification and comprised of more than 1500 nucleotides. A phylogenetic tree showed that PD-A10 fall within the radiation of the cluster comprising *Bacillus* species (Figure 15). PD-A10 exhibited more than 99% 16S rDNA similarity to *Bacillus* species whose 16S rRNA gene sequences are known. Levels of 16S rDNA similarity between PD-A10 and the type strains of some phylogenetically related *Bacillus* species were as follows: *Bacillus amyloliquefaciens*, 99.65; *B. velezensis*, 99.58; *B. vallismortis*, 99.43; *B. subtilis* subsp. *spizizenii*, 99.22; *B. axarquiensis* and *B. mojavensis*, 99.15 and *B. subtilis* subsp. *subtilis*, 99.08 (Table 21).

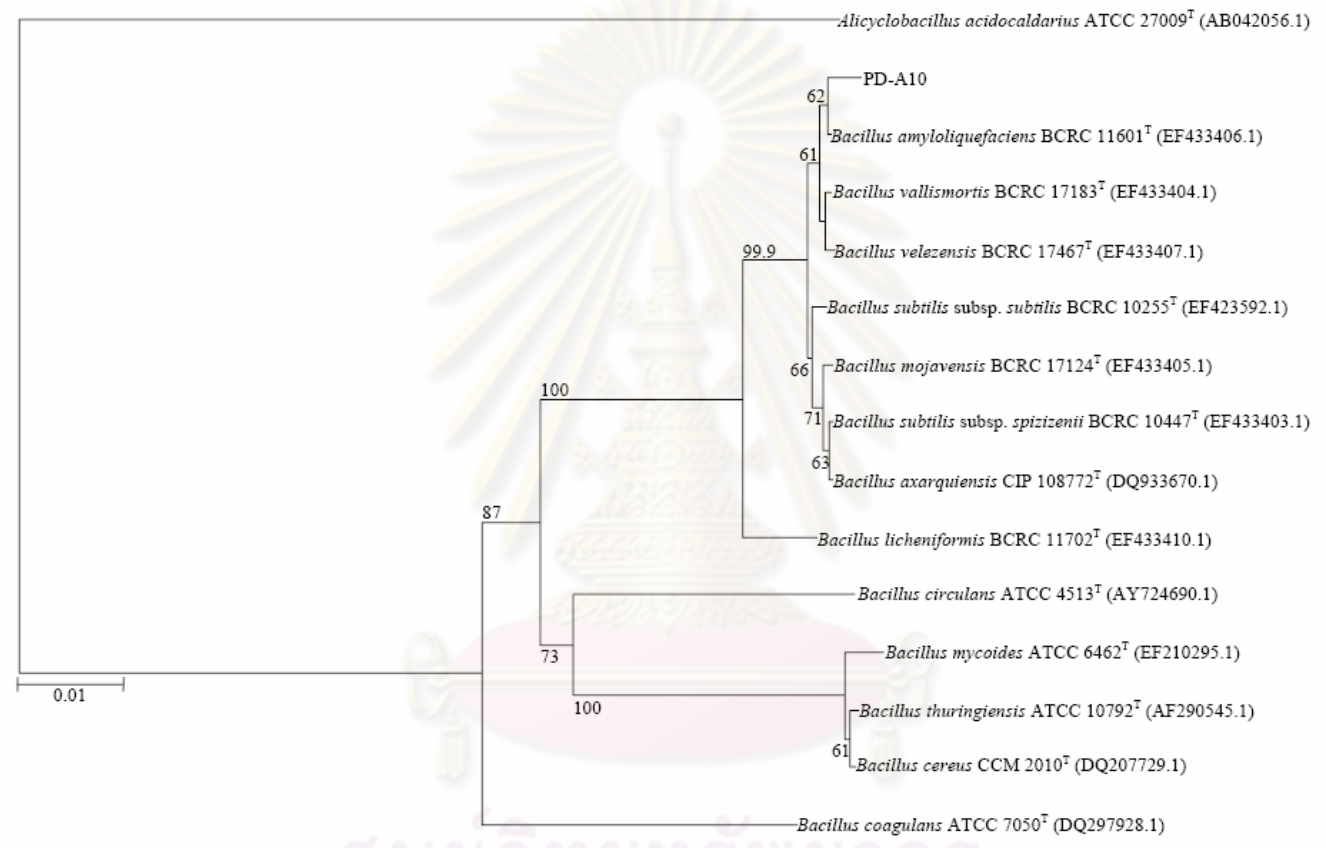


Figure 15. Neighbour-joining-tree showing the phylogenetic position of PD-A10 and related taxa based on 16S rRNA sequences. Bar 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; values greater than 60% were considered significant.

Table 21. Percentage similarities of PD-A10 and related taxa.

	B. mycoides	*B. cereus*	*B. thuringiensis*	*B. circulans*	*B. amyloliquefaciens*	*Bacillus* sp. PD-A10	*B. mojavensis*	*B. axarquiensis*	*B. subtilis subsp. subtilis*	*B. subtilis subsp. spizizenii*	*B. velezensis*	*B. vallismortis*	*B. licheniformis*	*B. coagulans*	*Alicyclobacillus acidocaldarius*
B. mycoides	100														
B. cereus	99.51	100													
B. thuringiensis	99.51	99.86	100												
B. circulans	94.20	94.43	94.28	100											
B. amyloliquefaciens	93.82	94.05	94.05	93.94	100										
Bacillus sp. PD-A10	93.52	93.75	93.75	93.63	99.65	100									
B. mojavensis	93.75	94.13	94.13	94.25	99.51	99.15	100								
B. axarquiensis	93.60	93.98	93.98	94.10	99.51	99.15	99.86	100							
B. subtilis subsp. subtilis	93.52	93.90	93.90	94.02	99.43	99.08	99.79	99.93	100						
B. subtilis subsp. spizizenii	93.60	93.98	93.98	93.94	99.43	99.22	99.65	99.65	99.72	100					
B. velezensis	93.67	94.05	94.05	93.79	99.65	99.58	99.43	99.43	99.51	99.65	100				
B. vallismortis	93.75	94.13	94.13	93.86	99.79	99.43	99.58	99.58	99.65	99.65	99.86	100			
B. licheniformis	93.75	93.98	93.98	94.10	98.58	98.22	98.51	98.51	98.43	98.43	98.22	98.36	100		
B. coagulans	92.66	93.05	92.89	93.04	93.41	93.17	93.33	93.33	93.33	93.48	93.56	93.56	93.41	100	
Alicyclobacillus acidocaldarius	81.90	81.72	81.63	81.91	82.08	81.78	81.90	81.90	81.91	81.81	81.90	81.91	82.47	82.44	100

PD-A10 could be distinguished from *B. amyloliquifaciens* KCTC 1660^T, *B. subtilis* subsp *subtilis* KCTC 3135^T and *B. vallismortis* KCTC 3707^T by the phenotypic characteristics as shown in Table 22. Negative for oxidase and utilization of citrate, maximum growth temperature (55ºC) and maximum growth in the presence of 15% NaCl were the trait that differentiated PD-A10 from *B. amyloliquifaciens* KCTC 1660^T, *B. subtilis* subsp *subtilis* KCTC 3135^T and *B. vallismortis* KCTC 3707^T. The ability to ferment D-lactose differentiated PD-A10 from *B. subtilis* subsp *subtilis* KCTC 3135^T and *B. vallismortis* KCTC 3707^T, while ability to ferment D-melibiose separated PD-A10 from *B. subtilis* subsp *subtilis* KCTC 3135^T *and B. amyloliquifaciens* KCTC 1660^T. On the basis of differences in phenotypic and genetic distinctiveness, PD-A10 should be recognized as a novel species of the genus *Bacillus* and the name *Bacillus siamensis* sp. nov. was purposed.

Table 22. Differential characteristics of PD-A10 and related *Bacillus*.

Characteristics	1	2	3	4
Oxidase activity	-	+	+	+
Utilization of citrate	-	+	+	+
Growth in the presence of				
12% NaCl	+	-	-	-
15% NaCl	+	-	-	-
Maximum growth temperature (°C)	55	50	50	50
Acid in API system from:				
D-Lactose	+	-	-	+
D-Melibiose	-	+	-	+
G+C content (%)	41.4	42.9	43.7	45.4

1, *Bacillus* sp. PD-A10; 2, *B. subtilis* subsp *subtilis* KCTC 3135^T; 3, *B. vallismortis* KCTC 3707^T; 4, *B. amyloliquifaciens* KCTC 1660^T.

-, negative reaction; +, positive reaction.

Characteristics of *Bacillus siamensis* sp. nov.

Cells were Gram-positive, rod-shaped, and motile. Central endospores were observed in swollen sporangia. Colonies were white, mucous, and 3-4 mm in diameter after 2 days of incubation at 37ºC on TSA. Optimal growth temperature was 37ºC and growth occurred at 4-55ºC. Optimal pH for growth was 6-7. Growth was observed at pH 4.5. Growth occurred in the presence of 0-15% (w/v) NaCl. Urease reaction was negative. Tyrosine and Tween 80 were not hydrolysed. H_2S and indole were not produced. Voges-Proskauer was positive but methyl-red reaction was negative. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase were present, but lipase (C14), acid phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, α–glucosidase, β-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were absent. Acids from glycerol, L-arabinose, D-ribose, D-glucose, cellibiose, amygdalin, glycogen, xylose, D-arabitol, arbutin, inositol, lactose, D-fructose, raffinose, sorbitol, maltose, esculin and D-saccharose were positive, while dulcitol, gluconate, gentibiose, trehalose, L-sorbitol, L-arabitol, turanose, galactose, inulin, erythritol, rhamnose, melibiose, salicin, mannose, D-mannitol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, N-acetylglucosamine, D-melezitose, xylitol, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. The cell wall peptidoglycan contained *meso*-diaminopimelic acid. The predominant menaquinone was MK-7. The major fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The DNA G+C content was 38.4 mol% (determined by HPLC). The type strain was PD-A10^T (= BCC 22614^T, KCTC 13613^T) that isolated from Poo-khem collected from local market in Nakhon Pathom Province, Thailand.

Identification of isolate K-A7

The almost complete 16S rRNA gene sequence of K-A7 was directly determined following PCR amplification and comprised of more than 1500 nucleotides. A phylogenetic tree showed that K-A7 fall within the radiation of the cluster comprising *Bacillus* species (Figure 16). K-A7 exhibited more than 99% 16S rRNA gene similarity to *Bacillus* species whose 16S rRNA gene sequences are known. Levels of 16S rRNA gene similarity between K-A7 and the type strains of some phylogenetically related *Bacillus* species were as follows: *Bacillus subtilis* subsp *subtilis*, *B. subtilis* subsp *spizizenii*, *B. velezensis*, *B. vallismortis*, *B. axarquiensis* 99.36; *B. mojavensis*, *B. amyloliquefaciens* 99.22 (Table 23). In addition, ITS-PCR fingerprinting of K-A7 and closely related *Bacillus* sp. was evaluated (Figure 17).

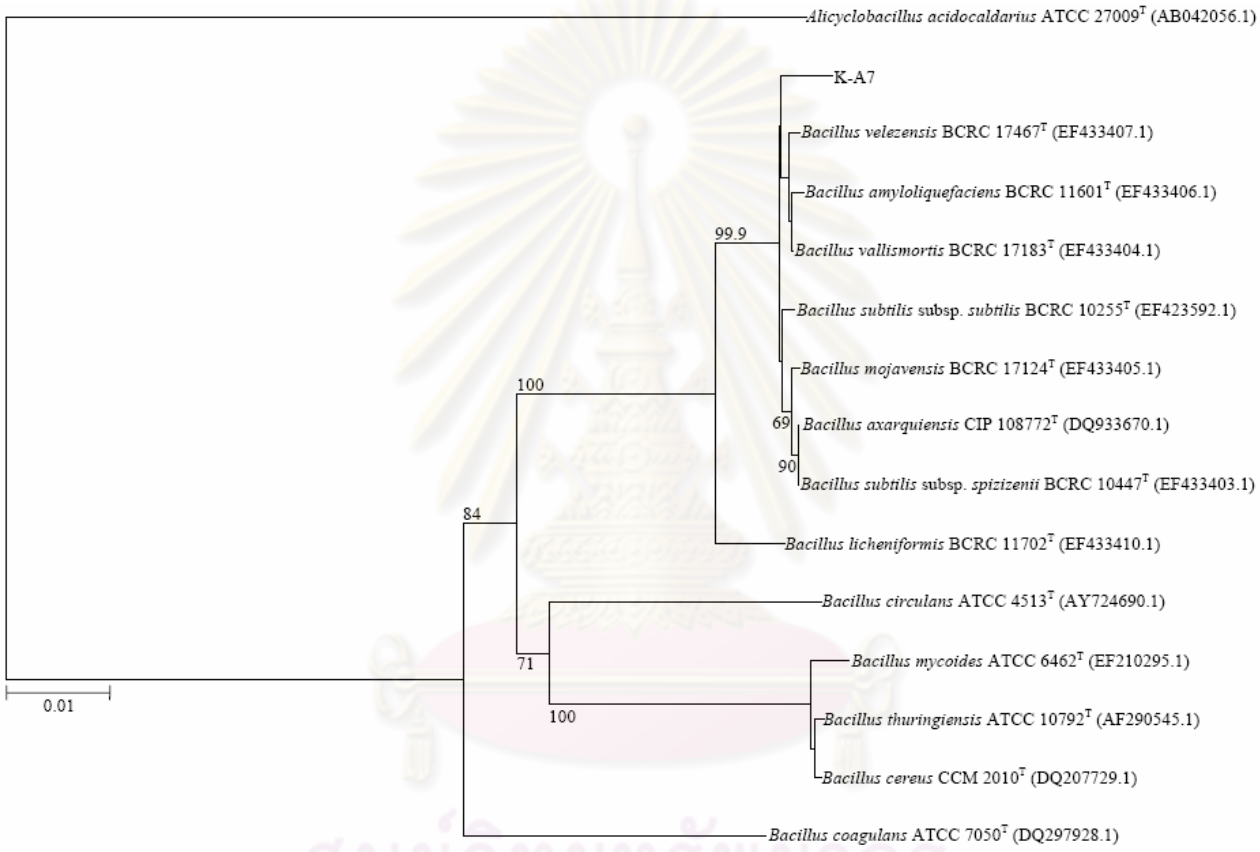


Figure 16. Neighbour-joining-tree showing the phylogenetic position of *Bacillus* sp. K-A7 and related taxa based on 16S rRNA sequences. Bar 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; values greater than 60% were considered significant.

Table 23. Percentage similarities of K-A7 and related taxa.

	B. mycoides	*B. cereus*	*B. thuringiensis*	*B. circulans*	*B. velezensis*	*B. vallismortis*	*B. amyloliquefaciens*	*B. subtilis subsp. subtilis*	*Bacillus* sp. K-A7	*B. subtilis subsp. spizzenii*	*B. mojavensis*	*B. axarquiensis*	*B. licheniformis*	*B. coagulans*	*Alicyclobacillus acidocaldarius*
B. mycoides	100														
B. cereus	99.51	100													
B. thuringiensis	99.51	99.86	100												
B. circulans	94.27	94.49	94.34	100											
B. velezensis	93.74	94.12	94.12	93.93	100										
B. vallismortis	93.81	94.19	94.19	94.01	99.86	100									
B. amyloliquefaciens	93.81	94.04	94.04	94.01	99.72	99.86	100								
B. subtilis subsp. subtilis	93.74	94.12	94.12	94.01	99.72	99.72	99.58	100							
Bacillus sp. K-A7	93.51	93.89	93.89	93.62	99.36	99.36	99.22	99.36	100						
B. subtilis subsp. spizizenii	93.66	94.04	94.04	94.08	99.58	99.72	99.58	99.72	99.36	100					
B. mojavensis	93.81	94.19	94.19	94.23	99.58	99.72	99.58	99.72	99.22	99.86	100				
B. axarquiensis	93.66	94.04	94.04	94.08	99.58	99.72	99.58	99.72	99.36	100	99.86	100			
B. licheniformis	93.81	94.04	94.04	94.09	98.36	98.50	98.65	98.50	98.14	98.50	98.50	98.50	100		
B. coagulans	92.72	93.11	92.95	93.18	93.63	93.62	93.47	93.63	93.39	93.47	93.47	93.47	93.55	100	
Alicyclobacillus acidocaldarius	81.97	81.79	81.70	82.07	81.96	81.97	82.15	81.96	81.32	82.06	82.06	82.06	82.63	82.51	100

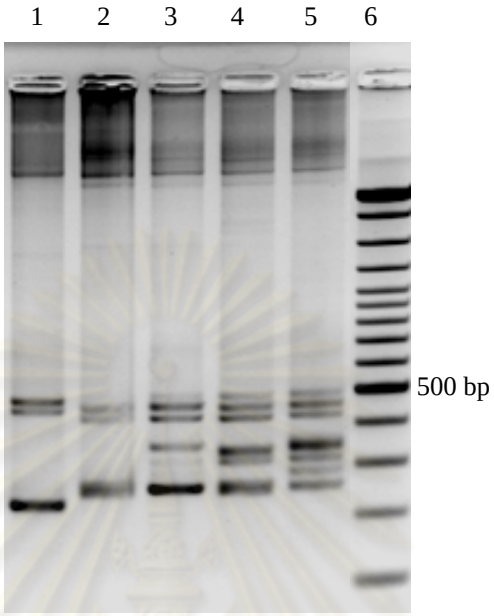


Figure 17. ITS-PCR fingerprinting of K-A7 and other *Bacillus* species. 1, *Bacillus cereus*; 2, *B. subtilis* subsp *subtilis* KCTC 3135^{T}; 3, *B. vallismortis* KCTC 3707^{T}; 4, *B. amyloliquefaciens* KCTC 1660^{T}; 5, K-A7; 6, 100 bp marker.

K-A7 could be distinguished from *B. subtilis* subsp *subtilis* KCTC 3135^T, *B. vallismortis* KCTC 3707^T and *B. amyloliquifaciens* KCTC 1660^T by the phenotypic characteristics as shown in Table 24. Negative for oxidase and utilization of citrate, maximum growth temperature (55ºC), maximum growth in the presence of 15% NaCl, and inability to ferment D-xylose were the traits that differentiated K-A7 from *B. subtilis* subsp *subtilis* KCTC 3135^T, *B. vallismortis* KCTC 3707^T and *B. amyloliquifaciens* KCTC 1660^T. The ability to ferment D-lactose differentiated K-A7 from *B. subtilis* subsp *subtilis* KCTC 3135^T and *B. vallismortis* KCTC 3707^T, while ability to ferment D-melibiose separated K-A7 from *B. vallismortis* KCTC 3707^T. On the basis of differences in phenotypic and genetic distinctiveness, K-A7 should be recognized as a novel species of the genus *Bacillus* and the name *Bacillus kapii* sp. nov. was purposed.

Table 24. Differential characteristics of K-A7 related *Bacillus*.

Characteristics	1	2	3	4
Oxidase activity	-	+	+	+
Utilization of citrate	-	+	+	+
Growth in the presence of				
12% NaCl	+	-	-	-
15% NaCl	+	-	-	-
Maximum growth temperature (°C)	55	50	50	50
Acid in API system from:				
D-Lactose	+	-	-	+
D-Xylose	-	+	+	+
D-Melibiose	+	+	-	+
G+C content (%)	40.2	42.9	43.7	45.4

1, *Bacillus* sp. K-A7; 2, *B. subtilis* subsp *subtilis* KCTC 3135^T; 3, B. *vallismortis* KCTC 3707^T; 4, *B. amyloliquifaciens* KCTC 1660^T.

+, Positive reaction; -, negative reaction and ND, not determined.

Characteristics of *Bacillus kapii* sp. nov.

Cells were Gram-positive, rod-shaped and motile. Subterminal ellipsoidal endospores were observed in swollen sporangia. Colonies were white, irregular, and 1-2 mm in diameter after 2 days of incubation at 37ºC on TSA. Optimal growth temperature was 37-40ºC; growth occurred at 4-50ºC. Optimal pH for growth was 6-7. Growth was observed at pH 4.5. Growth occurred in the presence of 0-15% (w/v) NaCl. Urease reaction was negative. Starch, gelatin, casein and tween-80 were hydrolysed but L-tyrosine was not hydrolysed. H_2S and indole were not produced. Voges-Proskauer was positive but methyl-red reaction was negative. When assayed with the API ZYM system, alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α–glucosidase were present, but lipase (C14), esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were absent. Acids from glycerol, L-arabinose, D-ribose, D-xylose, D-mannose, inositol, D-sorbitol, methyl-β-D-glucopyranoside, amygdalin, arbutin, salicin, gentibiose, turanose, raffinose, inulin, D-cellobiose, D-lyxose, D-maltose, D-trehalose, glycogen, D-glucose, D-fructose, D-mannitol, esculin and D-saccharose were positive, while L-rhamnose, lactose, galactose, dulcitol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, methyl-α-D-mannoside, N-acetylglucosamine, D-melezitose, xylitol, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, L-sorbose, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate were negative. The cell wall peptidoglycan contained *meso*-diaminopimelic acid. The predominant menaquinone was MK-7. The major fatty acids were anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The DNA G+C content was 40.2 mol% (determined by HPLC). The type strain was K-A7^T (= BCC 22608^T) that isolated from Ka-pi collected from local market in Surat Thani Province, Thailand.

4.2 Optimization of fibrinolytic protease production

Among 6 isolates, THY-C1 that showed the highest activity was chosen to study the optimal condition of fibrinolytic protease production. The condition for the enzyme production was optimized with a stepwise strategy. Firstly, the optimal nitrogen and carbon sources were screened with the one-at-a-time strategy followed by 3^3 full factorial design of the medium composition. Finally, the cultural conditions, such as inoculum level, growth temperature and fibrin supplementation, were studied with one-at-a-time strategy.

Effects of different nitrogen sources on fibrinolytic protease production

The effects of different nitrogen sources on fibrinolytic protease production by THY-C1 was investigated in the basal medium containing glucose (25 g/l) as a carbon source. Among the organic nitrogen sources, yeast extract gave the highest activity (24.6 unit/ml) while activities were slightly lower with meat extract, skim milk, tryptone, gelatin (Figure 18). Peptone and casein were poor for fibrinolytic protease production. Inorganic nitrogen source, such as ammonium sulfate and monosodium glutamate, appeared to be poor for fibrinolytic protease production.

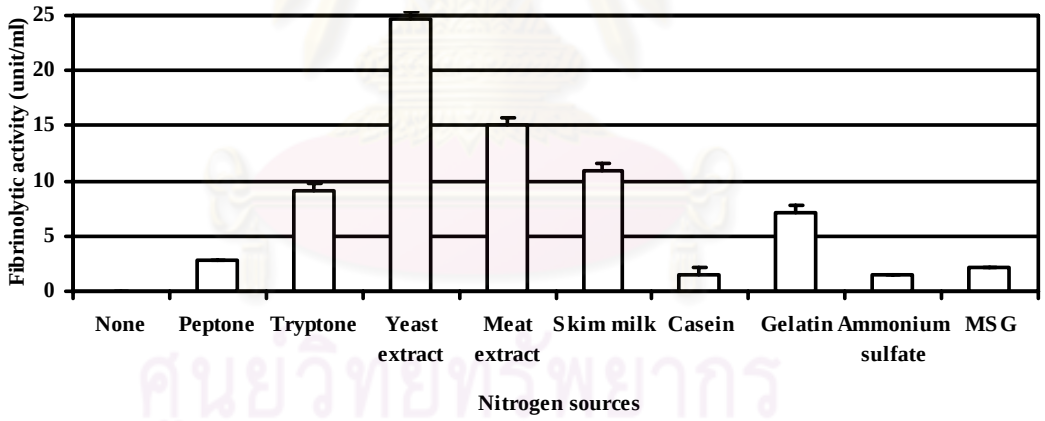


Figure 18. Effect of different nitrogen sources on fibrinolytic protease production by THY-C1.

Effects of different carbon sources on fibrinolytic protease production

The effect of carbon sources on the production of fibrinolytic protease by THY-C1 was investigated using D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, D-maltose, D-sucrose, D-lactose, D-mannitol, glycerol and soluble starch at concentration of 25 g/l in the basal medium containing 15.2 g/l yeast extract (Figure 19). As a result, maltose and sucrose were the best carbon sources for fibrinolytic protease production with activity 52.4 and 48.9 unit/ml respectively. Fibrinolytic protease production with the addition of mannose was found to be low when compared with not supplemented medium.

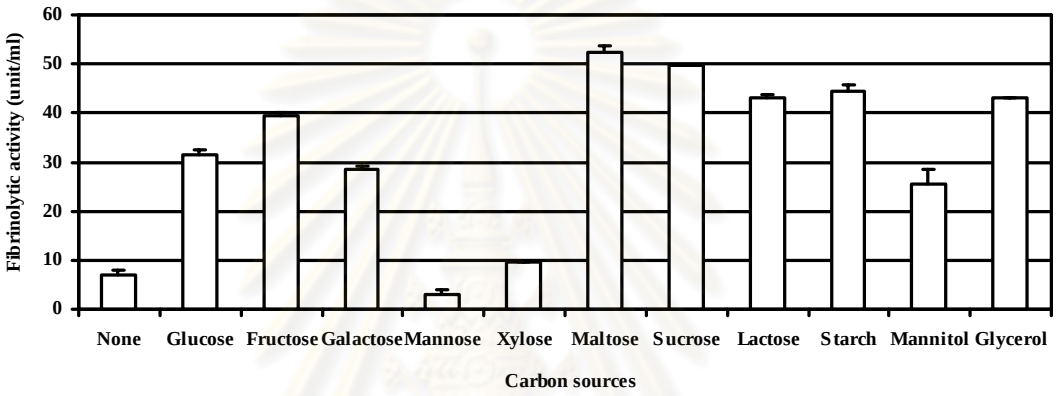


Figure 19. Effect of different carbon sources on fibrinolytic protease production by THY-C1.

For commercial production, the use of sucrose as carbon source to produce fibrinolytic protease seems to be the lower cost alternative. Therefore, sucrose was chosen for the further studies.

Optimization of medium compositions with 3^3 factorial designs

The effects of yeast extract concentration as nitrogen source, sucrose concentration as carbon source and pH on fibrinolytic protease production were studied using the statistical approach of 3^3 factorial design (Table 25). It can be noted that the fibrinolytic activity varied from 0.2 to 71.1 unit/ml. The maximum activity of these responses were attained at run number 3, employing 6 g/l yeast extract and 15 g/l sucrose concentration at pH 9.0. The lowest activity was obtained when the bacteria was grown in the medium containing 12 g/l yeast extract and 15 g/l sucrose at pH 5.0. According to Table 25, it can be observed that the fibrinolytic protease production was greatly influenced by the initial pH of medium.

Table 25. Experimental design and results of the 3^3 factorial design.

Run number	Coded levels			Fibrinolytic activity (unit/ml)	Cell growth (OD_{600})
	X_1	X_2	X_3		
1	-1	-1	-1	23.2 ± 0.0	4.1 ± 0.5
2	-1	-1	0	20.2 ± 0.0	3.2 ± 0.0
3	-1	-1	+1	71.1[a]± 1.9	2.7 ± 0.0
4	-1	0	-1	22.2 ± 0.2	3.6 ± 0.3
5	-1	0	0	16.2 ± 1.2	2.7 ± 0.1
6	-1	0	+1	40.7[d]± 0.7	2.4 ± 0.1
7	-1	+1	-1	9.3 ± 0.1	3.4 ± 0.2
8	-1	+1	0	31.7 ± 0.7	3.0 ± 0.1
9	-1	+1	+1	39.9[d]± 2.1	2.0 ± 0.2
10	0	-1	-1	17.7 ± 0.3	6.2[a] ± 0.3
11	0	-1	0	37.5 ± 1.2	3.7 ± 0.1
12	0	-1	+1	63.3[b]± 2.7	3.2 ± 0.0
13	0	0	-1	18.2 ± 0.7	5.9[a] ± 0.1
14	0	0	0	35.2 ± 0.4	3.2 ± 0.3
15	0	0	+1	57.8[c]± 0.7	2.7 ± 0.1
16	0	+1	-1	2.5 ± 0.1	5.1[b] ± 0.1
17	0	+1	0	60.6[c]± 0.7	2.8 ± 0.2
18	0	+1	+1	60.8[b]± 0.7	1.8 ± 0.1
19	+1	-1	-1	0.2 ± 0.0	7.7 ± 0.2
20	+1	-1	0	58.9[c]± 1.9	4.5 ± 0.2
21	+1	-1	+1	57.9[c]± 3.1	3.6 ± 0.2
22	+1	0	-1	0.8 ± 0.1	6.5[a] ± 0.2
23	+1	0	0	34.2 ± 3.7	3.7 ± 0.1
24	+1	0	+1	55.5[c]± 2.1	2.9 ± 0.3
25	+1	+1	-1	13.7 ± 0.4	5.5[b] ± 0.1
26	+1	+1	0	35.4 ± 2.6	3.3 ± 0.4
27	+1	+1	+1	40.1[d]± 3.3	2.7 ± 0.2

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

[a, b, c, d] Different letters in the same row denote significant differences ($P < 0.05$).

Effect of inoculum level on fibrinolytic protease production

The effect of inoculum level on the production of fibrinolytic protease was studied in the optimized medium (Table 26). It was observed that the specific activity of enzyme was highest at 5% (v/v) inoculums level with 29.6 unit/mg protein. It was noted that at 15% (v/v) inoculums level, the specific activity of enzyme was lowest.

Table 26. Effect of inoculum level on fibrinolytic protease production.

Inoculum level (%v/v)	Fibrinolytic activity (unit/ml)	Specific activity (unit/mg)	Cell growth (OD_{600})
1	$55.4^{d} \pm 0.7$	$20.9^{c} \pm 0.3$	$2.1^{b} \pm 0.0$
5	$80.5^{a} \pm 1.4$	$29.6^{a} \pm 0.2$	$2.3^{b} \pm 0.1$
10	$69.5^{b} \pm 0.0$	$22.9^{b} \pm 0.0$	$2.8^{a} \pm 0.1$
15	$56.6^{c} \pm 1.4$	$17.8^{d} \pm 0.2$	$2.7^{a} \pm 0.2$

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

$^{a, b, c, d}$ Different letters in the same row denote significant differences ($P < 0.05$).

Effect of growth temperature on fibrinolytic protease production

The optimum growth temperature for fibrinolytic protease production by THY-C1 was found to be 37°C with the highest yield (82.3 unit/ml) and specific activity of 28.7 unit/mg protein (Table 27). At 50 °C, less growth was observed and activities were found to be the lowest (48.7 unit/ml).

Table 27. Effect of temperature on fibrinolytic protease production.

Temperature (°C)	Fibrinolytic activity (unit/ml)	Specific activity (unit/mg)	Cell growth (OD_{600})
Control (37)	$82.3^{a} \pm 1.9$	$28.7^{a} \pm 0.1$	$2.4^{a} \pm 0.0$
30	$78.3^{b} \pm 0.7$	$27.3^{c} \pm 0.1$	$1.9^{b} \pm 0.1$
35	$81.7^{a} \pm 1.2$	$28.1^{b} \pm 0.1$	$1.9^{b} \pm 0.1$
40	$76.9^{c} \pm 3.3$	$26.9^{d} \pm 0.1$	$2.4^{a} \pm 0.1$
45	$53.6^{d} \pm 2.9$	18.7 ± 0.1	$1.8^{b} \pm 0.0$
50	48.7 ± 3.3	17.0 ± 0.6	$1.4^{c} \pm 0.0$

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

[a, b, c, d] Different letters in the same row denote significant differences ($P < 0.05$).

Effect of fibrin supplementation on fibrinolytic protease production

The effect of fibrin supplementation on fibrinolytic protease production was studied (Table 28). It was found that the addition of fibrin resulted in an increase in fibrinolytic protease production, and the maximum activity was obtained when the optimized medium was supplemented with 0.124 g/l fibrin. However, further increase in the amount of fibrin did not significantly affect the fibrinolytic protease production.

Table 28. Effect of fibrin supplement on fibrinolytic protease production.

Fibrin supplement (mg/ml)	Fibrinolytic activity (unit/ml)	Specific activity (unit/mg)	Cell growth (OD_{600})
Control	$82.0^{b} \pm 1.9$	$28.6^{b} \pm 0.3$	6.6 ± 0.1
0.124	$101.2^{a} \pm 1.9$	$35.3^{a} \pm 0.2$	6.7 ± 0.0
0.248	$102.0^{a} \pm 1.2$	$35.6^{a} \pm 0.1$	6.8 ± 0.0
0.372	$100.8^{a} \pm 1.2$	$35.3^{a} \pm 0.1$	6.8 ± 0.0

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

$^{a, b, c, d}$ Different letters in the same row denote significant differences ($P < 0.05$).

Time course of fibrinolytic protease production

Figure 20 shows the time course comparison of fibrinolytic protease production by THY-C1 in the original and optimized media. As the result, fibrinolytic protease production was found to start in late-log phase of bacterial growth in optimized medium, peak at 30 h of incubation (142.5 unit/ml) and stable upon further incubation. These results support the suggestion that the fibrinolytic protease production of THY-C1 is a manifestation of nitrogen and carbon limitation at the onset of death phase. In contrast, the results for fibrinolytic protease production in non-optimized medium found that enzyme activity reached its maximum at the end of exponential phase (69 unit/ml), which might indicate different mechanism of sporulation of this bacterium in these two different medium. Moreover, incubation time was the significant factor that can leading to a marked increase in fibrinolytic protease production.

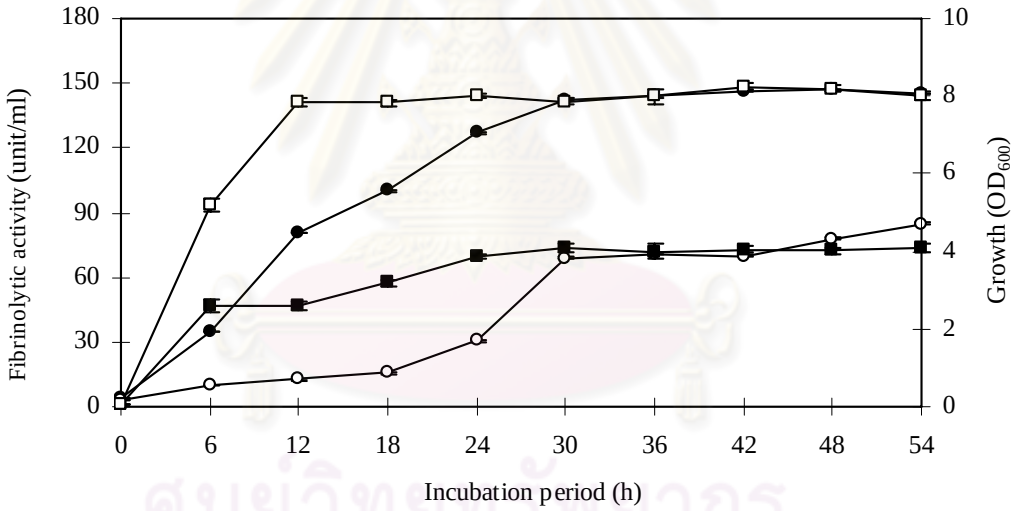


Figure 20. Time course of fibrinolytic protease production by THY-C1 in the original medium (○) fibrinolytic activity, (□) cell optical density at 600 nm and in the optimized medium, (●) fibrinolytic activity, (■) cell optical density at 600 nm.

4.3 Characterization of fibrinolytic protease produced by THY-C1

Under the optimal condition, the enzyme production by THY-C1 increased about 10 folds. Further study was conducted to elucidate the characteristic of crude fibrinolytic protease produced by THY-C1.

SDS-fibrin zymography

The changes in enzymatic pattern in the original and the optimized medium were observed by SDS-fibrin zymogram (Figure 21). Using a zymography analysis, it was found that THY-C1 secreted 9 extracellular fibrinolytic protease (EFPs; >165, 161.7, 156.6, 99.9, 80.3, 62.5, 58.4, 41.8 and 24.4 kDa) in the original culture medium while only 3 majors extracellular fibrinolytic protease (EFPs; 58.4, 41.8 and 24.4 kDa) was found in the optimized medium (Figure 21). However, the pattern of fibrinolytic protease in the optimized condition appeared to be more intense. Based on the results, changes in medium composition as well as fibrin supplementation selectively enhanced the expression of some protease.

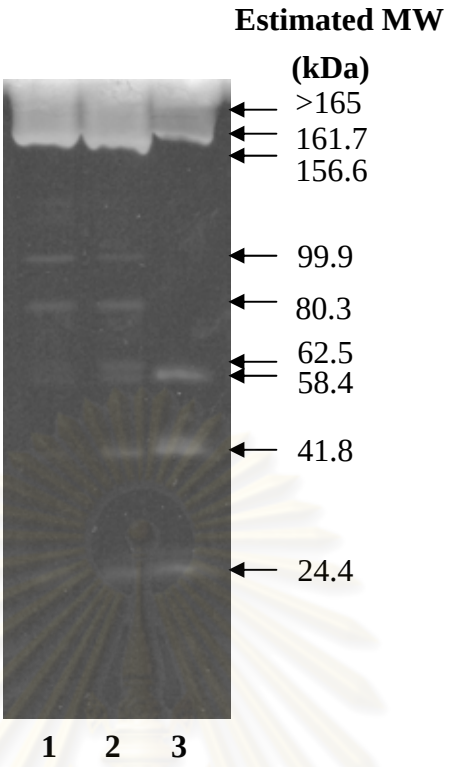


Figure 21. Zymographic pattern of fibrinolytic protease produced by THY-C1 in the original medium and in the optimized medium. Lane 1: crude extract from original medium; lane 2: crude extract from original medium that supplemented with fibrin clot and incubated for 30 h; lane 3: crude extract from optimized medium.

Effect of pH on the activity and stability of the fibrinolytic protease

The optimum pH for fibrinolytic activity was examined over pH ranges from 3-11 (Figure 22A). The optimum pH of the enzyme was around pH 7 to 8 and was relatively higher in the alkaline region. In contrast, the fibrinolytic activity in the acidic pH region decreased. The pH stability of the enzyme was investigated in a range of pH 3-11 by measuring the residual enzyme activity (Figure 22B). As a result, the fibrinolytic protease was found to be stable over a broad pH range (7-11) and retained approximately 60% of activity at pH 6 after 1 h incubation at 37°C.

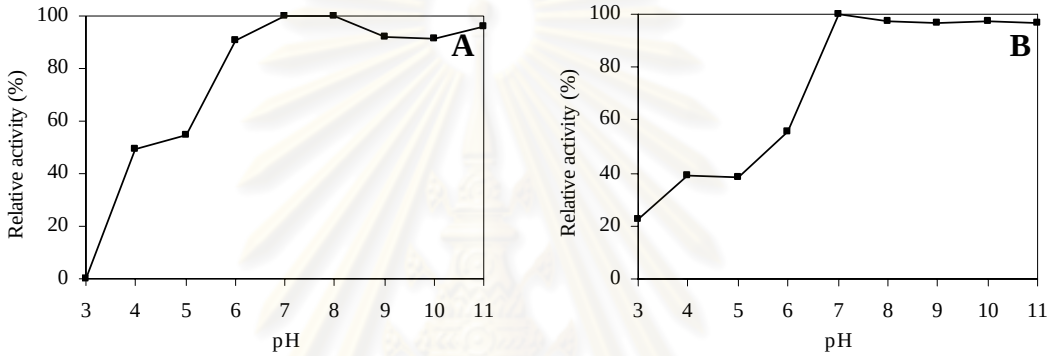


Figure 22. (A) Optimum pH of the fibrinolytic activity (B) pH stability of the fibrinolytic protease. Remaining activities were determined after incubating the enzyme in the Britton-Robinson universal buffer (citrate-phosphate-carbonate buffer) (Britton and Robinson, 1931) at different pH values ranging from 3-11 for 1 h at 37°C.

Effect of temperature on the activity and stability of the fibrinolytic protease

The effect of temperature on the activity and stability of the fibrinolytic protease was determined in a range of 30-70°C at pH 7.4 (Figure 23A). The optimum temperature for the fibrinolytic activity was approximately 50°C. The fibrinolytic protease was stable up to 50°C, and its stability decreased at high temperature (Figure 23B). The residual activities of 32% and 11% were shown at 60°C and 70°C, respectively.

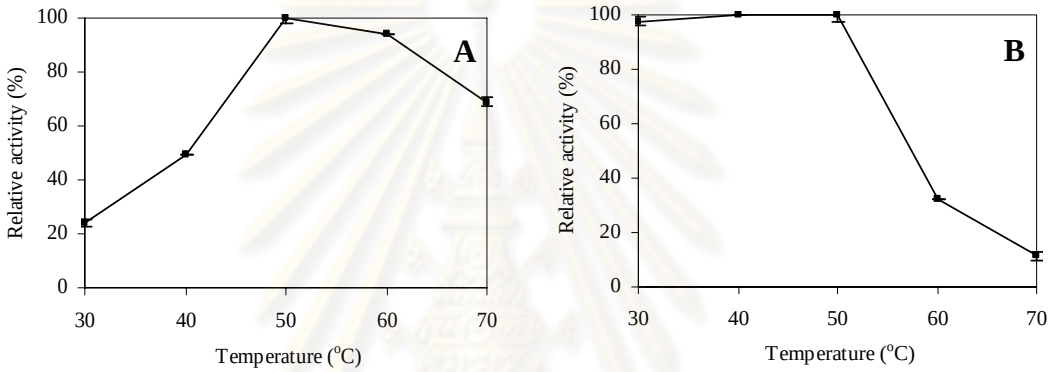


Figure 23. (A) Optimum temperature of the fibrinolytic activity (B) Heat stability of the fibrinolytic protease. Remaining activities were determined after incubating the enzyme at various temperatures ranging from 30 to 70°C for 1 h in 0.1 M phosphate buffer pH 7.4.

Protease inhibitors

The effect of natural and synthetic inhibitors on crude fibrinolytic protease was investigated (Table 29). Enzyme activity was inhibited by PMSF, while other serine protease inhibitors like soybean trypsin inhibitor, TLCK, TPCK, chymostatin and leupeptin did not significantly affect the enzyme activity. In addition, metalloproteinase inhibitors such as EDTA and phosphoramidon had no effect the enzyme activity suggesting that metal cofactors are not required for it activity. Moreover, cysteine protease inhibitor (E-64) and aspartic protease inhibitor (pepstatin A) showed no inhibitory effect on fibrinolytic protease. This inhibition profile suggests that the fibrinolytic protease from THY-C1 was belonged to a family of serine protease.

Table 29. Effect of various inhibitors on the activity of crude fibrinolytic protease from THY-C1.

Inhibitor	Concentration	Remaining activity (%)
None	-	100.0
PMSF	1 mM	58.3
Leupeptin	10 µM	117.0
Chymotrypsin	10 µM	95.4
TLCK	10 µM	114.0
TPCK	10 µM	118.0
Soybean trypsin inhibitor	0.1 mg/ml	109.7
EDTA	1 mM	126.0
Phosphoramidon	10 µM	117.2
E-64	1 µM	105.0
Pepstatin A	1 µM	116.3

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%. Statistical significance was assigned at 95% of confidence level.

Comparison of substrate specificity of fibrinolytic protease from THY-C1 and the commercial Nattokinase

The fibrinolytic activity of fibrinolytic protease produced by THY-C1 was compared with 3 brands of the commercial Nattokinase. It was found that crude enzyme showed stronger specific activity than commercial Nattokinase 1 and commercial Nattokinase 2, but lower than commercial Nattokinase 3 (Table 30).

Table 30. Fibrinolytic activity of enzyme from THY-C1 and commercial Nattokinase

Fibrinolytic compound	Fibrinolytic activity (unit/ml)	Protein (mg)	Specific activity (unit/mg)
Crude enzyme	197.53[c]	2.05[d]	96.35[b]
Commercial 1	575.82[a]	7.87[b]	73.13[c]
Commercial 2	409.14[b]	12.66[a]	32.31[d]
Commercial 3	592.06[a]	3.75[c]	157.84[a]

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

[a, b, c, d] Different letters in the same row denote significant differences ($P < 0.05$).

Moreover, substrate specificity of crude enzyme from THY-C1 was evaluated and compared with the commercial Nattokinase (Figure 24). Various synthetic substrates were used to evaluate, such as synthetic substrate for plasmin (H-D-Val-Leu-Lys-*p*NA) (Figure 24A), synthetic substrate for thrombin (H-D-Val-Leu-Arg-*p*NA) (Figure 24B) and synthetic substrate for urokinase (pyro-Glu-Gly-Arg-*p*NA) (Figure 24C). The results demonstrated that the activity of crude enzyme were specific to synthetic substrate for urokinase more than synthetic substrate for plasmin and thrombin. Compared to the commercial Nattokinase, crude enzyme from THY-C1 showed hydrolysis activity to synthetic substrate for plasmin and thrombin more than commercial Nattokinase 2 and commercial Nattokinase 3. The hydrolysis activity of crude enzyme to synthetic substrate for urokinase was also higher than commercial Nattokinase 2. However, the commercial Nattokinase 1 showed higher hydrolysis activity to all synthetic substrates than crude enzyme. These features of crude enzyme from THY-C1 make it attractive as a biomaterial agent for health promoting foods.

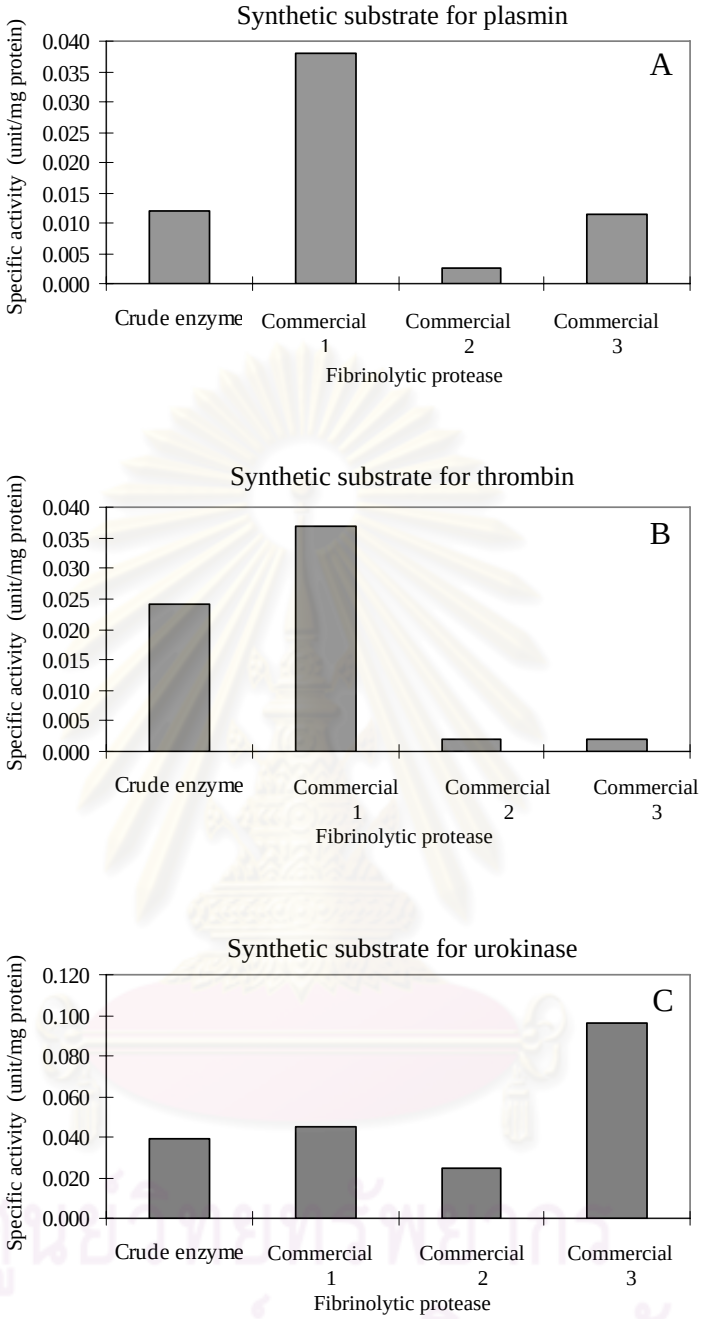


Figure 24. Hydrolysis activity of fibrinolytic protease from THY-C1 and the commercial Nattokinase on various synthetic substrates. A: synthetic substrate for plasmin (H-D-Val-Leu-Lys-*p*NA); B: synthetic substrate for thrombin (H-D-Val-Leu-Arg-*p*NA); C: synthetic substrate for urokinase (pyro-Glu-Gly-Arg-*p*NA).

The effect of digestive enzymes on fibrinolytic activity

Effect of pepsin on fibrinolytic activity The results from Table 31 showed that a decrease in fibrinolytic activity of crude enzyme incubated with pepsin in stomach condition compared to the control was found. The decrease in fibrinolytic activity of crude enzyme from THY-C1 could attribute to its stability in the acid condition.

Effect of pancreatin on fibrinolytic activity The results from Table 31 showed that a slightly decrease in fibrinolytic activity of crude enzyme incubated with pancreatin and bile salt in small intestine condition compared to the control was found. The result of crude enzyme stability could attribute to its stability in the alkaline condition. After the incubation with pancreatin, the fibrinolytic activity of crude enzyme was available from 86 - 87.7 %. According to these high values, it could be stated that to obtain good stability during digestion in stomach the encapsulation of crude enzyme should be considered. For this reason, the stability of crude enzyme in *in vitro* digestive system could be the main determinant for developing the food supplement product.

Table 31. The stability of crude enzyme on digestive enzymes in *in vitro* digestive model

Incubation condition	Remaining activity (%)
Crude enzyme	100
Crude enzyme incubated in stomach condition	
Crude enzyme without pepsin	35.72
Crude enzyme with 1 unit/ml pepsin	31.93
Crude enzyme with 10 unit/ml pepsin	30.31
Crude enzyme with 100 unit/ml pepsin	28.68
Crude enzyme incubated in small intestine condition	
Crude enzyme without pancreatin and bile salt	95.79
Crude enzyme with 1 unit/ml pancreatin and bile salt	86.05
Crude enzyme with 10 unit/ml pancreatin and bile salt	87.13
Crude enzyme with 100 unit/ml pancreatin and bile salt	87.67

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%. Statistical significance was assigned at 95% of confidence level.

4.4 Purification and characterization of fibrinolytic protease produced by PD-A10

According to the previous study, the optimal condition for fibrinolytic protease production were evaluated using THY-C1. Six representative bacteria from each group of enzymatic pattern grown in the optimal condition and fibrinolytic protease production were examined. The results showed that the fibrinolytic protease production by PD-A10 was increased and the level was higher than that of THY-C1 (data not shown). Thus, the present study was performed to conduct the purification and characterization of crude enzyme from PD-A10.

Characterization of crude fibrinolytic protease

Effect of pH and temperature on the fibrinolytic activity The enzyme was active in the pH range of 5-11 with the optimum pH at 7 and 10 (Figure 25A). It was noted that the crude enzyme showed 2 optimum pHs. It may be consumed that crude enzyme contained 2 major extracellular fibrinolytic protease. From the result, the enzyme was active in the alkaline region, and the activity was found to be decreased in the acidic pH region. This is a common feature of alkaline protease. The effect of temperature on the activity of crude enzyme was determined in a range of 30-70°C at pH 7.4 (Figure 25B). The optimum temperature for the fibrinolytic activity was approximately at 50°C.

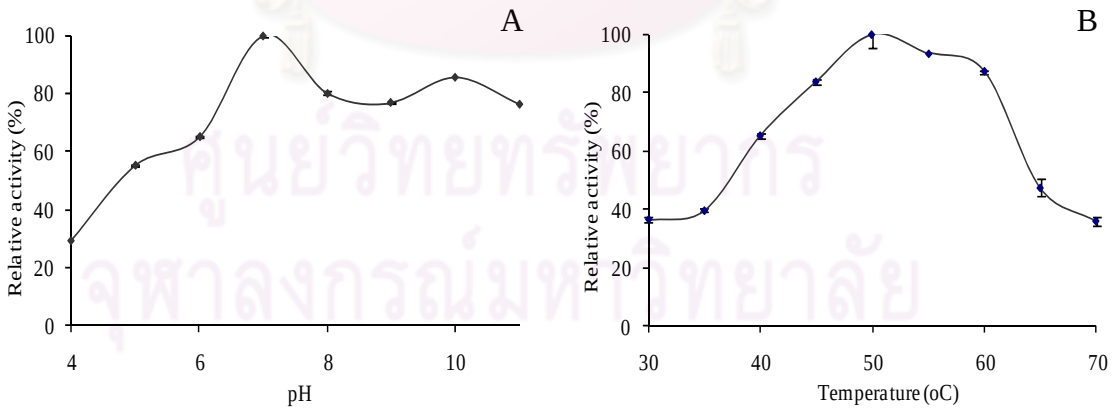


Figure 25. Effect of pH (A) and temperature (B) on fibrinolytic activity of crude fibrinolytic protease from PD-A10. (A) Fibrinolytic activity was assayed in the pH range of 5-11. (B) Fibrinolytic activity was measured by incubation in phosphate buffer pH 7.4 at temperatures from 30-70°C.

Effect of pH and temperature on the stability of crude enzyme The pH stability of the enzyme was investigated in a range of pH 4-11 by measuring the residual enzyme activity. As a result, the fibrinolytic protease was found to be stable over a broad pH range (5-11) and retained approximately 60% of activity at pH 5 after 1 h incubation at 37°C (Figure 26A). The fibrinolytic protease was stable up to 50°C, and its stability was decreased at high temperature (Figure 26B).

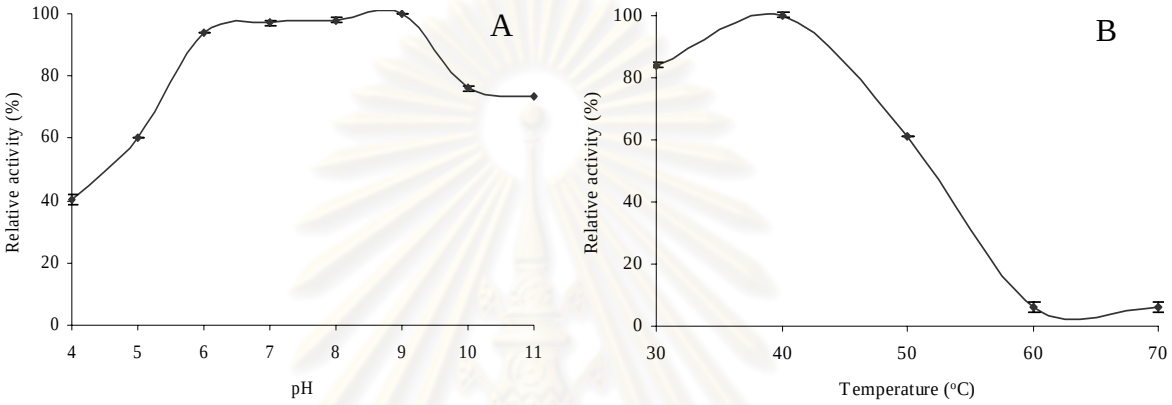


Figure 26. Effect of pH (A) and temperature (B) on the stability of the purified fibrinolytic protease from PD-A10. (A) Remaining activities were determined after incubating the enzyme in the Britton-Robinson universal buffer (citrate-phosphate-carbonate buffer) (Britton and Robinson, 1931) at different pH values ranging from 5-11 for 1 h at 37°C. (B) Remaining activities were determined after incubating the enzyme at various temperatures ranging from 30 to 70°C for 1 h in phosphate buffer pH 7.4.

Protease inhibitors The fibrinolytic protease was inhibited by PMSF, TPCK, chymostatin and EDTA while other protease inhibitors like soybean trypsin inhibitor, TLCK, leupeltin, phosphoramidon did not significantly affect the enzyme activity (Table 32). In addition, cysteine protease inhibitor (E-64) and aspartic protease inhibitor (pepstatin A) showed no inhibitory effect on fibrinolytic protease. This inhibition profile suggested that crude enzyme from PD-A10 was belong to family of serine and metalloprotease. As the previous result of pH optimum, crude enzyme may contain 2 major extracellular fibrinolytic protease. It may be proposed that one of them is serine protease and the other is metalloprotease.

Table 32. Effect of various inhibitors on enzyme activity of the crude fibrinolytic protease from PD-A10.

Protease inhibitor	Concentration	Remaining activity (%)
None	-	100.0
PMSF	1 mM	25.4
Leupeptin	10 µM	119.0
Chymostatin	10 µM	87.2
TLCK	10 µM	111.3
TPCK	10 µM	84.1
Soybean Trypsin inhibitor	0.1 mg/ml	108.5
EDTA	1 mM	84.0
E-64	1 µM	123.1
Pepstatin A	1 µM	104.2

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%. Statistical significance was assigned at 95% of confidence level.

Putification of fibrinolytic protease

Crude fibrinolytic protease was purified by anion-exchange chromatography on a Resource Q column. Table 33 showed that 4.81 mg of the enzyme was purified 12.19-fold, with a yield of 51.39%. The fact that 12.19-fold purification was sufficient to achieve homogeneity demonstrating that this protease is one of the major secreted proteins of PD-A10 in culture medium. The fibrinolytic protease preparation obtained by the procedure described here had a specific activity of 2037.3 unit/mg protein (Table 33).

Table 33. Purification of fibrinolytic protease from PD-A10[a].

Purification step	Total protein (mg)	Total activity[a] (unit)	Specific activity (unit/mg)	Yield[b] (%)	Purification (fold)
Culture supernatant	113.95	19049.19	161.17	100	1.00
Acetone precipitation	63.90	15210.14	238.03	79.85	1.42
Resource Q	4.81	9789.21	2037.30	51.39	12.19

[a]Fibrinolytic activity was determined by fibrinolytic activity assay. An enzyme unit was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per min per milliliter.

[b]The yield was calculated based on the total fibrinolytic activity

Figure 27 illustrated the purification profile and steps involved in the elution of desired protein. The crude broth was loaded on the column. The elution, after washing the column with the equilibration buffer, where a increasing linear gradient of sodium chloride was used. During this step, significant amount of active protein was eluted with low concentration of sodium chloride. The further eluting buffer with high concentration of sodium chloride eluted the undesired protein. It seem that contaminating proteins were retained on the column after the elution of the target protein.

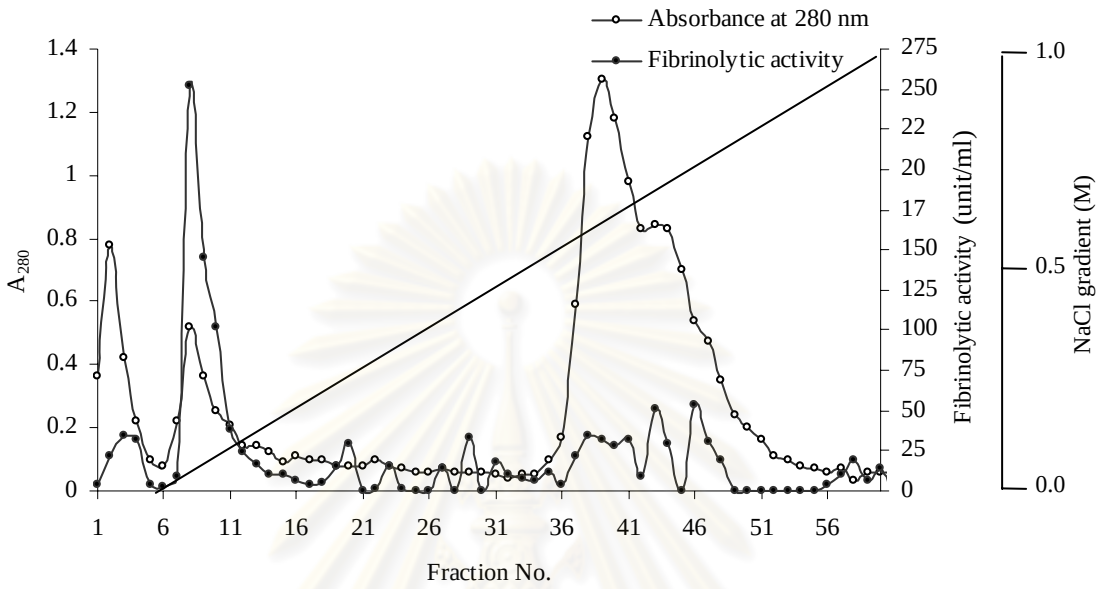


Figure 27. Purification of fibrinolytic protease from PD-A10 by using FPLC on Resource Q column equilibrated with 50 mM Tris-HCl (pH 8.0), then eluted at a flow rate of 0.2 ml/min with linear gradient of 0-1 M NaCl and 0.5 ml per fraction were collected. The elution profile was monitored by spectrophotometry at 280 nm. Fibrinolytic activity was measured by fibrinolytic activity assay and active fractions were pooled

Characterization of purified fibrinolytic protease

Molecular weight determination The molecular mass of purified enzyme was found to be 371.5 kDa as estimated by size exclusion on Superose 12 10/300 GL column using AKTA fast FPLC (Figure 28) and by SDS-PAGE (Figure 29A). The purified enzyme migrated as a single band in SDS-PAGE under reducing condition suggesting that the purified enzyme is monomeric. Fibrin zymogram activity staining also revealed one clear zone of fibrinolytic activity against a blue background (Figure 29B).

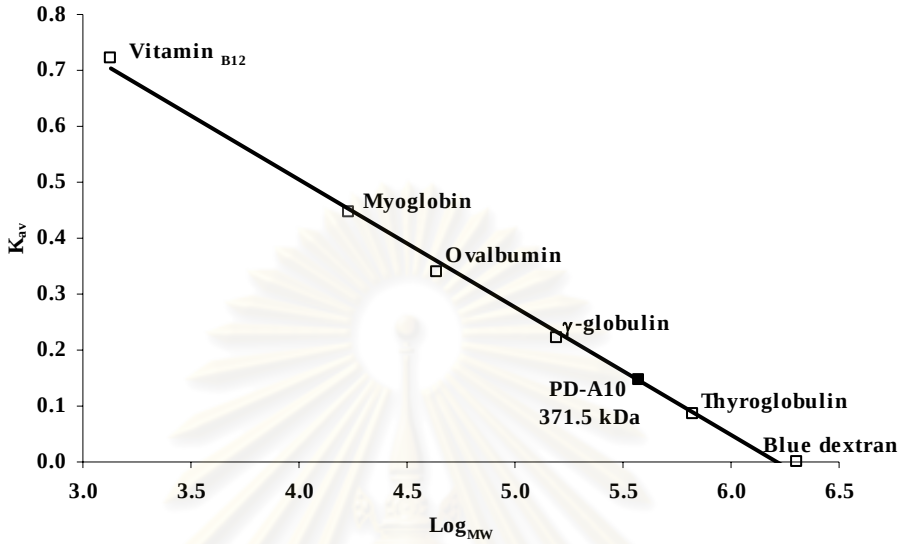


Figure 28. Size-exclusion of fibrinolytic protease purified from PD-A10 by FPLC on superpose 12 10/300 GL column. The standard marker was eluted through a Superpose 12 10/300 GL column equilibrated with 50 mM Tris-HCl buffer containing 0.1 M NaCl (pH7.4) at a flow rate of 0.1 ml/min. The elution profiles were monitored by spectrophotometry at 280 nm.

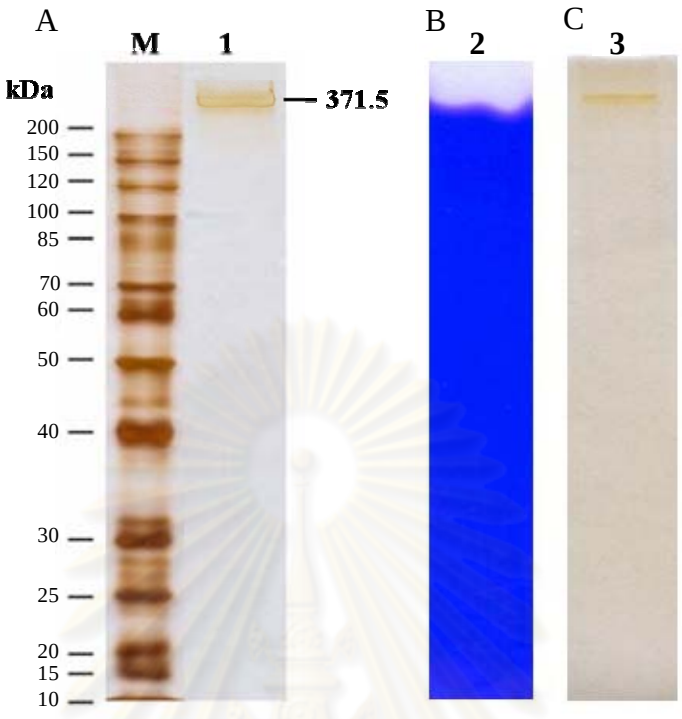


Figure 29. Purified fibrinolytic protease from PD-A10 visualized on SDS-PAGE (A), fibrin zymogram (B) and native-PAGE (C). Lane M is a protein standard marker, lane 1, 2 and 3 are purified fibrinolytic protease.

Amidolytic activity The amidolytic activity of purified fibrinolytic protease was assessed using several chromogenic substrates, such as H-D-Val-Leu-Lys-*p*NA (for plasmin), pyro-Glu-Gly-Arg-*p*NA (for urokinase) and H-D-Val-Leu-Arg-*p*NA (for thrombin). As shown in Table 34, the fibrinolytic protease exhibited a higher degree of specificity for the substrate for thrombin (H-D-Val-Leu-Arg-*p*NA). Therefore, the purified enzyme from PD-A10 was considered to have a fibrinogenolytic activity.

Table 34. Amidolytic activity on synthetic protease substrates

Substrate	A_{405}/min	A_{405}/min/mg	Relative activity (%)	Proteases[1]
H-D-Val-Leu-Lys-*p*NA	0.045[b]	46.78[b]	31[b]	Plasmin
Pyro-Glu-Gly-Arg-*p*NA	0.039[c]	40.54[c]	27[c]	Urokinase
H-D-Val-Leu-Arg-*p*NA	0.145[a]	150.73[a]	100[a]	Thrombin

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

[a, b, c, d] Different letters in the same row denote significant differences ($P < 0.05$).

[1]The substrate are cleaved by the respective protease listed.

When kinetic analysis of the purified fibrinolytic protease reaction was performed using H-D-Val-Leu-Arg-*p*NA as substrate, the enzyme showed the classical Michaelis-Menten kinetics. The following kinetic constants were obtained from the double reciprocal plots of the initial reaction rates and substrate concentrations between 0.05-1 mM: K_m 0.28 mM, V_{max} 0.295 mM/ml/min. The k_{cat} and k_{cat}/K_m values for purified enzyme were 2684.88 s^{-1} and 9663.33 mM/s, respectively (Table 35).

Table 35. Kinetic analysis of amidolytic activity of fibrinolytic protease from PD-A10

Synthetic substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
H-d-Val-Leu-Arg-*p*NA	0.28	2684.88	9663.33

Protease activity Substrate specificity of the purified enzyme was examined using various protein substrates. As shown in Table 36, bovine casein was the most susceptible substrate. Fibrin and fibrinogen were also highly susceptible toward the purified enzyme, at 47.1 and 31.6% relative activity, respectively. From these results, it was suggested that the purified enzyme was a potent protease with fibrinolytic and fibrinogenolytic activity.

Table 36. Protease activity on protein substrates

Protein substrate	Activity[3] (unit/ml)	Relative activity (%)
Fibrin[1]	148.3 ± 16.8^{b}	47.1^{b}
Bovine fibrinogen[2]	99.6 ± 6.9^{c}	31.6^{c}
Bovine serum albumin[2]	93.1 ± 7.7^{c}	29.6^{c}
Bovine haemoglobin[2]	97.9 ± 5.4^{c}	31.1^{c}
Bovine casein[2]	314.9 ± 7.7^{a}	100^{a}

All experiments were carried out in triplicates and repeated twice. The average values were presented. The standard deviation was within 10%.

[a, b, c, d] Different letters in the same row denote significant differences ($P < 0.05$).

[1]Fibrinolytic activity was determined by fibrinolytic activity assay.

[2]Protease activity was determined by protease activity assay.

[3]An enzyme unit was defined as the amount of enzyme releasing 1 nmol of tyrosine equivalent per min per milliliter.

Effect of pH and Temperature The effect of pH on the activity of purified fibrinolytic protease was determined with universal buffer at various pH values. As shown in Figure 30A, the purified enzyme was active over a wide pH range (pH 5-11), but exhibited maximum activity at pH 7.0.

The optimum temperature for fibrinolytic activity was determined by measuring the activity after the incubation of fibrinolytic protease at different temperatures (30-70°C) as showed in Figure 30B. The influence of temperature on the fibrinolytic activity showed that it was active between 30-60°C. The enzyme became less active when the temperature rose above 60°C, and it was completely denatured at temperatures 70°C.

The enzyme remained active at high pH values, but the relative activity was decreased to 60% at pH 5 after incubation for 1 h (Figure 30C). The enzyme was proved to be thermally stable up to a temperature of 50°C after an incubation time of 1 h. Its activity was decreased markedly over 50°C and was not detectable at temperature 70°C (Figure 30D).

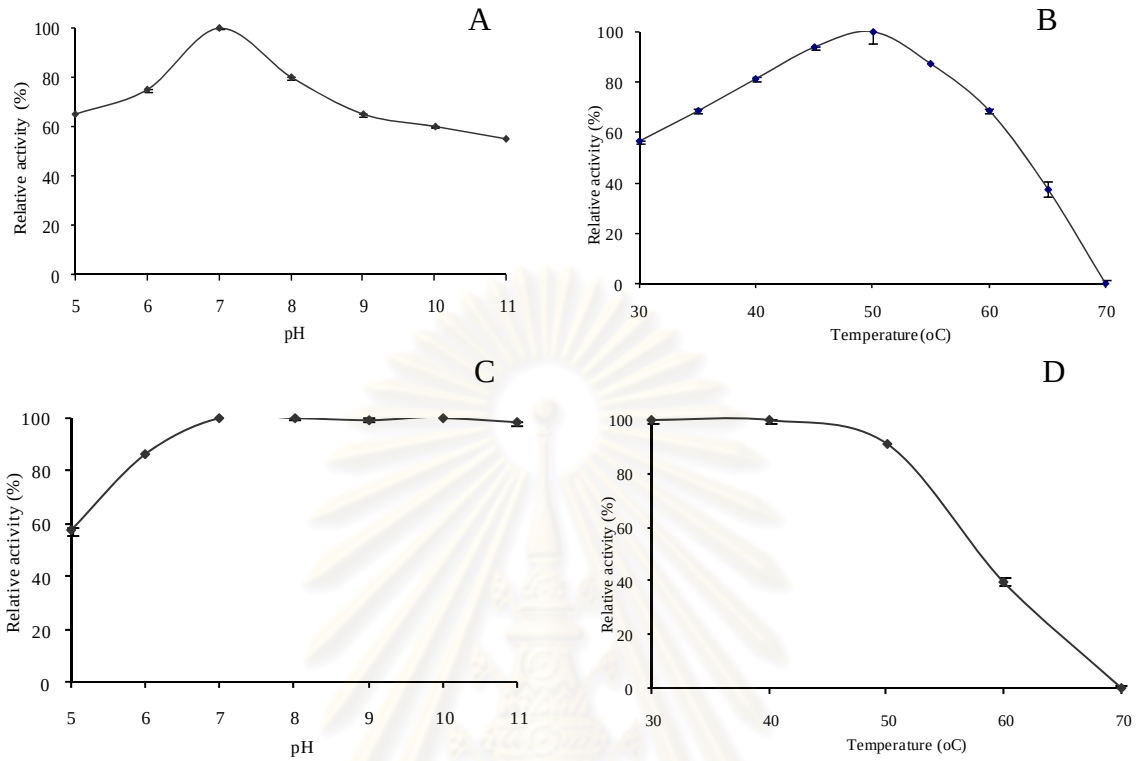


Figure 30. Effect of pH (A and C) and temperature (B and D) on the enzyme activity and stability of the purified fibrinolytic protease from PD-A10. A and B, effect of pH and temperature on the enzyme activity; C and D, effect of pH and temperature on the stability, respectively.

Effect of protease inhibitors on the enzyme activity To classify the purified enzyme, the fibrinolytic activity was assayed in the presence of various protease inhibitors. As shown in Table 37, no significant effects were found with PMSF, leupeptin, TLCK, soybean trypsin inhibitor, EDTA, E-64 and pepstatin A. Approximately 66.7 and 77.8% decrease in activity occurred after the addition of 10 μM of chymostatin and TPCK and the activity was fully inhibited by 100 μM of chymostatin and TPCK. Chymostatin and TPCK potentially inhibited enzyme activity, suggesting that the purified enzyme is a chymotrypsin-like serine protease.

Table 37. Effect of various inhibitors on fibrinolytic activity of the purified fibrinolytic protease from PD-A10[a].

Protease inhibitors	Concentration	Remaining activity (%)
None	-	100
Pepstatin A	1 μM	111.1
E-64	1 μM	97.8
EDTA	1 mM	111.1
PMSF	1 mM	100
Leupeptin	10 μM	111.1
Chymostatin	10 μM	66.7
	100 μM	0
TLCK	10 μM	100
TPCK	10 μM	77.8
	100 μM	0
Soybean trypsin inhibitor	0.1 mg/ml	100

[a]Purified enzyme (2 ug) was preincubated in 20 mM phosphate buffer (pH7.2) at room temperature for 1 h with several protease inhibitors. Fibrinolytic activity was determined by fibrinolytic activity assay. Values represent the mean of triplicate experiments.

Analysis of degradation products of fibrin and fibrinogen To elucidate the mode of reaction of purified protease, degradation products were separated using SDS-PAGE. As shown in Figure 31A, the purified protease rapidly hydrolyzed γγ-chains of fibrin, followed by α- and β-chains. After 2 h of incubation time, all chains were completely hydrolyzed by purified enzyme. Also, the purified enzyme has a fibrinogenolytic activity (Figure 31B). Results for the fibrinogenolysis pattern showed that the enzyme rapidly hydrolyzed the fibrinogen Aα and Bβ chains. It also hydrolyzed the γ-chain, but more slowly. However, all chains were completely hydrolyzed within 1 h. The purified fibrinolytic protease seemed to be a direct-acting fibrinolytic and fibrinogenolytic agent as it acted via direct cleavage of fibrin and fibrinogen and

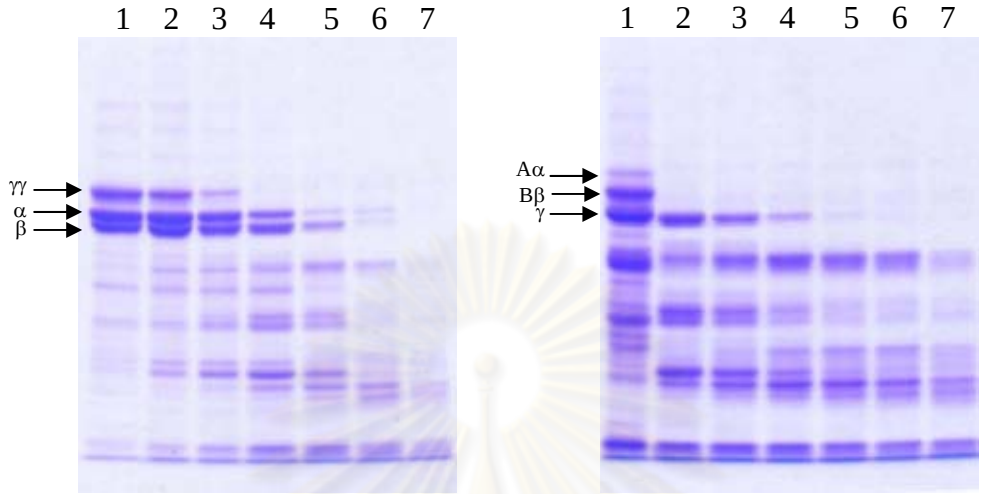


Figure 31. Fibrinolysis (A) and fibrinogenolysis (B) pattern exhibited by purified fibrinolytic protease from PD-A10 over time. The enzyme was incubated with fibrin and fibrinogen at 37°C for various time as indicated. Lane 1, control; lane 2, after 5 min of reaction; lane 3, after 10 min of reaction; lane 4, after 20 min of reaction; lane 5, after 30 min of reaction; lane 6, after 1 h of reaction; lane 7, after 2 h of reaction.

Fibrin and Fibrinogen-agarose plate assay According to the fibrin plate assay shown in Figure 32, the purified fibrinolytic protease was shown to have fibrinolytic activity. The fibrinolytic activity of the purified enzyme was compared with that of plasmin that was already known as standard fibrinolytic protease found in blood system. To further investigate the thrombin-like activity of the purified enzyme, the formation of fibrin was assessed using fibrinogen plate (Figure 33). Turbid fibrin ring was formed by thrombin, but no turbid ring was formed by purified enzyme, indicating that the purified fibrinolytic protease has fibrinogenolytic activity rather than thrombin-like activity.

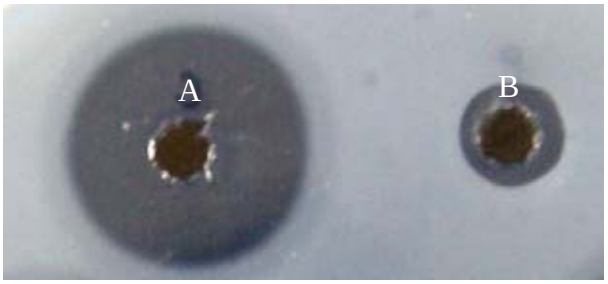


Figure 32. Analysis of fibrinolytic activity by fibrin plate method. Ten microliters of sample solution was placed in each hole and incubated at 37°C for 15 h. After incubation, the radius of clear zone was measured. A; purified fibrinolytic protease from PD-A10 and B; plasmin 1 unit/ml.

Figure 33. Analysis of fibrin formation using fibrinogen-agarose plate. Ten microliters of sample solution was placed in each hole and incubated at 37°C for 15 h. After incubation, the turbid fibrin rings formed around the hole, due to the polymerization of fibrinogen, were measured. A; purified fibrinolytic protease from PD-A10 and B; thrombin 10 NIH unit/ml.

CHAPTER V

DISCUSSION

5.1 Isolation, screening and identification of fibrinolytic protease producing bacteria

Bacteria were isolated from 20 kinds of traditional fermented food produced in Thailand by dilution plating technique on MLB medium. The result showed that all strains exhibited typical characteristics of *Bacillus*. In accordance with the previous report of Kim *et al*. (1997), they found that *Bacillus* sp. producing fibrinolytic protease could screened from a fermented fish known as *Jeot-Gal* by using MLB medium. As the previous reports, fibrinolytic proteases from the genus *Bacillus* that isolated from traditional fermented foods have attracted interests as thrombolytic agents because of their efficiency and safety in the fibrinolytic process (Nakamura *et al*., 1992; Kim *et al*., 1997; Peng *et al*., 2004; Wong and Mine, 2004). Thus, the isolated bacteria were screened for it fibrinolytic activity.

Primary screening was conducted with fibrin plate method. From 163 isolates, it was found that 141 isolates showed fibrinolytic activity. Therefore, 71 isolates from fermented fishery products and 68 isolates from fermented soybean products showed fibrinolytic activity while, 1 isolate from fermented fruits and 1 isolate from vegetable products showed fibrinolytic activity. The result of the present study revealed that the fermented fishery products, namely *Ka-pi, Plaa-chao, Plaa-jorm, Koong-jorm, Poo-khem, Tai-plaa, and Hoi-ma-laeng-poo-dorng*, and the fermented soybean products, namely *Toa-nao, Tao-jieo-khaw, Tao-jieo and Tao-hoo-yee*, were the potential source of microbial fibrinolytic protease. As a pioneer in evaluating the microbial fibrinolytic protease from fermented food, Wong and Mine (2004) revealed that fermented shrimp paste, a traditional and popular seasoning in Asia countries, showed the strong fibrinolytic activity. Later on, it was noted that such enzymes were excreted from *Bacillus* sp. nov. SK006 (Hua *et al*., 2008). In accordance to Nakamura *et al*., they found that fibrinolytic proteases presented in *natto* were from *B. natto* (Nakamura *et al*., 1992).

As the previous reports, potent fibrinolytic proteases have been discovered from fermented food products, such as Japanese *natto* (Sumi *et al*., 1987; Fujita *et al*., 1993), Korean *Chungkook-Jang* soy sauce (Kim *et al*., 1996; Noh *et al*., 1999),

skipjack shiokara (Nakajima *et al*., 1993; Sumi *et al*., 1995), fermented shrimp paste (Wong and Mine, 2004), and marine creatures (Sumi *et al*., 1992). In particular, oral administration of *natto* or its enzyme can effectively enhance the release of an endogenous plasminogen activator in both animal models and human subjects (Sumi *et al*., 1990). These finding imply the possibility of consuming fermented foods to prevent cardiovascular diseases. Indicating the benefit of consuming such Thai fermented foods and the potential to develop as a functional food.

Therefore, it was noted that 21 isolates with strong fibrinolytic activity were from fermented soybean products and fermented fishery products. In accordance with the previous study of Fujita *et al*. (1993), Kim *et al*. (1996), Kim *et al*. (1997), Chang *et al*. (2000), Lee *et al*. (2001), Peng *et al*. (2003), Ko *et al*. (2004), Seo and Lee (2004) and Choi *et al*. (2005), they reported the strong fibrinolytic proteases from *Bacilli* isolated from traditional fermented soybean and fermented fishery products. Therefore, it was presumed that fermented soybean and fermented fishery products were the potential source of fibrinolytic protease-producing *Bacillus*.

Furthermore, the selected 21 isolates were subjected to grouping and identify. From the result of internal transcribed spacers-PCR (ITS-PCR) fingerprinting, fibrinolytic protease-producing bacteria were divided into 4 groups. The result of ITS-PCR fingerprints clearly discriminated the different species, except those closely related like the members of the *B. subtilis* group (*B. subtilis*, *B. mojavensis* and *B. licheniformis*). Moreover, *B. velezensis* showed the same pattern with *B. amyloliquefaciens* as it purposed to be the heterotypic synonym of the follow. As the previous study of Daffonchio *et al*. (1998), the internal transcribed spacers between the 16S and the 23S ribosomal RNA genes were used to discriminate species of the 16S rRNA group I of the genus *Bacillus* by PCR.

In the secondary screening, fibrin zymography was conducted to analyze the pattern of extracellular fibrinolytic proteases (EFPs) from cell-free culture supernatant of 21 selected isolates. From the result, it was found that fibrinolytic protease producing bacteria were divided into 6 group base on enzymatic pattern on fibrin zymography. This is the first report that applied fibrin zymography to analyze and grouping the fibrinolytic protease producing bacteria.

Previously, the reverse fibrin autographic technique was used routinely for the assay of fibrinolytic activity; samples containing fibrinolytic proteases are electrophoresed in SDS-polyacrylamide gels and then placed on the surface of the

fibrin/agar indicator gel (Granelli-Piperno and Reich, 1978; Loskutoff *et al*., 1983; Sprengers *et al*., 1984). The major drawback of reverse fibrin autography is that it is time-consuming and low sensitivity for enzymatic activity. Fibrin zymographic techniques, which are based on SDS-polyacrylamide gel copolymerized with a fibrin substrate which degraded by the fibrinolytic proteases, which are restored during the incubation period after an electrophoretic separation. Enzymatic activities in the zymogram gel are visualized as clear bands of fibrinolysis against a dark-blue background of undigested fibrin substrate (Kim *et al*., 1998; Choi *et al*., 2001; Choi *et al*., 2002). This technique has been reported for the quantitation of extracellular fibrinolytic proteases from the culture media of *Bacillus* strains isolated from fermented foods (Kim *et al*., 1998). Moreover, Kim *et al*. (1998) stated that fibrin zymography technique is more convenient, sensitive and specific for fibrinolytic activity than the previous methods.

Furthermore, fibrinolytic activity of 21 isolates was evaluated by using the hydrolysis of fibrin clot assay. The result showed that fibrinolytic activity of the enzyme assayed by the hydrolysis of fibrin clot was correlative with the radius of clear zone on fibrin plate. According to the report of Astrup and Mullertz (1952), fibrin plate assay is useful for determining the effect of a given compound on fibrinolysis. Presently, this assay was used to screen for fibrinolytic activity of the compounds (Peng and Zhang, 2002a; Yoon *et al*., 2002; Choi *et al*., 2005; Kyung-Ju *et al*., 2007). However, the significant limitation of the fibrin plate is the diffusion capacity of such substance into agar/agarose gel. In this study, various concentrations of plasmin (0.5, 1, 2 and 4 unit/ml) were evaluated by fibrin plate assay and the correlation between the radius of clear zone and plasmin concentration were plotted. As showed in Appendix-C, the correlation between the radius of clear zone and plasmin concentration were not the linear. Thus, to calculate the activity of the test compound by this method would not reliable.

As the fibrin monomers continue to crosslink, the clot begins to retract, condensing the volume of the clot and finally degraded by fibrinolytic agents, the hydrolysis of fibrin clot assay may be a good approximation of *in vitro* clots lysis (Kim *et al*., 1997; Chang *et al*., 2000; Liu *et al*., 2005). This assay provides the natural morphology of the *in vitro* clot and demonstrated a dose-dependent in clot lyses with increasing the concentration of fibrinolytic compound. Therefore, the data obtained from this assay were more reliable than that of fibrin plate.

Next, 6 representative strains that choose based on the enzymatic pattern and the degree of fibrinolytic activity were subjected to 16S rRNA gene sequencing and phylogenetic analysis. From the result of 16S rRNA gene sequence analysis, 6 isolates were belonging to the members of the genera *Bacillus*. Therefore, relationship between 6 selected isolates and closely related *Bacillus* species was also determined by the rep-PCR fingerprinting method, a useful technique for determining inter- and intraspecies relatedness (Versalovic *et al*., 1994 and Gevers *et al*., 2001). $(GTG)_5$-PCR fingerprinting revealed that all isolates belong to the genus *Bacillus* and was similar to the result of 16S rRNA gene sequence analysis. In addition, the DNA-DNA similarity was assessed between isolates and the reference strain of *Bacillus*. From the result, it was found that isolate THY-C1, PD-A10 and K-A7 exhibited low levels of DNA-DNA similarity with the reference strains. Nick *et al*. (1999) and Rademaker *et al*. (2000) have compared rep-PCR genomic fingerprint analysis with DNA–DNA relatedness; they suggested that the two techniques yield results are in close agreement. Therefore, Heyrman *et al*. (2003) reported that rep-PCR fingerprinting can be used as a genomic screening method to differentiate at the species level and to select representatives for DNA–DNA reassociation experiments. Accordingly, on the basis of differences in phenotypic and genetic distinctiveness, THY-C1, PD-A10 and K-A7 should be recognized as representing a novel species of the genus *Bacillus* and the name *Bacillus thailandensis* sp. nov., *B. siamensis* sp. nov. and *B. kapii* sp. nov. were purposed, respectively.

5.2 Optimization of fibrinolytic protease production

Among 6 isolates, THY-C1 that showed the highest activity was chosen to study the optimal condition of fibrinolytic protease production. The condition for the enzyme production was optimized with a stepwise strategy. Firstly, the optimal nitrogen and carbon sources were screened with the one-at-a-time strategy followed by 3^3 full factorial design of the medium composition. Finally, the cultural conditions, such as inoculum level, growth temperature and fibrin supplementation, were studied with one-at-a-time strategy.

Effects of different nitrogen sources on fibrinolytic protease production

Among the organic nitrogen source, yeast extract gave the highest activity while activities were poor with peptone and casein. The type of nitrogen in the medium regulates the growth and fermentation process for fibrinolytic protease production (Liu *et al*., 2005; Chen *et al*., 2007; Deepak *et al*., 2008). Yeast extract, apart from acting as a nitrogen source also supplies vitamins and trace metals, thereby affecting the growth of the organism and thus increasing fibrinolytic protease production. Yeast extract alone or in combination with other nitrogen sources has been used for fibrinolytic protease production by a large number of *Bacillus* sp. (Liu *et al*., 2005; Chen *et al*., 2007; Deepak *et al*., 2008).

Johnvesly and Naik (2001) reported yeast extract was the best followed by casein while beef extract and peptone were poor organic nitrogen sources for protease production in thermophilic *Bacillus* sp. JB-99. Similarly, peptone was found to be poor for protease production in *B. brevis* (Banerjee *et al*., 1999). Therefore, inorganic nitrogen source, such as ammonium sulfate and monosodium glutamate, were poor for fibrinolytic protease production. Johnvesly and Naik (2001) reported that ammonium nitrogen completely inhibited protease production.

Effects of different carbon sources on fibrinolytic protease production

The effect of carbon sources on the production of fibrinolytic protease by THY-C1 was investigated using D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, D-maltose, D-sucrose, D-lactose, D-mannitol, glycerol and soluble starch in the basal medium containing yeast extract. As a result, maltose and sucrose were the best carbon sources for fibrinolytic protease production while fibrinolytic protease production with the addition of mannose was found to be low when compared with not supplemented medium.

Liu *et al*. (2005), working with a strain of *Bacillus natto* NLSSE, also found that maltose, sucrose and glucose had the strong effect on fibrinolytic protease production. Phadatare (1991) reported highest protease activity with arabinose followed by sucrose while Sutar *et al*. (1992) found sucrose, glucose and fructose to be equally good as carbon sources for protease production. Lactose was found to be the best carbon source for protease production by *Bacillus brevis* (Banerjee *et al*., 1999). Johnvesly and Naik (2001) reported that starch, raffinose, arabinose and fructose were good carbon sources while glucose totally repressed protease production. For commercial production, sugars like maltose will be prohibitive due to

their cost (Laxman *et al*., 2005) and hence the use of sucrose as carbon source to produce fibrinolytic protease seems to be the lower cost alternative. Therefore, sucrose was chosen for the further studies.

Optimization of medium compositions with 3^3 factorial designs

The effects of yeast extract concentration as nitrogen source, sucrose concentration as carbon source and pH on fibrinolytic protease production were studied using the statistical approach of 3^3 factorial design. The maximum activity of these responses were attained at run number 3, employing 6 g/l yeast extract, 15 g/l sucrose concentration and pH 9.0. While the lowest activity was obtained when the bacteria was growth in the medium containing 12 g/l yeast extract, 15 g/l sucrose and pH 5.0. From the results, it can be observed that the fibrinolytic protease production was greatly influenced by the initial pH of medium. According to the previously reports, the optimum pH for protease production by *Bacillus* sp. was found to be alkaline pH.

Effect of inoculum level on fibrinolytic protease production

The effect of inoculum level on the production of fibrinolytic protease was studied in the optimized medium. It can be observed that the specific activity of enzyme was highest at 5% (v/v) inoculums level. In accordance to Chauhan and Gupta (2004), the protease production was found to increase with the increase in inoculums percentage.

Effect of growth temperature on fibrinolytic protease production

The optimum growth temperature for fibrinolytic protease production by THY-C1 was found to be 37°C with the highest yield and specific activity while less growth was observed and hence activities were found to be lowest at 50°C. According to the previously report, increase in fermentation temperature caused an increase of fibrinolytic activity and maximum fibrinolytic activity was attained at around 37°C. However, incubation at the temperature higher than 37°C resulted in lower protease production (Ashipala and He, 2008).

Effect of fibrin supplementation on fibrinolytic protease production

The effect of fibrin supplementation on fibrinolytic protease production was studied and it was found that the addition of fibrin resulted in an increase in fibrinolytic protease production. According to the previously report, fibrin was found to be the inducer for fibrinolytic protease production by *Bacillus amyloliquefaciens*

DC-4 (Peng *et al*., 2003). However, further increase in the amount of fibrin did not significantly affect the fibrinolytic protease production.

Time course of fibrinolytic protease production

From the result, fibrinolytic protease production was found to start in late-log phase of bacterial growth in optimized medium, peak at 30 h of incubation and stable upon further incubation. These results support the suggestion that the fibrinolytic protease production of THY-C1 is a manifestation of nitrogen and carbon limitation at the onset of death phase. This phenomenon could be explained due to the fact that protease is expressed during sporulation of *Bacillus* sp. (Kobayashi *et al*., 1998). In contrast, the results for fibrinolytic protease production in non-optimized medium found that enzyme activity reached its maximum at the end of exponential phase (69 unit/ml), which might indicate different mechanism of sporulation of this bacterium in these two different medium.

Moreover, incubation time was the significant factor that can leading to a marked increase in fibrinolytic protease production. Incubation periods ranging from few hours to several days have been found to be best suited for maximum fibrinolytic protease production by *Bacillus* sp. (Liu *et al*., 2005; Chen *et al*., 2007; Deepak *et al*., 2008).

5.3 Characterization of fibrinolytic protease produced by THY-C1

Under the optimal condition, the enzyme production by THY-C1 increased about 10 folds. Further study was conducted to elucidate the characteristic of crude fibrinolytic protease produced by THY-C1.

SDS-fibrin zymography The changes in enzymatic pattern in the original and the optimized medium were observed by SDS-fibrin zymogram. Using a zymography analysis, it was found that THY-C1 secreted 9 extracellular fibrinolytic proteases in the original culture medium while only 3 majors extracellular fibrinolytic protease was found in the optimized medium. However, the pattern of fibrinolytic protease in the optimized condition appeared to be more intense. Based on the results, changes in medium composition as well as fibrin supplementation selectively enhanced the expression of some protease.

Effect of pH on the activity and stability of the fibrinolytic protease The optimum pH for fibrinolytic activity was examined over a pH ranges from 3-11. The optimum pH of the enzyme was around pH 7 to 8 and was relatively higher in the

alkaline region. In contrast, the fibrinolytic activity in the acidic pH region decreased. This is a common feature of alkaline proteases (Joo *et al*., 2003; Joo *et al*., 2004). The pH stability of the enzyme was investigated in a range of pH 3-11 by measuring the residual enzyme activity. As a result, the fibrinolytic protease was found to be stable over a broad pH range (7-11) and retained approximately 60% of activity at pH 6 after 1 h incubation at 37°C. Paik *et al*. (2004) found that fibrinolytic protease from *Bacillus subtilis* KCK-7 was very stable in a range of pH 7-10 and rapidly reduced at pH below 5.

Effect of temperature on the activity and stability of the fibrinolytic protease The effect of temperature on the activity and stability of the fibrinolytic protease was determined in a range of 30-70°C at pH 7.4. The optimum temperature for the fibrinolytic activity was approximately 50°C. A similar observation was found in the fibrinolytic protease produced by *Bacillus subtilis* KCK-7 (Paik *et al*., 2004). The fibrinolytic protease was stable up to 50°C, and its stability decreased at high temperature. The residual activities of 32% and 11% were shown at 60°C and 70°C, respectively. The similar heat stability of the enzyme was also reported in Nattokinase and Subtilin DFE (Kim and Choi, 2000; Peng *et al*., 2003).

Protease inhibitors The effect of natural and synthetic inhibitors on crude fibrinolytic protease was investigated. Enzyme activity was inhibited by PMSF, while other serine protease inhibitors like soybean trypsin inhibitor, TLCK, TPCK, chymostatin and leupeptin not significantly affect the enzyme activity. In addition, metalloproteinase inhibitors such as EDTA and phosphoramidon were not effected the enzyme activity suggested that metal cofactors are not required for it activity. Moreover, cysteine protease inhibitor (E-64) and aspartic protease inhibitor (pepstatin A) showed no inhibitory effect on fibrinolytic protease. This inhibition profile suggests that the fibrinolytic protease from THY-C1 belonged to a family of serine protease. Similar results have been observed in the fibrinolytic protease produced by *Bacillus natto* (Fujita *et al*., 1993), *Bacillus* sp. CK (Kim *et al*., 1996), *Bacillus* sp. DJ-4 (Kim and Choi, 2000), *B. amyloliquefaciens* DC-4 (Peng *et al*., 2003), *B. subtilis* QK02 (Ko *et al*., 2004).

Comparison of substrate specificity of fibrinolytic protease from THY-C1 and the commercial Nattokinase The fibrinolytic activity of fibrinolytic protease produced by THY-C1 was compared with 3 commercial Nattokinase. From the result,

it was found that crude enzyme showed stronger specific activity than the commercial Nattokinase 1 and the commercial Nattokinase 2, but lower than the commercial Nattokinase 3.

Moreover, substrate specificity of crude enzyme from THY-C1 was evaluated and compared with the commercial Nattokinase. Various synthetic substrates were used to evaluate, such as synthetic substrate for plasmin, synthetic substrate for thrombin and synthetic substrate for urokinase. The results demonstrated that the activity of crude enzyme were specific to synthetic substrate for urokinase more than synthetic substrate for plasmin and thrombin. Compared to the commercial Nattokinase, crude enzyme from THY-C1 showed hydrolysis activity to synthetic substrate for plasmin and thrombin more than the commercial Nattokinase 2 and the commercial Nattokinase 3. The hydrolysis activity of crude enzyme to synthetic substrate for urokinase was also higher than the commercial Nattokinase 2. However, the commercial Nattokinase 1 showed higher hydrolysis activity to all synthetic substrates than crude enzyme. These features of crude enzyme from THY-C1 make it an attractive agent as a biomaterial for health promoting foods.

The effect of digestive enzymes on fibrinolytic activity

Effect of pepsin of fibrinolytic activity The results showed that a decrease in fibrinolytic activity of crude enzyme compared to the control was found. The decrease in fibrinolytic activity of crude enzyme from THY-C1 could attribute to its stability in the acid condition.

Effect of pancreatine on fibrinolytic activity The results showed that a slightly decrease in fibrinolytic activity of crude enzyme compared to the control was found. The stability of crude enzyme could attribute to its stability in the alkaline condition. After the incubation with pancreatin, the fibrinolytic activity of crude enzyme was available from 86 - 87.7%. According to these high values, it could be stated that the encapsulation of crude enzyme in order to obtain good stability at the stomach digestion should be considered. For this reason, the stability of crude enzyme in *in vitro* digestive system could be the main determinant for developing to be the food supplement product.

5.4 Purification and characterization of fibrinolytic protease produced by PD-A10

According to the previous study, the optimal condition for fibrinolytic protease production was evaluated by using THY-C1. Later, six representative bacteria from each group of enzymatic pattern were growth in the optimal condition and fibrinolytic protease production were examined. The results showed that the fibrinolytic protease production by PD-A10 was increased and higher than that of THY-C1. Thus, the present study was performed to conduct the purification and characterization of crude enzyme from PD-A10.

Characterization of crude fibrinolytic protease

Effect of pH and temperature on the fibrinolytic activity The enzyme was active in the pH range of 5-11 with the optimum pH at 7 and 10. It was noted that the crude enzyme showed 2 pHs optimum. It may be consumed that crude enzyme contained 2 major extracellular fibrinolytic proteases. From the result, the enzyme was active in the alkaline region and the activity was found to be decreased in the acidic pH region. This is a common feature of alkaline protease. The effect of temperature on the activity of crude enzyme was determined in a range of 30-70°C at pH 7.4. The optimum temperature for the fibrinolytic activity was approximately at 50°C.

Effect of pH and temperature on the stability of crude enzyme The pH stability of the enzyme was investigated in a range of pH 4-11 by measuring the residual enzyme activity. As a result, the fibrinolytic protease was found to be stable over a broad pH range (5-11) and retained approximately 60% of activity at pH 5 after 1 h incubation at 37°C. The fibrinolytic protease was stable up to 50°C, and its stability was decreased at high temperature.

Protease inhibitors The fibrinolytic protease was inhibited by PMSF, TPCK, chymotrypsin and EDTA while other protease inhibitors like soybean trypsin inhibitor, TLCK, leupeltin, phosphoramidon not significantly affect the enzyme activity. In addition, cysteine protease inhibitor (E-64) and aspartic protease inhibitor (pepstatin A) showed non inhibitory effect on fibrinolytic protease. This inhibition profile suggests that crude enzyme from PD-A10 was belong to family of serine and metalloprotease. As the previous result of pH optimum, crude enzyme may contain 2

major extracellular fibrinolytic proteases. It may be proposed that one of them is serine protease and the other is metalloprotease.

Putification of fibrinolytic protease

Crude fibrinolytic protease was purified by anion-exchange chromatography on a Resource Q column. The result showed that 4.81 mg of the enzyme was purified 12.19-fold, with a yield of 51.39%. The fact that 12.19-fold purification was sufficient to achieve homogeneity demonstrates that this protease is one of the major secreted proteins of PD-A10 in culture medium. The fibrinolytic protease preparation obtained by the procedure described here had a specific activity of 2037.3 unit/mg protein.

Characterization of purified fibrinolytic protease

Molecular weight determination The molecular weight of purified enzyme was found to be 371.5 kDa as estimated by size exclusion on Superose 12 10/300 GL column using AKTA fast FPLC (Figure 26) and by SDS-PAGE. The purified enzyme migrated as a single band in SDS-PAGE under reducing condition suggesting that the purified enzyme is monomeric. Fibrin zymogram activity staining also revealed one clear zone of fibrinolytic activity against a blue background. In an earlier instance, molecular weight of the fibrinolytic protease from *Bacillus* sp. have been reported in range of 21-85 kDa (Peng *et al*., 2005). In this study, the molecular weight of the purified enzyme was found to be different.

Amidolytic activity The amidolytic activity of purified fibrinolytic protease was assessed using several chromogenic substrates, such as synthetic substrate for plasmin, synthetic substrate for urokinase and synthetic substrate for thrombin. The fibrinolytic protease exhibited a higher degree of specificity for the substrate for thrombin and considered to have a fibrinogenolytic activity. The other fibrinolytic protease from *Perenniporia fraxinea* mycelia and *Nephila clavata* spider (Joo *et al*., 2002) also exhibited the highest activity for synthetic substrate for thrombin.

When kinetic analysis of the purified fibrinolytic protease reaction was performed using synthetic substrate for thrombin as substrate, the enzyme showed the classical Michaelis-Menten kinetics. The following kinetic constants were obtained from the double reciprocal plots of the initial reaction rates and substrate concentrations between 0.05-1 mM: K_m 0.28 mM, V_{max} 0.295 mM/ml/min. The k_{cat} and k_{cat}/K_m values for purified enzyme were 2684.88 s^{-1} and 9663.33 mM/s, respectively. Catalytic efficiencies (k_{cat}/K_m) of purified enzyme were significantly higher than that of MEF-2 from Chinese mantis *Tenodera sinensis*, being 3148 mM/s

(Hahn *et al*., 2001). The higher activity of the purified enzyme from PD-A10 may be achieved by raising the turnover number (k_{cat}).

Protease activity Substrate specificity of the purified enzyme was examined using various protein substrates. From the result, bovine casein was the most susceptible substrate. Fibrin and fibrinogen was also highly susceptible toward the purified enzyme, at 47.1 and 31.6% relative activity, respectively. From these results, it was suggested that the purified enzyme was a potent protease with fibrinolytic and fibrinogenolytic activity.

Effect of pH and Temperature The effect of pH on the activity of purified fibrinolytic protease was determined with universal buffer at various pH values. The purified enzyme was active over a wide pH range (pH 5-11), but exhibited maximum activity at pH 7.0. The optimum pH of the enzyme is comparable with those of Jeot-gal enzyme from *Bacillus* sp. KA38 isolated from Korean *Jeot-gal* (Kim *et al*., 1997), Bacillokinase II (BKII) from *Bacillus subtilis* strain A1 (Jeong *et al*., 2004) and AMMP from culture mycelia of *Armillaria mellea* (Lee *et al*., 2005).

The optimum temperature for fibrinolytic activity was determined by measuring the activity after the incubation of fibrinolytic protease at different temperatures (30-70°C). The influence of temperature on the fibrinolytic activity showed that it was active between 30-60°C. Optimum activity was found to occur at 50°C, which was comparable with those of KCK-7 from *Bacillus subtilis* KCK-7 (Paik *et al*., 2004) and BK II from *Bacillus subtilis* strain A1 (Jeong *et al*., 2004). The enzyme became less active when the temperature rose above 60°C, and it was completely denatured at temperatures 70°C.

The enzyme remained active at high pH values, but the relative activity was decrease to 60% at pH 5 after incubation for 1 h. The enzyme proved to be thermally stable up to a temperature of 50°C after an incubation time of 1 h, its activity was decreased markedly over 50°C, and was not detectable at temperature 70°C. The temperature stability of the enzyme is comparable with those of CK from *Bacillus* sp. strain CK 11-4 (Kim *et al*., 1996) and Subtilisin DFE from *B. amyloliquefaciens* DC-4 (Peng *et al*., 2003).

Effect of protease inhibitors on the enzyme activity To classify the purified enzyme, the fibrinolytic activity was assayed in the presence of various protease inhibitors. From the result, no significant effects were caused by PMSF, leupeptin,

TLCK, soybean trypsin inhibitor, EDTA, E-64 and pepstatin A. Approximately 66.7 and 77.8% decrease in activity occurred after the addition of 10 μM of chymostatin and TPCK and the activity was fully inhibited by 100 μM of chymostatin and TPCK. Chymostatin and TPCK potentially inhibited enzyme activity, thus suggesting that the purified enzyme is a chymotrypsin-like serine protease. This feature is similar to other pre-reported microbial fibrinolytic protease, such as AMMP from culture mycelia of *Armillaria mellea* (Lee *et al*., 2005). It well known that the important enzyme in fibrinolysis system, t-PA, plasmin, and thrombin are chymotrypsin-like serine proteases.

Analysis of degradation products of fibrin and fibrinogen To elucidate the mode of reaction of purified protease, degradation products were separated using SDS-PAGE. The purified protease rapidly hydrolyzed γγ-chains of fibrin, followed by α- and β-chains. After 2 h of incubation time, all chains were completely hydrolyzed by purified enzyme. Moreover, the results of fibrinogenolysis pattern showed that the enzyme rapidly hydrolyzed the fibrinogen Aα and Bβ chains. It also hydrolyzed the γ-chain, but more slowly. However, all chains were completely hydrolyzed within 1 h. The fibrinogenolysis pattern of the purified enzyme is similar to that of MEF from praying mantis *Tenodera sinensis*, which preferentially hydrolyzed the Aα and Bβ chain of fibrinogen rather than γ-chain (Hahn *et al*., 1999). The purified fibrinolytic protease seems to be a direct-acting fibrinolytic and fibrinogenolytic agent as it acts via direct cleavage of fibrin and fibrinogen.

Fibrin and Fibrinogen-agarose plate assay According to the fibrin plate assay, the purified fibrinolytic protease was shown to have fibrinolytic activity. The fibrinolytic activity of the purified enzyme was compared with that of plasmin that was already known as standard fibrinolytic protease found in blood system. To further investigate the thrombin-like activity of the purified enzyme, the formation of fibrin was assessed using fibrinogen plate. Turbid fibrin ring was formed by thrombin, but no turbid ring was formed by purified enzyme, indicating that the purified fibrinolytic enzyme has fibrinogenolytic activity rather than thrombin-like activity.

CHAPTER VI

CONCLUSION

Because of the promising biological benefits from consuming food sources of fibrinolytic proteases, this study have extensively explored new sources of fibrinolytic protease form Thai fermented foods. Moreover, the fibrinolytic proteases from *Bacillus* sp., isolated from fermented foods, have been investigated.

Bacteria were screened from 20 kinds of Thai fermented food and 163 isolates were obtained. Initially, all bacteria were screened for it fibrinolytic activities by fibrin plate assay. It was found that 141 isolates showed the fibrinolytic activity: 71 isolates from fermented fishery products, 68 isolates from fermented soybean products, 1 isolate from fermented fruit and 1 isolate from vegetable products. It was concluded that the fermented soybean products and fermented fishery products were the potential sources of microbial fibrinolytic protease. In addition, it may be presumed that such fermented food products were the potential sources of fibrinolytic protease.

Twenty-one isolates with strong fibrinolytic activity were selected and subjected to identify. Based on their phenotypic characteristics, fibrinolytic protease-producing bacteria were identified as *Bacillus* sp. From the result of intergenic transcribed spacer-PCR (ITS-PCR) fingerprinting, bacteria were divided into four groups. Group I was closely related to *B. subtilis* and comprised of 12 isolates, Group II was closely related to *B. vallismortis* and comprised of 3 isolates and Group III was closely related to *B. amyloliquefaciens* and comprised of 4 isolates, respectively, while Group IV showed different pattern from type stains and comprised of 2 isolates.

Moreover, the enzymatic pattern of cell-free supernatant from 21 selected isolates was evaluated by using fibrin zymogram. Based on the enzymatic pattern, they were divided into 6 groups. In addition, fibrinolytic activity of all isolates was evaluated by the hydrolysis of fibrin clot assay. Then 6 representative bacteria, namely THY-C1, PD-Al0, K-A7, K-B16, TJW-A9 and TISTR 651, which showed the highest fibrinolytic activity in each group of enzymatic pattern were chosen and subjected to 16S rRNA gene sequencing. The results from 16S rRNA gene sequencing, they were identified as *Bacillus* sp. and closely related to the type strains of *B. licheniformis*, *B. mojavensis*, *B. subtilis*, *B. vallismortis*, *B. velezensis* and *B.*

amyloliquefaciens. Moreover, the results of DNA-DNA hybridization, rep-PCR fingerprinting, DNA G + C content, fatty acid profile and biochemical characteristics revealed that 3 isolates, namely, THY-C1, PD-A10 and K-A7, were identified as novel species in the genus *Bacillus* and the name *B. thailandensis* sp. nov., *B. siamensis* sp. nov. and *B. kapii* sp. nov., respectively, were purposed.

The optimal condition for the fibrinolytic protease production by THY-C1 that showed the highest fibrinolytic activity among 6 representative bacteria was evaluated. The maximal enzyme production (101.2 unit/ml) was achieved when the bacterium was inoculated at 5% (v/v) into a medium containing 0.6% (w/v) yeast extract and 1.5% (w/v) sucrose at pH 9.0 with fibrin supplementation and incubated for 30 h at 37°C. Under the optimal condition, the enzyme production increased about 10 folds compared to the original medium. The activity staining of crude enzymes on the fibrin zymogram indicated the changes in activity band patterns. The enzyme was optimally active at pH 7-8 and stable over a broad pH range from 6-11. Its optimum temperature was around 50°C. Enzyme was stable up to 50°C and strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), a specific inhibitor of serine protease. In addition, when compared with the commercial fibrinolytic enzyme, the cell free supernatant of THY-C1 showed stronger specific activity than the commercial Nattokinase 1 and the commercial Nattokinase 2, but lower than the commercial Nattokinase 3. Moreover, fibrinolytic protease showed its stability to pancreatin and bile salt solution, but lost its activity after incubating with pepsin solution in *in vitro* digestive model. These features of crude enzyme from THY-C1 make it an attractive agent as a biomaterial for health promoting foods.

Furthermore, Fibrinolytic proteases produced by 6 representative bacteria in the optimal condition were compared and the result showed that fibrinolytic protease produced by PD-A10 had the highest activity. Thus fibrinolytic protease produced by PD-A10 was purified and characterized. Crude extract of fibrinolytic protease from PD-A10 was purified to homogeneity by column chromatography on Resource Q. The molecular weight was 371.5 kDa as estimated by Gel filtration on sepharose GL12 10/30 and by SDS-PAGE. The enzyme was the most active at pH 7.0, and its optimum temperature was 50ºC. The enzyme activity was relative stable at pH 7.0-9.0 and temperature below 50ºC. The activity was inhibited by chymostatin and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), indicating that the chymotrypsin–like

serine protease. The enzyme exhibited a high affinity toward synthetic substrate for thrombin (H-D-Val-Leu-Arg-*p*NA) and V_{max} and K_m values of 0.295 mM/ml/min and 0.28 mM, respectively. The k_{cat} and k_{cat}/K_m values for purified enzyme were 2684.88 s^{-1} and 9663.33 mM/s. The purified enzyme could completely hydrolyze a fibrin and fibrinogen substrate *in vitro* within 2 h and 1 h, respectively. The result from fibrinogen plate assay showed that the purified enzyme possessed it fibrinogenolytic activity rather than thrombin-like activity.

Overall the results in this study suggested that traditional fermented foods produced in Thailand were the important source of microbial fibrinolytic protease, especially from the genus *Bacillus.* Fibrinolytic protease from these food grade-microorganisms has the potential to be developed as functional food additives and drug to prevent or cure cardiovascular diseases. Presently, development of microbial fibrinolytic protease is still ongoing and much work needs to be done intensively and extensively, especially concerning the thrombolytic effects *in vivo* and increasing the efficacy and fibrin specificity of the thrombolytic agents.

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APPENDICES

APPENDIX A

CULTURE MEDIA, REAGENT AND BUFFER PREPARATION

1. Gram's staining

Gram's staining was performed by using air-dried slides which were fixed and dried before staining by standard procedure (Dussault, 1955). Thin smear of bacterial conoly was prepared on a clean slide. Slide was fixed by passing through flame. The smear was covered with crystal violet solution for 30 sec, then rinsed with water and drained. Next, the smear was covered with iodine solution for 30 sec, then rinsed with water. Decolorized with 95% ethanol and washed with water, then it was counter stained about 30 sec with safranin solution. Blot slide was dried and examined under oil immersion (1,000X). Cell morphology was examined after incubated for 2 days.

2. Basal medium

2.1 Modified Luria-Bertani medium (Kim *et al*., 1997)

Tryptone	10	g
Yeast extract	5	g
Glucose	25	g
Sodium chloride	50	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

2.2 Nutrient medium

Peptone	5	g
Meat extract	3	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

2.3 Tryptic soy medium

Casein peptone	15	g
Soya peptone	5	g
Sodium chloride	5	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.3 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

2.4 Luria-Bertani medium

Tryptone	10	g
Yeast extract	5	g
Sodium chloride	5	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

2.5 Tyrosine medium

Peptone	5	g
Meat extract	3	g
L-tyrosine	5	g
Distilled water	1000	ml

Dissolved in distilled water and sterile by autoclaving at 115ºC for 10 min.

2.6 Potato dextrose medium (PD)

Potato dextrose broth (Difco)	24	g
Distilled water	1000	ml

Suspend the powder in 1000 ml of distilled water and mix thoroughly. Medium was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder then sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

2.7 Mannitol yeast-extract medium MYE)

Peptone	2.5	g
Yeast extract	2.5	g

Sodium chloride	2.5	g
Mannitol	5	g
Distilled water	950	ml

Dissolve and adjust pH to 7.0 with NaOH. Medium was sterile by autoclaving at 115ºC for 10 min.

3. Sulphide production, indole and motility test

3.1 Sulphide-indole-motility (SIM) medium

Tryptone	30	g
Meat extract	3	g
Sodium thiosulfate pentahydrate	0.5	g
Cysteine hydrochloride	0.2	g
Sodium chloride	5	g
Distilled	950	ml

Dissolved in and brought up the volume to 1000 ml with distilled water then sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

3.2 Kovac's reagent

p-Dimethylaminobenzaldehyde	5	g
Amyl alcohol	75	ml
Concentrate hydrochloric acid	25	ml

Dissolve the aldehyde in the alcohol by gentle warming in a water bath (about 50ºC) and kept in the amble bottle. Protect from the light and stored at 4ºC.

4. Catalase test

4.1 Hydrogen peroxide (H_2O_2) solution

Hydrogen peroxide	3	ml

Dissolve and adjust volume to 100 ml with distilled water.

5. Oxidase test

5.1 Tetramethyl-p-phenylenediamine solution

Tetramethyl-*p*-phenylenediamine	1	g

Dissolve and adjust volume to 100 ml with distilled water.

6. MR-VP test

6.1 Glucose phosphate (GP) medium

Peptone	5	g
Dipotassium phosphate	5	g
Glucose	5	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.5 with NaOH. Medium was sterile by autoclaving at 115ºC for 10 min.

6.2 Methyl red solution

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

Dissolve and mixed thoroughly, then adjust pH to 5.0

6.3 Creatine solution

Creatine monohydrate	0.5	g
Distilled water	100	ml

Dissolve and mixed thoroughly.

6.4 40% Potassium hydroxide (KOH) aqueous solution

Potassium hydroxide	40	g
Distilled water	90	ml

Dissolve and adjust the volume to 100 ml with distilled water.

7. Citrate utilization test

7.1 Simmon's citrate medium

Sodium chloride	5	g
Magnesium sulfate	0.2	g
Ammonium phosphate	1	g
Dipotassium hydrogen phosphate	1	g
Citric acid	2	g
Bromthymol blue, 40% aq. soln	20	ml

Dissolve and adjust pH to 6.8 with HCl. Medium was sterile by autoclaving at 115ºC for 10 min.

8. DNase test

8.1 DNase test agar (Difco)

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

Dissolve and adjust pH to 7.3 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

9. Indole test

9.1 Peptone water

Peptone	10	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

9.2 Kovac's reagent

p-Dimethylaminobenzaldehyde	5	g
Amyl alcohol	75	ml
Concentrate hydrochloric acid	25	ml

Dissolve the aldehyde in the alcohol by gentle warming in a water bath (about 50ºC) and kept in the amble bottle. Protect from the light and stored at 4ºC.

10. Nitrate reduction test

10.1 Nitrate broth

Peptone	10	g
Beef extract	10	g
Potassium nitrate	3	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

10.2 Nitrate reagent A (sulphanilic acid solution)

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

Dissolve and gentle heating in a fume hood.

10.3 Nitrate reagent B (*N*,*N*-dimethyl-l-naphthylamine solution)

N,*N*-dimethyl-l-naphthylamine	0.5	g
5 N Acetic acid	100	ml

Dissolve and gentle heating in a fume hood.

11. Urease activity test

11.1 SSR medium

Potassium phosphate monobasic	9.1	g
Disodium hydrogen phosphate	9.5	g
Yeast extract	0.1	g
Urea	20	g
Phenol red, 0.2% aq. soln.	5	ml
Distilled water	950	ml

Dissolve and adjust pH to 6.8 with HCl. Medium was sterile by filtration. Then aseptically distribute into sterile tubes.

12. Hydrolysis of tween 80, starch, gelatin, casein, tyrosine, and aesculin

12.1 Tween 80 agar plate

Tween 80	2	ml
Yeast extract	5	g
Glucose	25	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

12.2 Starch agar plate

Starch	10	g
Yeast extract	5	g
Glucose	25	g

Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

12.3 Lugol's iodine solution

Potassium iodide	40	g

Dissolve and adjust volume to 100 ml with distilled water.

12.4 Gelatin agar plate

Gelatin	10	g
Yeast extract	5	g
Glucose	25	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

12.5 10% Trichloroacetic acid (TCA) solution

Trichloroacetic acid	10	g

Dissolve and adjust volume to 100 ml with distilled water.

12.6 Casein agar plate

Casein	10	g
Glucose	25	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

12.7 Tyrosine agar plate

Tyrosine	10	g
Glucose	25	g
Distilled water	1000	ml

Dissolve and adjust pH to 7.2 with NaOH. Medium was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

12.8 Aesculin broth

Aesculin	1	g
Ferric citrate	0.5	g
Peptone water	1000	ml

Dissolve and adjust pH to 7.4 with NaOH. Medium was sterile by autoclaving at 115ºC for 10 min.

13. Phenylalanine deamination

13.1 Phenylalanine agar slope

DL-phenylalanine	2	g
Yeast extract	3	g
Disodium hydrogen phosphate	1	g
Sodium chloride	5	g
Agar	20	g
Distilled water	950	ml

Dissolve and adjust volume to 1000 ml with distilled water. Medium was sterile by autoclaving at 115ºC for 10 min.

13.2 10% Ferric chloride ($FeCl_3$) aqueous solution

Ferric chloride	10	g
Distilled water	90	ml

Dissolve and adjust the volume to 100 ml by distilled water.

14. Decarboxylation of L-arginine, L-lysine and L-ornithine

14.1 Falkow's medium

Peptone	5	g
Yeast extract	3	g
Glucose	1	g
Bromcresol purple, 0.2% aq. soln	10	ml
Distilled water	1000	ml

Dissolve and adjust pH to 6.7 with HCl, and added the indicator solution. Medium was sterile by autoclaving at 115ºC for 10 min. Then solution was divided into sterile bottles and added the following additions:

1. L-arginine hydrochloride 0.5%

2. L-lysine hydrochloride 0.5%
3. L-ornithine hydrochloride 0.5%
4. No addition

Re-adjusted pH to 6.7 if necessary. Dispense in small tubes and sterile at 115ºC for 10 min.

15. DNA extraction

15.1 Saline-ethylenediaminetetraacetic acid (S-EDTA) buffer pH 8.0

Ethylenediaminetetraacetic acid	37.22	g
Sodium chloride	8.76	g

Dissolve EDTA with distilled water and adjust pH to 8.0 with NaOH. Then NaCl was added and adjusted volume to 1 l by distilled water. Sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

15.2 10% (w/v) Sodium lauryl sulfate (SDS) solution

Sodium lauryl sulfate	10	g

Dissolve and adjust the volume to 100 ml by distilled water.

15.3 Phenol:chloroform (1:1)

Crystalline phenol was liquidfied in water bath at 65ºC and mixed with the equal volume of chloroform. The solution was stored in the amble bottle to protect from the light.

15.4 0.1X Saline-sodium citrate (SSC) solution

Sodium citrate	440	mg
Sodium chloride	87.6	mg

Dissolve with distilled water and adjust pH to 7.0 with NaOH. Then make up to 1 l with distilled water. 0.1X SSC solution was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

15.5 RNase T_1 and proteinase K solution

RNase T_1	80	µl
Proteinase K (sigma)	4	mg
50 mM Tris-HCl, pH 7.5	10	ml

Mix 80 µl of RNase T_1 with 10 ml of 50 mM Tris-HCl, pH 7.5 and heat at 95ºC for 5 min. After cool down the solution to 4ºC, 40 mg of Proteinase K was added and stored at -20ºC.

16. Intragenic transcription spacer-PCR (ITS-PCR)

16.1 6X DNA loading dye

Tris (hydroxymethyl)aminomethane	1.21	g
Bromophenol blue	0.03	g
Xylene cyanol FF	0.03	g
Glycerol	60	ml
Sodium lauryl sulfate	1	g
Ethylenediaminetetraacetic acid	37.22	g

Dissolve Tris (hydroxymethyl)aminomethane with distilled water and adjust pH to 8.0 with HCl. After dissolve EDTA and glycerol, pH was adjusted to 7.6 with HCl and bromophenol blue and xylene cyanol FF were added. Then the volume was brought up to 100 ml with distilled water. 6X DNA loading dye was stored at 4ºC.

16.2 2.5 % (w/v) Agarose gel

Agarose	2.5	g
Distill water	100	ml

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

16.3 10X Tris acetate- ethylenediaminetetraacetic acid (TAE) buffer

Tris (hydroxymethyl)aminomethane	48.4	g
Acetic acid (glacial)	11.4	g
Ethylenediaminetetraacetic acid	3.7	g
Sodium hydroxide	1	g

Mix Tris (hydroxymethyl)aminomethane and NaOH with distilled water. EDTA was added and mixed thoroughly, and then acetic acid was added and brought up the volume to 1000 ml with distilled water. Diluted 10 times before use.

16.4 Ethidium bromide solution

Ethidium bromide	50	mg

Dissolve with distilled water and make up to 100 ml with distilled water. The solution was stored in the amble bottle to protect from the light.

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

17.1 1.5% (w/v) Agarose gel

Agarose	1.5	g
Distill water	100	ml

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

17.2 10X Tris-borate buffer (TBE) buffer

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

Dissolve Tris (hydroxymethyl)aminomethane and boric acid with distilled water and adjust 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.

18. DNA-DNA hybridization

18.1 0.1 M Tris-Hydrochloric acid (Tris-HCl), pH 9.0

Tris (hydroxymethyl)aminomethane	1.21	g

Dissolve with distilled water and adjust pH to 9.0 with HCl. Then make up to 100 ml with distilled water. 0.1 M Tris-HCl buffer was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.2 2X Phosphate buffer-saline (2X PBS)

Potassium chloride	0.4	g
Potassium phosphate monobasic	0.24	g
Sodium phosphate (anhydrous)	1.82	g

Sodium chloride	4.0	g
Distill water	950	ml

Dissolve and adjust the volume to 1 l with distilled water. Phosphate-buffer saline was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.3 0.1 M Magnesium chloride ($MgCl_2$)

Magnesium chloride	9.52	g
Distill water	1000	ml

Dissolve and adjust to 1000 ml with distilled water. 0.1 M $MgCl_2$ was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.4 100X Denhardt solution

Bovine serum albumin	2	g
Polyvinyl pyrrolidone	2	g
Ficoll 400	2	g
Distill water	90	ml

Dissolve and adjust to 100 ml with distilled water.

18.5 10 mM Tris-Hydrochloric acid (Tris-HCl), pH 7.6

Tris (hydroxymethyl)aminomethane	1.21	g

Dissolve with distilled water and adjust pH to 7.6 with HCl. Then make up to 1 l with distilled water. 10 mM Tris-HCl buffer was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.6 10 mM Ethylenediaminetetraacetic acid disodium (Na_2-EDTA), pH 7.6

Na_2-EDTA	0.47	g
Distill water	950	ml

Dissolve with distilled water and adjust pH to 7.6 with HCl. Then bring up the volume to 1 l with distilled.

18.7 10 mM Tris-ethylenediaminetetraacetic acid (TE) buffer, pH 7.6

10 mM Tris-HCl, pH 7.6	100	ml
10 mM Na_2-EDTA, pH 7.6	100	ml

Adjust the volume to 1 l with distilled water.

18.8 10 mg/ml Salmon sperm DNA

Salmon sperm DNA	10	mg
10 mM TE buffer, pH 7.6	1	ml

Dissolve and boil for 10 min, immediately cool down on ice and sonicate for 3 min. The solution was kept at -20ºC.

18.9 20X Salin-sodium citrate (SSC) solution

Sodium citrate	8.8	g
Sodium chloride	17.5	g
Distilled water	950	ml

Dissolve with distilled water and adjust pH to 7.0 with NaOH, and then make up to 1 l with distilled water. 20X SSC solution was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.10 Prehybridization solution

100X Denhardt solution	5	ml
10 mg/ml Salmon sperm DNA	1	ml
20X SSC	10	ml
Formamide	50	ml
Distilled water	34	ml

The solution was mixed well and kept at -20ºC.

18.11 Hybridization solution

Prehybridization solution	100	ml
Dextran sulfate	5	g

The solution was mixed by sonicating and kept at -20ºC.

18.12 0.2X Saline-sodium citrate (SSC)

20X Saline-sodium citrate	1	ml
Distill water	99	ml

Dissolve 20X SSC with distilled water and adjust to 100 ml. 0.2X SSC solution was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

18.13 Solution I

Bovine serum albumin	0.25	g
Triton X-100	50	µl
Phosphate buffer-saline (PBS)	50	ml

Dissolved and mixed well, then kept at 4ºC.

18.14 Solution II

Streptavidin-POD	1	µl
Solution I	4	ml

Solution II was freshly prepared by dissolved streptavidin-POD in solution I before use.

18.15 Solution III

3,3',5,5'-Tetramethylbenzidine (TMB)		
(10 mg/ml DMFO)	100	ml
0.3% Hydrogen peroxide	100	ml
0.4 M Citric acid + 0.2 M Disodium hydrogen phosphate buffer,		
pH 6.2 in 10% DMFO	100	ml

Solution III was freshly prepared by dissolved all ingredients before use.

18.16 2 M Sulfuric acid (H_2SO_4)

Sulfuric acid	22	ml
Distill water	178	ml

The solution was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

19. Diaminopimelic acid (DAP) analysis

19.1 0.2% ninhydrin solution

Ninhydrin	0.2	g
1-Butanol saturated in water	100	ml

Solution was prepared by dissolved ninhydrin in n-butanol in water and keep in the amber bottle.

20. Polar lipids

20.1 Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	ml

Solution was prepared by dissolved ninhydrin in n-butanol in water and keep in the amber bottle.

20.2 Dittmer and Lester reagent

Solution A

Molybdenum trioxide (MoO_3)	4.011	g
25 N Sulfuric acid (H_2SO_4)	100	ml

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

Solution B

Molybdenum powder	0.178	g
Solution A	50	ml

Dissolved 0.178 g Molybdenum powder in 50 ml of solution A and boiled for 15 min. Cool and remove the precipitate by decantation.

Before spraying, mixed solution A (50 ml) with solution B (50 ml) and water (100 ml). The final solution was greenish yellow in color. If too little water was used, it will be blue; if too much, yellow. The spray was stable for months.

21. DNA base composition

21.1 The mixture of 40 mM sodium acetate (CH_3COONa) and 12 mM zinc sulfate ($ZnSO_4$) solution, pH 5.3

Sodium acetate	3.28	g
Zinc sulfate	1.94	g
Distill water	90	ml

Dissolve CH_3COONa 3.28 g and $ZnSO_4$ 1.94 g in distilled water and adjust to pH 5.3 with HCl. Brought the volume to 100 ml with distilled water.

21.2 Nuclease P_1 solution

Nuclease P_1	0.1	mg

The mixture of 40 mM sodium acetate

and 12 mM zinc sulfate (pH 5.3)	1	ml

Mix 0.1 mg of Nuclease P_1 with 1 ml of the mixture of 40 mM sodium acetate and 12 mM zinc sulfate, pH 5.3 and stored at -20ºC.

21.3 0.1 M Tris-hydrochloric acid (HCl), pH 8.1

Tris (hydroxymethyl)aminomethane	1.21	g

Dissolve with distilled water and adjust pH to 8.1 with HCl. Then make up to 100 ml with distilled water. 0.1 M Tris-HCl buffer was sterile by autoclaving at 121ºC, 15 pound/inch^2 pressure for 15 min.

21.4 Alkaline phosphatase solution

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

Mix 2.4 units of alkaline phosphatase with 1 ml of 0.1 M Tris-HCl, pH 8.1 and stored at -20ºC.

22. 0.1 M The Britton-Robinson universal buffer (Britton and Robinson, 1931)

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

Dissolve with distilled water and adjust to the desired pH with 1N HCl or 1N NaOH. Then make up to 1000 ml with distilled water.

APPENDIX B

STANDARD ASSAY METHODS

1. Lowry's method (Lowry *et al*., 1951)

1.1 Reagent

1.1.1 Reagent A

Sodium carbonate	2	g
Sodium hydroxide	4	g
Distill water	1000	ml

Dissolved and mixed well, then kept at room temperature.

1.1.2 Reagent B

Copper sulfate	0.5	g
Sodium tartrate	1	g
Distill water	100	ml

Dissolved and mixed well, then kept at 4ºC.

1.1.3 Reagent C

2N Folin-Ciocalteu's phenol reagent	10	ml
Distilled water	10	ml

The solution light-sensitive so it should be freshly prepared before use.

1.1.4 Reagent D

Reagent A	50	ml
Reagent B	1	ml

The solution should be freshly prepared before use.

1.2 Procedure

1.2.1 Place 0.1 ml of proper dilution of protein solution (for protein determination) or supernatant of reaction mixture (for soluble peptide determination) into clean tube.

1.2.2 Add 1 ml of reagent D into the tube and vortex immediately. Incubate at room temperature for 10 min, exactly. Then 0.1 ml of

reagent C was added to sample and vortex immediately. Incubate at room temperature for 30 min and the absorbance of sample was measured at 750 nm

1.2.3 Concentration of the sample was compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

2. Polyacrylamide gel electrophoresis (PAGE)

Running gel for SDS-PAGE

	10%	12%	
Monomer solution	3.333	4	ml
4X Running buffer	2.5	2.5	ml
Deionized water	4.012	3.345	ml
10% SDS	100	100	µl
10% Ammonium persulphate	50	50	µl
TEMED	5	5	µl

Note: Running gel for Native PAGE was prepared in a similar manner except that the addition of SDS was omitted.

Stacking gel for SDS-PAGE

	4%	
Monomer solution	0.666	ml
4X Stacking buffer	1.25	ml
Deionized water	3.053	ml
10% SDS	50	µl
10% Ammonium persulphate	25	µl
TEMED	5	µl

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

2.1 Monomer solution

Acrylamide	30	g
Bis-acrylamide	0.8	g

Brought up to 100 ml with deionized water and kept in amber bottle at 4ºC.

2.2 4X Resolving gel buffer

Tris (hydroxymethyl)aminomethane	18.15	g

Dissolved in deionized water and adjusted pH to 8.8 by 1 N HCl. Then brought up to 100 ml with deionized water and kept in amber bottle at 4ºC.

2.3 4X Stacking gel buffer

Tris (hydroxymethyl)aminomethane	6	g

Dissolved in deionized water and adjusted pH to 6.8 by 1 N HCl. Then brought up to 100 ml with deionized water and kept in amber bottle at 4ºC.

2.4 10X Tank buffer for Native PAGE

Tris (hydroxymethyl)aminomethane	30.28	g
Glycine	144.13	g
Distilled water	900	ml

Dissolved and brought up to 1000 ml with deionized water, kept at room temperature. Diluted 10 times before use.

2.5 10X Tank buffer for SDS-PAGE

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

Dissolved and brought up to 1000 ml with deionized water, kept at room temperature. Diluted 10 times before use.

2.6 10% Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulfate	10	g
Distilled water	90	ml

Dissolved and brought up to 1000 ml with deionized water, kept at room temperature.

2.7 2X Sample buffer for Native PAGE

4X Stacking gel buffer	2.5	ml
Glycerol	2	ml
Bromphenol blue (2 mg/ml)	1	ml

Dissolved and brought up to 10 ml with deionized water then filtered, kept at -20ºC.

2.8 2X Sample buffer for SDS-PAGE (non reducing agent)

4X Stacking gel buffer	2.5	ml
Glycerol	2	ml
10% (w/v) SDS	4	ml
Bromphenol blue (2 mg/ml)	1	ml

Dissolved and brought up to 10 ml with deionized water then filtered, kept at -20ºC.

For 2X sample buffer for SDS-PAGE (reducing agent), 0.2 ml of β-mercaptoethanol were added.

2.9 Staining solution

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

Dissolved and brought up to 1000 ml with deionized water, kept in amber bottle at room temperature. The solution should be filtered before use.

2.10 Destaining solution

Methanol	300	ml
Acetic acid	100	ml

Dissolved and brought up to 1000 ml with deionized water, kept at room temperature.

APPENDIX C

STANDARD CURVE

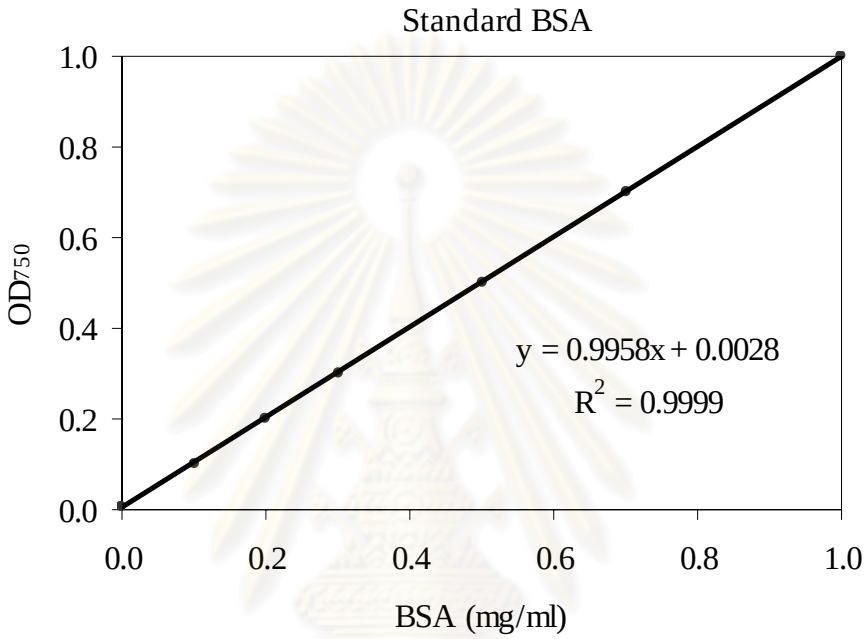

Figure 34. Standard curve of bovine serum albumin (BSA).

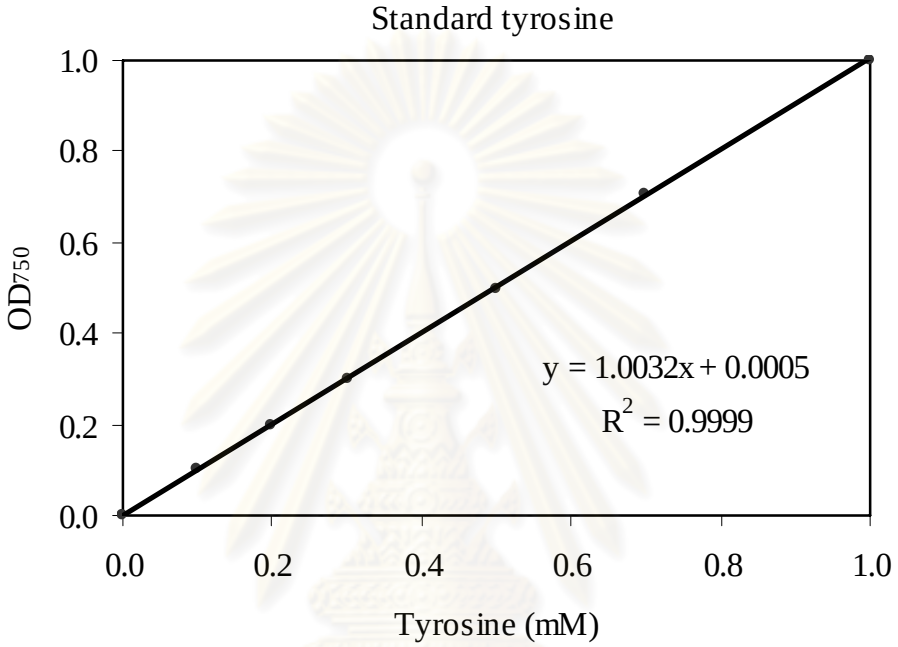

Figure 35. Standard curve of tyrosine.

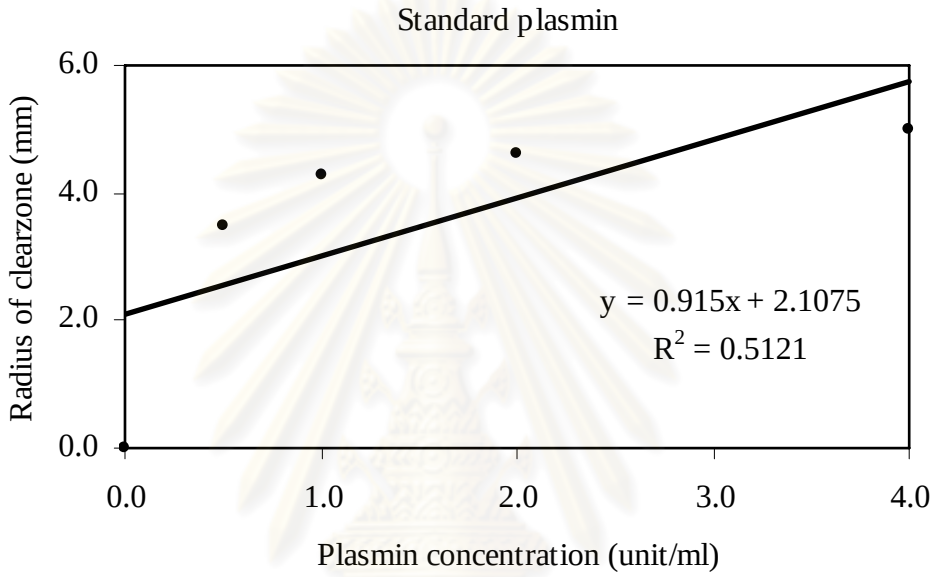

Figure 36. Standard curve of plasmin.

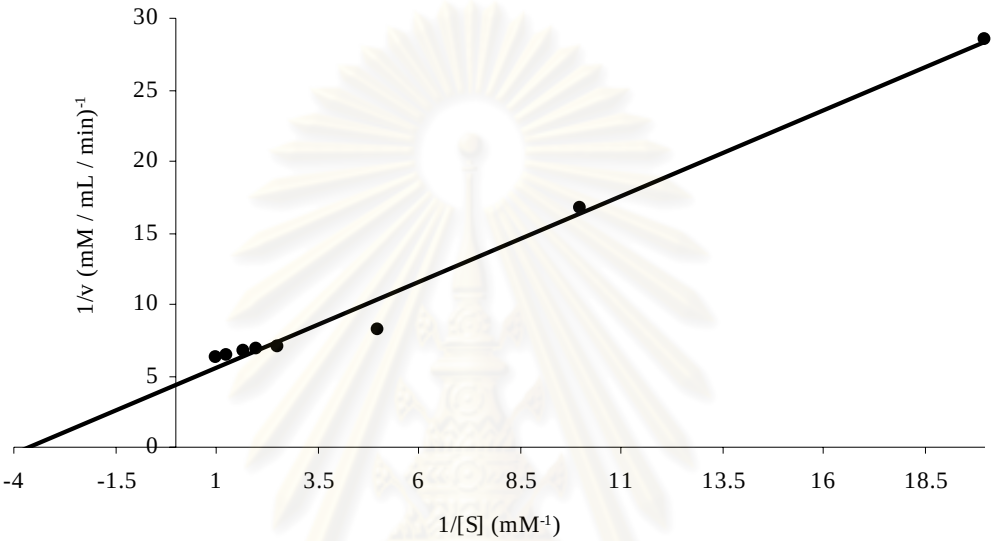

Figure 37. Lineweaver-Burk plot generated from the initial reaction rates obtained with H-D-Phe-Pip-Arg-*p*NA in the range of 0.05-1 mM and 2 μg of purified enzyme per assay.

APPENDIX D

CHEMOTAXONOMIC CHARACTERISTICS

Figure 38. Thin-layer chromatography of the diaminopimelic acid (DAP) of strain THY-C1^T, PD-A10^T and K-A7^T.

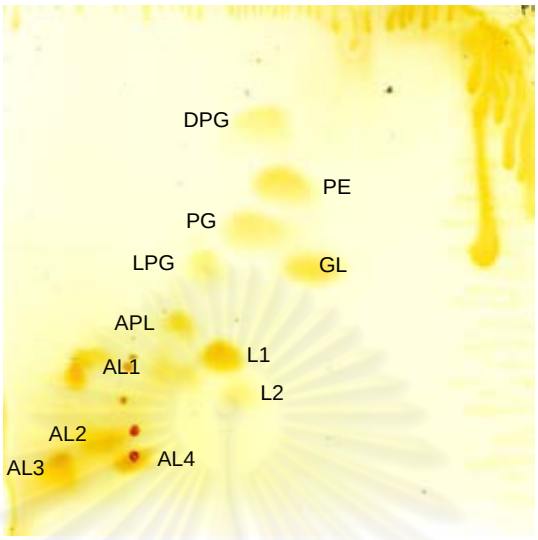

Figure 39. Two-dimensional thin layer chromatography of polar lipid of strain PD-A10^{T}. Chloroform-methanol-water (65:25:4) was used in the first direction, followed by chloroform-acetic acid-methanol-water (40:7.5:6:2) in the second direction. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPG, lysylphosphatidylglycerol; GL, glycolipid; APL, unknown aminophopholipid; AL1-4, unknown aminolipid; L1-2, unknown polar lipid.

Table 38. Cellular fatty acid profile (%) of strain THY-C1^T, PD-A10^T and K-A7^T. Strains: 1, THY-C1^T; 2, PD-A10^T; 3, K-A7^T. Fatty acid contents are given as percentages of the total amount. -, less than limit of detection.

Fatty acid	1	2	3
Straight-chain saturated			
$C_{14:0}$	0.48	0.56	0.50
$C_{15:0}$	0.26	-	0.24
$C_{16:0}$	5.78	6.41	5.47
$C_{18:0}$	0.63	0.54	0.48
Branch-chain saturated			
iso-$C_{13:0}$	0.14	-	0.18
iso-$C_{14:0}$	1.56	1.42	1.22
iso-$C_{15:0}$	19.32	9.80	21.27
iso-$C_{16:0}$	4.52	6.47	3.82
iso-$C_{17:0}$	17.53	9.81	11.15
anteiso-$C_{15:0}$	34.49	42.35	38.01
anteiso-$C_{17:0}$	14.67	22.65	15.04
Monounsaturated			
$C_{16:1}\omega 11c$	0.21	-	0.97
iso-$C_{17:1}\omega 10c$	0.22	-	0.79

Table 39. DNA base compositions of strain THY-C1^T, PD-A10^T and K-A7^T.

Strain	G+C content (mol%)
THY-C1^T	40.4
PD-A10^T	41.4
K-A7 ^{T}	40.2

BIOGRAPHY

Miss Punnanee Sumpavapol was born on January 23, 1981 in Suratthani, Thailand. She received her Bachelor's degree of Science in Public Health (Food and Nutrition) with the second class honors in 2001 from the Faculty of Public Health, Mahidol University. She graduated with the M.Sc. in Food and Nutritional Toxicology in 2004 from Institute of Nitrition, Mahidol University and continued studying for Ph.D. in Pharmaceutical Chemistry and Natural Products at Faculty of Pharmaceutical Sciences, Chulalongkorn University in that year.