การผลิตโปรติเนสและการปรับตัวในสภาวะที่มีโซเดียมคลอไรด์ของแบคทีเรียชอบเกลือความเข้มข้นปานกลางสายพันธุ์ *VIRGIBACILLUS* SP. SK37 ที่คัดแยกจากการหมักน้ำปลา

นายศรชัย สินสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PROTEINASE PRODUCTION AND SODIUM CHLORIDE ADAPTATION RESPONSE OF MODERATELY HALOPHILIC *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION

Sornchai Sinsuwan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

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PROTEINASE PRODUCTION AND SODIUM CHLORIDE ADAPTATION RESPONSE OF MODERATELY HALOPHILIC *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION

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

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ศรชัย สินสุวรรณ : การผลิตโปรติเนสและการปรับตัวในสภาวะที่มีโซเดียมคลอไรด์ของแบคทีเรียชอบเกลือความเข้มข้นปานกลางสายพันธุ์ *VIRGIBACILLUS* SP. SK37 ที่คัดแยกจากการหมักน้ำปลา (PROTEINASE PRODUCTION AND SODIUM CHLORIDE ADAPTATION RESPONSE OF MODERATELY HALOPHILIC *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 193 หน้า.

วัตถุประสงค์ของการศึกษานี้คือเพื่อหาสภาวะที่เหมาะสมขององค์ประกอบในอาหารเลี้ยงเชื้อต่อชีวมวลและการผลิตโปรติเนสภายนอกเซลล์ที่ทนเกลือของ *Virgibacillus* sp. SK37 ซึ่งมีศักยภาพในการเป็นกล้าเชื้อเพื่อการหมักน้ำปลา นอกจากนั้นเพื่อระบุสภาวะการเจริญที่กระตุ้นการตอบสนองเพื่อปรับตัวต่อโซเดียมคลอไรด์ และศึกษาพฤติกรรมการปรับตัวต่อโซเดียมคลอไรด์ดังกล่าวของแบคทีเรียอย่างสมบูรณ์ในระดับการสังเคราะห์อาร์เอ็นเอด้วยเทคนิคดีเอ็นเอไมโครอาร์เรย์

จากแบบการทดลองแพลคเก็ตต์-เบอร์แมน (Plankett-Burman design, PBD) พบว่า ปลากะตักแห้ง สารสกัดจากยีสต์ และพีเอช เป็นปัจจัยสำคัญที่ส่งผลต่อชีวมวลและการผลิต โปรติเนส การหาสภาวะที่เหมาะสมต่อมาด้วยแบบการทดลองโรทาเทเบิล เซ็นทรัลคอมโพสิทดีไซน์ (rotatable central composite design, RCCD) แสดงให้เห็นว่าการเจริญของแบคทีเรียเพิ่มขึ้นสูงสุดประมาณ 1.6 Log CFU/ml และการสร้างโปรติเนสเพิ่มขึ้น 1.4 เท่า ได้ด้วยอาหารเลี้ยงเชื้อที่ประกอบด้วยปลากะตักแห้ง 1.5 เปอร์เซ็นต์ สารสกัดจากยีสต์ 0.5 เปอร์เซ็นต์ โซเดียมคลอไรด์ 2.5 เปอร์เซ็นต์ ที่ค่าพีเอช 8 สมการการทำนายที่ประกอบด้วยสมการถดถอยแบบคิวบิคพิสูจน์ได้ว่ามีความเหมาะสม ปัจจัยปลากะตักแห้งและสารสกัดจากยีสต์ไม่มีอิทธิผลต่อรูปแบบการหลั่งโปรติเนสจากการวิเคราะห์ด้วยการย้อมกิจกรรมเอนไซม์ด้วยสารตั้งต้นเปปไทด์สังเคราะห์ (Suc-Ala-Ala-Pro-Phe-AMC) แต่พบขนาดโมเลกุลของโปรติเนสที่หลั่งออกมามีความผันแปรกับปริมาณโซเดียมคลอไรด์และพีเอชของอาหารเลี้ยงเชื้อ ระดับการย่อยสลายโปรตีนที่สูงกว่าในอาหารเลี้ยงเชื้อที่มีปริมาณโซเดียมคลอไรด์สูงสามารถตรวจวัดได้จากการวิเคราะห์เปปไทด์แมสฟิงเกอร์พรินทร์ (peptide mass fingerprint, PMF) โปรติเนสจาก *Virgibacillus* sp. SK37 ในสภาวะที่มีโซเดียมคลอไรด์สามารถย่อยสลายโปรตีนจากปลากะตักแห้งที่ตำแหน่งไลซีน อาร์จินีน และไทโรซีน ในขณะที่สภาวะปราศจากโซเดียมคลอไรด์พบการย่อยสลายที่จำเพาะต่อไลซีนและอาร์จินีนที่ตำแหน่ง P_1 ของเปปไทด์ตามผลวิเคราะห์ด้วย *de novo* sequencing

แม้ว่าแบคทีเรียสายพันธุ์ *Virgibacillus* sp. SK37 ไม่เจริญที่โซเดียมคลอไรด์เข้มข้น 25 เปอร์เซ็นต์ แต่เซลล์สามารถอยู่รอดได้ที่สภาวะโซเดียมคลอไรด์เข้มข้น 15 เปอร์เซ็นต์ เป็นเวลา 15 นาที ก่อนนำไปสัมผัสกับโซเดียมคลอไรด์เข้มข้น 25 เปอร์เซ็นต์ การวิเคราะห์ทรานสคริปโตม

แสดงให้เห็นว่ายีนที่ทำหน้าที่ปกป้องเซลล์จากโปรตีนที่โครงสร้างคลายตัว (*dnaK*, *grpE*, *groE*S, และ *groEL*) จากโปรตีนที่สูญเสียสภาพแบบไม่ผันกลับ (*clpP*, *clpC*, *clpE*, *clpX*, *lonA*, และ *ftsH*) และจากภาวะเครียดจากปฏิกิริยาออกซิเดชัน (*katE* และ *yfkM/yraA*) แสดงออกอย่างมีนัยสำคัญภายใต้โซเดียมคลอไรด์เข้มข้น 15 เปอร์เซ็นต์ การตอบสนองทางสรีระของแบคทีเรียมีความเชื่อมโยงกับการเปลี่ยนกลไกเมทาบอลิซึมจากวัฏจักรกรดไตรคาร์บอกซิลิก (tricarboxylic acid cycle, TCA) และปฏิกิริยาออกซิเดทีฟฟอสโฟรีเลชันเป็นวิถีการหมักเอทานอล และการเหนี่ยวนำยีนที่จำเพาะต่อการตอบสนองต่อภาวะเครียดจากปฏิกิริยาออกซิเดชันภายใต้โซเดียมคลอไรด์เข้มข้น 15 เปอร์เซ็นต์ มีการจัดเรียงตัวใหม่ของส่วนห่อหุ้มเซลล์ของแบคทีเรียในสภาวะเครียดจากโซเดียมคลอไรด์ ยีนที่บันทึกรหัสโปรตีนที่ทำหน้าที่นำเข้าสาร compatible solute สู่เซลล์ ได้แก่ sodium/proline symporter, OpuA, OpuB/C, และ OpuD มีการเหนี่ยวนำให้สังเคราะห์อาร์เอ็นเออย่างมีนัยสำคัญ องค์ความรู้จากงานวิจัยนี้เป็นประโยชน์ต่อความเข้าใจในกลไกภายในเซลล์ของแบคทีเรียในการตอบสนองเพื่อปรับตัวในโซเดียมคลอไรด์และศักยภาพในการใช้เป็นกล้าเชื้อที่ผลิตโปรติเนสเพื่อหมักน้ำปลา

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2556

ลายมือชื่อนักศึกษา _____
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SORNCHAI SINSUWAN : PROTEINASE PRODUCTION AND SODIUM CHLORIDE ADAPTATION RESPONSE OF MODERATELY HALOPHILIC *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 193 PP.

VIRGIBACILLUS/PROTEINASE PRODUCTION/RESPONSE SURFACE METHODOLOGY/SODIUM CHLORIDE ADAPTATION/DNA MICROARRAY/ FISH SAUCE

Objectives of this study were to optimize the medium components on biomass and extracellular NaCl-tolerant proteinase production of *Virgibacillus* sp. SK37, a potential starter culture strain of fish sauce fermentation. In addition, the growth condition to activate a NaCl-adaptive response was addressed. The NaCl adaptation behavior of the strain was thoroughly elucidated at the transcript levels using DNA microarray technique.

Based on Plankett-Burman design (PBD), dried anchovy, yeast extract, and pH were key factors affecting biomass and proteinase production. Further optimization using rotatable central composite design (RCCD) showed that the highest incremental yields of biomass of approximately 1.6 log CFU/ml and 1.4-fold increase in the proteinase production were achieved in the culture medium containing 1.5% dried anchovy, 0.5% yeast extract, and 2.5% NaCl, at pH 8. The predicted models obtained by cubic regression were proved to be adequate. Dried anchovy and yeast extract did not influence the proteinase secretion pattern based on the activity staining of a peptide synthetic substrate (Suc-Ala-Ala-Pro-Phe-AMC), but molecular weight (MW) of the

secreted proteinases varied with NaCl contents and pHs of the medium. A greater extent of proteolysis at high NaCl medium was observed based on peptide mass fingerprint (PMF). *Virgibacillus* proteinases in the presence of NaCl preferably hydrolyzed dried anchovy proteins at Lys, Arg, and Tyr positions, while in the absence of NaCl, they exhibited a specificity for the basic residues Lys and Arg, located at P_1 position on the peptide as evidenced by *de novo* sequencing.

Although *Virgibacillus* sp. SK37 did not grow at 25% NaCl, it can survive when cells were pre-incubated in 15% NaCl for 15 min prior to exposure of the extreme condition of 25% NaCl. Transcriptome analysis revealed that genes expressed for a protective function against improper protein folding (*dnaK*, *grpE*, *groES*, and *groEL*), irreversible denatured proteins (*clpP*, *clpC*, *clpE*, *clpX*, *lonA*, and *ftsH*), and oxidative stress (*katE* and *yfkM/yraA*) were significantly induced under 15% NaCl. The physiological responses were associated with a metabolic shift from the tricarboxylic acid cycle (TCA) cycle and oxidative phosphorylation to the ethanol fermentative pathway, and the induction of specific oxidative response genes. The cell envelop of the strain was rearranged during the NaCl-stress condition. The genes, encoding compatible solute transporters, namely sodium/proline symporter, OpuA, OpuB/C, and OpuD, were significantly transcribed. The outcome of this research would lead to the understanding of the cellular mechanisms of *Virgibacillus* sp. SK37 in NaCl-adaptive response and proteinase-producing starter culture with the potential application for fish sauce fermentation.

School of Food Technology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

Co-advisor's Signature_____

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Sornchai Sinsuwan

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LIST OF ABBREVIATIONS

AB	=	Anchovy broth
ACN	=	Acetronitrile
ADABA	=	γ-*N*-acetyl-α,γ-diaminobutyric acid
AhpC	=	Alkyl hydroperoxide
ALDH	=	Acetaldehyde dehydrogenase
AMC	=	4-Methyl-7-coumarylamides
ANOVA	=	Analysis of variance
APS	=	3-Aminopropyl-trimethoxysilane
ARDRA	=	Amplified rDNA restriction analysis
ASA	=	Aspartic β-semialdehyde
A.U.	=	Absorbance unit
BCCT	=	Betaine choline carnitine transporter
CCD	=	Central composite design
cDNA	=	Complementary DNA
CFU	=	Colony-forming unit
CV	=	Coefficient of variation
DABA	=	2,4-Diaminobutyrate
DEPC	=	Diethyl pyrocarbamate
DHB	=	2,5-Dihydroxybenzoic acid
DoE	=	Design of experiment
DPG	=	Diphophatidylglycerol

LIST OF ABBREVIATIONS (Continued)

dCTP	=	Dexoycytosine triphosphate
dUTP	=	Deoxyuridine triphosphate
ECF	=	Extracytoplasmic function
EctA	=	2,4-Diaminobutyrate acetyltransferase
EctB	=	2,4-Diaminobutyrate aminotransferase
EctC	=	Ectoine synthase
EctD	=	Ectoine hydroxylase
EDTA	=	Ethylenediaminetetraacetic acid
ETC	=	Aerobic electron transport chain
×g	=	Relative centrifugal fields
GbsA	=	Glycine betaine aldehyde dehydrogenase
GbsB	=	Type III alcohol dehydrogenase
GL	=	Glycolipid
GPx	=	Glutathione peroxidase
HEPES	=	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	=	Hydrogen peroxide
IAA	=	Isoamyl alcohol
IMG	=	Integrated Microbial Genomes
INH	=	Isoniazid
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
KatA	=	Catalase

LIST OF ABBREVIATIONS (Continued)

LC-ESI-MS/MS=		Liquid chromatography-electrospray ionization tandem mass spectrometry
MALDI-TOF	=	Matrix-assisted laser desorption/ionization-time of flight
Met-O	=	Methionine sulfoxide
Msrs	=	Methionine sulfoxide reductase
MW	=	Molecular weight
MWCO	=	Molecular weight cut-offs
m/z	=	Mass-to-charge ratio
NCBI	=	The National Center for Biotechnology Information
NHS ester	=	*N*-Hydroxysuccinylimidyl ester
$O_2^{\cdot-}$	=	Superoxide
$^{\bullet}OH$	=	Hydroxyl radical
$ONOO^-$	=	Peroxynitrite
ORF	=	Open reading frame
OVAT	=	One-factor-at-a-time
OD_{600}	=	Optical density at 600 nm
PBD	=	Plankett-Burman design
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PDC	=	Pyruvate decarboxylase
PE	=	Phosphatidylethanolamine
PerR	=	Peroxide repressor

LIST OF ABBREVIATIONS (Continued)

PG	=	Phosphatidyl glycerol
PMF	=	Peptide mass fingerprint or Proton motive force
ProR	=	Proline racemase
Prx	=	Peroxiredoxin
R^2	=	Determination coefficient
RCCD	=	Rotatable central composite design
ROOR′	=	Organic peroxide
ROS	=	Reactive oxygen species
RSM	=	Response surface methodology
RT	=	Reverse transcription
σ^A	=	The house-keeping sigma factor
σ^B	=	The alternative transcription factor
SAGE	=	Serial analysis of gene expression
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMM	=	Spizizen's minimal medium
SOD	=	Superoxide dismutase
SSC	=	Saline sodium citrate
Suc	=	Succinyl
t-buOOH	=	*tert*-Butyl peroxide
TCA	=	Tricarboxylic acid cycle
TFA	=	Trifluoroacetic acid

LIST OF ABBREVIATIONS (Continued)

Tpx	=	Thiol peroxidase
3D	=	Three dimensions
Trx	=	Thioredoxin
TSA	=	Tryptic soy agar
uAPL	=	Unknown aminophopholipid
uGL	=	Unknown glycolipid
uPL	=	Unknown phospholipid
Ym	=	Yeast extract medium
YugJ	=	NADH-dependent butanol dehydrogenase
2D-electrophoresis	=	Two-dimensional electrophoresis

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce is a condiment consumed in Southeast Asia and is an important source of nutrients (Sikorski and Ruiter, 1994). The production of fish sauce involves the addition of salt to uneviscerated fish at a ratio of approximately 1:3. It usually takes at least 6 months for fish to liquefy and another 1-3 months to ripen for the full flavor and aroma development (Jay, 2000). Therefore, this traditional fermentation is a very slow process required about 1-1.5 years, which is a major limitation for the growth of fish sauce industry. Moreover, the traditional fermentation requires large area and the fermentation process is difficult to control. Therefore, acceleration of natural fermentation process will reduce the capital cost and improve business profitability.

High salt content reduces water activity to prevent putrefactive microorganisms from spoilage. It simultaneously allows halophilic bacteria to proliferate. Proteinases from halophilic bacteria or fish viscera/muscle convert protein to peptides and amino acids (Beddows, 1998). These compounds contribute to an unique characteristic of fish sauce. Many reports have indicated the characteristics of proteinases produced by extremely and moderately halophilic bacteria. These bacteria exhibit optimum growth between 0.5-3.4 M NaCl (2.9-20.4%) (Ventosa, Nieto, and Oren, 1998). Proteinases produced from these groups of bacteria normally inherite halophilic property to be

active under moderate or high salt concentration. Activity of serine proteinase from *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3 isolated from fish sauce fermentation increased with the increase of NaCl concentration (Hiraga et al., 2005; Namwong et al., 2006). *Virgibacillus* sp. produced extracellular and cell-bound proteinases, which exhibited high proteolytic activities at high NaCl concentration (Sinsuwan, Rodtong, and Yongsawatdigul, 2007, 2008). *Halobacillus thailandensis* sp. produced serine proteinases with molecular weight (MW) of 100 and 17 kDa, and metalloproteinase with MW of 42 kDa (Chaiyanan et al., 1999). There are several proteinase-producing halophiles found during fish sauce fermentation. Hence, these bacteria could be used as a starter culture to accelerate fish sauce fermentation.

Secretion of proteinase from bacteria is dependent on available nutrients, media composition and physical factors, such as NaCl concentration, temperature and incubation time. For instance, casein supplemented in a medium induced maximum proteinase production of *Bacillus clausii*, while the supplementation of whey protein, malt extract, tryptone, peptone, soytone and yeast extract lower its proteinase production (Kumar, Joo, Koo, Paik, and Chang, 2004). Yeast extract was the best nitrogen source for proteinase production of *Bacillus stearothermophilus* (Razak et al., 1994). 0 Thus, in order to maximize bacterial proteinase production, optimization of such factors must be determined. Optimization of the factors by classical methods which involves in the change of single factor has some disadvantages, such as time consuming due to more required experiments and neglecting the interaction among factors (Kumar, Ananthan, and Prabhu, 2014). The classical method has been replaced by statistical optimization such as response surface methodology (RSM). The RSM is useful for understanding interactions among various physicochemical parameters using a minimal number of experiments, and detecting true optimal conditions (Haddar et al.,

2010). Although the optimization of medium components on proteinase production of non halophilic bacteria using RSM has been extensively studied (Reddy, Wee, Yun, and Ryu, 2008; Sen, Veeranki, and Mandal, 2009; Rai and Mukherjee, 2010), few studies have reported the optimal proteinase production of moderately halophilic bacteria, particularly those isolated from fish sauce fermentation. Five factors, namely yeast extract, casamino acid, NaCl, pH, and inoculum size, were optimized to obtain an overall 20-fold increase in the proteinase production of a haloalkaliphilic bacterium strain *Geomicrobium* sp. EMB2 (Karan, Singh, Kapoor, and Khare, 2011). Optimization of medium components, namely soybean flour and $FeCl_3$, for proteinase production by an extreme haloarchaeon, *Halobacterium* sp. SP1(1) (Akolkar, Bharambe, Trivedi, and Desai, 2009). Hence, it is greatly interesting to optimize the factors affecting proteinase production of the halophile, isolated from fish sauce fermentation, using RSM to obtain the maximum yield.

NaCl is used as a main component in fish sauce fermentation process. Bacteria have been isolated from fish sauce fermentation (Uchida et al., 2004; Hiraga et al., 2005; Namwong et al., 2006; 2010; Toyokawa et al., 2010). Bacteria employ several strategies to adapt to extreme condition. Exposure to mild NaCl concentration has enabled to induce the protective responses in *B. subtilis*, *B. cereus*, and *Coreynebacterium glutamicum*, which are subsequently able to survive under extreme condition (Höper, Bernhardt, and Hecker, 2006; den Besten et al., 2009; Fränzel et al., 2010). So far, little is known about the osmotic response in moderate halophiles. Ectoine production of *Virgibacillus salexigens* through an *ectABC* biosynthetic operon was increased at high salinity of growth medium (Bursy, Pierik, Pica, and Bremer, 2007). Exogenous ectoine and hydroxyectoine served as compatible solutes in *V. pantothenticus* (Kuhlmann, Hoffmann, Bursy, Jebbar, and Bremer, 2011).

In contrast, physiological and transcriptomic studies in NaCl-adaptive response of non-halophile, *Bacillus*, have been extensively studied (Steil, Hoffmann, Budde, Völker, and Bremer, 2003; Höper et al., 2006; den Besten et al., 2009). DNA microarray has been found as a powerful tool to investigate the bacterial stress response. It has main advantage that the transcript levels of an entire genome are measured simultaneously (Hughes and Shoemaker, 2001). However, this approach has never been applied in NaCl-adaptive response of halophilic bacteria.

Among many halophilic bacteria isolated in fish sauce fermentation, the moderate halophile, *Virgibacillus* sp. SK37, has shown potential to be used as a starter culture for acceleration of fish sauce fermentation. Addition of *Virgibacillus* sp. SK37 into anchovy that were previously hydrolyzed by Alcalase and Flavourzyme reduced fish sauce fermentation time to 4 months (Yongsawatdigul, Rodtong, and Raksakulthai, 2007). Addition of 0.2% $CaCl_2$ in conjunction with starter culture of *Virgibacillus* sp. SK37 significantly enhanced protein hydrolysis throughout fish sauce fermentation for 4 months (Sinsuwan, Rodtong, and Yongsawatdigul, 2012). This strain is Gram-positive/variable, long rod with 0.6 to 0.7 × 3.0 to 6.6 μm, nonmotility and terminal or subterminal ellipsoidal spores. The strain can grow at a wide pH range of 4 to 11 and 20 to 45 °C (Sinsuwan et al., 2007). More importantly, whole genome sequences of this strain have been established (Phrommao, 2010). The availability of whole genome information has made the possibility to study the gene transcription by DNA microarray. Hence, it is of interest to determine the gene transcripts of the halophilic bacteria under NaCl stress condition. It would reveal the regulatory system underlying NaCl adaption behavior of this potential starter culture.

1.2 Research objectives

The objectives of this study were:

1. To optimize the factors affecting the growth and proteinase production of *Virgibacillus* sp. SK37 using response surface methodology.
2. To establish a comprehensive understanding of gene transcription profile in response to high salinity condition of *Virgibacillus* sp. SK37.

1.3 Research hypotheses

Growth and proteinase production of *Virgibacillus* sp. SK37 are significantly affected by culture condition and medium composition. Statistical method, namely response surface methodology, can be applied to obtain the optimal condition of protein production. In addition, the habitat of the strain is found in an extremely high saline protein-rich environment could lead to the adaptation of the strain. A comprehensive profiling of all gene products and transcribed regulatory genes when the strain is exposed to high NaCl concentration can be achieved by DNA microarray technique.

1.4 Scope of study

The moderately halophilic bacterium, *Virgibacillus* sp. SK37, could be used as a starter culture to increase the degree of protein hydrolysis during fish sauce production. To achieve appropriate starter culture development, the cultivation conditions and medium constituents affecting growth and proteinase production of the candidate was optimized using statistical methods. The interactions among such factors were considered to understand the relationship between physiological behavior

and growth condition. Hydrolyzed peptide sequences by activity of *Virgibacillus* sp. SK37 proteinases at various growth conditions were investigated. To improve the resistance of the strain at extremely high salinity condition, the growth condition for inducing NaCl-adaptive response was addressed. The complex relationship between gene products and regulatory genes during NaCl stress condition was fully described.

1.5 Expected results

Proteinases from *Virgibacillus* sp. SK37 possess the halotolerant property, which is suitable to be applied for the acceleration of fish sauce fermentation. The true optimization condition for growth and proteinase production of *Virgibacillus* sp. SK37 was established, which can be applied for starter culture production. The condition activating the adaption of the strain is important information required to improve the survivals of the strain at an extreme NaCl stress of fish sauce fermentation. Result from transcriptomic analysis would provide a better understanding of how the halophilic bacterial cells function under the osmotic stress.

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CHAPTER II

LITERATURE REVEIWS

2.1 Fish sauce fermentation

2.1.1 Production of fish sauce

Fish sauce is prepared from whole, ungutted anchovy (*Stolephorus* spp.) by mixing fish with solar salt at a ratio 3:1 and stored in a concrete tank for about 12-18 months. In Thailand, anchovy are caught off the Gulf of Thailand and brought to the fermentation plant, practically taking several hours. Whole fresh fish with approximately 4 cm in length are mixed with solar salt, and the liquid is drained off. The mixtures are placed into an unsterilized and underground concrete tank with dimension approximately 2 × 2 × 2 m. During fermentation, the liquid, containing soluble protein, from the fish body, is leached out by osmotic effect and proteolysis, and reached to the top of the layers. To avoid contact with the air of fish particles, a piece of bamboo mat is used to weight down. The tank is not tightly coved with a sheet of galvanized steel to protect raining water and allow oxygen penetrating to the fermentation mixtures. Park et al. (2001) reported the chemical characteristics of Thai fish sauce in 100 mL that contained 63.7 g moisture, 21.4 g NaCl, and 1.68 g total nitrogen, and pH 5.63. Nitrogen recovery of Thai fish sauce was 64.3% (Park et al., 2001). Fish proteins, the main nitrogen source, are liquefied by action of endogenous proteinases, which are from muscle and digestive tracts, and proteinases from halophilic bacteria (Beddows, 1998; Thongthai, McGenity, Suninanalert, and Grant,

1992). Endogenous proteinases were observed throughout 12 months of fish sauce fermentation (Siringan, Raksakulthai, and Yongsawatdigul, 2006b). Thus, endogenous and halophilic bacterial proteinases play an important role in protein hydrolysis of fish sauce fermentation.

2.1.2 Bacteria in fish sauce fermentation

The action of endogenous proteinases results in amino acids and peptides, which are nutrients for bacterial growth during fermentation (Saisithi, 1994). Fukui et al. (2012) reported that the viable cell counts of non-halophile and halophilic bacteria, predominated by *Staphylococcus* spp., gradually decreased within the first 4 weeks during fermentation, while that of halophilic and highly halophilic bacteria, predominated by *Tetragenococcus halophilus*, remarkably increased thereafter. *Lantibacillus halophilus, Natrinema gari, Chromohalobacter salexigens, Halobacterium salinarum,* and *Halococcus saccharolyticus* have been isolated from Thai fish sauce (Tanasupawat et al., 2006, 2009; Tapingkae et al., 2008). *Micrococcus kritinae, Staphylococcus xylosus*, *S. equorum,* and *Bacillus* isolated from Pacific whiting (*Merluccius productus*) fish sauce have been reported (Lopetcharat and Park, 2002). Saisithi, Kasemsarn, Liston, and Dollar (1966) isolated *Bacillus, Streptococcus, Micrococcus, Staphylococcus* and coryneforms from Thai fish sauce fermented for 9 months. These bacteria produced volatile acids when grown in a medium prepared by hydrolyzing rockfish (*Sebastodes* sp.) with a mineral acid. Beddows and Ardeshir (1979b) claimed that bacteria were involved in aroma development of budu fermentation. Budu with addition of antimicrobial agents did not possess typical sensory characteristics of fish sauce. Inoculated fish sauce with *Virgibacillus* sp. SK37, isolated from Thai fish sauce, under reduced NaCl contents of 15-20% produced 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, which contributed

to stronger malty or dark chocolate notes (Lapsongphon, Cadwallader, Rodtong, and Yongsawatdigul, 2013). The use of *T. halophilus*, isolated from Thai fish sauce, as a starter culture, was able to reduce dimethyl disulfide, which is a compound contributing to a fecal note (Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul, 2011). *Staphylococcus xylosus* was demonstrated to improve fish sauce odor (Fukami, Satomi, Funatsu, Kawasaki, and Watabe, 2004). Bacteria found in fish sauce fermentation play a major role not only in aroma development but also protein hydrolysis. Numerous studies showed that bacteria isolated from fish sauce fermentation or sea salt can produce proteinase. Norberg and Hofsten (1969) showed that halobacterium strains isolated from sea salt produced an extracellular proteinase which could hydrolyze gelatin and casein. Strains of *Bacillus subtilis* JM-3 isolated from fish sauce fermentation produced acid proteinase (Kim and Kim, 2005). *Filobacillus* sp. RF2-5, *B. subtilis* CN2, *Bacillus licheniformis* RKK-04, *Virgibacillus* sp., and *Halobacillus* sp. SR5-3 produced serine proteinases (Uchida et al., 2004; Hiraga et al., 2005; Namwong et al., 2006; Sinsuwan, Rodtong, and Yongsawatdigul, 2007, 2010; Toyokawa et al., 2010). Chaiyanan et al. (1999) reported that strains of *Halobacillus* spp. secreted serine and metalloproteinases. These studies indicated that halophilic bacteria might contribute to protein hydrolysis during fish sauce fermentation.

2.1.3 Acceleration of fish sauce fermentation

Several methods have been proposed to accelerate fish sauce fermentation process. Raising fermentation temperature to about 45 ºC for a couple of week at low salt concentration reduced fermentation time from 1 year to 2 months (Gildberg, 1993). In addition, the fermentation time was reduced to 2 months by adjusting pH to 11 at low salt concentration to activate the alkaline digestive proteinases and inactivate

endogenous trypsin and chymotrypsin inhibitors. Flavor of the finished product was comparable to that of the traditional fish sauce (Díaz-López and García-Carreño, 2000). Beddows and Ardeshir (1979b) produced fish sauce using hydrochloric acid, however, the acidified fish sauce had very little aroma and flavor. Gildberg, Hermes, and Orejana (1984) accelerated autolysis at the initial stage by acidifying anchovies (pH 4) (*Stolephorus spp.*) in the presence of 5% NaCl at 40 °C for 5 days and it was found that flavor of the product was acceptable after incubation for 2 months. In addition, several researchers have studied the addition of exogenous enzymes for protein hydrolysis. Quaglia and Orban (1987) reported that Alcalase and commercial papain were suitable for protein hydrolysis of sardine (*Sardina pilchardus*), resulting in high solubility and nitrogen recovery. Rebeca, Peña-Vera, and Díaz-Castañeda (1991) used bacterial proteinases to hydrolyze fish (*Mugil cephalus*) proteins and suggested that proteins were solubilized faster with Pescalase 560 (*Bacillus* proteinase) than HT-200 (*Bacillus* proteinase) and Proteinase N (*Bacillus* proteinase). Beddows and Ardeshir (1979a) used bromelain to obtain fish sauce within 18 to 21 days. The accelerated product contained comparable nitrogen compounds to the traditional fish sauce, but had very little aroma. Male capelin (*Mallotus villosus*) fish sauce was produced rapidly by adding squid hepatopancreas tissue (Raksakulthai, Lee, and Haard, 1986). Squid hepatopancreas added fish sauce showed higher acceptance than commercial product from the Philippines. However, these approaches have never been successfully adopted by the industry due to the high cost of enzyme. Application of proteinases from bacteria isolated from fish sauce fermentation to accelerate the fermentation has been reported. Proteinase from *Bacillus megaterium* KLP-98, isolated from fish sauce, added to 6-month old fish sauce exhibited a greater extent of proteolysis during 2 days of fermentation (Fu, You, and Kim, 2008). Furthermore, the

application of starter culture for fish sauce fermentation has been investigated. Proteinase activity, peptide release, and α-amino acid content in the inoculated fish sauce with *Halobacterium* sp. SP1(1) were higher than those of uninoculated sample at day 10 (Akolkar, Durai, and Desai, 2010). Chinese silver carp (*Hypophthalmichthys molitrix*) fish sauce added the soy sauce koji (prepared from *Aspergillus oryzae* Biokku Co's strain) showed a high levels of organic material, total nitrogen, and organic acid, compared to the sample without adding the koji (Uchida et al., 2005). A greater extent of total free amino acids in the fish sauce added sucrose in conjunction with starter cultures (koji and *T. halophilus*), compared to control sample, was obtained (Shozen et al., 2012). Yongsawatdigul, Rodtong, and Raksakulthai (2007) reduced fish sauce fermentation time to 4 months by adding starter culture, *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, or *Staphylococcus* sp. SK1-1-5, into anchovy that was previously hydrolyzed by Alcalase (Novozymes) and Flavourzyme (Novozymes). Addition of 0.2% $CaCl_2$ in conjunction with starter culture of *Virgibacillus* sp. SK37 significantly enhanced protein hydrolysis throughout 4 months of fish sauce fermentation (Sinsuwan, Rodtong, and Yongsawatdigul, 2012).

2.2 NaCl-adaptative responses in bacteria

Bacteria employ certain mechanisms to adapt to adverse environments and those strategies are called "adaptative stress response", otherwise the condition is lethal (Kempf and Bremer, 1998b). Under moderate and high NaCl environments, water exists from the cells, resulting in a reduction in cell turgor and cessation of growth (Kempf and Bremer, 1998b). To cope in the unflavored condition, bacteria use a variety of solutes (organic and inorganic) to counter high osmotic pressure (Galinski,

1993; Kempf and Bremer, 1998b; Roberts, 2005). Although all of these molecules may reduce individual enzyme activities, they do not inhibit overall cellular functions (Galinski, 1993; Roberts, 2005). Organic osmolytes, which is amassed by bacteria to counteract the outflow of the water, are called compatible solutes (Kempf and Bremer, 1998b; Wood et al., 2001). The compatible solutes can either be synthesized by the cells or transported into the cells from environments (Kempf and Bremer, 1998a, b; Roberts, 2005). The initial response to high osmolarity is much more rapid if there is an availability of the compatible solutes in the environment (Poolman and Glaasker, 1998). After an osmotic downshift, the degradation of the compatible solutes is required (Poolman and Glaasker, 1998).

2.2.1 Compatible solutes and their characteristics

In general, the compatible solutes are polar, highly soluble, and do not carry a charge at physiological pH (Galinski, 1993; Kempf and Bremer, 1998b). A reduction in free water content in the bacterial cells is a key determination for cell growth under high-osmolarity condition (Kempf and Bremer, 1998b). The accumulated compatible solutes increase free water content of the NaCl-stressed cells, resulting in a restoration of cell proliferation at the stress condition (Kempf and Bremer, 1998b). In addition, the compatible solute also serves as a stabilizer of enzyme and cell component against the denaturing effects at high ionic strength (Kempf and Bremer, 1998a, b; Wood et al., 2001). It is explained by the theory of the preferential hydration, (Galinski, 1993; Roberts, 2005). The compatible solutes can be classified into three types: (1) zwitterionic, (2) uncharged, and (3) anionic. Glycine betaine, ectoine, hydroxyectoine, Nγ-acetyldiaminobutyrate, Nε-acetyl-β-lysine, and β-glutamine are zwitterionic compatible solutes. Glycine betaine is a derived from glycine with the primary amine methylated to form a quaternary amine (Roberts, 2005). Carboxyl group and

quaternary amine provide negative and positive charges of the molecule, respectively. Glycine betaine is able to be imported from environment by active transport system or synthesized either by oxidation of choline or methylate of glycine (Roberts, 2005). In halophilic bacteria, the average concentration of the accumulated betaine was 208, 650, and 972 mM at 3, 10, and 20% NaCl in growth medium, respectively, indicating that the betaine is major organic solute of halophilic bacteria (Imhoff and Rodriguez-Valera, 1984). Ectoine is a cyclic tetrahydropyrimidine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid). It is considered as a marker for halophilic bacteria, of which the endogenously synthesized ectoine is generally detected (Roberts, 2005). It has been reported that an accumulation of the ectoine in *V. pantothenticus* reached to approximately 330 μmol/g dry weight, while no detectable ectoine in the medium containing 1.3 M NaCl (~7.6%) (Kuhlmann, Bursy, Gimpel, Hoffmann, and Bremer, 2008). *B. subtilis* cells were unable to synthesize ectoine (Kempf and Bremer, 1998a). In growth medium without supplied compatible solutes, *Brevibacterium linens* exhibited the strict dependence between the intracellular ectoine content and the external NaCl content (up to 1 M) (Bernard et al., 1993). Hydroxyectoine has similar structure to ectoine, except addition of hydroxyl group in the structure. Hydroxyectoine was detected in *Halomonas elongata* cells after addition of NaCl at > 1.71 M and the amount of hydroxyectoine reached 45 μg/mg of dry cells at the medium containing 2.56 M NaCl (Ono et al, 1998). Carbohydrates (glycerol, α-glucosylglycerol, α-mannosylglyceramide, trehalose, and sucrose) and modified free amino acids and peptides (N-α-carbamoyl-L-glutamine 1-amide, N-acetylglutaminylglutamine amide) are uncharged compatible solute. Reactive ends of the sugar are normally formed a glycosidic bond with another neutral molecule, either glycerol or glyceramide (Roberts, 2005). Otherwise, the sugar could not act as the

compatible solute because its reducing ends are chemically reactive and would be likely to react with surface amino acids (Roberts, 2005). Carboxylic acids, phosphates, and sulfates supply a negative charge in anionic compatible solutes. L-α-glutamate, β-glutamate, hydroxybutyrate, poly-β-hydroxybutyrate, α-glucosylglycerate, α-mannosylglycerate, α-diglycerol phosphate, di-*myo*-inositol-1,1'-phosphate, manosyl-DIP, cyclic-2,3-diphosphoglycerate, and sulfotrehalose have been reported to be anionic compatible solutes (Roberts, 2005). Some strains of halophilic bacteria show the correlation between the intracellular glutamate content and the NaCl concentration in medium, indicating that halophiles are able to use glutamate as a compatible solute (Imhoff and Rodriguez-Valera, 1984).

2.2.2 Transport of potassium and compatible solutes

Two strategies of osomoadaptation in bacteria are the KCl-type and the compatible-solute type (Galinski, 1993; Galinski and Trüper, 1994; Kempf and Bremer, 1998b). Transportation of K^+ and the compatible solutes plays a significant role in the osmotic response. The compatible-solute type efficiently scavenges the compatible solutes from the ecosystem even at low concentration (Kempf and Bremer, 1998b; Roberts, 2005). Bacteria normally possess several transporter systems for uptake of the compatible solutes (Kempf and Bremer, 1998a, b; Wood et al., 2001; Heermann and Jung, 2004). These transports are very specific or broad specificity (Kempf and Bremer, 1998b). Basically, there are two basically two types including (1) secondary transporters that use either proton motive force (PMF) or sodium motive force to drive compatible solutes flow into the cells, and (2) ATP binding cassette (ABC) transporters that couple ATP hydrolysis to take up compatible solutes (Wood et al., 2001; Roberts, 2005). The Kdp, Kup, Trk, and Krt systems are the transport system for K^+ in bacteria (Imhoff, 1986; Kempf and Bremer, 1998b; Poolman and Glaasker,

1998). Kup transporter in *Escherichia coli* was not influenced by medium containing high NaCl concentration (Kempf and Bremer, 1998b). In contrast, K^+ uptake via the multiple component Kdp, Trk, and Krt systems was enhanced by high-osmolarity condition (Kempf and Bremer, 1998b). The induction of the *kdp* operon was mediated by the sensor kinase (KdpD) and the response regulator (KdpE) system (Poolman and Glaasker, 1998; Heermann and Jung, 2004). The transcription factor KdpE was phosphorylated, resulting in an increase in the transcription of the *kdpFABC* operon (Poolman and Glaasker, 1998; Heermann and Jung, 2004). The Kdp activity was not depended on the proton motive force, but represents a K^+-stimulated ATPase. It showed a very high substrate affinity for potassium (K_m = 2 μM) (Imhoff, 1986). Trk and Krt systems, containing transmembrane protein and the cytoplasmic NAD^+/NADH binding protein, are the main K^+-transporter in many bacteria (Heermann and Jung, 2004; Roberts, 2005). Trk system in *E. coli* and *H. elongate* (Roberts, 2005), while Krt system in *B. subtilis* and *Synechocystis*, was reported (Heermann and Jung, 2004). The Trk activity exhibited a low substrate affinity (K_m = 1.5 mM) with high rate of uptake (Imhoff, 1986). Betaine choline carnitine transporter (BCCT), belonging to family of secondary transporters, can specifically take up the betaine in the moderate halphilic lactic acid bacterium, *T. halophilus,* and the ectoine in moderate halophile, *V. pantothenticus,* (Kuhlmann, Hoffmann, Bursy, Jebbar, and Bremer, 2011; Roberts, 2005). The TeaABC transporter for ectoine and hydroxyectoine, a member of the tripartite ATP-independent periplasmic transporter family (TRAP-T), has been found in *H. elongata* (Roberts, 2005). Transport system ProP and ProU of *E. coli*, and BetP, EctP, LcoP, ProP, and PutP of *Corynebacterium glutamicum* are required for the uptake of proline, betaine, and ectoine (Imhoff, 1986; Kempf and Bremer, 1998b; Krämer and Morbach, 2004; Kunte, Crane, Culham,

Richmond, and Wood, 1999). The osmoregulated osmolyte transporter OpuA to OpuE (osmoprotectant uptake), which operated under high osmolarity, have been reported in *Bacillus subtilis* (Kempf and Bremer, 1998a; Kappes et al., 1999; Holtmann and Bremer, 2004). The *opuE* structural gene encodes a proline transport system that consists of a single component (OpuE) with high affinity to proline (Kempf and Bremer, 1998a, b). OpuE is a member of the sodium/solute symporter family, which obligatorily take up proline along with Na^+ (Kempf and Bremer, 1998a, b). The OpuE protein showed high similarity to the PutP proline permeases, which were used in *E. coli*, *Salmonella typhinurium*, and *Staphylococcus aureus* for the acquisition of proline as carbon and nitrogen sources, but not for osmoprotective proposes (Kempf and Bremer, 1998a, b). The activity of OpuE transporter was not enhanced by high osmolarity, but entirely dependent on its protein synthesis (Kempf and Bremer, 1998a). In *Bacillus*, the presence of two promoters, *opuE*-P1 and *opuE*-P2, was recognized by the house-keeping sigma factor (σ^A) and the alternative transcription factor (σ^B), respectively (Kempf and Bremer, 1998a, b). Thus, the activity of OpuE linked to the general stress regulon (σ^B). *B. subtilis* employs three transporters to scavenge glycine betaine from environment, including the multicomponent ABC transporters OpuA (OpuAA, OpuAB, and OpuAC) and OpuC (OpuCA, OpuCB, OpuCC, and OpuCD), and the single-component transporter OpuD (Kempf and Bremer, 1998a, b). Glycine betaine activity was completely abolished when all genes of these three transporters in *B. subtilis* were deleted (Kappes, Kempf, and Bremer, 1996). OpuA and OpuC systems were related to the binding protein-dependent glycine betaine transporter ProU from *E. coli* and *S. typhimurium* (Kempf and Bremer, 1998a, b). OpuD was related to glycine betaine transporter, BetP, in *C. glutamicum* (Kappes et al., 1996; Kempf and Bremer, 1998a, b). OpuA, OpuC, and OpuD exhibited high

substrate affinity with K_m values in the low micromolar range (2.4-13 μM) (Kappes et al., 1996). OpuA system was a predominant glycine betaine transporter of *B. subtilis*, as evidenced by the highest V_{max} at 282 nmol/min/mg protein (Kappes et al., 1996; Hoffmann et al., 2013). The OpuC system exhibited broad substrate specificity (Kempf and Bremer, 1998b). Glycine betaine can be synthesized from the precursor choline, which was imported from the ecosystem. The OpuB and OpuC possess a high efficiency of uptake choline into cells (Kempf and Bremer, 1998a). In addition, there was a report that the OpuC was used for ectoine uptake in *B. subtilis* (Jebbar, von Blohn, and Bremer, 1997).

2.2.3 NaCl stress response

In term of physiological response to high salinity, the composition of cell membrane of bacteria is altered (Galinski and Trüper, 1994; Heermann and Jung, 2004). It exhibited a rise in the proportion of anionic lipids, particularly phosphatidylglycerol (PG) and glycolipid (GL), relative to zwitterionic lipids (Galinski and Trüper, 1994). Genes participating in lipid biosynthesis were up-regulated in *C. glutamicum* (Fränzel et al., 2010). The membrane of *C. glutamicum* was more sticky and solution containing such membrane appeared to be more viscous (Fränzel et al., 2010). The genes involved in peptidoglycan biosynthesis of *Lactococcus lactis* subsp. *Lactis* IL1403 were induced during the osmotic stress (Xie, Chou, Cutler, and Weimer, 2004). However, those genes of *B. subtilis* were repressed (Steil, Hoffmann, Budde, Völker, and Bremer, 2003). Osmotic shift to low or high osmolarity caused to trigger a behavioral response (osmotaxis), allowing that the *Bacillus* cells can escape the NaCl-stressed condition (Kempf and Bremer, 1998b; Wood et al., 2001; Heermann and Jung, 2004). In contrast, den Besten, Mols, Moezelaar, Zwietering, and Abee (2009) reported that genes encoding protein of the flagellar apparatus (represented by *flgK*

and *flgB*) were down-regulated. Sporulation of *Bacillus* was defected (Heermann and Jung, 2004). The expression of many members of the general stress regulon controlled by the alternative transcription factor sigma B (σ^B) was induced by high osmolarity (Bernhardt et al., 1997; den Besten et al., 2009). Genes up-regulated when *B. subtilis* and *B. cereus* cells exposed to high NaCl concentration include *dnaK* (chaperone DnaK), *groEL* (chaperone GroEL), *grpE* (chaperone GrpE), *ctc* (general stress protein), *gspA* (general stress protein), *gsiB* (general stress protein), *gtaB* (UTP-glucose-1-phosphate uridylyltransferase), *clpC* (ClpC proteinase), *sod* (superoxide dismutase), *katE* (catalase), *clpP* (ClpP proteinase), *ahpC* (alkyl hydroperoxide reductase C), and *ahpF* (alkyl hydroperoxide reductase F) (Bernhardt et al., 1997; den Besten et al., 2009). These stress-specific proteins induced by the NaCl stress may explain a protective function of the bacterial cells against (i) improper protein folding by chaperone, (ii) irreversible protein denaturation by proteinase, and (iii) oxidative stress by antioxidative enzymes. Iron limitation-related genes are up-regulated under high-salinity condition (den Besten et al., 2009). In addition, the expression of several genes controlled by the two-component DegS-DegU regulatory system, composed of sensor kinase and response regulator, is negatively or positively affected by high-osmolariy condition (Kempf and Bremer, 1998b). The phosphorylated DegU (DegU-P) is increased during hyperosmotic media, and subsequently bound to DegU box, located on regulatory region of its regulated genes, to stimulate the expression (Ruzal and Sanchez-Rivas, 1998; Kayumov, Balaban, Mardanova, Kostrov, and Sharipova, 2006). Under the NaCl stress, the expression of *sacB* (levansucrase gene) and *aprBi* (subtilisin-like proteinase gene) were induced, while *aprE* (alkaline phosphatase gene) was repressed (Kunst and Rapoport, 1995; Kayumov et al., 2006). Under high salinity, osmotic equilibrium across the membrane and the cytoplasm to osmotic strength of the

environment is demanded (Galinski and Trüper, 1994). In general, the response of bacterial cells to survive at high osmolarity initiated the two-step adaptation strategies, which K^+ is firstly taken up into bacterial cells, and the compatible solutes are later acquired from exogenous sources through transporters or synthesized inside cells (Imhoff, 1986; Galinski and Trüper, 1994; Kappes et al., 1999; Wood et al., 2001; Heermann and Jung, 2004). In *Bacillus* cells, the osmotic upshock with 0.4 M NaCl caused an increase in the cellular K^+ level from 315 to 650 mM within 1 h (Whatmore et al., 1990). Under the NaCl stress, *B. subtilis* cells in the K^+-free medium cannot recover the cell turgor pressure, while the recovery of the cell turgor pressure is apparent when adding K^+ into the medium (Whatmore and Reed, 1990). The increased concentration of intracellular K^+ in *B. linens* did correlate to the external NaCl content (up to 1.5 M) (Bernard et al., 1993). The accumulation of K^+ at the initial phase accompanied by increase in the glutamate pool during growth at high osmolarity was related to maintain electroneutrality (Imhoff, 1986; Poolman and Glaasker, 1998; Kempf and Bremer, 1998b; Hoffmann et al, 2013). However, a high intracellular concentration of K^+ was deleterious for the bacterial cells such as disturbing vital cellular function of DNA replication, DNA-protein interaction, and the cellular metabolic machinery, and not an inadequate strategy when high osmolarity condition was prolonged (Whatmore et al., 1990; Kempf and Bremer, 1998a, b). Therefore, a large amount of intracellular K^+ was removed from the cells via specific (Kef) or nonspecific K^+ effect systems to allow the replacement by the compatible solutes (Kempf and Bremer, 1998b; Wood et al., 2001). In second phase, the compatible solute including proline, glutamate, glycine betaine, ectoine, and hydroxyectoine are accumulated (Whatmore, Chudek, and Reed, 1990; Kempf and Bremer, 1998a, b). If there were no exogenously supplied compatible solutes, proline was a major

osmoprotectant in *B. subtilis* cells (Whatmore et al., 1990). Proline is not only accumulated via *de novo* synthesis from glutamate, but also can be acquired from the environment by the OpuE (Kempf and Bremer, 1998a). The osmoprotectant symporter *opuE* showed a highly induced gene when *B. cereus* ATCC 14579 was exposed to NaCl stress (den Besten et al., 2009). The intracellular proline level of *B. subtilis* cells increased from 16 mM to approximately 700 mM within 7 h after the moderate osmotic upshock at 0.4 M NaCl (Whatmore et al., 1990). Glutamate, a compatible solute and a precursor of proline biosynthesis, was moderately increased from 103 to 167 mM (Whatmore et al., 1990). The biosynthesis of proline was controlled through a feed-back inhibition of *proB*-encoded γ-glutamyl kinase, which was the first enzyme of the proline biosynthetic pathway (Kempf and Bremer, 1998a, b). In addition, the biosynthetic pathway was permitted to produce the proline when the glutamate, the substrate, presented in the cells (Kempf and Bremer, 1998a). In *Bacillus*, there were two distinct sets of proline biosynthesis genes to response the different demands of the cells for protein under low- and high-osmolarity growth condition. The sequential reaction of proline biosynthesis required ProB, ProA, and ProC enzymes or ProJ, ProA, and ProH enzymes (Wood et al., 2001). The formation of endogenous proline was repressed by accumulation of glycine betaine (Brill, Hoffmann, Bleisteiner, and Bremer, 2011; Hoffmann et al., 2013), which conferred a greater degree of osmotolerance than proline (Imhoff, 1986). It was in agreement with the result of Whatmore et al. (1990) who found that the accumulated proline was replaced by glycine betaine if the medium contained the glycine betaine. Thus, size of the intracellular proline pool was dependent on external NaCl content and the presence of the glycine betaine (Hoffmann et al., 2013). Glycine betaine is a metabolically inert compound and highly effective compatible solute (Imhoff, 1986; Kempf and Bremer,

1998a; Wood et al., 2001). It can be taken up via three transporters, OpuA, OpuC, and OpuD (Kempf and Bremer, 1998a). In the presence of 1 mM glycine betaine and 0.4 M NaCl, the intracellular level of the glycine betaine increased from ~175 mM to ~700 mM (Whatmore et al., 1990). The expression of *opuA*, the major transporter gene for glycine betaine, was down-regulated when the intracellular glycine betaine pool was increased, regardless of whether the glycine betaine was imported or endogenously synthesized from choline (Hoffmann et al, 2013). This regulatory process might prevent wasteful overaccumulation of the glycine betaine (Hoffmann et al, 2013). Many bacteria can synthesize glycine betaine from the precursor choline via type III alcohol dehydrogenase (GbsB) and glycine betaine aldehyde dehydrogenase (GbsA) (Kempf and Bremer, 1998a; Kappes et al., 1999; Wood et al., 2001). The GbsB converts exogenous choline into glycine betaine aldehyde and subsequently GbsA oxidizes glycine betaine aldehyde to glycine betaine. In addition, GbsA has more the function to keep the level of highly toxic intermediate, glycine betaine aldehyde, very low (Kappes et al., 1999). The *gbsA* and *gbsB* were regulated to be expressed when the substrate choline was available in the growth media (Boch, Kampf, and Bremer, 1994; Boch, Kempf, Schmid, and Bremer, 1996; Kempf and Bremer, 1998a). The remaining activity of GbsA at 2.5 M KCl was 88% of its initial activity, indicating its ability to biosynthesize glycine betaine during high-osmolarity stress (Kempf and Bremer, 1998a). The precursor choline for glycine betaine biosynthesis is taken up by OpuC and OpuB, which exhibit a high affinity (Kempf and Bremer, 1998a; Wood et al., 2001). The OpuC exhibits wide substrate specificity, while the OpuB system is restricted to take up choline and glycine betaine (Kempf and Bremer, 1998a; Wood et al., 2001). In *Br. linens*, the synthesis of glycine betaine via *de no* synthesis was strongly inhibited by the presence of exogenous glycine betaine,

indicating that bacteria would rather use exogenous glycine betaine than endogenously synthesized glycine betaine (Bernard et al., 1993). Ectoine was able to be synthesized by *H. elongata*, *B. subtilis*, *B. pasteurii*, *B. alkalophilus*, *B. psychrophilus*, *V. pantothenticus*, and *V. selexigens*, of which the ectoine biosynthetic pathway composed of three enzymes including 2,4-diaminobutyrate (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), and ectoine synthase (EctC) (Jebbar et al., 1997; Cánovas, Vargas, Calderon, Ventosa, and Nieto, 1998; Ono et al., 1999; Kuhlmann et al., 2002; Kuhlmann et al., 2008). Aspartic β-semialdehyde (ASA) is converted to DABA with glutamate by DABA aminotransferase. DABA acetyltransferase catalyzes acetylation of DABA to γ-*N*-acetyl-α,γ-diaminobutyric acid (ADABA) with acetyl coenzyme A. Ectoine synthase catalyzes circularization of ADABA to ectoine (Cánovas et al., 1998; Ono et al., 1999). In *V. pantothenticus* cells, an amount of intracellular ectoine was increased in proportion to the increase in the external NaCl content and reached to maximum level at 1.3 M NaCl (Kuhlmann et al., 2008).

2.3 Bacteria in the genus *Virgibacillus*

2.3.1 Morphology and physiology of *Virgibacillus*

Bacteria in the genus *Virgibacillus* are motile, Gram-positive rods with varying dimensions (0·3-0·7 × 2-6 μm) that occur singly, in pairs, short chains, or filaments (Heyndrickx et al., 1998; Heyrman, De Vos, and Logan, 2005). They bear spherical to ellipsoidal endospores which lie in terminal or in swollen sporangia. On trypticase soy agar at 24 h incubation, colonies are small (0.5-2 mm in diameter), circular and slightly irregular, smooth, glossy or sometimes matt, flat, butyrous, creamy to

yellowish white and almost opaque (Heyndrickx et al., 1998). The genus *Virgibacillus* is closely related to the *Bacillus*. Species of *Virgibacillus* are catalase positive, hydrolysis of casein and gelatin generally positive, and utilization of citrate. Indole, urease, hydrogen sulfide are usually not produced, and nitrate (NO_3) reduction to nitrite (NO_2) is variable (Heyndrickx et al., 1998; Heyrman et al., 2005). The bacterial growth is stimulated by 4% NaCl but not inhibited by 10% NaCl, and growth may occur between 10 and 50 °C, with an optimum of about 28 or 37 °C (Heyrman et al., 2005). D-Raffinose can be used as sole carbon sources (Heyrman et al., 2005). No growth on D-arabinose, D-fructose or D-xylose when used as a single carbon source was observed (Heyrman et al., 2005). The major cellular fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$ (Heyndrickx et al., 1998), and major polar lipids are diphophatidylglycerol (DPG) and phosphatidyl glycerol (PG) (Heyndrickx et al., 1998). Physiological characteristics of some *Virgibacillus* species are shown in Table 2.1. The genus *Virgibacillus* is able to produce and accumulate compatible solutes under high osmolality conditions. *V. pantothenticus* preferentially synthesizes ectoine, glutamate, and proline, and a lesser extent, hydroxyectoine (Heyrman et al., 2005). Those compatible solutes could prevent water loss from osmotic pressure, allowing *V. pantothenticus* to survive at hyper-saline conditions (Heyrman et al., 2005).

2.3.2 Member of the genus *Virgibacillus*

Virgibacillus pantothenticus, the first identified species of the genus, was formerly classified as *Bacillus* (Heyndrickx et al., 1998, 1999). On the basis of the amplified rDNA restriction analysis (ARDRA) pattern, 16S rDNA sequences, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of whole-cell

Table 2.1 Physiological characteristics of some *Virgibacillus* species. 1, *V. pantothenticus*; 2, *V. proomii*; *3, V. zhanjiangensis*; *4, V. koreensis*; *5, V. siamensis*; *6, V. carmonensis*; *7, V. litoralis*; *8, V. halodenitrificans*; *9, V. salinus.*

Strain/Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Source	Soil	Soli, Infant bile, Water supply	Sea water	Salt field	Fermented Fish (pla-ra)	Deteriorated mural paintings	Saline soil	Marine solar saltern, fermented shrimp paste (*ka-pi*)	Saline lake
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram strain	+	+	+	+	+	+	+	V	+
Chain of cells and/or filaments	+	+	n.d.	-	n.d.	-	+	+	+
Pigmentation	-	-	-	-	Red	Pink	-	-	-
Spore shape	ES	ES	ES	E	E	E(S)	E or S	E	ES
Spore position	T(S)	T(S)	T	T	TS	S	T	T or S	T or S

Table 2.1 Physiological characteristics of some *Virgibacillus* species. 1, *V. pantothenticus*; 2, *V. proomii*; *3*, *V. zhanjiangensis*; *4*, *V. koreensis*; *5*, *V. siamensis*; *6*, *V. carmonensis*; *7*, *V. litoralis*; *8*, *V. halodenitrificans*; *9*, *V. salinus* (Continued).

Strain/Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Hydrolysis									
Gelatin	W	W	+	-	+	-	+	+	-
Casein	+	+	-	n.d.	W	+	-	+	-
Aesculin	+	+	-	+	n.d.	W	-	W	+
Anaerobic growth	+	+	-	+	+	-	-	+ (nitrate is required)	-
Catalase activity	+	+	+	+	+	+	+	n.d.	+
Reduction of nitrate to nitrite	V	-	+	-	-	+	+	+	+
H_2S production	W	-	-	-	-	-	-	-	-
Utilization of citrate	+	+	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
Production of indole	-	-	-	-	n.d.	-	-	-	-
Urease activity	n.d.	n.d.	-	-	n.d.	-	-	-	n.d.
Ratio of iso-$C_{15:0}$ and anteiso-$C_{15:0}$	1:3	1:1	1:6	1:7	1:5	1:14	1:2	1:35	1:3

Table 2.1 Physiological characteristics of some *Virgibacillus* species. 1, *V. pantothenticus*; 2, *V. proomii*; *3, V. zhanjiangensis*; *4, V. koreensis*; *5, V. siamensis*; *6, V. carmonensis*; *7, V. litoralis*; *8, V. halodenitrificans*; *9, V. salinus* (Continued).

Strain/Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Polar lipid	PG, DPG, PE, uPL, uAPL	PG, DPG, PE, uPL, uAPL	PG, DPG	PG, DPG, uPL	PG, DPG, uGL	PG, DPG	PG, DPG	PG, DPG	PE, DPE, uGL, uPL
Optimum temperature (°C)	37	37	30	25	37	25-30	30	35-40	37
Optimum pH	7	n.d.	7.5	7	7	n.d.	8	7.4-7.5	7.5
Growth at 0.5% NaCl	+	W	-	W	-	-	-	-	-
Growth at 10% NaCl	+	n.d.	+	+	+	+	+	+	+
Growth at 25% NaCl	-	-	-	-	-	-	+	-	-
G+C content (mol%)	36.9	37	39.5	41	38	38.9	40.2	38-39	38.8

Characteristics: +, positive; -, negative; W, weak; V, variable. Spore shape: E, ellipsoidal; S, spherical; (S), sometimes spherical. Spore position: T, terminal; S, subterminal; (S), sometimes subterminal; C, central. Polar lipids: PG, Phosphatidylglycerol; DPG, Diphosphatidylglycerol; PE, Phosphatidylethanolamine; uAPL, Unknown aminophopholipid; uGL, Unknown glycolipid; uPL, Unknown phospholipid; n.d., not determined.

proteins, biochemical test and phenotypic characteristics, Bacillus pantothenticus was reclassified as the new genus of *Virgibacillus* (Heyndrickx et al., 1998; 1999). In addition, *Salibacillus* was reclassified to *Virgibacillus* based on genotypic and phenotypic characteristics (Heyrman et al., 2003). Nowadays, 311 bacterial strains in the genus *Virgibacillus* have been reported in the National Center for Biotechnology Information (NCBI), but only 30 species have been validly described. Type strains in the genus *Virgibacillus* include (1) *V. pantothenticus*, (2) *V. proomii* (Heyndrickx et al., 1999), (3) *V. carmonensis*, (4) *V. necropolis*, (5) *V. marismortui*, (6) *V. salexigens* (Heyrman et al., 2003), (7) *V. albus* (Zhang et al., 2012), (8) *V. alimentarius* (Kim et al., 2011), (9) *V. arcticus* (Niederberger, Steven, Charvet, Barbier, and Whyte, 2009), (10) *V. byunsanensis* (Yoon et al., 2010), (11) *V. campisalis* (Lee, Kang, Oh, and Yoon, 2012), (12) *V. chiguensis* (Wang, Chang, Ng, Chen, and Shyu, 2008), (13) *V. dokdonensis* (Yoon, Kang, Lee, Lee, and Oh, 2005), (14) *V. halodenitrificans* (Yoon, Oh, and Park, 2005; Tanasupawat, Taprig, Akaracharanya, and Visessanguan, 2011), (15) *V. halophilus* (An, Asahara, Goto, Kasai, and Yokota, 2007), (16) *V. halotolerans* (Seiler and Wenning, 2013), (17) *V. kekensis* (Chen et al., 2008), (18) *V. litoralis* (Chen et al., 2009b), (19) *V. olivae* (Quesada, Aguilera, Morillo, Ramos-Cormenzana, Monteoliva-Sánchez, 2007), (20) *V. salarius* (Hua, Hamza-Chaffai, Vreeland, Isoda, and Naganuma, 2008), (21) *V. salinus* (Carrasco, Márquez, and Ventosa, 2009), (22) *V. sediminis* (Chen et al., 2009a), (23) *V. siamensis* (Tanasupawat, Chamroensaksri, Kudo, and Itoh, 2010), (24) *V. soli* (Kämpfer et al., 2011), (25) *V. subterraneus* (Wang, Xue, and Ma, 2010), (26) *V. xinjiangensis* (Jeon et al., 2009), (27) *V. marismortui* (Chamroensaksri, Akaracharanya, Visessanguan, and Tanasupawat, 2008), (28) *V. koreensis* (Lee et al., 2006), (29) *V. zhanjiangensis* (Peng et al., 2009), and (30) *V. natechei* (Amziane et al., 2013). Untill now, three *Virgibacillus* genomes,

contents are 37, 37.4, and 37.6%, respectively. The number of genes and proteins appeared on the genome is 4,250/4,182, 3,969/3,912, and 4,047/3,994 respectively. including *Virgibacillus* sp. CM-4, *V. halodenitrificans* 1806, and *Virgibacillus* sp. SK37, are availalble on NBCI and Integrated Microbial Genomes (IMG). Complete genome sizes of *Virgibacillus* sp. CM-4, *V. halodenitrificans* 1806, and *Virgibacillus* sp. SK37 are 4.18, 3.92, and 3.84 Mb, respectively. GC

2.3.3 Habitat of *Virgibacillus*

Members of *Virgibacillus* are either strictly aerobic or facultative anaerobic bacteria, and in some species, NaCl is required for growth. They are classified as moderately halophilic (*V. salaries*, *V. marismortui*, *V. xinjiangensis*, *V. subterraneus*, *V. sediminis*, *V. kekensis*, *V. halodenitrificans*, *V. salinus*, *V. natechei*, *V. albus*, *V. litoralis*, *V. siamensis*, and *V. arcticus*) and halotolerant bacteria (*V. olivae*) (Ouesada et al., 2007; Chamroensaksri et al., 2008; Chen et al., 2008; Hua et al., 2008; Carrasco et al., 2009; Chen et al., 2009a; Chen et al., 2009b; Jeon et al., 2009; Niederberger et al., 2009; Wang et al., 2010; Tanasupawat, et al., 2010; Tanasupawat, et al., 2011; Amziane et al., 2013; Seiler and Wenning, 2013). Species of *Virgibacillus* can be found in a wide variety of habitats, especially in hypersaline environments such as sea water, salt lake, saline soil, and traditional salt-fermented seafood (Yoon et al., 2005; Lee et al., 2006; Chamroensaksri et al., 2008; Hua et al., 2008; Chen et al., 2009b; Wang et al., 2010; Kim et al., 2011; Tanasupawat, et al., 2010; Tanasupawat, et al., 2011; Lee et al., 2012). Soil is also a habitat of several species (*V. soli*, *V. halophilus*, and *V. proomi*) (Heyndrickx et al., 1999; An et al., 2007; Kämpfer et al., 2011). Other habitats include dairy product (*V. halotolerans*) (Seiler and Wenning, 2013), olive-processing water (*V. olivae*) (Ouesada et al., 2007), permafrost (*V. arcticus*) (Niederberger et al., 2009), deteriorated murals (*V. carmonensis* and *V. necropolis*)

(Heyrman et al., 2003), water supply, and infant bile (*V. proomi*) (Heyndrickx et al., 1999).

2.4 Halophilic proteinases

Bacteria that can grow in saline environment are divided into two types. Firstly, halophilic archaea accumulate salt within their cytoplasms to maintain an osmotic balance against hypersaline environment. Their intracellular and extracellular enzymes function in the presence of salts. Secondly, halophilic or halotolerant eubacteria maintain an osmotic balance within cytoplasm by accumulating high concentration of various organic osmotic solutes (e.g. sugars, amino acids, and ectoines). Consequently, salt content within intracellular is low and intracellular enzymes are inactive at high salt content (Margesin and Schinner, 2001). For this reason, proteinases from halophilic and nonhalophilic bacteria showed different structural and biochemical characteristics.

2.4.1 Catalytic mechanism

Most halophilic proteinases have been identified as serine proteinase. Amino acid sequences of halophilic proteinase are homologous with subtilisin-type serine proteinase (Völkl, Markiewicz, Stetter, and Miller, 1994). The proteinase shows the conserved catalytic regions, especially catalytic triad of Ser221, His64 and Asp32. It might conclude that evolution of halophilic proteinase related to eubacterial subtilisin (Stepanov et al., 1992). Therefore, catalytic mechanism of halophilic proteinase could be similar to that of subtilisin. In subtilisin, Ser221 attacks a peptide bond to form tetrahedral intermediate. The tetrahedral intermediate decomposes to release the polypeptide chain on the amino side of peptide bond (C-terminal segment). The acyl-

enzyme intermediate is rapidly deacrylated to form a new tetrahedral intermediate. Subsequently, carboxyl group (as new C-terminal of product) of substrate dissociates from enzyme, resulting in N-terminal segment (Voet and Voet, 2004).

2.4.2 Structural feature under salt conditions

Nonhalophilic proteinase is usually inactive under high salt concentration. NaCl, an antichaotropic salt, tends to stabilize enzyme structure (Arakawa and Timasheff, 1982). NaCl increases the intramolecular hydrophobic interaction and surface tension of water lending to folded state. Surface tension of water increases with NaCl, resulting in exclusion of water around protein surface and protein aggregation. Degree of aggregation is correlated with an increase of NaCl concentration (Arakawa and Timasheff, 1982). Hence, active site of nonhalophilic proteinase would be disrupted by aggregation, resulting in reduction of catalytic activity in the presence of high NaCl concentration.

Proteinases from halophilic bacteria show different characteristics in the presence of NaCl. Typically, they require 1-4 M NaCl (5.8-23.3%) for catalytic activity and stability. The enzymes are irreversibly inactivated at lower salt concentrations. Halophilic proteinases usually contain more acidic amino acids than nonhalophilic proteinases. *Halobacterium halobium* proteinase contained ~23.1% aspartic, glutamic residues, and their amides per molecule, whereas subtilisin BPN' from *Bacillus amyloliquefaciens* contained only ~15.6% per molecule (Stepanov et al., 1992). Glu243Arg mutant halophilic malate dehydrogenase required higher NaCl for enzyme stability than did wild-type, indicating in a significance of acidic amino acids in halophilic protein characteristics (Madern, Pfister, and Zaccai, 1995). Excess acidic residues of halophilic proteinase are normally found on the surface (Rao and Argos, 1981). This attracts hydrated salts and water molecules into its surface (Rao and

Argos, 1981; Madern et al., 1995). This structure can maintain the intact halophilic proteinase at high NaCl content.

Generally, nonhalophilic protein shows high stability in the absence of salt because of hydrophobic effect. Nonpolar groups decrease the entropy of water creating hydrophobic interaction of enzyme structure. Halophilic proteins at low salt content undergo denaturation with loss of secondary and tertiary structure (Madern et al., 1995). This is because halophilic protein has a weak hydrophobic core compared to nonhalophilic protein (Lanyi, 1974). In the absence of salt, nonpolar interior of halophilic protein is exposed lending to loss stability. These suggest that hydrophobic core is insufficient to stabilize the native protein, thereby salting out agent is required to interact with protein surface resulting in an increase in hydrophobicity of core structure. Thus, halophilic proteinases require high NaCl concentration for their catalytic activity.

In addition, the two cysteine residues of halophilic proteinase from *Haloferax mediterranei* R4 (halolysin R4) located on C-terminal region which is not found in subtilisin show a significant role in catalytic activity. The activity of both mutated halolysin R4 cysteine 316 substituted with serine (Cys316Ser) and two cysteines 316/352 substituted with serine (Cys316Ser/Cys352Ser) proteinases decreased to ~30% of the original activity at 25% NaCl, whereas wild-type proteinase remained active (Kamekura, Seno, and Dyall-Smith, 1996). Therefore, cysteine residues on C-terminal region of halophile proteinase might play an important role in reinforcing conformation stability in the presence of NaCl.

2.5 Optimization of proteinase production by response surface methodology

The response surface methodology (RSM) is an empirical modeling approach for determining the relationship between various parameters and response, and searching the significance of these parameters on the response (Chiang and Chang, 2006). For building and optimizing the empirical model, a collection of mathematical and statistical procedures is applied (Neddermeijer, van Oortmarssen, Piersma, and Dekker, 2002; Chiang and Chang, 2006; Baş and Boyacı, 2007; Bezerra, Santelli, Oliveira, Villar, and Escaleira, 2008). The design of experiment (DoE) and application of regression analysis are used to gain the desired model (Chiang and Chang, 2006). The quantitative form of relationship between the independent input variables and the response can be represented as

$$y = f(x_1, x_2, x_3, \dots x_n) \pm \varepsilon$$

where *y* is the response, *f* is the response surface (or response function), x_1, x_2, x_3,… x_n are the independent input variables, and ε is the statistical error (Chiang and Chang, 2006; Aslan and Cebeci, 2007; Baş and Boyacı, 2007). The necessary data for building the response models are collected by DoE (Chiang and Chang, 2006). The appropriate approximation of *f* is required to determine whether the application of RSM is successful (Chiang and Chang, 2006; Aslan and Cebeci, 2007). In general, the approximation of *f* in RSM is proposed using the fitted second-order polynomial regression model which is called the quadratic model (Aslan and Cebeci, 2007; Baş and Boyacı, 2007; Bezerra et al., 2008). The quadratic model of *f* can be written as follows

$$f = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i<j}^{n} a_{ij} x_i x_j + \varepsilon$$

where a_0 represents the linear effect of x_i, a_{ii} represents the quadratic effect of x_i, and a_{ij} represents the linear interaction between x_i and x_j. The f contains the linear terms, squared terms, and cross product (Chiang and Chang, 2006). In practical application, using the response model of f is to investigate over the entire factor space and to locate region within the space where the response approaches its optimal value (Chiang and Chang, 2006). It is possible to summarize the optimization study using RSM into seven steps as follows:

(1) Defining the independent input variables and their levels, and the response

(2) Selecting the type of DoE.

(3) Performing the regression analysis with the quadratic model, or cubic model if necessary, of response surface *f*.

(4) Calculating the statistical analysis of variance (ANOVA) for the independent input variables and to find which parameter exhibits a significant effect on the response.

(5) Determining the situation of the model of response surface f in order to decide whether the model of RSM needs screening variables or not.

(6) Obtaining the response surface plot and predicting the optimal design parameters.

(7) Verifying the optimal design setting with the experiment

The DoE technique is a powerful method to investigate multiple factors simultaneously during the experiments. It is more applicable than one-factor-at-a-time (OVAT) experiment (Baş and Boyacı, 2007; Bezerra et al., 2008; Mandenius and Brundin, 2008). The OVAT experiment does not provide information on how the

response is altered when other factors are present (Baş and Boyacı, 2007; Bezerra et al., 2008). If two or more factors are included, the relationship among factors, called an *interaction*, appeared. In term of DoE, the dependent variable is called the *response* and the independent variables (parameters) are called *factors* (Bezerra et al., 2008). The experiments of DoE are run at different values of the factors, called *levels* (Bezerra et al., 2008). Each run of the experiment is performed by a combination of the levels of all factors, called a *treatment*. The number of treatments of the DoE is determined on the basis of the number of factor levels. If all combinations are run, the experiment is a full factorial. If some of the combinations are run, the experiment is a fractional factorial. The main objective of both full and fractional factorials is to identify the vital factor affecting a product or process (Ferreira et al., 2007b; Mandenius and Brundin, 2008; Tarley et al., 2009). A first-order model is used in the factorial designs and its assumption is that all interactions are negligible (Tarley et al., 2009). The identification of the vital factors and their levels is the first and most important step to achieve the optimization using RSM (Baş and Boyacı, 2007; Witek-Krowiak et al., 2014). However, the linear model is generally not sufficient to represent the experimental data if the relationship of the response and levels of each factor has a curvature (Tarley et al., 2009). Therefore, the response surface method design used to investigate the optimum value of the vital factors on the response is applied. The purpose of the response surface method design is to obtain the experiment fitted to the second-order model with minimizing the number of runs (Ferreira et al., 2007b; Bezerra et al., 2008; Tarley et al., 2009). The response surface method design contains four major types including central composite design (CCD), rotatable design, face-centered design, and Box-Behnken design. The CCD is obtained when runs at four points (0, -1), (0, 1), (-1, 0), and (1, 0) are added to the experimental design. 0 is

referred as a center point and 1, -1 are represented as a factorial point. The design contains other points, called an axial point, located at the -α and +α position on each axis. The α values for two, three, and four factors are 1.41, 1.68, and 2.00, respectively (Ferreira et al., 2007b; Bezerra et al., 2008). Therefore, each factor in this design is run at five levels (-α, -1, 0, 1, α) (Bezerra et al., 2008). The rotatable design is referred to CCD, when is set the levels of each factor depending upon the same distance of the point from the center point, regardless of the direction (Neddermeijer et al., 2002). For instance, each factor applied on the rotatable design is run at five levels with -2, -1, 0, 1, 2. The face-centered design is also referred to CCD, when the distance of the axis point (α) is 1. Hence, each factor is run at three levels (-1, 0, 1). Theoretically, the CCD is better to capture strong curvature and cubic response behavior than the face-centered design, because the CCD has five levels of each factor (Mandenius and Brundin, 2008). The Box-Behnken design assigns that each factor is run at three levels (-1, 0, 1) with equally spaced intervals between these levels (Bezerra et al., 2008). The low (-1) and high (+1) levels of each factor are not combined in the same treatment (Ferreira et al., 2007a, b; Tarley et al., 2009). The Box-Behnken design is used to avoid setting up the experiment under harsh condition (Ferreira et al., 2007a, b; Tarley et al., 2009). After the data for RSM are collected, multiple linear regression analysis is used to construct the suitable response surface (*f*). If two factors, x_1 and x_2, are studied, the multiple linear regression model can be written as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon$$

where β_0 represents the intercept, β_1 and β_2 represent the linear coefficients, and ε represents the statistical error (Mandenius and Brundin, 2008). If the contour plot is constructed, a straight contour line on the plot is obtained. In addition, the linear

regression model may include the interaction term if the interaction term shows statistical significances. However, the response surface is generally fitted to other kinds of the multiple linear regression model, called a *polynomial regression model*. The polynomial regression model contains squared and higher order terms to obtain the response surface curvilinear (Bezerra et al., 2008).

The application of RSM to optimize the high yield of bacterial proteinase production has been extensively studied as shown in Table 2.2. Media components and culture conditions are considered as important factors affecting proteinase production. Glycerol, casein, KH_2PO_4, $MgSO_4 \bullet 7H_2O$, NaCl, initial pH, incubation time, incubation temperature, and inoculum size were initially screened to determine which ones were the vital factors for the organic solvent-stable proteinase production of *Bacillus sphaericus* DS11 (Liu, Fang, Lv, and Chen, 2010). On the basis of the calculated *P*-values, three main factors, including glycerol, $MgSO_4 \bullet 7H_2O$, and initial pH were pronounced to be the most significant factors affecting proteinase productivity (Liu et al., 2010). Among numerous factors tested including *carbon sources:* glucose; glucose syrup; fructose corn syrup; maltose; glucose-fructose; natural corn starch; potato starch; molasses, *nitrogen source:* casein; corn steep liquor; corn meal; soybean meal (fine); soybean (crude); whey, and *elements and surfactants:* Mg_2SO_4; KH_2PO_4; $CaCl_2$; Tween80, the main factors affecting the proteinase production of *Bacillus* sp. L21 were soybean meal, maltose50 (50% maltose), Tween80, and initial pH (Tari, Genckal, and Tokatlı, 2006). Alternatively, if the general vicinity of the optimum levels of the main factors has been unknown, the method of steepest ascent can be applied to find the proper direction to change the concentration of the main factors to improve the productivity (Liu et al., 2010). The

Table 2.2 Optimization of bacterial proteinase production using response surface methodology.

Strain	Method	Input	Response	Improvement	Reference
Bacillus subtilis DM-04	Central composite design (20 treatments, 3 factors × 3 levels)	F1: Agro-industrial waste materials, the mixture between *Imperata cylindriaca* grass and potato peels (1:1) (%) F2: Beef extract (%) F3: Incubation time (h)	Alkaline proteinase yield	Optimum determined	Rai and Mukherjee (2010)
Bacillus sp. RKY3	Central composite design (20 treatments, 4 factors × 5 levels)	F1: Corn starch (g/L) F2: Corn steep liquor (g/L) F3: Inoculum size (%)	Alkaline proteinase yield	2.3-fold yield increase	Reddy, Wee, Yun, and Ryu (2008)
Bacillus pseudofirmus SVB1	Central composite design (20 treatments, 3 factors × 3 levels)	F1: initial pH F2: Temperature (°C) F3: Shaking speed (rpm)	Alkaline proteinase yield	6.2-fold yield increase	Sen, Veeranki, and Mandal (2009)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Bacillus sp. PE-11	Central composite design (20 treatments, 3 factors × 5 levels)	F1: Glucose (g/L) F2: Peptone (g/L) F3: Salt solution (0.5% KH_2PO_4, 0.01% $MgSO_4 \bullet 7H_2O$, and 0.01% $FeSO_4 7H_2O$) (g/L)	Alkaline proteinase yield	Optimum determined	Adinarayana and Ellaiah (2002)
Bacillus licheniformis BA17	Central composite design 2(12 treatments, 3 factors × 3 levels)	F1: Temperature (°C) F2: pH	Alkaline proteinase yield	3-fold yield increase	Nikerel, Ateş, and Öner (2008)
Bacillus clausii	Central composite design (20 treatments, 3 factors × 5 levels)	F1: Sucrose (g/L) F2: Yeast extract (g/L) F3: KNO_3 (g/L)	Alkaline proteinase yield	6-fold yield increase	Oskouie, Tabandeh, Yakhchali, and Eftekhar (2008)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Bacillus cereus SV1	Central composite design (43 treatments, 5 factors × 3 levels)	F1: Shrimp shell power (g/L) F2: NH_4Cl (g/L) F3:$CaCl_2$ (g/L) F4: K_2HPO_4 (g/L) F5: Speed of agitation (rpm)	Proteinase yield	Optimum determined	Ghorbel-Bellaaj, Manni, Jellouli, Hmidet, and Nasri (2012)
Bacillus sp. JER02	Central composite design (30 treatments, 4 factors × 5 levels)	F1: Temperature (°C) F2: pH F3: NaCl (mM) F4: $MgSO_4$ (mM)	Organic solvent-tolerant proteinase yield	2.5-fold yield increase	Badoei-Dalfard and Karami (2013)
Bacillus mojavensis A21	Central composite design (30 treatments, 4 factors × 5 levels)	F1: Hulled grain of wheat (g/L) F2: NaCl (g/L) F3: KH_2PO_4 (g/L) F4: K_2HPO_4 (g/L)	Alkaline proteinase yield	14-fold yield increase	Haddar et al. (2010)
Paenibacillus tezpurensis AS-S24-II	Central composite design (20 treatments, 3 factors × 5 levels)	F1: pH F2: Casein (%) F3: Ammonium sulfate (%)	Alkaline proteinase yield	Optimum determined	Rai, Roy, and Mukherjee (2010)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Streptomyces sp7	Central composite design (32 treatments, 5 factors × 5 levels)	F1: Feather meal (g/L) F2: K_2HPO_4 (g/L) F3: KH_2PO_4 (g/L) F4: NaCl (g/L) F5: pH	Keratinase proteinase yield	Optimum determined	Tatineni, Doddapaneni, Potumarthi, and Mangamoori (2007)
Streptomyces sp. A6	Central composite design (30 treatments, 4 factors × 5 levels)	F1: pH F2: Shrimp waste (g/L) F3: $FeCl_3$ (g/L) F4: $ZnSO_4$ (g/L)	Proteinase yield	4.96-fold yield increase	Singh and Chhatpar (2010)
Geomicrobium sp. EMB	Central composite design (36 treatments, 5 factors × 5 levels)	F1: NaCl (%) F2: pH F3: Inoculum size (%) F4: Yeast extract (%) F5: Casamino acid (%)	Organic solvent-tolerant proteinase yield	20-fold yield increase	Karan, Singh, Kapoor, and Khare (2011)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Microbacterium sp. kr10	Central composite design (17 treatments, 3 factors × 5 levels)	F1: Initial pH F2: Temperature (°C) F3: Feather meal (g/L)	Proteinase yield	3.6-fold yield increase	Thys, Guzzon, Cladera-Olivera, and Brandelli (2006)
Colwellia sp. NJ341	Central composite design (30 treatments, 4 factors × 5 levels)	F1: Casein (g/L) F2: Sodium citrate (g/L) F3: Temperature (°C) F4: Tween-80 (g/L)	Cold-active proteinase yield	Optimum determined	Wang, Hou, Xu, Miao, and Li (2008)
Pseudomonas aeruginosa B-2	Central composite design (20 treatments, 3 factors × 5 levels)	F1: Albumin (%) F2: Ammonium sulfate (%) F3: Glucose (%)	Alkaline proteinase yield	1.5-fold yield increase	Khan, Misra, Tripathi, Mishra, and Bihari (2006)
Pseudomonas aeruginosa san-ai	Central composite design (30 treatments, 4 factors × 5 levels)	F1: Sunflower oil (%) F2: Triton® X-100 (%) F3: Temperature (°C) F4: Incubation time (h)	Proteinase yield	0.6-fold yield increase	Grbavčić et al. (2011)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Pseudomonas sp. RAJR 044	Central composite design (20 treatments, 3 factors × 3 levels)	F1: pH F2: Temperature (°C) F3: Incubation volume (ml)	Proteinase yield	Optimum determined	Dutta, Dutta, and Banerjee (2004)
Pseudoalteromonas sp. IND11	Central composite design (20 treatments, 3 factors × 3 levels)	F1: pH F2: Maltose (%) F3: Sodium dihydrogen phosphate (%)	Fibrinolytic enzyme yield	3-fold yield increase	Vijayaraghavan and Vincent (2014)
Marinobacter sp. GA CAS9	Central composite design (31 treatments, 4 factors × 5 levels)	F1: NaCl (g/L) F2: Beef extract (g/L) F3: $CuSO_4$ (g/L) F4: pH	Alkaline proteinase yield	3.3-fold yield increase	Kumar, Anathan, and Prabhu (2014)
Exiguobacterium sp. CFR26M	Central composite design (18 treatments, 3 factors × 5 levels)	F1: Shrimp waste (%) F2: Sugar and D-glucose (%) F3: Phosphate dibasic (%)	Proteinase yield	6.3-fold yield increase	Kumar and Suresh (2014)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Shewanella oneidensis MR-1	Box-Behnken design (46 treatments, 5 factors × 3 levels)	F1: Incubation time (h) F2: pH F3: Temperature (°C) F4: Glucose (g/L) F5: Tryptone (g/L)	Alkaline proteinase yield	Optimum determined	Anbu, Annadurai, Lee, and Hur (2008)
Bacillus sp. L21	Box-Behnken design (27 treatments, 4 factors × 3 levels)	F1: Soybean meal (g/L) F2: Maltose50 (g/L) F3: Tween80 (g/L) F4: Initial pH	Alkaline proteinase yield	Optimum determined	Tari, Genckal, and Tokatlı (2006)
Exiguobactertium profundum BK-P23	Box-Behnken design (46 treatments, 5 factors × 3 levels)	F1: Lactose (%) F2: Corn steep solid (%) F3: pH F4: Temperature (°C) F5: Incubation period (h)	Alkaline proteinase yield	Optimum determined	Anbu, Annadurai, and Hur (2013)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Bacillus sphaericus DS11	Box-Behnken design (17 treatments, 3 factors × 3 levels)	F1: $MgSO_4 \cdot 7H_2O$ (g/L) F2: Glycerol (g/L) F3: pH	Organic solvent-stable proteinase yield	2.5-fold yield increase	Liu, Fang, Lv, Wang, and Chen (2010)
Pseudomonas putida SKG-1	Box-Behnken design (30 treatments, 4 factors × 3 levels)	F1: Glucose (%) F2: Yeast extract (%) F3: $MgSO_4$ (%) F4: pH	Thermoalkaline proteinase yield	13.5-fold yield increase	Singh, Singh, Tripathi, Khare, and Garg (2011)
Bacillus subtilis (*aprE::lacZ* expression)	Box-Behnken design (13 treatments, 3 factors × 3 levels)	F1: Peptone (g/L) F2: $MgSO_4 \cdot 7H_2O$ (g/L) F3: KCl (g/L)	Proteinase (*aprE* promoter expression) yield	5-fold yield increase	El-Helow, Abdel-Fattah, Ghanem, and Mohamad (2000)
Escherichia coli BL21 (Expression host)	Spherical CCD (19 treatments, 3 factors × 3 levels)	F1: Isopropyl β-D-1-thiogalactopyranoside (IPTG) (mM) F2: Ca^{2+} (mM) F3: Induction time (h)	Zinc-metalloprotease (SVP2) from *Salinivibrio proteolyticus* AF-2004 yield	16-fold yield increase	Beigi, Karbalaei-Heidari, and Kharrati-Kopaei (2012)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Bacillus sp. RGR-14	Face Centered CCD (30 treatments, 4 factors × 3 levels)	F1: Starch (mg/ml) F2: Peptone (mg/ml) F3: Inoculum (%) F4: Incubation time (h)	Proteinase yield	2.6-fold yield increase	Puri, Beg, and Gupta (2002)
Bacillus sp. RGR-14	Face Centered CCD (32 treatments, 5 factors × 3 levels)	F1: Starch (%) F2: Casamino acid (%) F3: Phosphate (%) F4: Inoculum size (%) F5: Incubation time (h)	Alkaline proteinase yield	12.8-fold yield increase	Chauhan and Gupta (2004)
Bacillus mojavensis MTCC 3606	Face Centered CCD (32 treatments, 5 factors × 5 levels)	F1: Casamino acid (mg/mL) F2: Glucose (mg/mL) F3: Inoculum age ($A_{550\ nm}$) F4: Incubation time (h) F5: Agitation (rpm)	Alkaline proteinase yield	4.2-fold yield increase	Beg, Sahai, and Gupta (2003)

Table 2.2 Optimization of bacterial proteinase production using response surface methodology (Continued).

Strain	Method	Input	Response	Improvement	Reference
Bacillus licheniformis RG1	Face Centered CCD (32 treatments, 5 factors × 3 levels)	F1: Soy flour (%) F2: Cornstarch (%) F3: Phosphate (%) F4: Inoculum density (%) F5: Incubation time (h)	Alkaline proteinase yield	2.3-fold yield increase	Ramnani, Kumar, and Gupta (2005)
Bacillus sp. SBP-66	Face Centered CCD (32 treatments, 5 factors × 3 levels)	F1: Starch (g/L) F2: Soybean meal (g/L) F3: $CaCl_2$ (mM) F4: Agitation rate (rpm) F5: Inoculum density (%)	Alkaline proteinase yield	1.5-fold yield increase	Saran, Isar, and Saxena (2007)
Bacillus subtilis	Face Centered CCD (30 treatments, 4 factors × 3 levels)	F1: Fructose (%) F2: Casein (%) F3: Inoculum size (%) F4: Agitation size (rpm)	Milk-clotting protease yield	2.08-fold yield increase	Dutt, Gupta, Saran, Misra, and Saxena (2009)

yield plateau at certain concentration of each factor was obtained and can be used to define the levels of each main factor for further optimization. Secondly, the type of the response surface method design with the main factors and their levels is designed.

For instance, the Box-Behnken design with three factors and three levels used for fitting a second-order polynomial was used for optimization of the organic solvent-stable proteinase production of *B. sphaericus* DS11 (Liu et al., 2010). Thirdly, the data were analyzed by ANOVA and following quadratic regression equation was obtained in term of proteinase yield. The high *F*-value and non-significant lack of fit indicated the model is a good fit. The significant *P*-value suggested the obtained experiment data is a good fit with the model. When determination coefficient (R^2) was reported as 0.9996, it is indicated that 0.04% of the total variations were not explained by the model (Liu et al., 2010). The high R^2 (> 0.75) value indicates the accuracy and general ability of the polynomial model (Oskouie, Tabandeh, Yakhchali, and Eftekhar, 2008). Subsequently, a significance of regression coefficient of all linear, quadratic, and interaction terms is considered. The statistical results showed that the linear terms of three factors (glycerol, $MgSO_4 \bullet 7H_2O$, and pH) had significant positive effects on the organic solvent-stable proteinase production of *B. sphaericus* DS11, but the interaction terms between $MgSO_4 \bullet 7H_2O$ and glycerol, and glycerol and initial pH exhibited negative effects (Liu et al., 2010). The results may reveal non significances of the interaction terms between all factors. None of interaction terms among the main factors, including glucose, peptone, $MgSO_4$, and $CaCl_2$, affecting the Nattokinase production from *B. subtilis* was statistically significant (Deepak et al., 2008). It was reported that the concentration of the factor tended to be a direct relationship to the response (Reddy, Wee, Yun, and Ryu, 2008). In addition, the larger magnitude of the

t-value and smaller *P*-value of each regression coefficient (linear, quadratic, and interaction terms) indicates the more significance of the factor on the response (Thys, Guzzon, Cladera-Olivera, and Brandelli, 2006). Among the main factors, including casein, sodium citrate, temperature, and Tween-80, affecting the proteinase production of psychrophilic bacterium, *Colwellia* sp. NJ341, the coefficient of the quadratic effect of casein was the largest, implying in the most significance on the productivity (Wang, Hou, Xu, Miao, and Li, 2008). Plus-minus sign of each regression coefficient should also be considered. Casein exhibited a significantly negative coefficient for linear and interaction effects on proteinase production of *Paenibacillus tezpurensis* AS-S24-II, indicating that a decrease in its concentration can improve its proteinase production (Rai, Roy, and Mukherjee, 2010). In some cases, the statistical result may show insignificances of the major factors on the response. Among four factors tested, the two factors, namely sunflower oil and incubation time, did not have any significant effect on the proteinase production of *Pseudomonas aeroginaosa* san-ai and were excluded from the predictive equation (Grbavčić et al., 2011). Three dimensional response surface plots are usually constructed to demonstrate relationships between the proteinase yield (response) and experimental levels of each factor. The shape of three dimensional plots may not appear curvature if the less interaction among factors is obtained (Reddy et al., 2008). Importantly, the plots can be used to predict where the optimum levels of each factor are required for proteinase production (Puri, Beg, and Gupta, 2002). The optimum condition, predicted by the model, for proteinase production of *B. sphaericus* DS11 was 0.73 g/L $MgSO_4 \bullet 7H_2O$, 12.47 g/L glycerol, and initial pH 8.25 with the maximum predicted yield of 1,079.55 U/ml (Liu et al., 2010). To confirm the optimization result, the proteinase yield at the predicted

condition must be verified. In addition, to validate the model, a random set of treatments assigned within the design space can be carried out. Chauhan and Gupta (2004) validated the model for optimization of alkaline proteinase production from *Bacillus* sp. RGR-14 with 10 random combinations of each factors. The ten treatments were prepared and tested for the proteinase production. The nine treatments of experimentally determined production values were in close agreement with the predicted value of the model.

2.6 Microarrays

The analysis of gene expression has globally received a great deal of attention for unraveling of cellular signaling pathways and the response of cells on genetic stimulus. When transcriptomic analyses are succeeded, their outcome is likely to describe the regulation of gene-expression networks. Various methods are available to monitor the quantity of expression levels including northern blots, S1 nuclease protection, differential display, sequencing of cDNA libraries, and serial analysis of gene expression (SAGE) (Duggan et al., 1999). The advantage of microarray is the allowance to simultaneously monitor the expression of all genes in the cells (Harrington et al., 2000; Ehrenreich, 2006). The use of proteomics combined protein separation by two-dimensional electrophoresis (2D-electrophoresis), followed by trypsinolysis and protein identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is one of the most powerful approaches (Kuipers et al., 2002; Greenbaum et al., 2003), however it cannot detect low-abandant proteins, too acidic or basic proteins, and has problems in analyzing membrane proteins and unstable proteins (Kuipers et al., 2002; Greenbaum et al., 2003). In contrast to protein-

based approaches, microarray is a genomic tool to offer the comprehensive data of all mRNAs transcribed in cells or tissues which response to the environmental stimulus (Kuipers et al., 2002). The crucial principle of a microarray experiment is the hybridization of two complementary single-stranded DNAs, which properly formed hydrogen bonds with each other. After washing, non-specific binding is removed and the hybridized molecules are still remained. Practically, mRNA from interested cells or tissues is used to generate a labeled sample, called as a *target*, which is hybridized specifically to a short part of its corresponding DNA sequences known as a *probe* (or reporter or oligos), that is immobilized on a solid phase in an ordered array (Schulze and Downward, 2001). Tens of thousands of transcript products can be detected and quantified simultaneously in the single experiment (Harrington et al., 2000; Schulze and Downward, 2001; Ehrenreich, 2006). The knowledge of functional genomics results in a better understanding of physiological processes and regulatory networks in microorganisms during growth (Kuipers, et al., 2002).

2.6.1 Array platforms

On the basis of an array material, the microarray system nowadays can be divided into three groups that are (1) complementary DNA (cDNA), (2) oligonucleotide, and (3) double-strand DNA microarrays. The array material is meant to the probe, which is equivalent to a probe, used in northern blot analysis, immobilized on a solid surface, normally called a microarray chip (Harrington et al., 2000; Schulze and Downward, 2001).

The probes for cDNA microarray are the nucleotide fragments from the polymerase chain reaction (PCR), generated from cDNA libraries or clone collections, using either vector-specific or gene-specific primers (Duggan et al., 1999; Schulze and Downward, 2001). By using cDNA for probe production, gene products are easily

identified since this method allows to detect fully transcribed mRNA and excludes enhancers, introns, and other regulatory elements. Alternatively, the probes can be generated by PCR amplification and using genomic DNA as a template, since there are the completely predicted data of all open reading frames (ORFs) that enables to generate the huge PCR products used as a probe (Kuipers et al., 2002). The use of a software program such as GenomePrimer or PrimeArray for design of primer pairs selected for all ORFs is doable (Kuipers et al., 2002). After amplicons are obtained from the first round of PCR, they are re-amplified with an aminated forward primer and non-aminated reverse primers, and single-aminated amplicons are then used as a probe (Kuipers et al., 2002). The probes are printed onto glass slides or nylon membranes as spots at defined locations (Schulze and Downward, 2001; Ehrenreich, 2006). Size of each spot is typically 100-300 μm and the distance apart among spots is the same. This kind of technique can generate the array containing more than 30,000 cDNAs on the slides (Schulze and Downward, 2001).

The probes for oligonucleotide microarray are short 20-25 single nucleotides, synthesized *in situ*, either by photolithography onto silicon wafers (high-density-oligonuclotide arrays from Affymetrix, http://www.affymetrix.com) or by ink-jet technology (licensed to Agilent Technologies) (Harrington et al., 2000; Schulze and Downward, 2001). The high-density oligonucleotide array typically contains 400,000 spots, while the printed array by ink-jet technology has about 10,000-30,000 spots on a single chip (Hughes and Shoemaker, 2001; Ehrenreich, 2006). Complete genome sequences alone is adequate to synthesize a large number of DNA to be arrayed, which offers the advantage such as no time-consuming handling of cDNA libraries and a probe design to represent the most unique part of a given transcript and to be able to specifically detect a closely related gene or splice variants (Lipshutz et al., 1999;

Schulze and Downward, 2001). Although the short probes (20-25 nucleotides) may result in less specific hybridization and reduced sensitivity (Schulze and Downward, 2001), nowadays the synthesized longer probes (50-100 nucleotides) are established to counteract these drawbacks (Schulze and Downward, 2001; Ehrenreich, 2006). In addition, the probes for oligonucleotide microarray from Affymetrix have been developed to contain 11-15 probe pairs, called a probe set, used to represent a single gene. The probe set is consisted of match probes and mismatch probes, which are a single different base in the middle of the probe, in order to allow cross-hybridization with mismatch probes and estimate local background to be subtracted from intensity of match probe, resulting in discrimination between real and fake signals from non-specific hybridization (Lipshutz et al., 1999; Harrington et al., 2000; Ehrenreich, 2006). When probes are successfully generated, they are required to be modified at either 5'or 3'end to covalently bind with slide surface coated with compounds providing aldehyde or epoxy functional group (Ehrenreich, 2006). Backbone of oligonucleotide probes is not fixed to the surface, resulting in the more availability of probe sequences able to be hybridized with the target (Ehrenreich, 2006).

The probes for double-stranded DNA microarrays is a 200- to 800-base pair length of amplified DNA resulted from PCR amplification (Ehrenreich, 2006). The amplicons are printed on slides with positively charged coating, which is normally poly-L-lysine or 3-aminopropyl-trimethoxysilane (APS) (Duggan et al., 1999; Ehrenreich, 2006). Probes are bound to the chip by electrostatic interaction with negatively charged phosphate backbone of nucleic acid, and subsequently the interaction treated with 80 ºC or UV allows to covalently form between thymine residues in the DNA and amino groups of the coating surface. This method is required an additional blocking step to prevent non-specific binding of the coating surface with

the target. The advantage of this array is their higher hybridization specificity and sensitivity (Ehrenreich, 2006).

2.6.2 Target preparation

In general, mRNA from given cells or tissues is extracted and converted to cDNA, which is labeled with fluorescent dyes and subsequently hybridized to the probes on the array chip. The hybridization is detected by phosphor-imaging or fluorescence scanning (Schulze and Downward, 2001). Quality of isolated RNA should be checked on agarose gel whether there is degradation and ratio of 16S to 23S rRNA should be approximately 1:2 (Kuipers et al., 2002). In labeling step, isolated mRNA from two different cells or tissues allows to be labeled with two different fluorescent dyes giving different colors. The labeled samples from control and test samples are mixed together and hybridized to the same array, resulting in competitive binding of the targets to the arrayed sequences. After hybridization and washing, the array chip is scanned with different wavelength, corresponding to the dye used, and the intensity of the same spot is compared and reported in the ratio of transcript levels for each gene, referred to up- and down-regulated genes (Brown and Botstein, 1999; Harrington et al., 2000; Schulze and Downward, 2001). Microarray allows to measure the same reference sample, compared to a large number of test samples in one experiment (Schulze and Downward, 2001).

Once total RNA from eukaryotic cells is purified, their mRNA is easily converted to cDNA with oligo(dT) primer, a short sequence of deoxy-thymine nucleotides, bound to the poly(A) tail at free 3'-end of the purified mRNA to extend a complementary single stranded-DNA. In contrast, prokaryotic mRNA lacks the poly(A) tail so that it is required to use random primers either hexamers or nonamers to be transcribed to cDNA. Therefore, prokaryotic total RNA is transcribed. Only 4%

of the total RNA from prokaryotic cells is mRNA and the rest is rRNA and tRNA. Hence, a higher amount of total RNA (approximately 20 to 25 µg) from prokaryotic cells is required to be used in a labeling reaction (Ehrenreich, 2006). In addition, prokaryotic mRNA is more difficult to handle, compared to eukaryotic one, because of its instability and rapid transcription (Ehrenreich, 2006). A half-life of bacterial mRNA is in range of 40 s to 20 min, because of high activity of bacterial RNase for rapid turnover in bacterial cells. Extreme care during RNA extraction is needed to circumvent its degradation. Additionally, apparently large amount proteins such as β-galactosidase from lacZ gene appear within 1 min after the initial signal for gene induction occurred. Hence, traditional protocol for cell disruption such as lysozyme treatment, French pressing, or sonication is inappropriate for DNA array experiment, because it takes a lot of time (Ehrenreich, 2006). Typically, cells harvested with very short time are resuspended with a high concentration of a strong protein denaturant, guanidinium isothocyanate, to inhibit any RNAase activity or buffer containing ethanol and phenol at an acidic pH to terminate any transcription immediately and prevent RNA degradation, and frozen then with liquid nitrogen. The frozen cells are ground in a cooled ball mill and total bacterial RNA is then prepared by phenol-chloroform extraction or commercial kits (Ehrenreich, 2006).

Cyanine dyes Cy-3 and Cy-5 are common fluorescent dyes used since they provide strong signal, good photostability, well-separated fluorescence spectra, and less adherence to chip surface (Duggan et al., 1999; Ehrenreich, 2006). There are two main protocols to incorporate the dyes into cDNA by reverse transcription (RT) that are (1) direct and (2) indirect labeling. In the direct labeling strategy, RT-PCR is incorporated with the derivative of nucleotide triphosphates such as Cy-3 deoxyuridine triphosphate (dUTP) or Cy-5 dexoycytosine triphosphate (dCTP). One cDNA is

labeled with Cy-3-dUTP and the other with Cy-5-dCTP. Ideally, roughly equal amount of Cy-3 and Cy-5 labeled with each cDNA is subjected to hybridization. Practically, they are, however, integrated in different yield because of a different molecular mass (Ehrenreich, 2006). Cy-5-dCTP has a low rate of incorporation, compared to Cy-3-dUTP. Thereby, analysis of the result is required to normalization to eliminate this artificial bias (Ehrenreich, 2006). To overcome the different yield of integration, the protocol of indirect labeling is established. Firstly, both mRNAs are reverse-transcribed to cDNA incorporated with an aminoallyl-modified dUTP or dCTP. Both modified nucleotides are better integrated into cDNA than Cy dyes, and result in equal amount. Secondly, *N*-hydroxysuccinylimidyl ester (NHS ester) derivatives of Cy-3 or Cy-5 are coupled to the aminoallyl-modified cDNA by chemical reaction. This protocol is less sensitive to the different molecular mass of dyes (Ehrenreich, 2006). In contrast to two-channel microarray (or two-color microarray), some platforms such as high-density-oligonuclotide arrays from Affymetrix are single-channel microarray (Hughes and Shoemaker, 2001). This array designed for estimating the absolute levels of gene expression is required only single dye. After hybridization, biotinylated cDNA bound to the array is stained with a fluorophore conjugated to avidin and detected by laser scanning (Harrington et al., 2000). The absolute transcript levels are estimated by comparing the intensity of individual genes to cDNA spikes, whose concentration is exactly known in the hybridization reaction (Harrington et al., 2000).

2.6.3 Hybridization and data acquisition

The targets, labeled cDNA with Cy-3 and Cy-5, from test and reference samples are mixed together with equal quantities of cDNA prior to hybridization to the array (Harrington et al., 2000; Ehrenreich, 2006). They are denatured at high

temperature and placed on a slide with carefully covering with a coverslip to avoid trapped air bubbles and prevent gradients in hybridization. The slide is then placed in the hybridization oven and incubated at the hybridization temperature for at least 16 h. The hybridization temperature ranging from 40-65 ºC depending on the organism studied (Ehrenreich, 2006). Following hybridization step, the slide is washed several times with the mixture between saline sodium citrate (SSC) and sodium dodecyl sulfate (SDS) to remove unspecific bound targets. This step is achieved with gradual decreasing the ionic strength and concentration of SDS until without SDS. The washed slide is finally dried by centrifugation. The slide is then scanned with microarray scanners. The scanner equipped with a laser beam able to excite the fluorescent dyes emitting with specific wavelengths. The data are stored in image file format (TIFF) for quantification by image analysis (Ehrenreich, 2006).

Image analysis is used to quantify the fluorescence of the *spots* or *features*. The pixels of the image are assigned either as the spot or the background. The spots are theoretically circular shape, however their shape might be irregular. Typically, all pixels inside the spot are defined as foreground, while all adjacent pixels surrounding the radius are defined as local backgrounds. The local background value should be subtracted from the foreground intensity or known as background correction. Then, an intensity-based filtering of the data should be done to eliminate artifacts and assess the quality of the signal. Importantly, the filtering step to assign the spots with smaller intensity than the background as a floor value allows the interpretation of genes, that are either switched on or off at certain condition, where is generally found in bacteria. Some operons in bacteria are required an inducer to turn them on. The filtered data are accepted the spots only expressing the intensity above the background and assign the rest (lower intensity than background) as the floor value. After the filtrating, the ratio

of means is calculated from the background-corrected intensities of the test and reference samples, and results in the actual raw data from DNA microarray experiment (Harrington et al., 2000; Ehrenreich, 2006).

2.6.4 Data analysis and validation

Before data analysis, the data should be transformed to logarithms in the base 2 because of confusion over the expression ratios of the untransformed data. For instance, a fourfold up-regulated gene is expressed ratio of 4, while the down-regulated gene is expressed ratio of 0.25. The transformed data are expressed as 2 and -2 for a fourfold up- and down-regulated gene, respectively (Ehrenreich, 2006). In addition, the data normalization is necessary to eliminate the systematic biases such as an amount of RNA used for labeling, an efficiency of dye incorporation, and a detection efficiency. The assumption of normalization is considered to equal the total sum of intensities in both channels (test and reference samples). The normalization factor is calculated from overall ratio within each channel, and the ratio of all spots is re-calculated and scaled accordingly (Ehrenreich, 2006).

Many errors originating from probe interchange, array production, labeling reaction, hybridization, and data acquisition, can be contained in microarray data (Ehrenreich, 2006). Thus, validation of data is required by using alternative methods such as northern hybridization, RNase protection or PCR with reverse transcription (RT-PCR), real-time RT-PCR (also called quantitative RT-PCR or qRT-PCR) (Schulze and Downward, 2001).

2.6.5 Design of microarray experiment

Microarray technique is normally used to compare transcriptional patterns of two cell populations (Ehrenreich, 2006). The major concern for reproducible and reliable microarray results is a huge variation in biological system. Bacteria are

extremely sensitive and rapidly changed in transcriptional levels when environment is altered. Many genes in bacterial cells are growth-rate-dependent, so that it should be aware to compare the gene expressions when the growth rate of the cultures is too different. To circumvent this problem, the growth rate of bacteria cells at conditions studied should be strictly controlled (Ehrenreich, 2006). In addition, technical replication is inevitable to achieve good data for microarray. There are three kinds of *technical replications* for microarray experiment. First, microarray can be done to spot the same probes multiple times on the single chip. This technique allows to calculate the "on chip variance" and to improve the data quality because of the calculation of average expression ratio on the same chip. Second, the preparation of the probes from single biological experiment with several labeling reactions is carried out. The variation from this case is calculated from the differences in the labeling reaction. Third, this technique is called "dye switch" or "dye swap". The RNA sample is labeled with Cy-3 and competitively hybridized with Cy-5 labeled reference sample, and vice versa. This is the most important in microarray replication because there are gene-specific dye effects and the fluorescent intensity depends on an amount of dyes bound to the probes (Ehrenreich, 2006).

2.6.6 Application of DNA microarray in bacteriological research

The microarray experiment can be assigned to compare the gene expression profiles in two samples such as wild-type bacteria with a mutant strain. To identify the target genes of the extracytoplasmic function (ECF) sigma factor of *B. subtilis*, including SigM, SigV, SigW, SigX, SigY, SigZ, and YlaC, strains carrying a plasmid pDG148 derivation with each sigma factor were constructed (Asai et al., 2003). Cells harboring those plasmids were grown in the presence and absence of isopropyl β-D-1-thiogalactopyranoside (IPTG) and their RNAs were subjected to DNA microarray

analysis. The genes exhibiting more than 3-fold changes under the addition of IPTG (compared to no IPTG) were considered (Asai et al., 2003). The result revealed that the each ECF sigma factor gene was transcribed by its own sigma factor, and *sigM*, *sigV*, *sigW*, *sigX*, *sigY*, *sigZ*, and *ylaC* upregulated 50, 98, 183, 10, 17, 3, and 6 genes, respectively (Asai et al., 2003). However, up-regulated genes of each ECF sigma factor, obtained from DNA microarray analysis, could not be concluded either to be direct or indirect target genes (Asai et al., 2003). DNA microarray technique was applied to the detection of the target genes of *B. subtilis* two-component regulator DegU, ComA, and PhoP (Ogura, Yamaguchi, Yoshida, Fujita, and Tanaka, 2001). The two-component regulatory system consists of a sensor kinase and its cognate response regulator, which usually functions as a transcriptional factor. The response regulator genes (*degU*, *comA*, and *phoP*) were cloned downstream of IPTG-inducible promoter (P*spac*) in plasmid pDG148 and the constructed plasmids were introduced into *B. subtilis* with disruption in the cognate sensor kinase genes (*degS*, *comP*, and *phoR*) (Ogura et al., 2001). As the mutant was deficient in the sensor kinase genes and overexpressed the response regulator genes in the presence of IPTG, DNA microarray can detect all target genes without signal transduction (Ogura et al., 2001). Target genes of other 24 *B. subtilis* two-component systems, including CitS-CitT, DesK-DesR, LytS-LytT, YbdK-YbdJ, YcbA-YcbB, YcbM-YcbL, YccG-YccH, YclK-YclJ, YdbF-YdbG, YdfH-YdfI, YesM-YesN, YfiJ-YfiK, YhcY-YhcZ, YkoH-YkoG, YrkQ-YrkP, YtsB-YtsA, YufL-YufM, YvcQ-YvcP, YvfT-YvfU, YvqB-YvqA, YvqE-YvqC, YvrG-YvrH, YxdK-YxdJ, and YxjM-YxjL, were identified following the means of Ogura et al.(2001) (Kobayashi et al, 2001). The role of CcpA in regulation of carbon metabolism and virulence in *Bacillus cereus* has been investigated using DNA microarrays (van der Voort, Kuipers, Buist, de Vos, and Abee, 2008). The gene

expression of *ccpA* deletion strain compared to that of the wild-type revealed that the CcpA regulated the catabolism of glucose with concomitant repression of gluconeogenesis and alternative metabolic pathways (van der Voort et al., 2008). In addition, the CcpA was involved in the expression of *nhe* and *nbl* operons, encoding non- and hemolytic enterotoxin, respectively (van der Voort et al., 2008).

DNA microarray was used to monitor all genes of *B. subtilis* transcribed at 10-minute intervals with 40 consecutive time points (Blom, Ridder, Lulko, Roerdink, and Kuipers, 2011). Expression profiles were distinguished into 5 groups including lag phase, exponential phase, transition point, early and late stationary phases (Blom et al., 2011). The result revealed that stress responses at specific times were occurred although no external stresses were applied. The transcript of SigB regulon was induced at the transition point, while that of SigW regulon was highly upregulated at the onset of the late stationary phase (Blom et al., 2011). In addition, the metabolic pathway at the transition point switched from glycolysis to gluconogensis and acetate production (Blom et al., 2011). DNA microarray has been also applied to study the response of bacteria under unflavoured conditions. The cellular response of *B. cereus* to be treated with disinfectants was demonstrated (Ceragioli et al., 2010). Genes involved in fatty acid metabolism were significantly induced when the cells exposed to benzalkonium chloride, which known to induce membrane damage (Ceragioli et al., 2010). An oxidation of sulfhydryl group is increased in the presence of sodium hypochloride. As expected, the induction of genes involved in metabolism of sulfur and sulfur-containing amino acid was observed, when *B. cereus* cells exposed to sodium hypochloride (Ceragioli et al., 2010). Exposure to hydrogen peroxide and peracetic acid induced genes involved in DNA damage repair and SOS responses (Ceragioli et al., 2010). Hyperthermophilic archaeon, *Pyrococcus furiosus*, exposed to gamma

irradiation, showed an induction of genes involved in DNA repair and oxidative stress (Williams, Lowe, Savas, and DiRuggiero, 2007). Transcriptional responses for mild and severe NaCl stresses (den Besten, Mols, Moezelaar, Zwietering, and Abee, 2009), organic and inorganic acids (Mols et al., 2010), low-temperature nitrogen gas plasma (Mols, Mastwijk, Groot, and Abee, 2013) in *B. cereus*, high pressure in *Lactobacillus sanfranciscensis* (Pavlovic, Hörmann, Vogel, and Ehrmann, 2005), aerobic and anaerobic condition in *Thermoplasma acidophilum* (Sun et al., 2010), and heat shock treatment in *Bifidobacterium longum* (Rezzonico et al., 2007) have been reported. One of the drawbacks of applying the DNA microarray to study the gene expression profiles in some bacteria possessing extensive post-transcriptional regulatory mechanisms would lead to incorrect conclusions on cellular mechanisms. Sun et al. (2010) found a low correlation between mRNA and protein level changes in *T. acidophilum*, which utilizes post-transcriptional mechanism to alter protein abundances. Furthermore, DNA microarray has been used to monitor changes in *Mycobacterium tuberculosis* gene expression in response to the antituberculoous drug isoniazid (INH). The result revealed that genes potentially involved in mycolic acid metabolism, of which role in this pathway has never been recognized (Wilson et al., 1999). These newly identified genes may be used to define new drug targets.

DNA microarray has been developed for simultaneous detection and discrimination between multiple bacterial species. Wang, Beggs, Erickson, and Cerniglia (2004) designed three 40-mer oligonucleotide specific for each bacteria, including seven species of *Bacteroides* and *Clostridium*, six species of *Ruminococcus*, five species of *Bifidobacterium*, four species of *Eubacetrium*, two species of *Fusobacterium*, *Lactobacillus*, and *Enterococcus*, and single species of *Collinsella*, *Eggerthella*, *Escherichia*, *Faecalibacterium*, and *Finegoldia*, based on the 16S rRNA

gene sequences. The method achieved to identify the species from fecal sample (Wang et al., 2004). Detection and identification of intestinal pathogenic bacteria has been accomplished using the DNA microarray. Based on the 16S rRNA gene sequences, oligonucleotide probes were designed for detection of *Escherichia coli*, *Shigella* sp., *Salmonella* sp., *Staphylococcus aureus*, Coagulase-negative *Staphylococcus*, *Clostridium botulinum*, *Clostridium perfringens*, *Camylobacter jejuni*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Proteus* sp., *Pseudomonas aeruginosa*, *Listeria monocytogens*, *Vibrio cholerae*, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Enterococcus faecalis*, *Aeromonas hydrophila*, and *B. cereus* (Jin et al., 2005). When the method was applied to identify 26 cultures, 25 strains were distinguished (Jin et al., 2005). Based on nucleotide sequences of 16S-23S ribosomal DNA internal transcribed space containing genes for $tRNA^{Ile}$, 42 oligonucleotide probes were designed for specific detection of *Bacillus anthracis* (Nübel et al., 2004).

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CHAPTER III

STATISTICAL OPTIMIZATION OF GROWTH AND PROTEINASE PRODUCTION OF *VIRGIBACILLUS* SP. SK 37

3.1 Abstract

The objectives of this study were to obtain the optimal condition providing high yields of biomass and NaCl-tolerant extracellular proteinases of *Virgibacillus* sp. SK37, and to investigate the effect of medium compositions (dried anchovy, yeast extract, $MgSO_4 \cdot 7H_2O$, glucose, NaCl, initial pH of the medium, and incubation temperature) on growth and proteinase production of this bacterium. The optimized medium attaining the high yield of both responses contained 1.5% dried anchovy, 0.5% yeast extract, and 2.5% NaCl, at pH 8. Under this condition, an increase of biomass of 1.6 LogCFU/ml and 1.4-fold increase in the proteinase production were accomplished as compared to the original medium (0.5% dried anchovy, 0.5% yeast extract, and 5% NaCl, at pH 7). The cubic regressions adequately described growth and proteinase production. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) proteinase activity staining towards synthetic substrate (Suc-Ala-Ala-Pro-Phe-AMC) revealed that secretion of extracellular proteinases varied with NaCl and initial pH of the medium. Peptide mass fingerprint (PMF) of the 10% NaCl cultured media showed higher abundance and intensity of lower mass peptides of 500-

1,000 m/z as compared to 0% NaCl, indicating higher proteolytic activity of high-salt medium. *Virgibacillus* sp. SK37 proteinases showed a remarkable preference towards Lys, Arg, and Tyr in the presence of NaCl, and towards Lys and Arg in the absence of NaCl.

3.2 Introduction

Bacterial growth and proteinase production are greatly influenced by medium composition, chemical, and physical factors. To achieve efficient fermentations, the optimization of those parameters is therefore needed. The optimization by the classical method carried out by changing one independent variable and keeping other variable constant is time-consuming, costly, and might not be accurate as interactions among factors are neglected. Statistical methods are powerful approache to investigate multiple factors simultaneously at once. In general, Plankett-Burman design (PBD) is applied to fairly reduce the large number of variables to manifest key factors affecting response (Myers and Montgomery, 2002). Subsequently, those key factors are optimized using response surface method (RSM). Advantage of RSM enables to predict the true optimization as well as simultaneously understand the interaction among the key factors with the small number of the experiments. This optimization method has been successfully applied on the production of cold-active proteinase from *Colwellia* sp. NJ341 (Wang, Hou, Xu, and Miao, 2008), alkaline proteinase from *Bacillus* sp. RXY3 (Reddy, Wee, Yun, and Rye, 2008), Nattokinase from *Bacillus subtilis* (Deepak et al., 2008), and organic solvent tolerant proteinase from *Geomicrobium* sp. EMB2 (Karan, Singh, Kapoor, and Khare, 2011), however the

optimization of bacterial growth and NaCl-tolerant proteinases from moderately halophilic bacteria has never been reported.

Moderately halophilic bacteria are able to grow well over a wide range of salt concentration (3 to 15% NaCl) (Ventosa, Neito, and Oren, 1998). They have diverse potential application including being a source of hydrolytic enzymes and compatible solutes, degrading toxic industrial residues, and forming biopolymer for oil recovery (Ventosa and Nieto 1995). The genus *Virgibacillus*, classified as a moderate halophile, was proposed by Heyndrickx et al. (1998) and there are 30 species validly named thus far. The numerous *Virgibacillus* were isolated from different samples such as saline environments (Yoon et al., 2010), soil (Kämpfer et al., 2011), and traditional salt fermentation (Tanasupawat, Chamroensaksri, Kudo, and Itoh, 2010). Recently, the proteinase-producing bacterium, *Virgibacillus* sp. SK37, isolated from fish sauce fermentation showed a great promise to be used as a starter culture (Yongsawatdigul, Rodtong, and Raksakulthai, 2007; Sinsuwan, Rodtong, and Yongsawatdigul, 2012). Interestingly, its cell-bound and extracellular proteinases exhibited high activities at high salt concentration (Sinsuwan, Rodtong, and Yongsawatdigul 2007; 2008). Thus, optimization to gain high yields of biomass and extracellular proteinases from *Virgibacillus* sp. SK37 would have a direct impact to technological development of fish sauce fermentation.

Fish sauce is a popular condiment consumed in Southeast Asia. It is produced by a natural fermentation by mixing uneviscerated anchovy and solar salt at a ratio of 3 to 1, yielding approximately 27-30% NaCl in the fermentation. Protein hydrolysis proceeds slowly due to extremely high salt content, resulting in at least 1 year for complete fermentation. Application of a starter strain possessing high activity of NaCl-tolerance proteinases would be a feasible means to accelerate fish sauce fermentation

(Sinsuwan et al., 2012). The success of the starter culture technology would rely mainly on the efficient biomass and proteinase production. The optimization of both critical criteria for production of *Virgibacillus* sp SK37 has never been investigated.

Peptides and free amino acids contribute to unique characteristic of fish sauce. The proteinases of *Virgibacillus* sp. SK37 showed different activities towards anchovy proteins at various NaCl concentrations (Sinsuwan et al., 2012), which presumably might lead to different peptide products. However peptide products under varied condition of culture conditions have not been systematically characterized. In this study, anchovy was used as a major nitrogen source for biomass and enzyme production. Hydrolyzed anchovy peptides would better resemble to those of fish sauce fermentation.

The aim of this research was to optimize the critical parameters affecting the bacterial growth and proteinase production of *Virgibacillus* sp. SK37 using rotatable central composite design (RCCD). In addition, the pattern of proteinase secretion and peptide fragments derived from *Virgibacillus* sp. SK37 was elucidated.

3.3 Materials and methods

3.3.1 Inoculum preparation and cultivation condition

Single colony of *Virgibacillus* sp. SK37 grown on tryptic soy agar (Merck KGaA, Darmstadt, Germany) containing 2.5% NaCl at 40 °C for 2 days was transferred into 30 ml of yeast extract broth (Ym) (1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% $MgSO_4 \cdot 7H_2O$) containing 2.5% NaCl (Sinsuwan et al., 2008) and incubated at 40 °C, at a shaking speed of 100 rpm, for 1 day. Cultured medium was diluted to a final optical density of 0.25 absorbance unit

(A.U.) at 600 nm (OD_{600}) with sterile 0.85% NaCl. Diluted samples were subsequently used as an inoculum.

Inoculum (2%) was transferred into the experimental media as described below. The inoculated samples having approximately 10^6 CFU/ml were incubated at a shaking speed of 150 rpm, for 2 days. After incubation, total viable cell count was measured using a drop plate technique (Hoben and Somasegaran, 1982) grown on tryptic soy agar containing 2.5% NaCl. The cell-free supernatant was collected by centrifugation at 15,000×g, 4 °C, for 15 min (Sorvall Legend Micro 21 Microcentrifuge, Thermo Fisher Scientific, Bremen, Germany) and the extracellular proteinase activity was determined as described below.

3.3.2 Plackett-Burman design (PBD)

The effect of independent factors, namely dried anchovy, yeast extract, $MgSO_4 \cdot 7H_2O$, glucose, NaCl, initial pH, and incubation temperature, on bacterial growth and NaCl-tolerant proteinase production were evaluated. Two levels, low (-1) and high (+1), of all factors were investigated. Their concentrations at -1 and +1 were set at 0.2, 2% dried anchovy; 0.1, 0.5% yeast extract; 0.1, 1% $MgSO_4 \cdot 7H_2O$; 0.1, 1% glucose; 0.1, 1% NaCl; initial pH 7, 9; and incubation temperature at 30, 40 °C. Eleven independent variables including four dummy variables were screened in twelve experiments according to the experimental design of the Plackett and Burman (Plackett and Burman, 1946). A linear regression analysis of the data was carried out by the following first-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i$$

in which Y is the predicted response (bacterial growth, LogCFU/ml or proteinase production, mU/ml), β_0 is the intercept, β_i is the linear constant coefficient, X_i is the coded independent variables, and k is the number of involved variables. The statistical package (SPSS for Windows, version 17.0; Chicago, IL, USA) was used to analyze the experimental design. Dried anchovy powder was prepared by drying anchovy in an air oven set at 70 °C for 35 h, grinding with an IKA M20 universal laboratory mill (IKA-Werke GmbH & Co, Staufen, Germany), and sieving through 140 mesh (Fritsch vibratory sieve shaker (Fritsch GmbH, Idar-Oberstein, Germany).

3.3.3 Response surface methodology (RSM)

Four factors, namely dried anchovy, yeast extract, NaCl, and initial pH, were fed to a rotatable central composite design (RCCD) for optimization. The factors were analyzed at five levels (-2, -1, 0, +1, +2) and 32 experiments with 8 central points were set. The experimental media were incubated at 40 °C, growth and proteinase production were measured as a response. The empirical model of the third-order polynomial equation is:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j}^{3} \sum_{j=1}^{4} \beta_{ij} X_i X_j + \sum_{i=1}^{4} \beta_{iii} X_i^3 + \sum_{i<j}^{3} \sum_{j=1}^{4} \beta_{iij} X_i^2 X_j + \sum_{i<j}^{2} \sum_{j<k}^{3} \sum_{k=1}^{4} \beta_{ijk} X_i X_j X_k$$

where Y is the predicted response (bacterial growth, LogCFU/ml or proteinase production, mU/ml), β_0 is the intercept, β_i is the linear constant coefficient, β_{ii} is the coefficient of the quadratic single term, β_{ij} is the coefficient of the quadratic cross product term, β_{iii} is coefficient of the cubic single term, β_{iij} is coefficient of the cubic two cross product terms, β_{ijk} is the coefficient of the cubic three cross product terms,

and X_i, X_j and X_k are the coded independent variables. The experiment was performed in three replicates. Response data were analyzed by Design expert (Design Expert version 8.0.5, stat-Ease Corporation, USA). To check the validity of the models, experiments selected within the design space were conducted.

3.3.4 Proteinase assay

Proteinase activity was assayed following the method of Sinsuwan, Rodtong, and Yongsawatdigul (2010) with some modifications. The reaction mixture (1 ml) contained 50 µl crude extracellular proteinases, 1 µM succinyl (Suc)-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (AMC), 200 mM Tris-HCl (pH 8.0), 30 mM $CaCl_2$ and incubated at 65 ºC for 5 min. The activities of proteinases were terminated by adding 1.5 ml of the mixture containing 30% butanol, 35% methanol, and 35% deionized water (v/v). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan). Unit activity was expressed as a release of AMC in µmole per min.

3.3.5 Zymogram

The effect of medium composition on extracellular proteinase secretion was evaluated using fluorogenic peptide zymogram (Laemmli, 1970; Yasothornsrikul and Hook, 2000). Two ml of the inoculum, prepared as described above, was transferred into 18 ml of various media, and incubated at 40 °C, shaking speed of 150 rpm, for 2 days. Media used were: 1) dried anchovy (0.5, 1.25, and 2%), 5% NaCl, pH 8; 2) yeast extract (0.5, 1.25, and 2%), 5% NaCl, pH 8; 3) NaCl (0, 5, and 10%), 0.5% dried anchovy, 0.5% yeast extract, pH 8; 4) initial pH (6, 7.5, and 9), 0.5% dried anchovy, 0.5% yeast extract, 5% NaCl. The cell-free supernatant was collected by centrifugation at 15,000×g, 4 °C, for 15 min (Sorvall Legend Micro 21 Microcentrifuge, Thermo

Fisher Scientific, Bremen, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 4 and 12.5% acrylamide stacking and separating gels, respectively. The separating gel was prepared by copolymerization with 500 µM Suc-Ala-Ala-Pro-Phe-7-AMC in dark at 4 ºC for 1 h. Crude enzymes were mixed with a treatment buffer (4% SDS, 10% 2-mercaptoethanol (β-ME), 20% glycerol, 125 mM Tris-HCl (pH 6.8), 0.1% bromophenol blue) at a ratio of 1 to 1. Ten µl of the mixtures was loaded into the gel. Electrophoretic separation was carried out in dark at 4 ºC and constant current at 10 mA. Subsequently, the gel was washed with cold 2.5% Triton X-100 at 4 ºC for 5 min and washed twice in cold deionized water. Proteinases was activated by incubating the gel with 200 mM Tris-HCl (pH 8.0), 30 mM $CaCl_2$ at 65 ºC for 5 min. Fluorescent bands indicating the existence of proteinases were detected immediately using a Gel Doc™ XR system (Bio-Rad Laboratories, Hercules, CA, USA). Pre-stained SDS-PAGE standard including myosin (209 kDa), β-galactosidase(124 kDa), serum albumin (80 kDa), ovalbumin (49.1 kDa), carbonic anhydrase (34.8 kDa), trypsin inhibitor (28.9 kDa), and lysozyme (20.6 kDa) (Bio-Rad Laboratories, Hercules, CA, USA) was used for molecular mass estimation.

3.3.6 Mass spectrometry

The cultured media containing 0.5% dried anchovy, 0.5% yeast extract, pH 8, at either 0 or 10% NaCl were selected for mass spectrometry studies. The cell-free supernatant was collected by centrifugation at 15,000×g, 4 °C, for 15 min. A fraction containing peptides smaller than 10 kDa was collected by Nanosep® centrifugal devices with Omega™ membrane, 10K molecular weight cut-offs (MWCO) (Pall Corporation, Ann Arbor, MI, USA) and then subjected desalting using the desalting spin column (Thermo Fisher Scientific Inc., Waltham, MA, USA). Desalted samples

were dried by vacuum centrifugation and dissolved in a mixture of 0.1% acetronitrile (ACN) and 0.1% trifluoroacetic acid (TFA) (1:2). The peptide samples were mixed with the matrix mixture containing 2,5-dihydroxybenzoic acid (DHB), and applied onto a sample holder. The peptide mass fingerprint (PMF) of samples was determined by MALDI-TOF (MS model reflex V, Bruker Daltonik GmbH, Bremen, Germany) equipped with a 2 GHz LeCroy digitizer and 337 nm nitrogen laser. The MALDI-TOF spectrum was obtained by the positive ion mode at acceleration voltage of 20 kV and 400 ns extraction delay.

The *de novo* peptide sequencing was carried out using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with the Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Co., Waltham, MA, USA). Peptides were separated using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Coventry, UK) coupled to an UltiMate 3000 LC System (Dionex Ltd., Surrey, UK) equipped with a nanocolumn (PepSwift monolithic column 100 µm i.d. × 50 mm). Eluent A was 0.1% formic acid and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was performed with a linear gradient from 10% to 70% B for 13 min at a flow rate of 300 nl/min, including a regeneration step at 90% B and an equilibration step at 10% B. The *de novo* peptide sequencing was achieved using program Pepnovo without the assistance of a sequence database (Frank and Pevzner, 2005).

3.4 Results and discussion

3.4.1 Plackett-Burman design (PBD)

Growth of *Virgibacillus* sp. SK37 increased with dried anchovy, yeast extract, and glucose, while reduced with the increased incubation temperature (P<0.05, Table 3.1). As expected, the content of carbon and nitrogen sources greatly influenced growth of *Virgibacillus* sp. SK37. Yeast extract was also found to favor the growth of *Bacillus amyloliquefaciens* (Alam et al., 1989). Yeast extract is a rich source of free amino acids, vitamins, minerals, and other growth factors essential for bacterial growth. Although dried anchovy is not a rapidly metabolized nitrogen source, *Virgibacillus* sp. SK37 possesses a promising proteolytic system to breakdown and absorb fish proteins (Sinsuwan et al., 2007; 2012).

Table 3.1 Linear regression analysis for Plackett-Burman experiment.

Variables	Bacterial growth[1]			Proteinase production[2]		
	Regression coefficient value	*t*-value	*P*-value[3]	Regression coefficient value	*t*-value	*P*-value[3]
Dried anchovy	0.286	5.099	0.000	0.021	0.609	0.551
Yeast extract	0.154	2.747	0.014	0.259	7.496	0.000
$MgSO_4 \cdot 7H_2O$	0.048	0.852	0.407	0.143	4.145	0.001
Glucose	0.175	3.118	0.007	-0.290	-8.389	0.000
NaCl	-0.003	-0.052	0.959	-0.059	-1.702	0.108
Initial pH	0.015	0.273	0.788	0.140	4.051	0.001
Incubation temperature	-0.197	-3.516	0.003	0.020	0.575	0.573

[1] R^2 = 0.883; [2] R^2 = 0.954; [3] Significant differences at P <0.05

Factors affecting proteinase production were different from those affecting growth. Yeast extract, $MgSO_4 \cdot 7H_2O$, and initial pH promoted proteinase production, but glucose showed suppressive effect (Table 3.1). Yeast extract seemed to be critical component for both growth and proteinase production (Table 3.1). Glucose appeared to limit proteinase production of *Virgibacillus* sp. SK37 due to carbon catabolite repression. Carbon catabolite repression in Gram-positive bacteria involves the metabolite-activated HPr(ser)-kinase, the phosphocarrier protein HPr, and a transcription factor CcpA (Saier et al., 1995). These key proteins of the repression are also found in the whole genome sequences of the strain (Phrommao, 2010). When the levels of glycolytic intermediates fructose-1,6-bisphosphate are elevated, the HPr(ser)-kinase is activated and phosphorylates the HPr protein (HPr~P). Subsequently, the HPr~P forms a complex protein with CcpA protein, acting as a repressor of many protein synthesis (Saier et al., 1995). This regulation is, therefore, most likely to repress the synthesis of *Virgibacillus* sp. SK37 proteinases. An increase in $MgSO_4 \cdot 7H_2O$ in the medium positively increased proteinase production (Table 3.1). The secretion of α-amylase from *Bacillus subtilis* decreased dramatically when cells grew in the medium containing EDTA, despite of no differences in the growth (Leloup, Haddaoui, Chambert, and Petit-Glatron, 1997). Mg^{2+} can neutralize the negative charges on cell wall of Gram-positive bacteria (Beveridge and Murray, 1980). Major secreted proteinases from *Virgibacillus* sp. SK37 showed acidic pI, implying the presence of numerous negative charges in their structures (Phrommao, Rodtong, and Yongsawatdigul, 2010). The repulsive forces between negative charges of secretory proteinases and cell wall might be minimized during protein translocation by the presence of Mg^{2+}, resulting in an improvement of the proteinase secretion.

NaCl was considered to be insignificant factor for growth and proteinase production (Table 3.1). However, halophile characteristics of growth and proteinase production of this strain have been reported (Lapsongphon, Rodtong, and Yongsawatdigul, 2013). Therefore, it was selected for optimization. The PBD assumes that a linear relationship between factors and response exists, and all interactions of main effects are negligible. The insignificant result might be obtained. For this reason, optimization by RSM is normally required. Therefore, NaCl was chosen along with dried anchovy, yeast extract, and initial pH, for further optimization by RSM.

3.4.2 Optimization of culture conditions and validation of models

The experimental design was present in Table 3.2 and 3.3. The ANOVA suggested that the cubic regression models for both responses were satisfactory (Table 3.4). High *F-value* generally indicates a good fit of a model (Table 3.4). *F-values* and the significant *P-values* implied that the obtained experimental data fit well with the models. The R^2 indicated that the cubic regression models could explain 85.7% and 90.1% of total variations for growth and the proteinase production ability, respectively. The value of the adjusted regression coefficient (Adj R^2) was correlated to R^2 advocating a high significance of the models. Low value of coefficient of variation (CV) indicated that deviations between experimental and predicted values were low. Lack of fit was significant for growth model, but insignificance for the model of proteinase production (Table 3.4). Many appropriate models for bacterial enzyme production which expressed accepted R^2, despite of the significance of lack of fit, were reported (Wang et al., 2008; Deepak et al., 2008; Badoei-Dalfard and Karami, 2013). The result implied that the model may not accurately predict the growth of *Virgibacillus* sp. SK37 in some regions of the response surface. The adequate precision value is an index of the signal-to-noise ratio and the prerequisites for good-

fitting model are considered as the value of higher than 4. The adequate precision values of both models were higher than 4, suggesting that both models were satisfactorily used to navigate the design space. The regression equation coefficients were determined as shown in equation 1 and 2 for the bacterial growth and the proteinase production, respectively.

Table 3.2 Experimental levels of the independent variables studied using a rotatable central composite design.

Independent variables	Symbols	Code levels				
		-2	**-1**	**0**	**+1**	**+2**
Dried anchovy (%)	A	0	0.5	1	1.5	2
Yeast extract (%)	B	0	0.5	1	1.5	2
NaCl (%)	C	0	2.5	5	7.5	10
Initial pH	D	6	7	8	9	10

Table 3.3 Experimental design and results of a rotatable central composite design.

Standard run	Coded value				Experimental value	
	Dried anchovy (%)	**Yeast extract (%)**	**NaCl (%)**	**Initial pH**	**Bacterial growth (LogCFU/ml)**	**Proteinase production (mU/ml)**
1	-1	-1	-1	-1	7.83	0.338
2	1	-1	-1	-1	8.69	0.259
3	-1	1	-1	-1	7.35	0.457
4	1	1	-1	-1	8.22	0.234
5	-1	-1	1	-1	8.36	0.039
6	1	-1	1	-1	8.63	0.164
7	-1	1	1	-1	9.15	0.029
8	1	1	1	-1	9.47	0.053
9	-1	-1	-1	1	8.36	0.212

Table 3.3 Experimental design and results of a rotatable central composite design (Continued).

Standard run	Coded value				Experimental value	
	Dried anchovy (%)	Yeast extract (%)	NaCl (%)	Initial pH	Bacterial growth (LogCFU/ml)	Proteinase production (mU/ml)
10	1	-1	-1	1	9.11	0.360
11	-1	1	-1	1	9.92	0.060
12	1	1	-1	1	9.44	0.010
13	-1	-1	1	1	7.66	0.030
14	1	-1	1	1	9.72	0.330
15	-1	1	1	1	8.90	0.021
16	1	1	1	1	8.11	0.005
17	-2	0	0	0	7.95	0.508
18	2	0	0	0	8.60	0.042
19	0	-2	0	0	8.43	0.051
20	0	2	0	0	8.81	0.261
21	0	0	-2	0	7.77	0.037
22	0	0	2	0	8.39	0.030
23	0	0	0	-2	9.15	0.201
24	0	0	0	2	0.00	0.018
25	0	0	0	0	8.50	0.420
26	0	0	0	0	8.14	0.462
27	0	0	0	0	8.03	0.376
28	0	0	0	0	8.64	0.417
29	0	0	0	0	7.77	0.252
30	0	0	0	0	8.57	0.336
31	0	0	0	0	8.23	0.517
32	0	0	0	0	8.09	0.244

Table 3.4 Analysis of variance (ANOVA) of the cubic regression models for the experiments.

	Bacterial growth	**Proteinase production**
F-value	11.13	17
P-value	<0.0001	<0.0001
R^2	0.8566	0.9012
Adjusted R^2	0.7797	0.8482
Coefficient of variance (CV)	9.30%	31.75%
P-value of lack of fit	<0.0001	0.4630
Adequate precision	19.89	13.08

LogCFU/ml = 8.246631 + 0.163399A + 0.096053B + 0.153604C - 2.28651D - 0.25154AB - 0.00858AC- 0.04914AD + 0.019236BC + 0.052795BD - 0.37111CD + 0.223632A^2 + 0.308831B^2 + 0.174391C^2 - 0.7026D^2 - 0.09798ABC - 0.25835ABD + 0.132404ACD - 0.30249BCD + 0.041614 A^2B -0.086A^2C + 2.506881A^2D + 0.078097 AB^2 *(equation 1)*

Proteinase production (mU/ml) = 0.378085 - 0.11648A + 0.05254B - 0.0019C - 0.04562D - 0.05324AB + 0.045765AC + 0.039388AD - 0.00893BC - 0.05642BD + 0.052548CD - 0.02155A^2 - 0.05134B^2 - 0.08202C^2 - 0.06294D^2 - 0.01056ABC - 0.02264ABD - 0.01073ACD + 0.017915BCD - 0.11246A^2B - 0.07092A^2C + 0.017406A^2D + 0.136538AB^2 *(equation 2)*

with LogCFU/ml and proteinase production (mU/ml), the predicted responses; A, dried anchovy B, Yeast extract; C, NaCl; and D, initial pH.

In order to confirm the validity of the cubic regression equations, some conditions within the design space were tested. The predicted values were comparable with those of the experimental ones (Table 3.5), suggesting the validity of these models. These results reflected that RSM is able to be applied to optimize the growth

Table 3.5 Validation of the cubic model for bacterial growth and proteinase production within the design space.

Validated levels				Bacterial growth (LogCFU/ml)		Proteinase production (mU/ml)	
Dried anchovy (%)	**Yeast extract (%)**	**NaCl (%)**	**Initial pH**	**Experimental**	**Predicted[1]**	**Experimental**	**Predicted[2]**
0.9	1	5	7.9	8.83	8.43	n.d.	n.d.
1	0.25	0.125	8	8.81	8.76	n.d.	n.d.
1	0.25	8.75	8	8.78	9.33	0.029	0.016
1	1.75	0.125	8	9.15	9.11	0.052	0.059
1	1.75	8.75	8	8.76	9.21	0.140	0.139
1	0.9	4.9	8	10.25	9.01	0.358	0.365
1.75	1	5	6.5	n.d.	n.d.	0.051	0.038
0.25	1	5	6.5	n.d.	n.d.	0.089	0.142

[1] Prediction value was calculated according to *equation (1)*

[2] Prediction value was calculated according to *equation (2)*

n.d., not determined

and proteinase production of halophilic bacteria with satisfactory reliability. The significances of each coefficient were determined as shown in Table 3.6. Initial pH exhibiting a very low *P-value* (<0.001) of linear (D) and quadratic effects (D^2) indicated the profound effect on the bacterial growth (Table 3.6). In addition, the interaction of the initial pH (D) with other factors including dried anchovy (A^2D), NaCl (CD), and yeast extract/NaCl (BCD) was significant (Table 3.6). These results revealed that initial pH appeared to be the most important factor on the growth of *Virgibacillus* sp. SK37. The results were in agreement with Lapsongphon et al. (2013), indicating that pH exhibited a strong effect on growth of *Virgibacillus* sp. SK37. Figure 3.1 represented the three dimensional (3D) response surface of the bacterial growth as a function of NaCl and initial pH and holding other two variables at zero level. It showed that optimum initial pH for growth of *Virgibacillus* sp. SK37 was pH 7-8 and the growth of *Virgibacillus* sp. SK37 was gradually declined at initial pH >8 (Figure 3.1). The doubling times of *B. subtilis*, at pH 6 and 8.5 were 18 and 42 min, respectively (Wilks et al., 2009). *B. subtilis* could maintain its cytoplasmic pH when pH of medium was in a range of 5.5 to 7.5 (Martinez et al., 2012). There was a report on different gene responses of *B. subtilis* to acidic pH medium, which upregulated dehydrogenases and decarboxylases to possibly consume acids and generate basic amines, while alkaline pH medium upregulated acid-generated catabolism (Wilks et al., 2009).

Table 3.6 Results of regression analysis of the cubic model for optimizing of growth and proteinase production of *Virgibacillus* sp. SK37.

Factor	Coefficient estimate	*F-value*	*P value*
Bacterial growth			
A (Dried anchovy)	0.163	0.73	0.3992
B (Yeast extract)	0.096	0.25	0.6192
C (NaCl)	0.154	0.64	0.4278
D (initial pH)	-2.287	142.12	<0.0001
AB	-0.252	3.44	0.0708
AC	-0.009	0.00	0.9499
AD	-0.049	0.13	0.7190
BC	0.019	0.02	0.8879
BD	0.053	0.15	0.6991
CD	-0.371	7.49	0.0091
A^2	0.224	5.02	0.0305
B^2	0.309	9.57	0.0035
C^2	0.174	3.05	0.0881
D^2	-0.703	49.55	<0.0001
ABC	-0.098	0.52	0.4741
ABD	-0.258	3.63	0.0638
ACD	0.132	0.95	0.3347
BCD	-0.302	4.97	0.0313
A^2B	0.042	0.03	0.8603
A^2C	-0.086	0.13	0.7162
A^2D	2.507	113.89	<0.0001
AB^2	0.078	0.11	0.7412
Proteinase production			
A (Dried anchovy)	-0.116	48.04	<0.0001
B (Yeast extract)	0.053	9.77	0.0032
C (NaCl)	-0.002	0.01	0.9107
D (initial pH)	-0.046	7.37	0.0097

Table 3.6 Results of regression analysis of the cubic model for optimizing of growth and proteinase production of *Virgibacillus* sp. SK37 (Continued).

Factor	Coefficient estimate	*F-value*	*P value*
Proteinase production			
AB	-0.047	15.87	0.0003
AC	0.040	11.26	0.0017
AD	0.033	7.95	0.0074
BC	-0.003	0.07	0.7997
BD	-0.051	18.08	0.0001
CD	0.047	15.42	0.0003
A^2	-0.023	6.64	0.0137
B^2	-0.052	35.79	<0.0001
C^2	-0.083	90.07	<0.0001
D^2	-0.064	53.42	<0.0001
ABC	-0.005	0.15	0.6962
ABD	-0.017	1.99	0.1661
ACD	-0.017	1.96	0.1693
BCD	0.024	4.01	0.0518
A^2B	-0.107	26.81	<0.0001
A^2C	-0.077	13.93	0.0006
A^2D	0.012	0.31	0.5789
AB^2	0.131	40.29	<0.0001

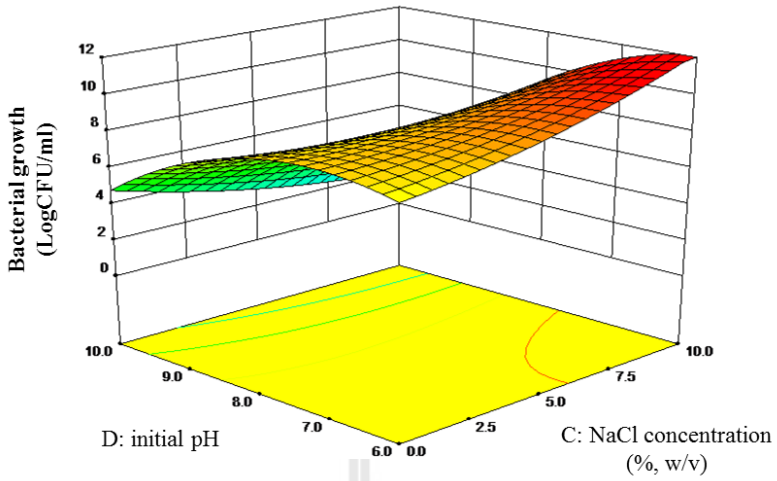

Figure 3.1 The three-dimension (3D) surface plot of the growth of Virgibacillus sp. SK37 as function of NaCl and initial pH, when the other factors were held at zero level.

The quadratic effects of dried anchovy (A^2), yeast extract (B^2), NaCl (C^2), and initial pH (D^2) were significant on proteinase production (Table 3.6). The interactions, AB, AC, AD, BD, CD, A^2B, A^2C, and AB^2 were significant. With an increase in initial pH value, the proteinase production gradually increased to maximum at pH 7.5-8 (Figure 3.2A). This result was similar to that of Lapsongphon et al. (2013). The maximum proteinase yield was predicted at 2.5-5% NaCl (Figure 3.2A). In addition, the higher amount of yeast extract favored an increase in the proteinase production of *Virgibacillus* sp. SK37 (Figure 3.2B). Based on the models and the response surface plots, the optimal levels to attain both high bacterial growth and proteinase production were: 1.5% dried anchovy, 0.5% yeast extract, and 2.5% NaCl, at pH 8. Under this optimized condition, the predicted responses for the growth and proteinase production were 9.2 LogCFU/ml and 0.36 mU/ml, respectively. These conditions were shown to

increase biomass about 1.6 Log and 1.4-fold increase in the proteinase production, as compared to the original medium (0.5% dried anchovy, 0.5% yeast extract, and 5% NaCl, at pH 7). It should be noted that a total nitrogen and an α-amino group content originated from the dried anchovy in the optimized medium was approximately 0.12% and 21.6 mM leucine equivalent, respectively, while those in the original medium were ~3 times less. Optimized medium accomplished by means of RSM was reported to increase the proteinase production in *Bacillus* of 1.5-12.9 times, compared to unoptimized medium (Chauhan and Gupta, 2004; Saran, Isar, and Saxena, 2007).

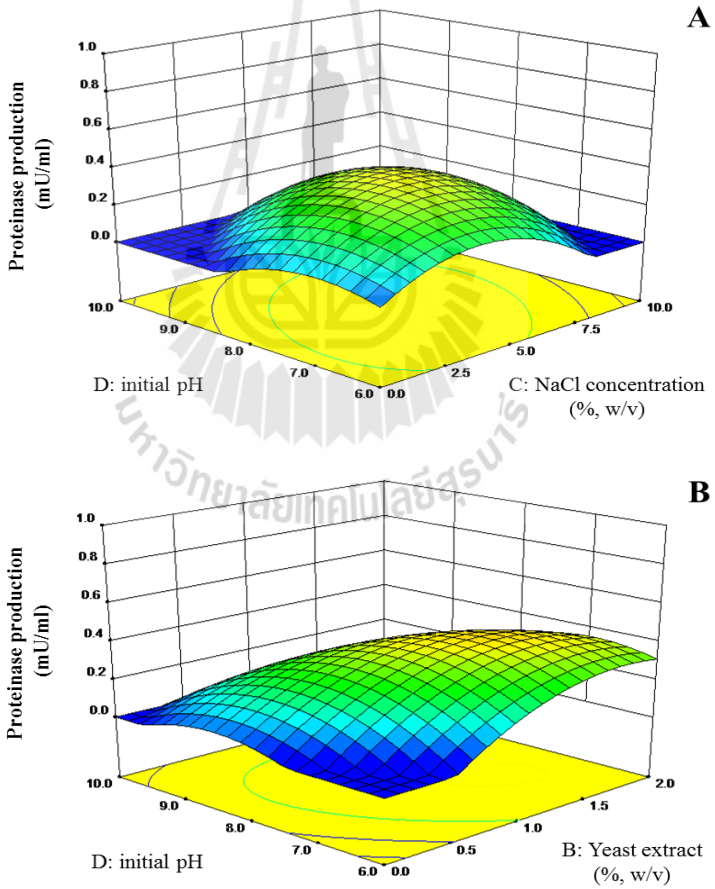

Figure 3.2 The 3D surface plot of the proteinase production of SK37 (A) as function of NaCl and initial pH, and (B) yeast extract and initial pH, when the other factors were held at zero level.

3.4.3 Zymogram

Dominant proteinases showed molecular weights (MW) of 19, 34, 35, and 44 kDa based on zymogram, which were essentially secreted at all studied concentrations of dried anchovy (Figure 3.3A). Larger MWs were detected as minor proteinases (Figure 3.3A). This was in agreement to that of Phrommao et al. (2010) who reported that major proteinases of *Virgibacillus* sp. SK37 was 19, 34, and 44 kDa. Activity based on band intensity appeared to increase with dried anchovy level in the medium and corresponded to growth of *Virgibacillus* sp. SK37 (Figure 3.3A). Similar results when yeast extract was increased were also observed (data not shown). Hence, the production of proteinases from *Virgibacillus* sp. SK37 in medium containing dried anchovy or yeast extract proportionally varied with biomass.

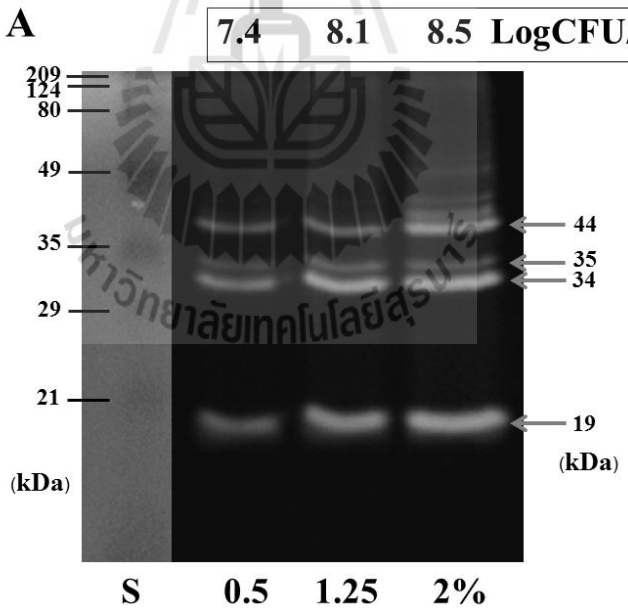

Figure 3.3 Zymogram (12.5%T) of Virgibacillus sp. SK37 using the synthetic substrate, Suc-Ala-Ala-Pro-Phe-AMC as a substrate. The strain cultivated in various media varying concentration of dried anchovy (A), NaCl (B), and initial pH value (C). S, Molecular weight standard; Total viable cell count was measured by a drop plate technique and expressed as LogCFU/ml.

NaCl strongly affected the activity pattern (Figure 3.3B). In the absence of NaCl, proteinases with MW of 22 and 42 kDa were detected, while proteinases with MW of 19, 34, 35, and 44 kDa were predominant in the presence of NaCl. This result indicated that the secretion of extracellular proteinases from *Virgibillus* sp. SK37 was markedly NaCl-dependent. The DegS-DegU two-component system, controlling the expression of degradative enzymes, involved in the NaCl response of *B. subtilis* (Kunst and Rapoport, 1995). Two major component genes, *degS* (encoding membrane-associated histidine kinase) and *degU* (encoding cytoplasmic response regulator), were also found in whole genome of *Virgibacillus* sp. SK37 (Phrommao 2010). Phosphorylated DegU (DegU~P) recruited RNA polymerase at promoter region of genes to stimulate or inhibit a transcriptional process (Murray et al., 2009). The expression of alkaline proteinase, *aprE*, was repressed, but stimulated for levansucrase, *sacB*, at high NaCl concentration by the DegU~P (Kunst and Rapoport, 1995). It might be postulated that DegU~P might regulate the level of expression of *Virgibacillus* sp. SK37 proteinase genes at high-NaCl medium.

The highest activity was observed at initial pH 7.5 (Figure 3.3C). The same activity pattern at initial pH 7.5 and 9 (Figure 3.3C) indicated no specific inducers on the proteinase secretion. However, activity bands at initial pH 6 were not detected, despite apparent bacterial growth of 6.1 LogCFU/ml (Figure 3.3C). The expression of glutamate dehydrogenase (GDH) attributed to an efficiency of secretory enzyme production in *B. subtilis* (Manabe et al. 2012). The production of α-amylase from *B. subtilis* was greatly decreased at acidic pH of growth medium because of the lower expression of GDH (Manabe et al. 2012). It is speculated that GDH of *Virgibacillus* sp. SK37 might be down-regulated at pH 6, inhibiting transcriptional process of

proteinase genes. This finding clearly demonstrated that the medium composition not only affected yield of proteinase production, but also a type of the secreted proteinase.

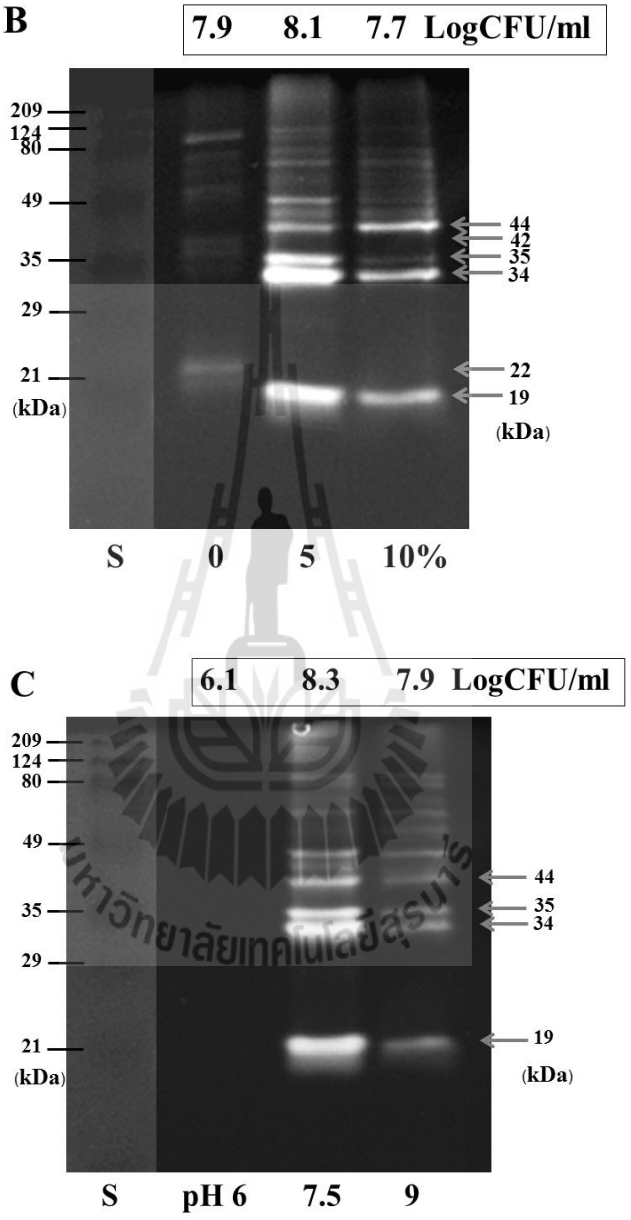

Figure 3.3 Zymogram (12.5%T) of Virgibacillus sp. SK37 using the synthetic substrate, Suc-Ala-Ala-Pro-Phe-AMC as a substrate. The strain cultivated in various media varying concentration of dried anchovy (A), NaCl (B), and initial pH value (C). S, Molecular weight standard; Total viable cell count was measured by a drop plate technique and expressed as LogCFU/ml (Continued).

3.4.4 Mass spectrometry

Peptide mass fingerprint (PMF) of cultured media at 0 and 10% NaCl was evaluated (Figure 3.4). The uninoculated samples showed different PMF patterns at varied NaCl of 0 and 10% (Figure 3.4A, C). This could be due to different protein extractability at varied NaCl contents. The inoculated sample at 10% NaCl apparently exhibited higher mass intensities and the greater number of mass-to-charge ratio (m/z) (Figure 3.4D), indicating that the larger extent of proteolysis occurred at 10% NaCl with high abundance and intensity of mass around 500-1,000 m/z, whereas lower those of mass ranging 600-700 m/z in the medium without NaCl (Figure 3.4C, D). Peptides generated from hydrolysis varied with NaCl content, suggesting differences in active proteinases (Table 3.7). This was in concomitant with zymogram, showing different dominant proteinases at varied salt content (Figure 3.3B). The proteinases in medium without NaCl preferably hydrolyzed Lys and Arg, while those in high-salt medium significantly hydrolyzed Lys, Arg, and Tyr (Table 3.7). The detection of trypsin cleavage sites in the medium without NaCl might assume that either the apparent MW of 22 and 42 kDa, as detected by the specific substrate for subtilisin (Figure 3.3B), possessed a broad substrate specificity or another trypsin-like proteinase was produced. These results demonstrated that NaCl in the cultured media affected yield and characteristics of resulting peptides. It might consequently affect the quality of the fermented product.

Table 3.7 *De novo* sequencing of peptides in the cultured media in absence and presence of 10% NaCl, generated by the halophile, *Virgibacillus* sp. SK37.

0% NaCl [1]	**10% NaCl** [1]
ALVPK	GAVAFSK*
EEGEFLR*	GSLVLAH
ELGSQFLQQK*	HALLAR
ELLFR	LLPKYDR*
ELTETRR*	QDLLAH
ELVEEER*	SGVVGPY*
ELVHAKP	SHLFR
ENQGYGR	YLLGQDLLLLTK*
FNEKAR	
GFLQAER*	
HAVNLCR*	
LDQAWHR*	
LEGNEQFLNAAK*	
LEQAHVPK	
MLNYR	
SFAK	
SHVEEER	

[1] De novo peptide sequences were not observed in the non-inoculated samples.

[*] Abundant peptide fragments based on an intensity >900 a.u.

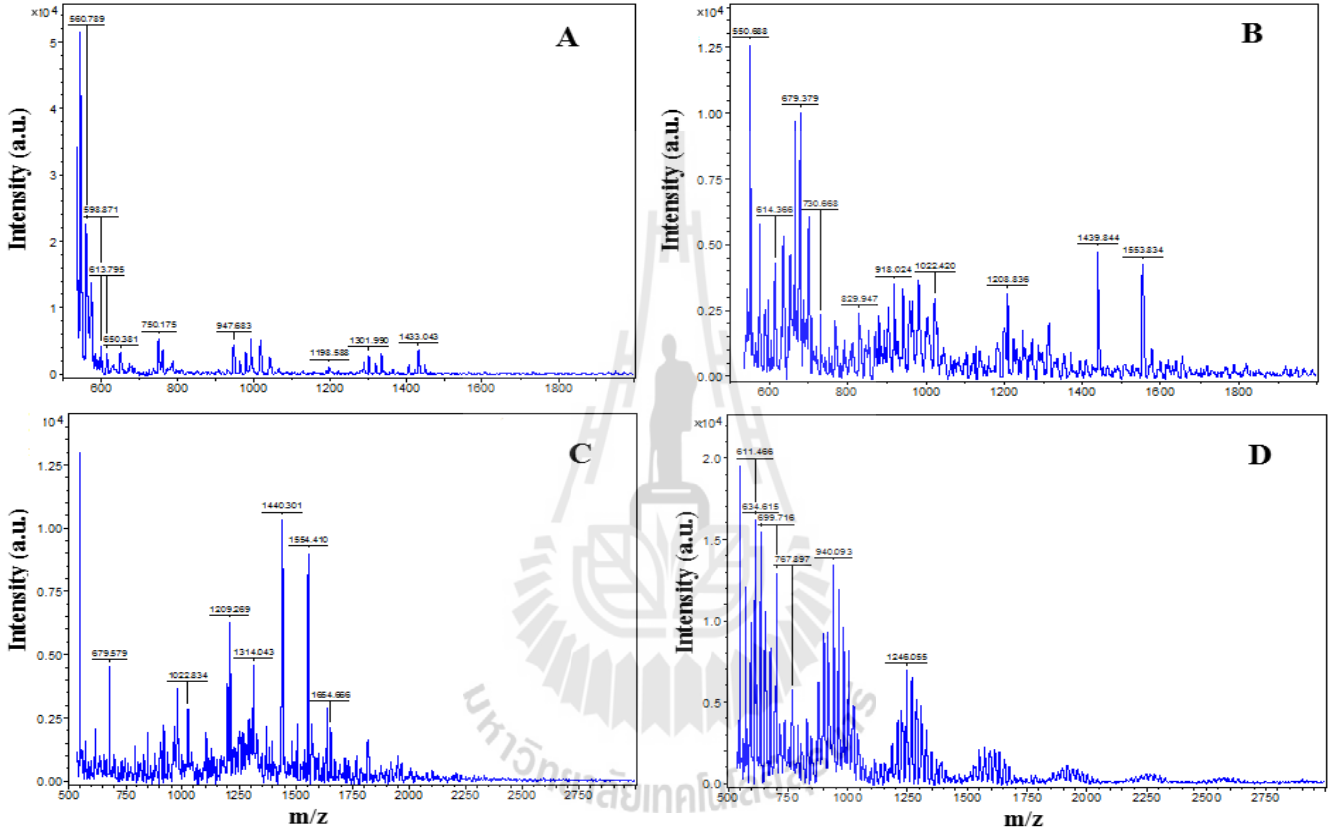

Figure 3.4 Peptide mass fingerprint (PMF) of Virgibacillus sp. SK37 cultivated in media (0.5% dried anchovy, 0.5% yeast extract, initial pH 8) in the absence (A, B) and presence (C, D) of 10% NaCl. Non-inoculated samples (A, C) were determined as a control.

3.5 Conclusions

Four key variables, namely dried anchovy, yeast extract, NaCl, and initial pH were important for biomass and proteinase production of *Virgibacillus* sp. SK37 based on PBD and RSM experiments. A significant increase in growth of 1.6 LogCFU/ml and 1.4-fold increase in proteinase production of *Virgibacillus* sp. SK37 were obtained under the medium containing 1.5% dried anchovy, 0.5% yeast extract, and 2.5% NaCl, at pH 8. The acidic medium inhibited the growth and secretion of proteinases. Proteinases with MW of 22 and 42 kDa were detected in the medium without NaCl, while those of 19, 34, 35, and 44 kDa were predominant in the NaCl-contained medium. In medium containing dried anchovy as a protein substrate, Lys and Arg were preferably hydrolyzed by the proteinases from *Virgibacillus* sp. SK37 in the absence of NaCl, while the enzymes showed a specificity to hydrolyze Lys, Arg, and Tyr in presence of 10% NaCl.

3.6 Reference

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CHAPTER IV

ADAPTATION OF MODERATE HALOPHILE, *VIRGIBACILLUS* SP. SK37, TO NaCl-STRESS CONDITION

4.1 Abstract

Virgibaillus sp. SK37 overcame against high osmolality of 25% NaCl, pH 7 when pre-exposed to sublethal condition (15% NaCl, pH 7) for at least 15 min, otherwise its proliferation was terminated. Transcriptomic approaches to provide a comprehensive view of the dynamic changes during the NaCl-stress condition were applied. It was revealed that external compatible solutes including glutamate, proline, glycine betaine, and ectoine would be taken up through Na^+/glutamate symporter, OpuA, OpuB, OpuC/D, and OpuE, whereas the biosynthesis of those compatible solutes, except glutamate, was less important. Reactive oxygen species (ROS) were enormously generated as detected by flow cytometry and fluorescence microscopy images. ROS was declined concomitantly with the up-regulations of the oxidative response genes, namely *perR*, *fur*, *mntR*, *ohrA/B*, and *spx*. The transcription of the regulons, including *hrcA*, *sigB*, and *ctsR*, which involved the general stress response, was induced. The mRNA of *codY* was enhanced corresponding to the down-regulation of its regulated genes. Metabolic rerouting from TCA cycle and oxidative phosphorylation to ethanol fermentative pathway was taken place. Activation of fatty

acid biosynthesis towards the production of cardiolipin and phosphatidylglycerol as well as cell well biosynthesis was demonstrated, implying an alteration of cell envelope during the NaCl-stress condition. This is the first study to reveal the transcriptomic view of NaCl-adaptive response of the moderate halophiles, *Virgibaillus* sp. SK37, leading to a cell survival at high-osmolality condition.

4.2 Introduction

Fish sauce is a hydrolysate used as a condiment in Southeast Asia and worldwide. The production of fish sauce involves in the addition of solar salt to uneviscerated fish at a ratio of approximately 1:3 and storing in an underground concrete tank at ambient temperature (~35-40 °C). The complete fermentation is required for about 1-1.5 year. Thus far, a traditional process and extremely long fermentation time mainly cause to lack a consistency of product quality and face high production cost. Application of a stature culture producing high NaCl-tolerant proteinase activities could be feasible means to shorten a process of protein hydrolysis, reduce fermentation time, and ensure to obtain a consistent product. *Virgibacillus* sp. SK37 obtained from fish sauce mash at 1st month, suggesting its existence in the natural fermentation (Sinsuwan, Rodtong, and Yongsawatdigul, 2007). The strain produced cell-bound and secreted proteinases exhibiting high activities at high NaCl concentration (Sinsuwan et al., 2007; 2008a; Phrommao, Rodtong, and Yongsawatdigul, 2010). It is nowadays a promising strain to be a candidate for starter culture of fish sauce fermentation.

Moderately halophilic bacteria can be found in high salinity ecology. It is defined as bacteria growing optimally between 0.5 and 2.5 M NaCl (2.9-14.6%)

(Ventosa, Nieto, and Oren, 1998). *Virgibacillus* sp. SK37 isolated from fish sauce fermentation was categorized as a moderate halophile. It was a Gram-positive facultative anaerobic rod with 0.6 to 0.7 × 3.0 to 6.6 μm, nonmotility and terminal or subterminal ellipsoidal spores (Sinsuwan et al., 2007). The strain in yeast slude medium exhibited optimal growth at 40 °C, pH 7.5, and 7.5% NaCl (Lapsongphon, Rodtong, and Yongsawatdigul, 2013). Apparently, while living in the fish sauce fermentation, the strain is subject to stressful environmental conditions, which are a high salinity (~27% NaCl) and acidic pH (pH ~5.5). These conditions would threaten bacterial cells with dehydration under hypertonic condition, resulting in loss of cell turgor, and depressing cytoplasmic pH, respectively (Kempf and Bremer, 1998; Mols and Abee, 2011). To survive in harsh conditions, bacteria required to be able to adapt efficiently to a growth-limiting condition. The study of inducible cellular stress responses system for survivals under unfavored conditions has been well-established in *Bacillus* (Helmann et al., 2001; den Besten, Mols, Moezelaar, Zwietering, and Abee, 2009; Mols, van Kranenburg, van Melis, Moezelaar, and Abee, 2010a; Schroeter et al., 2011). The expression of a protective gene was typically induced by mild stress condition, enabling the bacterial cells to survive at extreme condition. Völker, Mach, Schmid, and Hecker (1992) demonstrated that *Bacillus subtilis* cells survived under lethal NaCl condition (6% NaCl) when pre-incubated at mild NaCl stress condition (2% NaCl), otherwise the cells would be killed. Furthermore, the cellular mechanisms underlying the NaCl stress response in *Bacillus* have been extensively investigated (Steil, Hoffmann, Budde, Völker, and Bremer, 2003; Höper, Bernhardt, and Hecker, 2006; den Besten et al., 2009; Hahne et al., 2010). Under the NaCl stress condition, *B. subtilis* cells initially mediated by taking up extracellular K^+ via K^+ uptake system (KtrAB and KtrCD) (Kempf and Bremer, 1998; Holtmann, Bakker, Uozumi, and

Bremer, 2003). Subsequently, the initial phase of osmotic adaptation was followed by a replacement of accumulated K^+ with exo- and endogenous compatible solutes. The exogenous compatible solutes were imported through transport systems. *B. subtilis* possesses five osmotically regulated transport systems (OpuA, B, C, D, and E) (Kempf and Bremer, 1998). Endogenously synthesized compatible solutes, including proline, glycine betaine, and ectoine, were synthesized via dedicated osmostress-responsive pathways (Kempf and Bremer, 1998; Kuhlmann and Bremer, 2002). The high osmolality caused the activation of alternative sigma B (σ^B) factor, by which the regulated genes were, in turn, induced the expression (Boylan, Redfield, Brody, and Price, 1993; Petersohn et al., 2001). The alternative sigma W (σ^W), M (σ^M), and X (σ^X) regulons, which specifically influenced the physiological response to cell envelop stress, were induced in *B. subtilis* (Hahne et al., 2010). Catalase gene (*katA* and *katE*) and alkyl hydroxide reductase gene (*ahpC*) were up-regulated during the NaCl stress in *B. cereus* (den Besten et al., 2009). Obviously, a comprehensive perspective of the response of *Bacillus* to high salinity has been accomplished. However, a little is currently known about the mechanisms in the moderate halophile, *Virgibacillus.* In response to high salinity of *V. pantothenticus*, the exogenous ectoine and hydroxyectoine were imported into the cells through betaine-choline-carnitine transporter (BCCT)-type carrier EctT (Kuhlmann, Hoffmann, Bursy, Jebbar, and Bremer, 2011). Additionally, endogenously synthesized compatible solutes, namely ectoine, proline, and glutamate, were detected in *V. pantothenticus* (Kuhlmann and Bremer, 2002). Recently, the whole-genome sequences of *Virgibacillus* sp. SK37 has been established (Phrommao, 2010). It offers the opportunity to identify the global gene expression profile of the bacterium under a certain condition. Hence, it is a great interesting topic to elucidate a transcriptomic view of global changes of *Virgibacillus*

sp. SK37 in response to the osmotic stress. The outcome would lead to better understanding of the cellular mechanisms to defend the high-osmolality stress. In this study, the physiological result indicated that the strain exhibited the NaCl-adaptive response. The objective was to perform transcriptional analyses of the moderate halophile, *Virgibacillus* sp. SK37, in response to the pre-exposure at 15% NaCl. In addition, the observed several responses were linked to describe the cellular stress adaption strategies of the moderate halophile.

4.3 Materials and methods

4.3.1 Bacterial strain and growth condition

The strain SK37 was isolated from 1st-month fish sauce fermentation and identified to be *Virgibacillus* sp. according to the 16S rRNA gene sequence (GenBank/NCBI no. DQ910840). The strain is deposited at the Culture Collection Center at Suranaree University of Technology (Nakhon Ratchasima, Thailand). A single colony of the strain growing on tryptic soy agar (TSA) (Merck KGaA, Darmstadt, Germany) containing 10% NaCl at 35 °C for 1 day was transferred into 30 ml of yeast extract medium (Ym) (1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% $MgSO_4 \cdot 7H_2O$) containing 10% NaCl (pH 7) (Sinsuwan et al., 2008b) and incubated at 35 °C, shaking speed of 100 rpm, for 24 h. *Virgibacillus* sp. SK37 culture (300 µl) was inoculated into 30 ml Ym containing 10% NaCl (pH 7) and the bacterial cells were allowed to propagate in the same condition described previously. The 24-h culture was transferred to Ym at a ratio of 1:9 to attain a final concentration of 1, 5, 10, and 15% NaCl (pH 7). The samples were pre-incubated at 35 °C, shaking speed of 100 rpm, for 2 h. The pre-incubated samples were diluted to

optical density at 600 nm (OD_{600}) of 0.15 absorbance unit (A.U.) with 0.85% NaCl and the diluted culture (600 µl) was subsequently transferred into 30 ml of anchovy broth (AB) containing 25% NaCl (pH 7). Bacterial growth was determined by a spread plate technique on TSA containing 10% NaCl.

Anchovy (*Stolephorus* spp.) was lyophilized in a freeze dryer (LYOVAC GT2-S, SRK Systemtechnik GmbH, Riedstadt, Germany) and the dried fish were ground in an IKA M20 universal laboratory mill (IKA-Werke GmbH & Co, Staufen, Germany). The ground samples were sieved through 140 mesh (Fritsch GmbH, Idar-Oberstein, Germany). Total nitrogen content of 8.18% in anchovy power was obtained. AB containing 0.1% total nitrogen of anchovy powder and 25% NaCl, pH 7 was sterilized by autoclaving for 121 ºC for 15 min.

4.3.2 RNA isolation

Cultures (30 ml) at 15% NaCl were pre-incubated at 15, 30, and 120 min. Cell pellets were collected by centrifugation at 14,000×g, 25 ºC, for 1 min (Eppendorf centrifuge 5810 R, Eppendorf, Hamburg, Germany). The pellets were resuspended in 400 µl of autoclaved TE buffer (10 mM Tris, 1 mM EDTA, pH 8) treated with diethyl pyrocarbamate (DEPC). The resupended samples in 2-ml screw cap tubes were rapidly frozen in liquid nitrogen and kept in -80 ºC until RNA extraction. RNA was extracted following the modified method of den Hengst et al. (2005). The frozen samples were thawed on ice and added 0.5 g of 50-100µm-diameter glass beads, 50 µl 10% SDS, and 500 µl phenol/chloroform/isoamyl alcohol (IAA). Phenol was mixed with chloroform:IAA (24:1) in a ratio of 1:1. The cells were disrupted by pulsing twice for 1 min at 4 ºC in a mini bead beater (Mini-Beadbeater-16, Model 607EUR, BioSpec Products, Bartlesville, OK, USA) and subsequently centrifuged at 20,000×g, 4 ºC, for 10 min (Eppendorf centrifuge 5417 R, Eppendorf, Hamburg, Germany). The upper

layer was transferred into a new tube containing 0.5 ml of chloroform:IAA (24:1), and then centrifuged at 20,000×g, 4 ºC, for 5 min. The upper phase containing extracted total RNA was subject to isolation and purification based on a high pure RNA isolation kit (Roche Applied Sciences, Mannheim, Germany). This isolation protocol was accomplished by adding 40 U/ml of RioboLock™ RNase inhibitor (Thermo Scientific-Fermentas, Waltham, MA, USA). RNA concentration was measured by Nanodrop® spectrophotometer (ND-1000, NanoDrop Technologies, Inc, Wilmington, DE, USA). The quality of extracted total RNA was determined using Agilent RNA 6000 Nano assay and Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). The extracted RNA samples were stored at -80 ºC until use.

4.3.3 cDNA synthesis, labeling, and microarray hybridization

Complementary DNA (cDNA) incorporated with amino-allyl-labelled dUTP (Ambion Inc, Austin, TX, USA) was prepared from the extracted total RNA by the modification procedure of reverse transcription (RT) reaction, described by den Hengst et al. (2005). The mixture (30 µl) of the RT reaction consisted of 0.85 µg the extracted total RNA, 0.1 µg/µl random nonamers, 0.5 mM each of dATP, dCTP, dGTP, 0.3 mM dTTP, 0.2 mM 5-(3-aminoallyl)-dUTP, 10 mM DTT, 1× Superscript® III buffer, and 12 U/µl SuperScript® III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and was incubated in a thermocycler (Bio-Rad Laboratories In, Hercules, CA, USA) at 42 ºC for 16 h. Residual RNA was eliminated by incubating the sample with 2.5 M NaOH at 37 ºC for 15 min and then neutralizing with 2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Amino allyl-modified cDNAs were purified using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co, Duren, Germany) according to the manufacturer's instruction, except that 0.1 M sodium carbonate (pH 9) was used as an elution buffer instead.

Cy3 and Cy5 labeling of the cDNAs were performed with DyLight 550 and 650 NHS esters (Thermo Fisher Scientific Inc., Rockford, IL USA). The purified amino allyl-modified cDNAs were mixed with Cy3 and Cy5 and incubated in a dark condition at room temperature for 90 min. The samples (65 µl) were added with 15 µl of 4 M hydroxylamine and incubated in the same condition as described previously for 15 min. The labeled cDNAs were purified using the NucleoSpin® Gel and PCR clean-up kit according to the provided protocol. Loop design was applied to the microarray experiment for the comparison of the transcriptomes of the pre-incubated samples (15% NaCl, incubated for 0, 15, 30, and 120 min, Figure 4.1). Each sample was performed in two independent biological replicates with the dye swaps (Figure 4.1). The labeled samples at 0.3 µg were hybridized onto the custom-made Agilent *Virgibacillus* sp. SK37 microarray (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instruction. The samples were hybridized at 60 ºC, 10 rpm, for 17 h in a hybridization oven (Sheldon Manufacturing, Inc., Cornelius, OR, USA). The microarray wash was performed in a microarray wash station (Arrayit Corporation, Sunnyvale, CA, USA). The microarray slide was washed with 6× Saline sodium citrate (SSC) (0.9 M NaCl and 0.09 M sodium acetate) supplemented with 0.005% Triton X-102 at room temperature for 10 min. Subsequently, the slide was washed at 4 ºC for 5 min with pre-chilled 0.1× SSC containing 0.005% Triton X-102. The washed slide was immediately dried with nitrogen gas.

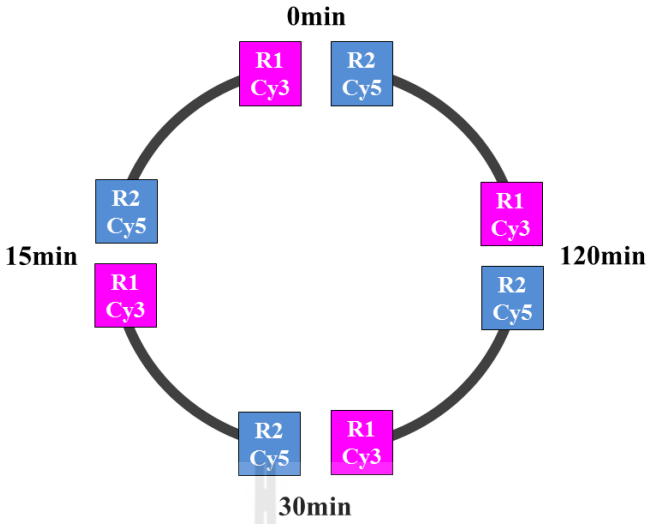

Figure 4.1 Loop design for DNA array experiment. The DNA array experiment was performed that the sample at 0 min, biological replication 2, labeled with Cy5 was hybridized to that of 120 min, biological replication 1, labelled with Cy3, and so on.

4.2.4 Microarray scanning and data analysis

The microarray slide was scanned using an Axon Genepix 4200AL microarray scanner (Axon Instruments, Inc., Union City, CA, USA). The differentially expressed microarray spots were identified. Subsequently, the expression ratios of the spots representing the same open reading frame were averaged. The transcriptional expression data for 15, 30, and 120 min were compared to those for the control (0 min).

4.2.5 Reactive oxygen species (ROS) measurement

CellROX® green reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used to detect generated reactive oxygen species (ROS). The pre-incubated samples (30 ml) at 15% NaCl for 15, 30, 60, and 120 min were prepared as described

previously. The cultures (0.5 ml) were mixed with the green reagent at a final concentration of 5 µM, and incubated at 37 ºC for 10 min. The cell pellets were collected by centrifugation at 14,000×g for 30 s (Eppendorf centrifuge 5417, Eppendorf, Hamburg, Germany) and washed with 0.5 ml of filtered sterile 1× phosphate buffered saline (PBS Buffer). The washed pellets were resuspended with 0.5 ml PBS buffer. The cell resuspension was used as a sample for flow cytometry and microscopy. The generated ROS were detected by using BD FACSCanto™ Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using WinMDI 2.9 (Joseph Trotter, Scripps Research Institute, San Diego, CA, USA) and graphically presented using Adobe Illustrator CS2 (version 12.0.1). For fluorescence images, the samples were spotted on agarose to immobilize the cells. The images were observed using a DeltaVision microscope and recorded with the DV Elite™ Imaging System (Applied Precision, Inc, Issaquah, WA, USA). The result was graphically presented using ImageJ (version 1.47) (National Institute of Mental Health, Bethesda, MD, USA).

4.4 Results and discussion

4.4.1 Physiological response in NaCl adaptation

AB containing 20 and 25% NaCl (pH 7) was pronounced to be a lethal condition for *Virgibacillus* sp. SK37 (data not shown). An increased NaCl concentration of the medium to 15% NaCl (pH 7) apparently extended a lag phase period of the strain, thus, it was defined as a sublethal condition. NaCl-adaptive response of the strain was tested by pre-incubating aerobically log-phase cultures at various concentrations of NaCl ranging from 1 to 15% for 2 h, and subsequently

exposed them to the lethal medium (AB containing 25% NaCl, pH 7) (Figure 4.2). The pre-incubation at 1-10% NaCl for 2 h did not activate the NaCl-adaptive response (Figure 4.2). In contrast, the pre-exposure at 15% NaCl dramatically affected viability of *Virgibacillus* sp. SK37 cells under the lethal condition (Figure 4.2). The number of viable cell count sharply declined for ~2 Log CFU/ml within 4 h and remained constant afterwards (Figure 4.2). A greater extent of survival in *B. subtilis* pretreated with a mild NaCl stress (4%) for 30 min before exposure to 10% NaCl, has been demonstrated (Völker, Maul, and Hecker, 1999). Furthermore, survival cells in 25% NaCl after pre-incubated at 15% NaCl for 15, 30, 60, and 120 min were comparable, indicating that the stress for 15 min was enough to activate the NaCl adaption response. It was in agreement with the previous report in *B. subtilis* which showed maximal stress-induced transcription when exposed to the NaCl stress condition for 10 min (Petersohn et al., 2001). These results revealed that *Virgibacillus* sp. SK37 exhibited the adaptive response to withstanding the extreme condition. This would be a simple means to improve the resistance of the strain at high-NaCl stress in fish sauce fermentation.

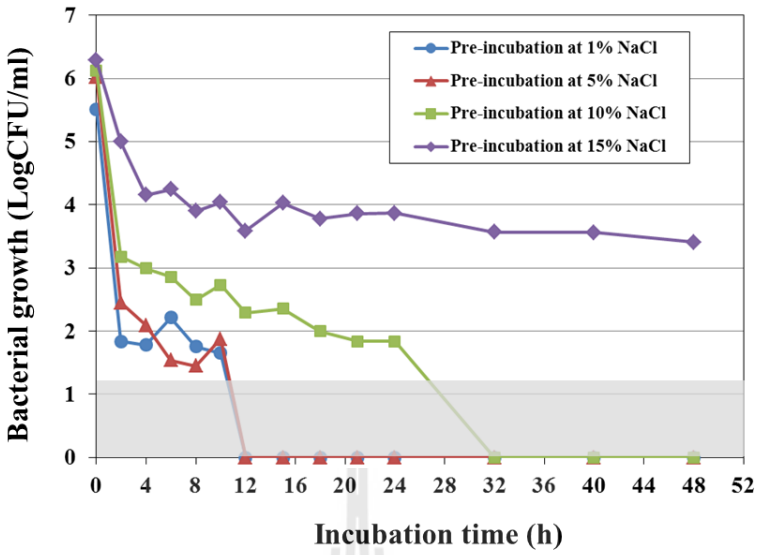

Figure 4.2 Bacterial growth (LogCfu/ml) of Virgibacillus sp. SK37 in anchovy broth (AB) containing 25% NaCl (pH 7) after pre-incubation in yeast extract medium (Ym) adding 1, 5, 10, and 15% NaCl (pH 7). Gray shaded area indicates below detection limits.

4.4.2 Transcriptomic response

The study of transcriptional response of *Virgibacillus* sp. SK37 under NaCl stress using DNA microarray-based measurement was carried out. The total RNA samples obtained 15, 30, and 120 min after the cells were pre-exposed to 15% NaCl were compared to that of sample without pre-incubation. On the basic of the putative roles in the stress responses of Gram-positive bacteria, all genes in regards with transcriptional regulators, transporters, compatible solute synthesis, and metabolic pathway were considered.

4.4.2.1 Osmotic stress

During the pre-exposure at 15% NaCl, the mRNA levels of *ktrC* and *ktrD*, encoding NAD^+/NADH binding protein and the transmembrane protein,

respectively, were slightly down-regulated (Figure 4.3). Although there was no evidence to support whether the Ktr system (K+ uptake channel) was essential for the adaptation of *Virgibacillus* sp. SK37 to salinity stress (Figure 4.3, Table 1S), the role of Ktr system on the NaCl stress response could not be ignored. In *B. subtilis*, the osmotic stress induced an increase in the cellular K^+ concentration, but did not influence the mRNA levels of *krt* transcripts (Holtmann et al., 2003). Typically, the accumulation of K^+ is an initial response of the cellular defense in non- and halophilic bacteria against a suddenly osmotic increase in their environment (Kraegeloh and Kunte, 2002; Corratgé-Faillie et al., 2010). The concentration of intracellular K^+ in *B. subtilis* rapidly increased from 350 to 650 mM within 1 h after osmotic stress (Whatmore, Chudek, and Reed, 1990). Bacteria frequently proceeded to synthesize and take up other compatible solutes to replace accumulated K^+, because the large amount of K^+ might be detrimental to many physiological functions (Whatmore et al., 1990; Kuhlmann and Bremer, 2002; Strom and Kaasen, 1993). Therefore, after initial phase of the response, the intracellular K^+ was expelled. *B. subtilis* exhibited the maximal up-regulation of the *yugO* (K^+ efflux channels) after 30 min of exposure to NaCl (Hahne et al., 2010). It was in agreement to this study that the mRNA levels of *yeaB* and *yugO*, encoding relevant K^+ efflux channels, were slightly enhanced at 15 min of the pre-exposure (Figure 4.3, Table 1S). It seemed likely that at 15 min after the NaCl stress, *Virgibacillus* sp. SK37 would rather take the cation out from the cells. The strain might modulate its intracellular K^+ levels in a short time (≤15 min) to respond to high-osmolality medium. After that, the mRNA levels of transporter genes for uptake other compatible solutes were significantly induced as detected at 15 min (Figure 4.3, Table 1S).

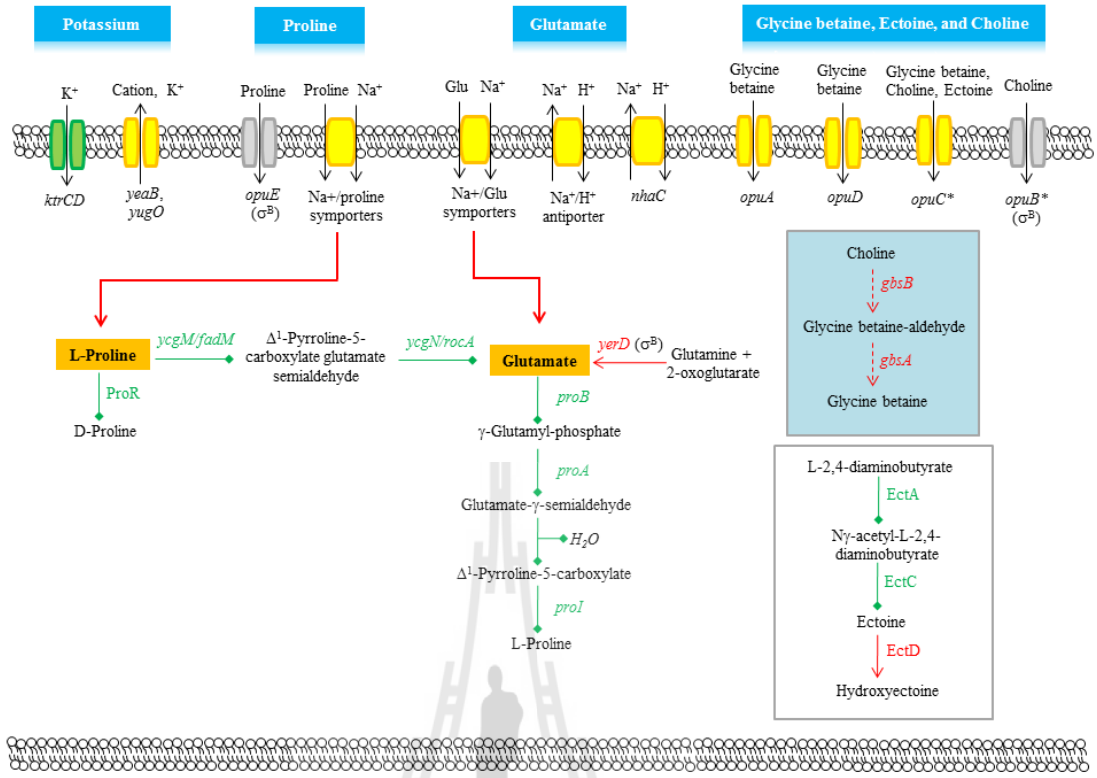

Figure 4.3 Schematic representation of the NaCl stress response upon exposure of Virgibacillus sp. SK37 cells to 15% NaCl. *, the genes corresponding to 2 genes, *opuB* and *opuC*; gray, insignificantly changed genes; green/–♦, down-regulated genes; red/→, up-regulated genes; - -> , slightly up-regulated genes; (σ^B), regulated by sigma B transcriptional factor as reported by Price et al. (2001).

The single-component transporter *opuE* transcript was essentially unchanged during the pre-exposure (Figure 4.3). *B. subtilis* can acquire the exogenous proline by *opuE*, which presented in two proline transporters (von Blohn, Kempf, Kappes, and Bremer, 1997; Spiegelhalter and Bremer, 1998). Although the *Virgibacillus* sp. SK37 *opuE* did not respond under the NaCl stress, at least four unidentified genes corresponding to Na^+/proline symporters, were strongly up-

regulated (Figure 4.3, Table 1S). The response to osmotic stresses of these genes implied the role in the acquisition of proline for osmoprotective purpose. The concentration of intracellular proline in *B. subtilis* remarkably increased from 16 to 188 mM after 2 h of exposure to osmotic stress (Whatmore et al., 1990). It was uncovered from this study that the mRNA levels of *ycgM/fadM* and *ycgN/rocA* were substantially decreased (Figure 4.3). Their gene products, proline dehydrogenase (PRODH) and 1-pyrroline-5-carboxylate dehydrogenase (P5CDH), respectively, provided function in the proline degradation (Luo, Singh, and Tanner, 2013). In addition, the mRNA levels of genes encoding proline racemase (ProR) significantly decreased (Figure 4.3). The ProR catalyzed the interconversion of L- and D- proline (Hartya et al., 2014), which the enantiomer D-proline did not show a role of compatible solute (Sasaki, Takaki, Oshima, Ishida, and Nagata, 2007). It might suggest that the strain would maintain the accumulated L-proline under high salinity. There was the evidence that the L-proline served as an osmoprotectant in *V. pantothenticus* (Kuhlmann and Bremar, 2002; Kuhlmann, Bursy, Gimpel, Hoffmann, and Bremer, 2008). Exogenous proline served as an osmoprotectant for *Virgibacillus* sp. SK37, and was maintained in its active form by keeping amount of the proline-degrading enzymes at low levels. Additionally, the *yerD* (glutamate synthase gene) as well as the unidentified gene name, of which gene product was Na+/glutamate symporter, were up-regulated over a period of 30 min after the pre-exposure (Figure 4.3, Table 1S). It indicated an accumulation of exo- and endogenous glutamates in *Virgibacillus* sp. SK37 cells during the NaCl stress. The concentration of intracellular glutamate of *B. subtilis* cells at 2 h after osmotic stress was moderately increased from 103 to 158 mM (Whatmore et al., 1990). *V. pantothenticus* also accumulated endogenously synthesized glutamate under osmotic growth condition (Kuhlmann and

Bremer, 2002). The mRNA levels of five unidentified genes corresponding to Na^+/H^+ antiporters were significantly enhanced (Figure 4.3), implying that *Virgibacillus* sp. SK37 , while proceeded to take up the desirable glutamate along with Na^+, would concomitantly control the levels of intracellular Na^+ in the osmotic challenge through the Na^+ extrusion system. In addition to their osmoprotective function, glutamates were the precursor for the synthesis of proline. In this study, the transcriptions of *proB*, *proA*, and *proI*, encoding γ-glutamyl kinase, γ-glutamyl phosphate reductase, and Δ^1- pyrroline-5-carboxylate reductase, respectively, belonging to proline synthesis pathway, were slightly down-regulated (Figure 4.3). In contrast, endogenously synthesized proline of *V. pantothenticus* was accumulated when the cells were grown in Spizizen's minimal medium (SMM) containing 1 M NaCl (~5.8%) (Kuhlmann and Bremer, 2002). In this experiment, the Ym used as the pre-exposure medium probably contained prolines, therefore the bacterium would rather use exogenous proline than endogenously synthesized proline under the NaCl stress. Hence, the glutamates and exogenous prolines would play significant roles in the NaCl stress condition for*Vigibacillus* sp. SK37, but the endogenous synthesized proline was less important.

Glycine betaine in *B. subtilis* was taken up by multicomponent ABC transporters OpuA and OpuC, and the single-component transporter OpuD (Kappes, Kempf, and Bremer, 1996; Kempf and Bremer, 1998). For *Virgibacillus* sp. SK37, the mRNA level of the *opuD* was significantly increased throughout the pre-exposure for 2 h, while that of the *opuA* and *opuC*/*opuB* was enhanced at 15-30 min after the pre-exposure (Figure 4.3, Table 1S). Glycine betaine was more effective to alleviate the growth inhibition in *B. subtilis* under high NaCl stress, compared to proline and ectoine (von Blohn et al., 1997; Jebbar, von Blohn, and Bremer, 1997). Choline, a compatible solute and a precursor of the glycine betaine synthesis, was transported into

the *Bacillus* cells by multicomponent ABC transporters OpuB and OpuC (Boch, Kampf, and Bremer, 1994; Kempf and Bremer, 1998; Kappes et al., 1999). The amount of the channel proteins (OpuB/OpuC) to take up the choline into *Virgibacillus* sp. SK37 cells was apparently increased (Figure 4.3, Table 1S). However, the transcriptions of *gbsB* and *gbsA*, encoding type III alcohol dehydrogenase and glycine betaine aldehyde dehydrogenase, respectively, were slightly up-regulated (Figure 4.3, Table 1S). The GbsB converts exogenous choline into glycine betaine aldehyde, and subsequently glycine betaine is synthesized by GbsA activity. Due to insignificant up-regulation of biosynthesis pathway of glycine betaines, it was ambiguous to conclude whether the synthesis of the glycine betaine from the choline was an important facet in the NaCl stress response of *Virgacillus* sp. SK37. The result indicated that exogenous glycine betaine and choline would be used as a compatible solule in *Virgibacillus* sp. SK37 during the NaCl stress.

OpuC system in *B. subtilis* served as uptake route for ectoine (Jebbar et al., 1997). It is inconclusive to pinpoint whether *opuC* transcript was enhanced in *Virgibacillus* sp. SK37 due to uncertain gene identification between *opuC* and *opuB* (Table 1S). The sequence relatedness of structural genes of *opuB* and *opuC* in *B. subtilis* was closely related with ~69-85% similarity (Kappes et al., 1999). However, the uptake of the ectoine [^{14}C] in *V. pantothenticus* reached to approximately 32 nmol/mg protein in 0.4 M NaCl medium (Kuhlmann et al., 2011). It was expected that the up-regulated genes was supposed to be the *opuC*, while the other down-regulated one might be the *opuB* (Figure 4.3, Table 1S). The genes, encoding the L-2,4-diaminobutyrate acetyltransferase (EctA) and the ectoine synthase (EctC), were down-regulated (Figure 4.3), whereas the gene, encoding the ectoine hydroxylase (EctD), was strongly up-regulated (Figure 4.3). In *B. subtlilis*, the intermediate L-2,4-

diaminobutyrate was converted into the intermediate, Nγ-acetyl- L-2,4-diaminobutyrate, by the activity of EtcA and subsequently converted into ectoine by EctC activity (Bursy, Pierik, Pica, and Bremer, 2007). Under the NaCl stress, *Virgibacillus* sp. SK37 would not prefer to accumulate the endogenous synthesized ectoine. Although the expression of EctD, catalyzing in a conversion of the precursor ectoine to hydroxyectoine, was apparently induced, the hydroxyectoine biosynthesis basically depended on the existence of ectoine in the cells. In addition, the full activity of EctD would depend on Fe^{2+} (Bursy et al., 2007), of which the amount might be depleted by the oxidative defense. *V. pantothenticus*, has been reported to synthesiz ectoine, but hydroxyectoine was not produced, at high osmolality growth medium (Kuhlmann and Bremer, 2002; Bursy et al., 2007). It might be concluded that the strain did not synthesize both ectoine and hydroxyectoine during the NaCl stress. Results certainly demonstrated that the glutamate and exogenous compatible solutes including proline, glycine betaine, and ectoine, played important role in the osmotic response of *Virgibacillus* sp. SK37, but further studies to answer some ambiguities in respect with the biosynthesis of the compatible solutes at high-salinity medium are required.

4.4.2.2 Radical formation and oxidative response

During the pre-exposure at 15% NaCl, *Virgibacillus* sp. SK37 cells significantly increased in the transcription of the genes corresponding to radical detoxifying proteins to maintain intracellular redox balances (Figure 4.4). It might be reasonable to assume that reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($^{\bullet}OH$) would be greatly generated. Therefore, the formation of ROS during the pre-exposure was studied in more details using fluorescence microscopy and flow cytometry. Cells exposed to 15% NaCl for 0,

15, 30, 60, 120 min were stained with the green fluorescent probe, CellROX® green reagent. The molecular probe, which can penetrate through cell envelope and subsequently bind to DNA, is very weakly fluorescent in the reduced state, but it becomes highly green-fluorescent upon an oxidized state inductively generated by ROS. The green-fluorescent signal was quite weak in the non-stressed control cells (Figure 4.5A, t = 0 min), implying a very low amount of the generated ROS. The strong green-fluorescent cells appeared when the cultures were exposure to 15% NaCl for 15 min (Figure 4.5A, t = 15 min); however, the prolonged exposure resulted in a decrease in the fluorescent signal (Figure 4.5A, t = 60 and 120 min). The level of the generated ROS somewhat decreased at prolonged exposure time. The majority of the non-stressed control cells exhibited a low fluorescent intensity (Figure 4.5B). Apparently, the amount of ROS were maximally observed at 30 min and declined afterwards, as evidenced by a shift of the florescent peak to left (Figure 4.5B). The exposure to NaCl stress at 6% did not induce the formation of hydroxyl ($^{\bullet}OH$) and peroxynitrite ($ONOO^{-}$) in aerobically grown *B. cereus* cells (Mols, Pier, Zwietering, and Abee, 2009). The formation of ROS may be caused by a perturbation of the aerobic electron transport chain (ETC) in *Virgibacillus* sp. SK37 under NaCl-stress condition, as previously described in other bacteria exposed to acid stress (Mols, van Kranenburg, van Melis, Moezelaar, and Abee, 2010b) and bactericidal antibiotics (Kohanski, Dwyer, Hayete, Lawrence, and Collins, 2007). A large supply of available oxygen to NaCl-stressed cells in the experimental could reinforce the formation of ROS in *Virgibacillus* sp. SK37. Low pH stresses of aerobically grown *B. cereus* cells induced a formation of OH and $ONOO^{-}$, but no detection of both radicals if the strain grew in anaerobic condition at similar low pH values (Mols et al., 2010b). The aerobically-exponential *Virgibacillus* cells exposed to the NaCl stress rapidly

generated numerous ROS and subsequently the stressed cells could reduce the generated ROS.

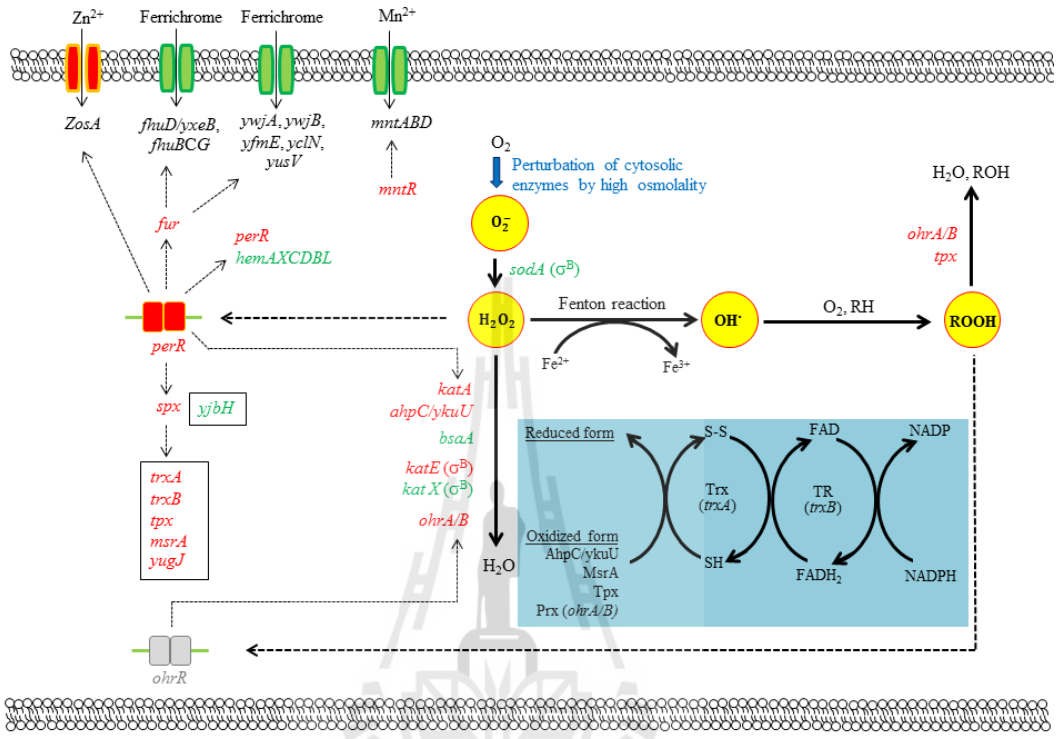

Figure 4.4 Schematic representation of the oxidative stress response upon exposure of *Virgibacillus* sp. SK37 cells to 15% NaCl. (σ^B), regulated by sigma B transcriptional factor as reported by Price et al. (2001) and Höper, Völker, and Hecker. (2005), *ohrR*, not detected in genome sequence data.

Changes of mRNA levels of several genes relevant to oxidative defenses of *Virgibacillus* sp. SK37 during exposure to 15% NaCl were observed. The transcription of the genes encoding peroxide-sensing regulators, namely peroxide repressor (*perR*) and organic hydroperoxide resistance (*ohr*), in Gram-positive bacteria has been well-reported (Fuangthong, Atichartpongul, Mongkolsuk, and Helmann, 2001; Fuangthong, Herbig, Bsat, and Helmann, 2002; Helmann et al., 2003). PerR was

induced when *B. licheniformis* cells treated with H_2O_2 (Schroeter et al., 2011). PerR regulon in *B. subtilis* consisting of *katA* (catalase), *ahpCF* (alkyl hydroperoxide reductase), *hemAXCDBL* (heam biosysthesis operon), *zosA* (a zine uptake system), *fur* (ferric uptake repressor), *spx* (Spx), *perR* itself sensed in the presence of H_2O_2 (Chen, Keramati, and Helmann, 1995; Bsat, Herbig, Martinez, Setlow, and Helmann, 1998; Fuangthong et al., 2002, Helmann et al., 2003; Leelakriangsak , Kobayashi, and Zuber, 2007). Transcription of *katA*, ah*pC/ykuU*, *zosA*, *fur*, *spxA*, and *perR* (Figure 4.4) increased with slight changes in *hemAXCDBL* (Table 1S) under salt stress of *Virgibacillus* sp. SK37. These results were in agreement with the response in *B. subtilis* showing that not all members of PerR regulon were stimulated by oxidative stress (Fuangthong et al, 2002). Some of PerR-regulated genes (*katA*, *mrgA*, and *zosA*) were strongly induced by H_2O_2, however, others (*hemeA* operone, *ahpCF*, and *perR*) showed less induction (Fuangthong et al, 2002; Helmann et al, 2003). It could be speculated that the de-repression of PerR in *Virgibacillus* sp. SK37 cells would be taken place during the pre-exposure. In the absence of peroxide, Fe^{2+}-bound PerR bound to the promoters of the oxidative resistance genes, leading to the repression (Giedroc, 2009). The bound Fe^{2+} was effectively oxidized by H_2O_2 inside the cells to generate $^{\bullet}OH$, which directly oxidized amino residues on metal site ligands. Consequently, the dissociation of the ferrous iron and subsequent release of PerR from the DNA operator resulted in the PerR de-repression and the transcription of its regulated genes (Giedroc, 2009). Catalase (*katA*) and alkyl hydroperoxide reductase (ah*pC/ykuU*) play important role in a bacterial defense against ROS. Catalase (KatA) and alkyl hydroperoxide (AhpC) can reduce the content of the toxic compounds, H_2O_2 and hydroperoxides, respectively, to a level that permits growth (Storz, Tartaglia, Farr, and Ames, 1990). ZosA would perform the function of Zn^{2+} uptake (Gaballa and

Helmann, 2002). An elevated intracellular Zn^{2+} may protect thiols from oxidation (Gaballa and Helmann, 2002). In *B. subtilis*, the resistance to high concentration of H_2O_2 was significantly increased when intracellular Zn^{2+} increased (Gaballa and Helmann, 2002). A gradually decreased transcription of PerR-regulated genes when the pre-incubation time was extended (Table 1S) suggested a restoration of DNA-binding ability of PerR with simultaneously decreasing in a level of ROS.

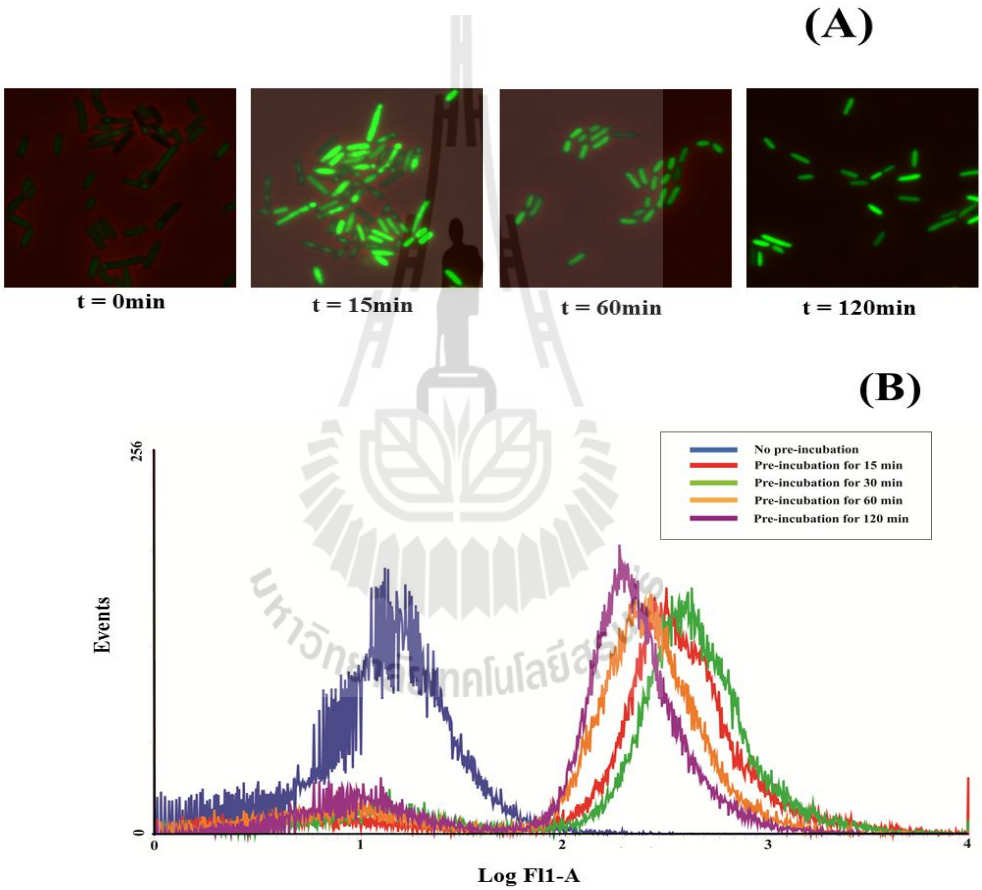

Figure 4.5 Radical formation in *Virgibacillus* sp. SK37 cells upon exposure to 15% NaCl. (A) fluorescence microscopy images (B) fluorescent signal pattern detected by FACS. Samples were taken at 0, 15, 30, 60, and 120 min. Subsequently, the cells were strained with CellROX® green reagent. The green fluorescent cells (A) or the shift in fluorescent signal to the right (B) indicate the formation of reactive oxygen species.

The greater extent of the transcribed *fur* (Figure 4.4) corresponded to the down-regulation of the *fur*-regulated genes, including *fhuD/yxeB*, *fhuB*, *fhuC*, *fhuG*, *ywjA*, *ywjB*, *yfmE*, *yclN*, and *yusV* (Figure 4.4). Those regulated genes encoded relevant siderophore uptake proteins. This was similar to a previous report in *B. subtilis* (Baichoo, Wang, Ye, and Helmann, 2002). Typically, the Fur repressor is able to bind the promoter and subsequently blocks the access of RNA polymerase binding to DNA. It resulted in a decrease in the mRNA levels of *fur*-regulated genes. Both iron forms, free intracellular iron (Fe^{2+}) and Fe^{3+}-siderophore complex, can react with H_2O_2 via Fenton reaction to generate reactive $^{\bullet}OH$ (Barbusiński, 2009; Wang et al., 2013). This was implied that up-regulation of *fur* in *Virgibacillus* sp. SK37 to limit exogenous iron would avoid to chemically form the radicals during salt stress condition. Although the free endogenous ferrous iron (Fe^{2+}) plays a significant role in Fenton-mediated hydroxyl radical formation (Kohanski et al., 2007), the transcription of the genes encoding relevant iron-sulfur cluster proteins (represented by *yhbA*, *yutM*, *sdaAA*, *sdaAB*, and *sdhB*) and the iron-sulfur cluster repair slightly changed in *Virgibacillus* sp. SK37 (Table 1S). Those newly synthesized proteins did not require to replace the damaged ones, implying that the amount of endogenous ferrous irons released from damaged iron-sulfur clusters would be small. Furthermore, genes, corresponding to Mn^{2+} homeostasis of *Virgibacillus* sp. SK37, exhibited a response to the NaCl stress (Figure 4.4). The metalloregulatory protein MntR from *B. subtilis* acted as a transcriptional repressor, which was activated by binding to Mn^{2+}, and the Mn^{2+}-bound MntR was able to repress the transcription of the *mntABCD* (an ABC transporter) and *mntH* (a proton-coupled metal ion transporter) (Lieser, Davis, Helmann, and Cohen, 2003; Moore and Helmann, 2005). Under the NaCl stress condition, the mRNA of *mntR* was significantly enhanced in concomitant with down-

regulated *mntABD* (Figure 4.4). Under high osmolality, the uptake of exogenous Mn^{2+} in *Virgibacilllus* sp. SK37 was minimized. Mn^{2+} might generate $^{\bullet}OH$ (Ito, Yamamoto, and Kawanishi, 1992) and amino acid oxidation (Berlett, Chock, Yim, and Stadtman, 1990). The *mntR* mutant strain of *B. subtilis*, a constitutive expression of the Mn^{2+} transporter, exhibited sensitivity to H_2O_2 (Que and Helmann, 2000). Therefore, the limitation of exogenous Mn^{2+} in *Virgibacillus* cells faced the NaCl stress condition might be required to response to oxidative stress.

The *ohrA/B* encoding peroxiredoxin (Prx) showed the up-regulation during the pre-incubation at 15% NaCl (Figure 4.4). In *B. subtilis*, it was induced by the formation of organic peroxide (ROOR′) (Helmann et al., 2003). The *ohrA* of *B. subtilis* was strongly induced by *tert*-butyl peroxide (t-buOOH), but not by H_2O_2 (Helmann et al., 2003). The expression of *ohrA* was controlled by the action of the transcriptional repressor, OhrR. In the presence of ROOH, the OhrR, of which cysteine residue was oxidized into cysteine sulfenate (Cys-SOH), would be lost the DNA binding activity for the *ohrA* promoter (Fuangthong et al., 2001; Mongkolsuk and Helmann, 2002; Giedorc, 2009). The *ohrR* (formerly *ykmA*) was absent in the annotation of *Virgibacillus* sp. SK37 genome, it is likely to be present. The de-repression of putative OhrR in *Virgibacillus* sp. SK37 would appear, and elevate the transcription of *ohrA*. This mechanism defense would be a part of adaptation process to high osmolality stress of *Virgibacillus* sp. SK37. The role of Prx is proposed as a physiological reductant to eliminate a hydroperoxide (Rhee, Chae, and Kim, 2005a). Upon exposure to the radicals, the cysteine residue (Cys-SH) of Prx would be readily oxidized to Cys-SOH, which maybe subsequently reacted to Cys-SH of the other subunits to form an intermolecular disulfide if the molecular structure contained two

active site cysteine residues (Rhee et al., 2005a, b). Prxs were remarkably expressed when *B. stearothermophilus* TLS33 cells treated with H_2O_2 (Topanurak, Sinchaikul, Phutrakul, Sookkheo, and Chen, 2005). Furthermore, glutathione peroxidase (GPx) has been reported to eliminate H_2O_2 (Ng, Schafer, Buettner, and Rodgers, 2007). Small magnitude of the transcriptional changes during the pre-incubation (Figure 4.4, Table 1S), suggested insignificant role of GPx (*bsaA*) during the NaCl adaptation response of *Virgibacillus* sp. SK37.

The *spx*, encoding the transcription regulator SpX, of *Virgibacillus* sp. SK37 appeared to be up-regulated along with other Per-regulated genes (Figure 4.4). Spx-regulated genes including *trxA* (Trx), *trxB* (Trx reductase, TrxR), *tpx* (Tpx), *msrA* (Peptidyl Msrs), and *yugJ* (NADH-dependent butanol dehydrogenase) also were up-regulated (Figure 4.4). Thus, it would be expected that Spx was activated during the pre-exposure to 15% NaCl. In *B. subtilis*, the Spx-regulated genes were significantly up-regulated after addition of H_2O_2 or paraquat to the medium (Mostertz, Scharf, Hecker, and Homuth, 2004). The transcription regulator Spx regulates the genes involved in thiol homeostasis in response to unwanted disulfide bonds, generated by oxidative stress (Newberry, Nakano, Zuber, and Brennan, 2005). In *B. subtilis* growing under oxidized condition, an intracellular disulfide bond in a Cys-X-X-Cys (CXXC) motif of Spx was spontaneously formed and the oxidized Spx directly interacts, in turn, with the C-terminal domain of the α subunit (αCTD) of RNA polymerase, resulting in an induction of the Spx-regulated genes (Nakano, Erwin, Ralle, and Zuber, 2005; Newberry et al., 2005). Furthermore, a down regulation of *yjbH*, encoding a negative effector, was observed in *Virgibacillus* sp. SK37 (Figure 4.4). It might refer to an accumulation of Spx in the cells during pre-exposure at 15% NaCl. In *B. subtilis*, YjbH-bound Spx would facilitate degradation by the activities of ClpXP (ATP-

dependent proteinases), resulting in low amount of Spx during normal growth condition (Larsson, Rogstam, and Wachenfeldt, 2007). On the other hand, upon oxidized condition, YjbH would lose a Zn atom coordinated by its N-terminal histidine-rich region and disassemble from Spx, leading to a slow degradation and subsequent accumulation of Spx (Garg, Kommineni, Henslee, Zhang, and Zuber, 2009). Thioredoxin (Trx) is typically required for regeneration of Prx, methionine sulfoxide reductase (Msrs), and thiol peroxidase (Tpx), which decompose ROS (Comtois, Gidley, and Kelly, 2003; Rhee et al., 2005a; Kwak, Hwang, and Kim, 2012). NADH-dependent butanol dehydrogenase (YugJ) functions in the production of NADPH (Schroeter et al., 2011), which acts as an electron donor for Trx. Msrs is able to recognize methionine sulfoxide (Met-O) and catalyzes the reduction of free or protein-based Met-O into methionine (Kwak et al., 2012). Tpx also functions in the breakdown of peroxides (Veal, Day, and Morgan, 2007). Therefore, it would be envisaged that during the pre-exposure at 15% NaCl, Trx of *Virgibacillus* sp. SK37 would be maintained in a reduced state by activity of TrxR with the expense of NADPH, when the Trx received the electron from Prx, Peptidyl Msrs, and Tpx.

4.4.2.3 General stress response

The same set of proteins was induced to respond to a variety of stress such as heat shock, salt stress, ethanol, starvation for oxygen or nutrients, etc., called general stress proteins (Hecker, Schumann, and Völker, 1996). Genes associated with general stress responses in *Virgibacillus* sp. SK37 were observed in a response to NaCl stress condition. Although the mRNA of the transcriptional repressor gene, *hrcA* (HrcA repressor) was increased after 30 min of the pre-exposure (Figure 4.6, Table 1S), the transcription levels of CIRCE (controlling inverted repeat of chaperone expression)-dependent genes (Class I stress genes) were slightly enhanced, especially

at 120 min (Figure 4.6). It implied that the HrcA repressor of the strain was likely to be inactive under the NaCl stress condition, allowing to up-regulation of CIRCE-regulated gene. The CIRCE-dependent genes of *Virgibacillus* sp. SK37 included *dnaK* (chaperone protein DnaK), *hrcA* (heat shock gene repressor HrcA), *grpE* (chaperone GrpE), *groES* (HSP10 protein) and *groEL* (chaperonin GroL) (Figure 4.6). Their products prevent misfolding and aggregating of partially denatured proteins (Mogk, Homuth, Scholz, Kim, Schmid, and Schumann, 1997). Typically, the active *B. subtilis* HrcA repressor binds the conserved CIRCE element and specifically blocks the transcription of its regulated genes (Reischl, Wiegert, and Schumann, 2002). Because of a tendency to aggregate, the *B. subtilis* HrcA repressor required the GroE chaperone for preventing its protein aggregation (Reischl et al., 2002). Under stress condition, the presence of several unfolding proteins in the cells was able to interact with the GroE chaperone, resulting in a depletion of GroE and an increase in the inactive HrcA (Mogk et al., 1997). The GroEL and DnaK in *B. subtilis* were not induced by salt stress (Völker et al., 1994). In contrast to *B. subtilis*, this finding might be expected that the non-native proteins in the stressed *Virgibacillus* SK37 cells would be formed during the NaCl stress condition, leading to unavailable GroE chaperone to maintain the active form of HrcA repressor.

The expression of the σ^B general stress regulon (Class II stress genes) was relevant for stress survival in Gram-positive bacteria (Völker et al., 1999). The transcription levels of *sigB*, *rbsW*, and *rsbV*, encoding σ^B, anti-sigma factor, and anti-anti sigma factor, respectively, were rapidly enhanced within 30 min and gradually decreased afterwards (Figure 4.6, Table 1S). The transcription of σ^B-controlled genes of *Virgibacillus* sp. SK37 was also simultaneously enhanced (Figure 4.6), as reported previously in the salt stress response of *B. subtilis* (Price et al., 2001; Petersohn et al.,

2001). It would be speculated the activation of σ^B in response to the pre-exposure of binding to its anti-sigma factor, RsbW, rendering inactive σ^B. Under stress condition,

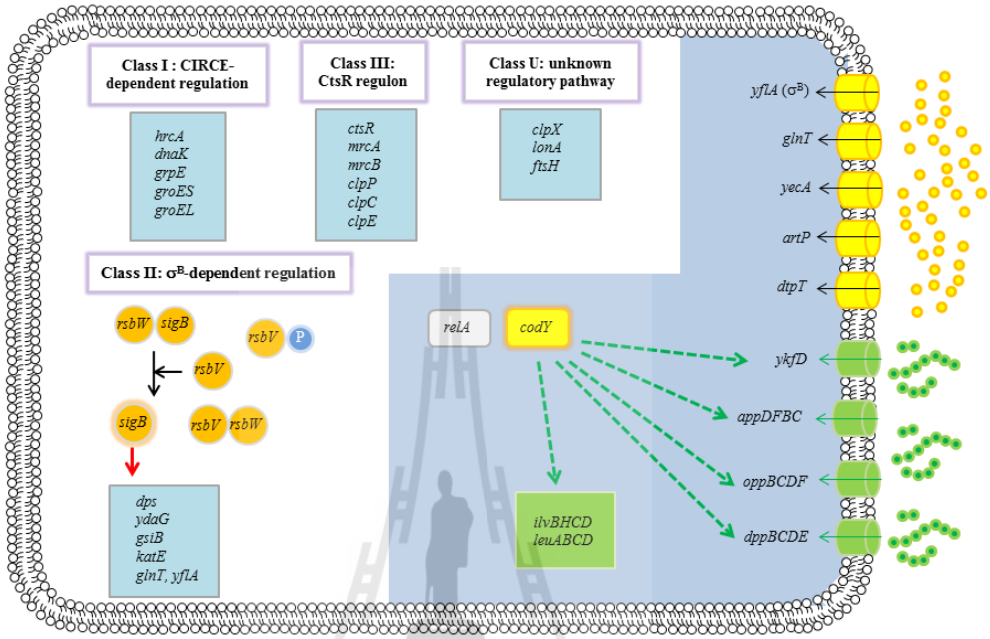

Figure 4.6 Schematic representation of the general stress response and CodY upon exposure of Virgibacillus sp. SK37 cells to 15% NaCl.

a dephosphorylated anti-anti sigma factor, RsbV, can interact with RsbW to concomitantly release active σ^B, which consequently triggers the gene transcripts (Redfield and Price, 1996). The *yfkM/yraA*, encoding putative intracellular proteinase was remarkably up-regulated (Figure 4.6). As previously reported in *B. subtilis*, the *yfkM* transcript in the salt stress sample was increased by 17 times, compared to that of untreated sample (Petersohn et al., 2001). The denatured proteins generated during the NaCl stress would be required to be eliminated. The mRNA level of *dps* and *ydaG*, encoding a DNA-protecting protein and general stress protein, was increased (Figure 4.6). Dps was essential for resistance against oxidative stress in *B. subtilis* (Antelmann

et al., 1997). The mRNA of *gsiB*, encoding general stress protein, was significantly enhanced over 30 min (Figure 4.6, Table 1S). The *katE* (catalase E gene), was remarkably up-regulated (Figure 4.6), whereas transcription of *katX* (catalase X gene) and *sodA* (superoxide dismutase gene) was slightly down-regulated (Table 1S). Superoxide dismutase (Sod) is certainly required for detoxification of superoxide (O_2^-) to H_2O_2 (Scott, Meshnick, and Eaton, 1987). The generated H_2O_2 was eliminated by activity of catalase. The σ^B regulon of *B. subtilis* was not highly sensitive to H_2O_2. It was activated by the intermediate level of H_2O_2 (58 µM), but not by the low level of H_2O_2 (8 µM) (Helmann et al., 2003). It should be noted that under NaCl stress, the two catalase genes, *katA* and *katE*, which regulated by a different mechanism, were significantly up-regulated in *Virgibacillus* sp. SK37. In contrast, the regulatory system in *B. subtilis*, only KatA played an important role in the oxidative stress (Engelmann and Hecker, 1996).

This results uncovered the enhanced transcription of *clpP*, *clpC* and *clpE* from *Virgibacillus* sp. SK37 during the pre-exposure at 15% NaCl (Figure 4.6), whereas the *ctsR* (CtsR transcriptional repressor gene), *mcsA* (modulator gene), and *mcsB* (arginine kinase gene) transcripts were slightly changed (Figure 4.6). In *B. subtilis*, the *clpP*, *clpC* and *clpE* transcripts, encoding ATP-dependent Clp proteinases, exhibited stress inducible after the CtsR repressor was inactivated (Class III stress genes) (Derré, Rapoport, and Msadek, 1999; Petersohn et al., 2001; Schroeter et al., 2011). The CtsR repressor in Gram positive bacteria was also regulated by post-translational process (Elsholz, Michalik, Zühlke, Hecker, and Gerth, 2010; Elsholz et al., 2011). Upon oxidative stress, the *B. subtilis* McsA, acting as a redox-sensing protein, was oxidized and no longer formed a complex with McsB and ClpC proteinase. The free oxidized McsA was, in turn, able to remove the CtsR repressor

from the promoter of the regulated genes (Elsholz et al., 2011). Additionally, under stress condition, other ATP-dependent proteinases, encoded by *clpX*, *lonA*, and *ftsH*, were able to be induced by σ^B -independent regulation (Class U stress genes) (Gerth, Krüger, Derré, Msadek, and Hecker, 1998; Helmann et al., 2001). These genes of *Virgibacillus* sp. SK37 were also up-regulated during the NaCl stress condition (Figure 4.6). Many induced intracellular proteinases of *Vrigibacillus* sp. SK37 could play important role of the strain coping with unfavored growth condition. It was in agreement with reports in *B. lichenisformis* and *B. pumilus*, of which the CtsR-regulated genes were induced when the cells were treated with H_2O_2 (Schroeter et al., 2011; Handtke et al., 2014). These results suggested that NaCl stress condition in *Virgibacillus* sp. SK37 would lead to formation of irreversibly damaged proteins, which were required to be degraded by intracellular proteinases.

4.4.2.4 CodY

The up-regulation of *codY* was detected in *Virgibacillus* sp. SK37 during the NaCl stress (Figure 4.6). It was in agreement with *B. subtilis*, of which CodY was up-regulated when the strain propagated at high salinity (Steil et al., 2003). Typically, CodY acted as a transcriptional repressor during rapid growth in nutrient-rich conditions (Sonenshein, 2005). In the presence of the high intracellular concentrations of GTP and branched-chain amino acids (BCAA), the DNA binding activity of CodY was increased by interaction with those two effectors, resulting in the repression of secretion of proteases, expressions of amino acid transporters, catabolic pathway, and biosynthesis of BCAA (Sonenshein, 2005; Handke, Shivers, and Sonenshein, 2008; Lindbäck et al., 2012). Expression of *codY* was low in rich-nutrient medium (containing BCAA) (Belitsky and Sonenshein, 2011). The cultures of this study were prepared in the rich-nutrient medium (Ym). It would be speculated that in the

exponentially growing culture (before exposed to 15% NaCl), the *codY* transcripts of *Virgibacillus* sp. SK37 would be low. After exposure to high salinity, the mRNA levels of *relA*, encoding (p)ppGpp synthetase, was insignificantly changed (Figure 4.6), implying that observed up-regulated *codY* did not respond to a decrease in GTP by RelA activity, by which the stringent response was subjected to encounters a nutrient-limiting condition. It might be conceivable that CodY of the moderate halophile would directly response to the NaCl stress condition. A decrease in mRNA levels of *dppBCDE* (dipeptide permease), *oppBCDF* (oligopeptide ABC transporter), *appDFBC* (oligopeptide ABC transporter), *ykfD* (oligopeptide ABC transporter), *ykfB* (L-alanine-DL-glutamate epimerase), *ykfA* (microcin-resistance protein), *ilvBHCD* (valine/isoleucine biosynthesis), *leuABCD* (leucine biosynthesis), and *yufNOPQ* (unidentified ABC transporter) was detected (Figure 4.6, Table 1S). Those genes in *Bacillus* have been reported to be inhibited their transcriptions by CodY activity (Molle et al, 2003; Lindbäck et al., 2012). In addition, the genes encoding the amino acid transporters (*yflA*, *glnT*, *yecA*, *artP*, and one unidentified gene name) and the di- and tripeptide carrier (*dtpT*) were significantly up-regulated (Figure 4.6, Table 1S). Hence, the conceivable explanation for *codY* up-regulation during the NaCl stress was that the strain would utilize free amino acids or di/tri-peptides rather than oligopeptides, by taking up via the certain transporters. The strain would not allow to expend the limited energy for biosynthesis of proteinases in order to liberate amino acids from protein molecules and the relevant BCAA biosynthesis pathway.

4.4.2.5 Metabolic rearrangement

The NaCl-stress condition caused the metabolic shift in *Virgibacillus* sp. SK37 when exposed to the NaCl stress (Figure 4.7). The transcription of the genes (*citZ*, *icd*, *odhB*, *sucD*, *sucC*, *sdhA*, *sdhB*, *sdhC*, *fumC*, and *mdh*, except *citB*)

belonging to TCA cycle pathway was down-regulated, especially at 15 min of the pre-exposure (Figure 4.7, Table 1S). In addition, the several genes involved in the electron transport chain (ETC) (represented by *yumB* (NADH dehydrogenase, complex I), *cccA, cccB, resA, resB, resC, ccdA, qcrB, qcrC* (cytochrome b-c_1 complex, complex III), *qoxA, qoxB, qoxC, qoxD, ctaE,* unidentified gene names (cytochrome oxidase, complex IV), *atpA, atpB, atpC, atpD, atpE, atpF, atpG, atpH,* and *atpI* (F_1F_0-ATPase, complex V)) were generally down-regulated (Figure 4.7, Table 1S). However, the mRNA levels of the genes involved in the Embden-Meyerhof pathway for glucose assimilation (represented by *pgi, pfkA, fbaA, tpiA, pgk, pgm,* and *pyk,* except *gapA*) were insignificantly changed (Figure 4.7). The genes, encoding pyruvate dehydrogenase (*pdhA, pdhB, and pdhC*), that contributes to transform the pyruvate into acetyl-CoA, were up-regulated after 30 min of the pre-exposure (Figure 4.7, Table 1S). In addition, the respiratory pathway of the strain did not switch to utilize nitrate as an alternative electron acceptor (Figure 4.7). The mRNA levels of genes encoding anaerobic regulator (*fnr*), response regulator (*resD*), histidine kinase (*resE*), nitrite extrusion (*narK*), nitrate reductase (*narG, narH, narJ,* and *narI*), and nitrite reductase were down-regulated (Figure 4.7, Table 1S). Nitrate is reduced by respiratory nitrate reductase to nitrite, which is, in turn, reduced further to ammonia by nitrate reductase (Nakano and Zuber, 1998). The result revealed that during the pre-exposure at 15% NaCl, the metabolic pathways of TCA cycle and ETC were negatively affected, whereas the pyruvate and acetyl-CoA were concurrently formed. It might be expected that, to avoid the formation of by-products, ROS, via electron transport chain and to maintain the redox balance, the strain may use the fermentation pathway to form the end products, such as ethanol and acetate. No significant changes in mRNA levels of the gene involved in the production of acetate (represented by *ackA,* encoding acetate

kinase) from pyruvate were found (Figure 4.7, Table 1S). On the other hand, genes involved in both ethanol fermentation pathway found in yeast (pyruvate decarboxylase gene (PDC) and *adhB* (alcohol dehydrogenase, ADHII)), and in other *Bacillus* (represented by *ydaD* (alcohol dehydrogenase)) were clearly up-regulated (Figure 4.7). It indicated the metabolic shift in *Virgibacillus* sp. SK37 from TCA cycle and oxidative phosphorylation into alcohol fermentation under the NaCl stress condition. The metabolic rerouting to fermentation pathways was observed in *B. cereus* when treated with bactericidal acid stress condition (Mols et al., 2010b).

4.4.2.6 Cell envelope rearrangement

Cell physiology in term of structural integrity of the bacterial cells in high osmolality might be altered. The σ^B-regulated *gtaB*, encoding UDP-glucose pyrophosphorylase, was up-regulated at 15 min of the pre-incubation (Figure 4.7, Table 1S). The enzyme catalyzes the synthesis of UDP-glucose, which considered the precursor for cell wall biosynthesis (Varón, Boylan, Okamoto, and Price, 1993; Ruffing and Chen, 2006). It was also found a significant up-regulation of *sigW* (RNA polymerase sigma-W factor, σ^W) (Figure 4.7), of which its regulated genes might involve in cell wall homeostasis (Cao, Wang, Ye, and Helmann, 2002). The result was in agreement to that in *B. subtilis*, of which σ^W was also clearly induced to be transcribed by salt shock (Hahne et al., 2010; Petersohn et al., 2001). The σ^W-regulated *pbpE*, encoding penicillin-binding protein Pbp4, was up-regulated at 120 min of the pre-exposure (Table 1S). The Pbp4 was expected to play an important role in the cell wall rearrangement (Hahne et al., 2010). In addition, the genes corresponding to cell wall synthesis (represented by *murAA, murB, murC, murD, murE, murF, mraY, ponA, dacA*, and *ddl*) were either slightly or significantly up-regulated during the pre-exposure at 15% NaCl (Figure 4.7, Table 1S). It was understandable, if the

occurrences of cell wall rearrangements upon the NaCl stress condition, because the cell wall provides the cell with structural support and protection. This result was supported by a report of López, Heras, Ruzal, Sánchez-Rivas, and Rivas (1998) who found that cell wall of *B. subtilis* increased in thickness, but less compact when the strain was exposed to NaCl stress condition. In addition, the genes involving in fatty acid biosynthesis (represented by *accA*, *accD*, *fabD*, *fabF*, *fabHA*, *fabG*, *fabZ*, *fabI* *plsX*, *yneS*, *plsC*, *cdsA*, *pgsA*, and *clsA*) were generally up-regulated, especially at 120 min after the pre-exposure (Figure 4.7, Table 1S). It might be expected that the adaptation of *Virgibacillus* sp. SK37 to high osmolality was accomplished by rearrangement of cell membrane. Cellular phospholipid compositions of halotolerant bacterium, *Halomonas elongate*, were modified to contain more negatively charged lipids (cardiolipin) and less neutral species (phosphatidylethanolamine) (Vreeland, Anderson, and Murray, 1984). Likewise, the membrane lipids, cardiolipin and glycoprotein, of the NaCl-stressed *B. subtilis* cells, were increased (López et al., 1998). The *clsA* mutant *B. subtilis* showed the extended lag phase in medium containing 1.5 M NaCl (8.8%) (López, Alice, Heras, Rivas, and Sánchez-Rivas, 2006). The presence of the negatively charged lipids in membrane layers might influence on activation threshold of the OpuA transporter and/or an increase in the uptake of K^+ into cytoplasm via enhanced electrostatic attraction between external cation and cell surface (López et al., 2006).

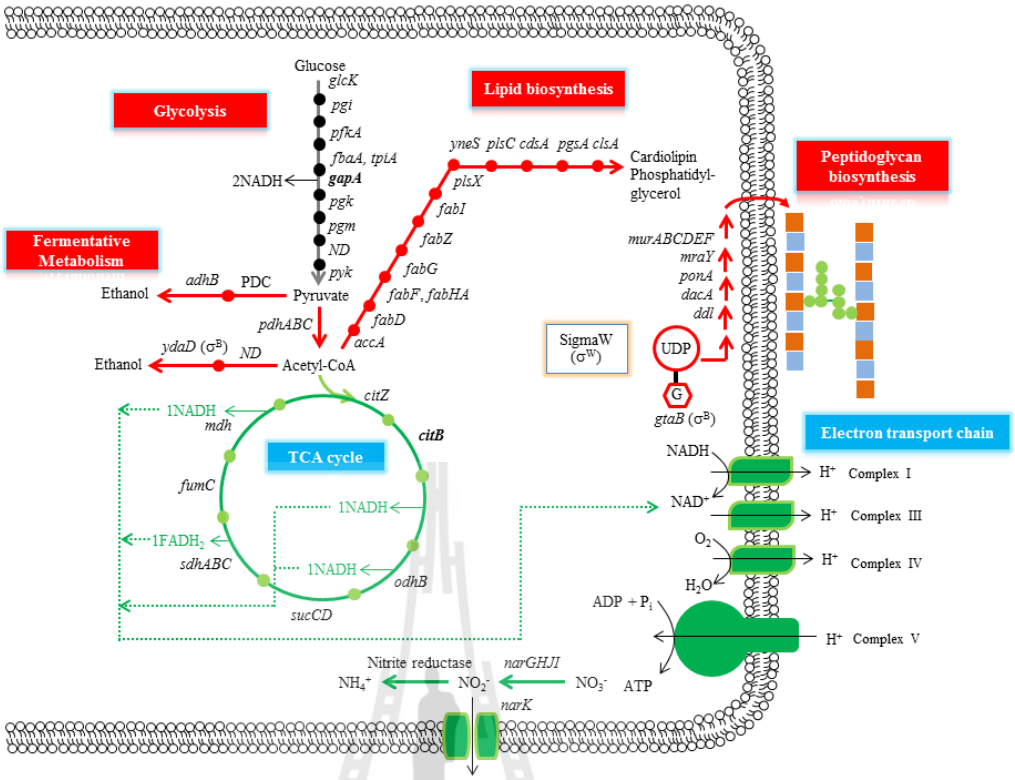

Figure 4.7 Schematic representation of the metabolic and cell envelope rearrangements upon exposure of Virgibacillus sp. SK37 cells to 15% NaCl. Gray line, insignificant changes; Green, down-regulated; Red, up-regulated; ND, Acetaldehyde dehydrogenase (ALDH) gene was undetected in genome annotation of Virgibacillus sp. SK37; (σ^B), regulated by sigma B transcriptional factor as reported by Price et al. (2001)

4.5 Conclusions

The NaCl adaption of the moderate halophile, *Virgibacillus* sp. SK37, was activated by pretreatment with the sublethal condition (15% NaCl), resulting in a cell survival at high salinity (25% NaCl). The uptake of compatible solutes, including glutamate, proline, glycine betaine, and ectoine, from environmental sources would

play an important role in the osmotic response. Free radical was generated during the NaCl stress condition. It induced the oxidative stress response to eliminate an excess of the free radicals. General stress responses were induced under high salinity. Ethanol fermentative pathway was activated under the NaCl stress. Cell membrane and cell wall rearrangements would be a part of the adaptive response strategies of the moderate halophile. For the cellular mechanism arranged during sublethal condition, the *Virgibacillus* sp. SK37 cells were readily exposed and able to survive at high osmolality condition.

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CHAPTER V

SUMMARY

Moderate halophile, *Virgibacillus* sp. SK37, was subjected to optimization for high yield of biomass and NaCl-tolerant proteinase production. Among 7 factors tested, dried anchovy, yeast extract, and initial pH of the medium were significant factors for the production of NaCl-tolerant proteinases. Rotatable central composite design (RCCD) provided an appropriate predicted models. A significant improvement in growth of 1.6 LogCFU/ml increment and a 1.4-fold increase in proteinase production of *Virgibacillus* sp. SK37 was accomplished. The pattern of secreted proteinases from *Virgibacillus* sp. SK37 was greatly affected by NaCl and initial pH. Lys, Arg, and/or Tyr were preferably hydrolyzed by these proteinases secreted by *Virgibacillus* sp. SK37 in the medium containing dried anchovy as a protein substrate.

The strain was able to cope with the challenges of a sudden increase in high NaCl concentration by activating adaption mechanism. The pre-exposure of exponential growing cells at 15% NaCl for 15 min was addressed to activate the NaCl-adaptive response of *Virgibacillus* sp. SK37. Upregulation of osmoprotectant transporter, including Na+/glutamate symporter, OpuA, OpuB, OpuC/D, and OpuE, and activation of an oxidative stress response were noticeable. Metabolic shift from TCA cycle and oxidative phosphorylation to ethanol fermentation pathway, cell envelop rearrangements, and general stress responses were observed. Since *Virgibacillus* sp. SK37 is the first moderate halophile strain to be established a

comprehensive study in NaCl-adaptive response, the linkage of transcriptomes and phenotypic responses would contribute to a better understanding on what this strain possesses the cellular stress adaption strategies to cope in hypersaline ecosystem.

APPENDIX

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl.

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
	NaCl stress response				
	Potassium transportation				
2503218895	BSU14510|ktrC|NP_389334.1|GeneID:939429	K+ transport systems, NAD-binding component	-1.0	-1.0	1.1
2503218926	BSU13500|ktrD|NP_389233.1|GeneID:939342	Trk-type K+ transport systems, membrane components	-2.1	-1.1	1.0
2503217333	BSU06320|yeaB|NP_388513.1|GeneID:936032	cation diffusion facilitator family transporter	1.3	1.2	-1.3
2503219022	BSU31322|yugO|NP_391010.3|GeneID:938842	K+ transport systems, NAD-binding component	1.3	-1.3	-1.2
	Proline/Glutamate uptake, biosynthesis pathway, and degradation				
2503217046	BSU03220|ycgO|NP_388204.2|GeneID:938330, BSU06660|opuE|NP_388548.1|GeneID:939439	sodium/proline symporter	1.2	-1.1	-1.5
2503219603	NA	Na+/proline symporter	1.4	1.3	1.8
2503219236	NA	sodium/proline symporter	4.9	-1.2	-1.1
2503217830	NA	Na+/proline symporter	1.1	2.5	-1.4
2503218924	NA	Na+/proline symporter	-1.5	-1.1	1.9
2503219078	BSU03200|ycgM|NP_388202.1|GeneID:938338, BSU32850|fadM|NP_391164.1|GeneID:937064	Proline dehydrogenase (EC:1.5.99.8)	-1.8	-1.6	-2.2
2503217043	BSU03210|ycgN|NP_388203.2|GeneID:938333, BSU37780|rocA|NP_391658.1|GeneID:937222	delta-1-pyrroline-5-carboxylate dehydrogenase, group 2, putative (EC:1.5.1.12)	-2.7	-5.9	-8.9
2503219556	NA	Proline racemase	-3.2	-3.7	-3.1
2503219557	NA	Proline racemase	-3.4	-1.3	-2.3
2503218963	NA	Na+/glutamate symporter	2.5	1.6	-1.4
2503219892	BSU06590|yerD|NP_388541.1|GeneID:936058	Glutamate synthase domain 2	2.3	1.3	1.1
2503217330	BSU09680|nhaC|NP_388849.1|GeneID:936274	Na+/H+ antiporter NhaC	-1.1	2.2	1.3
2503218962	NA	NhaP-type Na+/H+ and K+/H+ antiporters	2.1	2.1	1.2
2503218213	NA	Na+/H+ antiporter NhaD and related arsenite permeases	1.8	1.5	1.1
2503219056	NA	Na+/H+ antiporter	1.5	1.9	1.2
2503219910	NA	NhaP-type Na+/H+ and K+/H+ antiporters	1.3	2.8	3.0
2503217239	BSU13120|proB|NP_389195.2|GeneID:936790	glutamate 5-kinase (EC:2.7.2.11)	-1.0	-1.1	-1.1
2503217240	BSU13130|proA|NP_389196.2|GeneID:936166	gamma-glutamyl phosphate reductase (EC:1.2.1.41)	-1.1	1.4	1.2
2503216347	BSU23800|proI|NP_390261.1|GeneID:938697	pyrroline-5-carboxylate reductase (EC:1.5.1.2)	-1.1	-1.3	1.7

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
	Glycine betaine, Ectoine, and Choline uptake				
	opuA				
2503217356	BSU02980|opuAA|NP_388180.2|GeneID:938362	glycine betaine/L-proline transport ATP binding subunit (EC:3.6.3.32)	1.4	1.1	-1.3
2503217357	BSU02990|opuAB|NP_388181.1|GeneID:938360, BSU33820|opuCB|NP_391262.1|GeneID:937105, BSU33720|opuBB|NP_391252.1|GeneID:936225	ABC-type proline/glycine betaine transport system, permease component	1.6	1.1	-2.4
2503217359	BSU03000|opuAC|NP_388182.1|GeneID:938354	ABC-type proline/glycine betaine transport systems, periplasmic components	2.3	1.3	-2.3
	opuD				
2503216990	BSU30070|opuD|NP_390885.1|GeneID:937986	choline/carnitine/betaine transport	4.5	2.8	1.9
2503219345	BSU30070|opuD|NP_390885.1|GeneID:937986	choline/carnitine/betaine transport	1.7	3.1	3.1
2503216757	NA	Choline-glycine betaine transporter	2.0	4.5	4.5
2503217903	NA	Choline-glycine betaine transporter	1.7	1.4	1.2
	opuC				
2503218624	BSU33710|opuBC|NP_391251.1|GeneID:938475, BSU33810|opuCC|NP_391261.1|GeneID:936248	Periplasmic glycine betaine/choline-binding (lipo) protein of an ABC-type transport system (osmoprotectant binding protein)	3.4	1.5	-2.0
2503218625	BSU33730|opuBA|NP_391253.1|GeneID:936230, BSU33830|opuCA|NP_391263.1|GeneID:937136	glycine betaine/L-proline transport ATP binding subunit	2.7	1.2	1.2
	opuB				
2503218980	BSU33700|opuBD|NP_391250.1|GeneID:936226, BSU33800|opuCD|NP_391260.1|GeneID:937199	ABC-type proline/glycine betaine transport systems, permease component	-1.0	1.1	-1.2
2503218981	BSU33710|opuBC|NP_391251.1|GeneID:938475, BSU33810|opuCC|NP_391261.1|GeneID:936248	Periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transport system (osmoprotectant binding protein)	1.0	-1.1	1.1
2503218983	BSU33730|opuBA|NP_391253.1|GeneID:936230, BSU33830|opuCA|NP_391263.1|GeneID:937136	glycine betaine/L-proline transport ATP binding subunit	1.2	-1.0	-1.3
2503220241	BSU31060|gbsA|NP_390984.1|GeneID:938829	Alcohol dehydrogenase (EC:1.2.1.8)	1.5	1.0	1.2

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
	Biosynthesis of glycine betaine				
2503219515	BSU31050|gbsB|NP_390983.2|GeneID:938832	Alcohol dehydrogenase, class IV (EC:1.1.1.1)	1.0	1.1	1.2
	Biosynthesis of ectoine/hydroxyectoine				
2503218978	NA	L-2,4-diaminobutyric acid acetyltransferase (EC:2.3.1.178)	1.1	1.2	-3.8
2503218976	NA	Ectoine synthase. (EC:4.2.1.108)	-1.0	-1.0	-2.9
2503219922	NA	Ectoine hydroxylase (EC:1.14.11.-)	6.9	8.3	1.1
	Oxidative stress response				
	perR				
2503217140	BSU08730|perR|NP_388753.1|GeneID:939227	Fe^{2+}/Zn^{2+} uptake regulation proteins	3.0	2.4	-1.2
2503216934	BSU08820|katA|NP_388762.2|GeneID:939240	Catalase	12.9	9.2	1.3
2503218900	BSU40090|ahpC|NP_391889.1|GeneID:938147, BSU14220|ykuU|NP_389305.1|GeneID:938810	Peroxiredoxin	1.1	1.5	3.4
2503218173	BSU28170|hemA|NP_390695.1|GeneID:937443	Glutamyl-tRNA reductase	1.3	1.5	-1.6
2503218174	BSU28160|hemX|NP_390694.2|GeneID:937489	ABC-type transport system involved in cytochrome c biogenesis, permease component	-1.6	1.1	1.5
2503218175	BSU28150|hemC|NP_390693.1|GeneID:937488	Porphobilinogen deaminase	-1.1	1.2	-1.1
2503218176	BSU28140|hemD|NP_390692.1|GeneID:937287	Uroporphyrinogen-III synthase	-1.1	-1.2	-1.0
2503218177	BSU28130|hemB|NP_390691.1|GeneID:936972	Delta-aminolevulinic acid dehydratase	-1.2	-1.2	-1.4
2503218178	BSU28120|hemL|NP_390690.1|GeneID:937490	Glutamate-1-semialdehyde-2,1-aminomutase	1.0	-1.1	-1.1
2503217101	BSU13850|zosA|NP_389268.1|GeneID:939268, BSU33490|copB|NP_391229.2|GeneID:936034	Heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating P-type ATPase	9.2	4.7	-1.2
2503218468	BSU23520|fur|NP_390233.2|GeneID:938727	Fe^{2+}/Zn^{2+} uptake regulation proteins (Transcriptional repressor of iron uptake)	3.0	2.0	1.0
2503220189	BSU33320|fhuD|NP_391213.1|GeneID:935996, BSU39610|yxeB|NP_391840.2|GeneID:937581	ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component (Siderophore uptake)	1.3	-1.1	1.8

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503220191	BSU33310|fhuB|NP_391212.1|GeneID:937085	ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component (Siderophore uptake)	-1.1	-1.7	-1.3
2503220188	BSU33290|fhuC|NP_391210.1|GeneID:935994	ABC-type cobalamin/Fe^{3+}-siderophores transport systems, ATPase components (Siderophore uptake)	-1.4	-1.2	1.2
2503220192	BSU33300|fhuG|NP_391211.1|GeneID:938619	ABC-type Fe^{3+}-siderophore transport system, permease component (Siderophore uptake)	-1.2	1.0	-1.6
2503220301	BSU37230|ywjA|NP_391604.1|GeneID:937049	ABC-type multidrug transport system, ATPase and permease components	-1.1	-1.1	-1.1
2503216555	BSU37220|ywjB|NP_391603.1|GeneID:938455	ABC-type transport system, ATPase and permease components Dihydrofolate reductase	1.3	-1.5	-1.3
2503217923	BSU07500|yfmE|NP_388631.1|GeneID:939692	ABC-type Fe^{3+} -siderophore transport system, permease component (Siderophore uptake)	-1.8	-1.3	1.2
2503216835	BSU03800|yclN|NP_388262.1|GeneID:938273	ferrichrome ABC transporter (permease) (Siderophore uptake)	-1.1	-1.1	-1.0
2503219008	BSU32940|yusV|NP_391173.1|GeneID:935921	ABC-type cobalamin/Fe^{3+}-siderophores transport systems, ATPase components (Siderophore uptake)	-1.0	-1.2	-1.1
2503216810	BSU08480|yfhC|NP_388729.1|GeneID:939221	Nitroreductase	-1.0	1.1	1.7
2503216720	BSU03310|nasC|NP_388213.2|GeneID:938321	Nitrate reductase (catalytic subunit)	-1.0	-1.2	1.1
2503217442	BSU11500|spxA|NP_389032.1|GeneID:936407	conserved hypothetical protein (EC:1.20.4.1)	1.5	1.3	1.1
2503218147	BSU28500|trxA|NP_390728.1|GeneID:938187	thioredoxin	2.0	3.1	2.2
2503219143	BSU34790|trxB|NP_391359.1|GeneID:936549	thioredoxin-disulfide reductase (EC:1.8.1.9)	1.3	2.4	1.5
2503218059	BSU29490|tpx|NP_390827.1|GeneID:937688	Peroxiredoxin (EC:1.11.1.15)	1.2	-1.3	-1.4
2503217017	BSU21690|msrA|NP_390052.1|GeneID:939099	methionine-S-sulfoxide reductase (EC:1.8.4.11)	1.3	1.6	-1.4

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503219933	BSU31360|yugK|NP_391014.1|GeneID:938844, BSU31370|yugJ|NP_391015.1|GeneID:937164	Uncharacterized oxidoreductases, Fe-dependent alcohol dehydrogenase family	1.1	1.3	-1.0
	other				
2503217447	BSU11550|yjbH|NP_389037.2|GeneID:939365	Predicted dithiol-disulfide isomerase involved in polyketide biosynthesis	-1.1	-1.7	-1.1
	iron-sulfur cluster proteins				
2503217293	BSU08910|yhbA|NP_388772.2|GeneID:939243	iron-sulfur cluster binding protein, putative	1.0	-1.1	1.2
2503219038	BSU32160|yutM|NP_391096.1|GeneID:936627	Iron-sulfur cluster assembly accessory protein	-1.0	-1.2	1.1
2503218914	NA	iron-sulfur cluster repair di-iron protein	-1.1	-1.2	-3.8
2503218781	BSU15860|sdaAA|NP_389468.1|GeneID:937109	L-serine dehydratase, iron-sulfur-dependent, alpha subunit (EC:4.3.1.17)	1.4	-1.0	-1.2
2503218152	BSU28430|sdhB|NP_390721.1|GeneID:937460	succinate dehydrogenase and fumarate reductase iron-sulfur protein (EC:1.3.99.1)	1.2	-1.1	1.1
2503218782	BSU15850|sdaAB|NP_389467.1|GeneID:935964	L-serine dehydratase, iron-sulfur-dependent, beta subunit (EC:4.3.1.17)	1.1	-1.3	1.3
	Mn^{2+}				
2503218410	BSU24520|mntR|NP_390332.2|GeneID:938554	Mn-dependent transcriptional regulator	1.1	2.3	1.6
2503219105	BSU30770|mntA|NP_390955.1|GeneID:937219	ABC-type metal ion transport system, periplasmic component/surface adhesin	-1.4	-1.5	-1.9
2503219107	BSU30760|mntB|NP_390954.1|GeneID:937196	ABC-type Mn/Zn transport systems, ATPase component	-1.8	-1.5	-4.0
2503219106	BSU30740|mntD|NP_390952.1|GeneID:937149	ABC-type Mn2+/Zn2+ transport systems, permease components	-1.8	-2.2	-11.5
	ohr				
2503218801	BSU13140|ohrA|NP_389197.1|GeneID:939848, BSU13160|ohrB|NP_389199.1|GeneID:936734	Peroxiredoxin, Ohr subfamily	16.4	12.1	-2.7
	General stress response				
	Class I heat-shock proteins				
2503218278	BSU25490|hrcA|NP_390427.1|GeneID:937843	Heat shock gene repressor HrcA	-1.1	1.6	2.0
2503218280	BSU25470|dnaK|NP_390425.1|GeneID:937849	Chaperone protein DnaK	1.1	1.0	1.1
2503218279	BSU25480|grpE|NP_390426.1|GeneID:937846	Molecular chaperone GrpE (heat shock protein)	-1.2	-1.4	1.0

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503216964	BSU06020|groES|NP_388483.2|GeneID:938006	Co-chaperonin GroES (HSP10)	1.3	-1.1	1.3
2503216965	BSU06030|groEL|NP_388484.1|GeneID:938045	chaperonin GroL	1.1	1.3	1.3
	Class II: sigma B-dependent regulation				
2503216931	BSU04730|sigB|NP_388354.2|GeneID:939937	RNA polymerase sigma-B factor	5.8	4.0	1.3
2503216929	BSU04710|rsbV|NP_388352.1|GeneID:939930	anti-anti-sigma factor	2.6	1.1	-1.3
2503216930	BSU04720|rsbW|NP_388353.1|GeneID:938167	serine-protein kinase RsbW (EC:2.7.11.1)	2.6	1.6	-1.4
2503216358	BSU07850|yfkM|NP_388666.1|GeneID:938808, BSU27020|yraA|NP_390580.2|GeneID:937603	intracellular protease, PfpI family (EC:3.2.-)	12.4	6.5	-2.0
2503218654	BSU30650|dps|NP_390943.1|GeneID:937211	DNA-binding ferritin-like protein (oxidative damage protectant)	-2.2	1.7	1.8
2503220050	BSU04220|ydaG|NP_388303.1|GeneID:939958	Uncharacterized stress protein (general stress protein 26)	1.3	1.1	-1.2
2503217008	BSU04400|gsiB|NP_388321.1|GeneID:938235	Small hydrophilic plant seed protein.	2.4	2.4	-3.1
2503219997	BSU39050|katE|NP_391784.2|GeneID:937481	Catalase (EC:1.11.1.6)	3.3	2.6	1.1
2503216736	BSU38630|katX|NP_391742.1|GeneID:937399	Catalase (EC:1.11.1.6)	-1.4	-1.1	-1.6
2503218325	BSU25020|sodA|NP_390381.3|GeneID:938052	Superoxide dismutase (EC:1.15.1.1)	-1.1	-1.0	-1.1
2503219995	BSU07750|yflA|NP_388656.1|GeneID:939686	amino acid carrier protein	1.8	2.6	-2.0
	Class III: CtsR regulon				
2503216432	BSU00830|ctsR|NP_387964.1|GeneID:936883	Transcriptional repressor of class III stress genes	-1.4	1.6	1.0
2503216433	BSU00840|mcsA|NP_387965.1|GeneID:936845	Uncharacterized protein with conserved CXXC pairs	1.3	1.1	-1.5
2503216434	BSU00850|mcsB|NP_387966.1|GeneID:936939	Arginine kinase	-1.1	1.1	-1.4
2503219137	BSU34540|clpP|NP_391334.1|GeneID:938625	ATP-dependent Clp protease, proteolytic subunit ClpP (EC:3.4.21.92)	1.6	3.1	1.1
2503216435	BSU00860|clpC|NP_387967.1|GeneID:938481	ATPases with chaperone activity, ATP-binding subunit	1.4	2.5	1.6
2503217111	BSU13700|clpE|NP_389253.1|GeneID:939289	ATPases with chaperone activity, ATP-binding subunit	5.6	9.2	1.2
	Class U				
2503218165	BSU28220|clpX|NP_390700.2|GeneID:937482	Endopeptidase Clp ATP-binding regulatory subunit (clpX)	2.0	2.9	2.1
2503218166	BSU28200|lonA|NP_390698.1|GeneID:937486	ATP-dependent protease La (EC:3.4.21.53)	1.9	1.5	1.6

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

enes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
2503216413	BSU00690|ftsH|NP_387950.1|GeneID:938094	ATP-dependent metalloprotease FtsH (EC:3.4.24.-)	2.4	3.3	4.1
	CodY				
2503218749	BSU16170|codY|NP_389499.1|GeneID:936491	GTP-sensing transcriptional pleiotropic repressor CodY	2.5	2.3	2.4
2503218222	BSU27600|relA|NP_390638.2|GeneID:936753	(p)ppGpp synthetase, RelA/SpoT family (EC:2.7.6.5)	-1.4	1.2	1.2
2503219818	BSU12930|dppB|NP_389176.1|GeneID:939881	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-1.1	-1.3	-1.1
2503219817	BSU12940|dppC|NP_389177.1|GeneID:938038	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-1.0	-1.1	-1.2
2503219816	BSU12950|dppD|NP_389178.1|GeneID:938628	oligopeptide/dipeptide ABC transporter, ATP-binding protein, C-terminal domain	-1.1	-1.1	-1.2
2503219815	BSU12960|dppE|NP_389179.2|GeneID:938099	ABC-type oligopeptide transport system, periplasmic component	-1.7	-3.5	-2.8
2503219945	BSU11440|oppB|NP_389026.1|GeneID:936397	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-1.8	-2.2	-1.0
2503219944	BSU11450|oppC|NP_389027.1|GeneID:936396	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-1.9	-1.7	-1.2
2503219943	BSU11460|oppD|NP_389028.1|GeneID:939814	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	-1.4	-1.6	-1.4
2503219942	BSU11470|oppF|NP_389029.1|GeneID:936410	ATPase components of various ABC-type transport systems, contain duplicated ATPase	-1.1	1.1	1.2
2503219282	BSU11390|appB|NP_389021.2|GeneID:936400	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-1.3	1.2	-1.0
2503219549	BSU11390|appB|NP_389021.2|GeneID:936400	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	1.1	-1.3	-1.2
2503219568	BSU11400|appC|NP_389022.1|GeneID:936394	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	1.1	-1.9	-1.9
2503219548	BSU11400|appC|NP_389022.1|GeneID:936394	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	1.1	-2.6	-1.9

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503219566	BSU11370|appF|NP_389019.2|GeneID:936390	oligopeptide/dipeptide ABC transporter, ATP-binding protein, C-terminal domain	1.5	-1.2	-1.0
2503219551	BSU11370|appF|NP_389019.2|GeneID:936390	oligopeptide/dipeptide ABC transporter, ATP-binding protein, C-terminal domain	1.4	-1.2	1.2
2503219567	BSU11360|appD|NP_389018.1|GeneID:936391	oligopeptide/dipeptide ABC transporter, ATP-binding protein, C-terminal domain	1.1	1.1	-1.1
2503219552	BSU11360|appD|NP_389018.1|GeneID:936391	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	1.1	1.1	1.1
2503219811	BSU13000|ykfD|NP_389183.2|GeneID:939863	oligopeptide/dipeptide ABC transporter, ATP-binding protein, C-terminal domain	-1.3	-1.0	1.0
2503219814	BSU12970|ykfA|NP_389180.2|GeneID:938175	Uncharacterized proteins, homologs of microcin C7 resistance protein MccF (EC:3.4.17.13)	-1.1	1.1	-1.1
2503219813	BSU12980|ykfB|NP_389181.1|GeneID:939862	L-alanine-DL-glutamate epimerase and related enzymes of enolase superfamily	1.1	-1.2	-1.1
2503218677	BSU31540|yufN|NP_391032.2|GeneID:938870	Uncharacterized ABC-type transport system, periplasmic component/surface lipoprotein	1.1	-1.1	1.4
2503218676	BSU31550|yufO|NP_391033.1|GeneID:937172	ABC-type uncharacterized transport systems, ATPase components (EC:3.6.3.17)	1.1	1.2	1.0
2503218675	BSU31560|yufP|NP_391034.1|GeneID:937175	ABC-type uncharacterized transport system, permease component	-1.1	1.1	-1.0
2503218674	BSU31570|yufQ|NP_391035.1|GeneID:937176	Uncharacterized ABC-type transport system, permease component	1.1	1.5	-1.2
2503219302	BSU28310|ilvB|NP_390709.1|GeneID:936792	acetolactate synthase, large subunit, biosynthetic type (EC:2.2.1.6)	-1.2	-1.2	-2.2
2503219301	BSU28300|ilvH|NP_390708.2|GeneID:937477	acetolactate synthase, small subunit (EC:2.2.1.6)	1.1	-1.9	-1.3
2503219300	BSU28290|ilvC|NP_390707.1|GeneID:937475	ketol-acid reductoisomerase (EC:1.1.1.86)	-1.4	-2.7	-2.1
2503219303	BSU21870|ilvD|NP_390070.2|GeneID:939084	dihydroxy-acid dehydratase (EC:4.2.1.9)	-1.1	-1.4	-1.4

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503219299	BSU28280|leuA|NP_390706.1|GeneID:936316	2-isopropylmalate synthase, bacterial type (EC:2.3.3.13)	-2.5	-1.9	-2.7
2503219298	BSU28270|leuB|NP_390705.2|GeneID:936221	3-isopropylmalate dehydrogenase (EC:1.1.1.85)	-1.4	-1.5	-1.1
2503219297	BSU28260|leuC|NP_390704.1|GeneID:937478	3-isopropylmalate dehydratase, large subunit (EC:4.2.1.35,EC:4.2.1.33)	-1.4	-1.5	-3.0
2503219296	BSU28250|leuD|NP_390703.1|GeneID:937683	3-isopropylmalate dehydratase, small subunit (EC:4.2.1.35,EC:4.2.1.33)	-1.5	-1.7	-1. 9
	Amino acid transporter				
2503219995	BSU07750|yflA|NP_388656.1|GeneID:939686	amino acid carrier protein	1.8	2.6	-2.0
2503219914	BSU02420|glnT|NP_388124.2|GeneID:938419, BSU18120|alsT|NP_389694.1|GeneID:938191	amino acid carrier protein	2.2	1.2	-1.3
2503217782	BSU06550|yecA|NP_388537.2|GeneID:936051	Amino acid transporters	1.8	1.2	-1.1
2503216869	BSU23980|artP|NP_390278.1|GeneID:938682	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	1.0	1.4	3.0
2503219774	NA	Amino acid transporters	6.0	1.7	-1.1
	Peptide transporter				
2503218002	BSU03670|dtpT|NP_388249.2|GeneID:938295	amino acid/peptide transporter (Peptide:H+ symporter), bacterial	1.0	1.8	3.1
	Metabolic rearrangement				
2503218337	BSU24850|glcK|NP_390365.2|GeneID:938206	ROK family protein (putative glucokinase) (EC:2.7.1.2)	2.6	1.1	1.4
	Glycolysis				
2503219026	BSU31350|pgi|NP_391013.2|GeneID:937165	Glucose-6-phosphate isomerase (EC:5.3.1.9)	1.5	-1.1	1.3
2503218094	BSU29190|pfkA|NP_390797.1|GeneID:937376	6-phosphofructokinase (EC:2.7.1.11)	1.0	-1.1	-1.2
2503219857	BSU37120|fbaA|NP_391593.1|GeneID:937040	fructose-1,6-bisphosphate aldolase, class II, various bacterial and amitochondriate protest (EC:4.1.2.13)	1.1	-1.2	-1.0
2503219117	BSU33920|tpiA|NP_391272.1|GeneID:938626	triosephosphate isomerase (EC:5.3.1.1)	-1.0	1.1	1.1
2503219119	BSU33940|gapA|NP_391274.1|GeneID:938627	glyceraldehyde-3-phosphate dehydrogenase, type I (EC:1.2.1.12,EC:1.2.1.12)	4.2	6.2	1.6
2503219118	BSU33930|pgk|NP_391273.1|GeneID:938572	3-phosphoglycerate kinase (EC:2.7.2.3)	-1.3	1.1	1.6

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
2503219116	BSU33910|pgm|NP_391271.1|GeneID:938574	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC:5.4.2.1)	1.1	1.6	1.0
2503218095	BSU29180|pyk|NP_390796.1|GeneID:936596	pyruvate kinase (EC:2.7.1.40)	1.3	1.1	-1.0
	Acetyl CoA				
2503218888	BSU14580|pdhA|NP_389341.1|GeneID:936005	pyruvate dehydrogenase E1 component, alpha subunit (EC:1.2.4.1)	1.6	1.9	1.5
2503218887	BSU14590|pdhB|NP_389342.1|GeneID:939496	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, beta subunit (EC:1.2.4.1)	-1.0	1.2	1.4
2503218886	BSU14600|pdhC|NP_389343.1|GeneID:936010	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide acyltransferase (E2) component, and related enzymes (EC:2.3.1.12)	1.2	1.3	1.5
	TCA cycle				
2503218098	BSU29140|citZ|NP_390792.1|GeneID:937381	2-methylcitrate synthase/citrate synthase II (EC:2.3.3.1)	-1.1	-1.1	1.4
2503216783	NA	aconitate hydratase, putative, Aquifex type (EC:4.2.1.3)	-1.9	-2.6	-3.1
2503218633	BSU18000|citB|NP_389683.1|GeneID:938140	aconitate hydratase 1 (EC:4.2.1.3)	1.8	3.2	5.6
2503218099	BSU29130|icd|NP_390791.1|GeneID:938183	isocitrate dehydrogenase, NADP-dependent, prokaryotic type (EC:1.1.1.42,EC:1.1.1.41)	-1.8	1.1	1.4
2503217300	BSU19360|odhB|NP_389818.2|GeneID:939505	2-oxoglutarate dehydrogenase complex dihydrolipoamide succinyltransferase (E2 component) (EC:2.3.1.61)	-4.0	-1.4	1.1
2503218757	BSU16090|sucC|NP_389491.1|GeneID:938521	Succinyl-CoA synthetase, beta subunit (EC:6.2.1.5)	-2.4	-1.6	3.1
2503218756	BSU16100|sucD|NP_389492.1|GeneID:937797	Succinyl-CoA synthetase, alpha subunit (EC:6.2.1.4,EC:6.2.1.5)	-1.3	1.1	1.8
2503217337	BSU33040|fumC|NP_391184.1|GeneID:938591	fumarate hydratase, class II (EC:4.2.1.2)	-1.0	1.1	1.3
2503218100	BSU29120|mdh|NP_390790.1|GeneID:937385	malate dehydrogenase, NAD-dependent (EC:1.1.1.37)	-1.5	-1.3	-1.0
	Electron transport chain (ETC)				
	NADH dehydrogenase(Complex I)				

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
2503218956	BSU32100|yumB|NP_391090.1|GeneID:937062	NADH dehydrogenase, FAD-containing subunit (EC:1.6.99.3)	-1.2	1.3	1.5
	Cytochrome bc1 complex (Complex III)				
2503218309	BSU25190|cccA|NP_390398.1|GeneID:937902	Cytochrome c, mono- and diheme variants	1.3	1.0	1.4
2503219166	BSU35270|cccB|NP_391407.1|GeneID:936703	Cytochrome c, mono- and diheme variants	1.0	-1.5	-5.6
2503218492	BSU23150|resA|NP_390196.2|GeneID:938958	Peroxiredoxin	-1.1	1.2	-1.7
2503218493	BSU23140|resB|NP_390195.1|GeneID:938961	ResB protein required for cytochrome c biosynthesis	-1.2	1.0	-1.0
2503218494	BSU23130|resC|NP_390194.2|GeneID:938962	cytochrome c-type biogenesis protein CcsB	-1.3	1.1	1.2
2503218639	BSU17930|ccdA|NP_389676.1|GeneID:937140	Cytochrome c biogenesis protein	-1.3	1.4	-1.4
2503218543	BSU22550|qcrB|NP_390136.1|GeneID:939019	Cytochrome b subunit of the bc complex	-1.1	1.0	1.9
2503218544	BSU22540|qcrC|NP_390135.1|GeneID:939021	Cytochrome b subunit of the bc complex	-1.0	1.1	2.3
	Cytochrome oxidase (Complex IV)				
2503217060	BSU38170|qoxA|NP_391696.2|GeneID:937295	cytochrome aa3 quinol oxidase, subunit II/cytochrome c oxidase, subunit II (EC:1.10.3.12)	1.1	1.3	1.0
2503217059	BSU38160|qoxB|NP_391695.1|GeneID:937303	cytochrome aa3 quinol oxidase, subunit I (EC:1.10.3.12)	1.2	-2.3	-2.0
2503217058	BSU38150|qoxC|NP_391694.1|GeneID:937299	cytochrome aa3 quinol oxidase, subunit III (EC:1.10.3.12,EC:1.10.3.-)	1.2	-2.0	-2.3
2503217057	BSU38140|qoxD|NP_391693.1|GeneID:937285	cytochrome aa3 quinol oxidase, subunit IV (EC:1.10.3.12)	1.1	-2.3	-2.4
2503217056	NA	Prokaryotic Cytochrome C oxidase subunit IV.	1.4	-1.3	-1.6
2503219349	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 1 (EC:1.9.3.1)	1.5	1.1	-1.3
2503218577	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 1 (EC:1.9.3.1)	-12.4	-16.5	-16.7
2503217390	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 1 (EC:1.9.3.1)	-1.0	-1.1	-2.6
2503217389	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 2 (EC:1.9.3.1)	1.2	-1.2	-7.1
2503218578	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 2 (EC:1.9.3.1)	-2.8	-3.9	-11.7

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
2503217389	NA	Heme/copper-type cytochrome/quinol oxidases, subunit 2 (EC:1.9.3.1)	1.2	-1.2	-7.1
2503219350	NA	Heme/copper-type cytochrome/quinol oxidase, subunit 3 (EC:1.9.3.1)	2.0	1.7	1.1
2503218861	BSU14910|ctaE|NP_389374.1|GeneID:935993	Heme/copper-type cytochrome/quinol oxidase, subunit 3 (EC:1.9.3.1)	-1.1	1.3	3.8
	F1F0-ATPase (Complex V)				
2503219829	BSU36830|atpA|NP_391564.1|GeneID:936995	Proton translocating ATP synthase, F1 alpha subunit (EC:3.6.3.14)	-2.4	-1.1	2.8
2503219833	BSU36870|atpB|NP_391568.1|GeneID:937004	F0F1-type ATP synthase, subunit a (EC:3.6.3.14)	-1.2	1.3	1.8
2503219826	BSU36800|atpC|NP_391561.1|GeneID:936994	ATP synthase, F1 epsilon subunit (delta in mitochondria) (EC:3.6.3.14)	-2.0	-1.1	1.4
2503219827	BSU36810|atpD|NP_391562.1|GeneID:936992	ATP synthase, F1 beta subunit (EC:3.6.3.14)	-2.0	-1.1	1.4
2503219832	BSU36860|atpE|NP_391567.1|GeneID:936999	ATP synthase, F0 subunit c (EC:3.6.3.14)	-1.1	-1.3	1.2
2503219831	BSU36850|atpF|NP_391566.1|GeneID:936998	ATP synthase, F0 subunit b (EC:3.6.3.14)	-1.6	1.1	2.4
2503219828	BSU36820|atpG|NP_391563.1|GeneID:936989	ATP synthase, F1 gamma subunit (EC:3.6.3.14)	-2.1	-1.1	2.7
2503219830	BSU36840|atpH|NP_391565.1|GeneID:936997	ATP synthase, F1 delta subunit (EC:3.6.3.14)	-1.9	-1.1	1.6
2503219834	BSU36880|atpI|NP_391569.1|GeneID:937003	ATP synthase I chain.	-1.2	1.0	2.3
	Nitrite reduction				
2503217421	BSU37310|fnr|NP_391612.1|GeneID:937050	cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases	-1.8	2.4	-1.6
2503218495	BSU23120|resD|NP_390193.2|GeneID:938960	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain	-1.0	1.2	-1.2
2503218496	BSU23110|resE|NP_390192.1|GeneID:938965	Signal transduction histidine kinase (EC:2.7.13.3)	1.0	-1.1	-1.1
2503216726	BSU37320|narK|NP_391613.2|GeneID:937055	PAS domain S-box	-1.3	-1.5	-1.5
2503218603	BSU37280|narG|NP_391609.2|GeneID:938358	respiratory nitrate reductase, alpha subunit (EC:1.7.99.4)	-3.7	-9.0	-12.4
2503218604	BSU37270|narH|NP_391608.1|GeneID:937047	nitrate reductase, beta subunit (EC:1.7.99.4)	-11.9	-17.0	-33.9
2503218605	BSU37260|narJ|NP_391607.1|GeneID:937051	nitrate reductase molybdenum cofactor assembly chaperone (EC:1.7.99.4)	-4.1	-5.2	-6.8

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name	LogFC		
			t_{15min}	t_{30min}	T_{120min}
2503218606	BSU37250|narI|NP_391606.1|GeneID:938454	respiratory nitrate reductase, gamma subunit (EC:1.7.99.4)	-2.4	-1.3	-4.4
2503216722	NA	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	1.0	-1.3	-1.3
	Acetate production				
2503218061	BSU29470|ackA|NP_390825.1|GeneID:937347	acetate kinase (EC:2.7.2.1)	-1.2	-1.4	1.6
	Ethanol fermentation via heterologous pathway				
2503217670	NA	Pyruvate decarboxylase and related thiamine pyrophosphate-requiring enzymes (EC:4.1.1.74,EC:4.1.1.1)	8.7	3.9	-1.1
2503216771	BSU26970|adhB|NP_390574.2|GeneID:937604	Threonine dehydrogenase and related Zn-dependent dehydrogenases (EC:1.1.1.1,EC:1.1.1.284)	1.5	2.4	-1.1
	Ethanol fermentation via Embden Meyerhof pathway				
2503220299	BSU04190|ydaD|NP_388300.1|GeneID:938251, BSU10400|yhxC|NP_388921.1|GeneID:939316, BSU09450|yhdF|NP_388826.1|GeneID:939749	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	1.2	1.1	-1.8
2503217404	BSU04190|ydaD|NP_388300.1|GeneID:938251, BSU10400|yhxC|NP_388921.1|GeneID:939316, BSU09450|yhdF|NP_388826.1|GeneID:939749	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	1.4	2.0	-1.3
	Cell envelope rearrangement				
	Cell wall				
2503219686	BSU35670|gtaB|NP_391447.1|GeneID:936797	UTP-glucose-1-phosphate uridylyltransferase (EC:2.7.7.9)	1.6	-1.4	1.2
2503216571	BSU01730|sigW|NP_388054.1|GeneID:938868	RNA polymerase sigma-W factor	2.4	1.6	-1.1
2503217699	BSU34440|pbpE|NP_391324.1|GeneID:938615	Beta-lactamase class C and other penicillin binding proteins (EC:3.4.11.-,EC:3.4.16.4)	-1.7	-1.1	1.6
2503219822	BSU36760|murAA|NP_391557.1|GeneID:936980	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC:2.5.1.7)	3.2	2.8	2.1
2503216853	BSU15230|murB|NP_389406.1|GeneID:939804	UDP-N-acetylenolpyruvoylglucosamine reductase (EC:1.1.1.158)	-1.1	1.3	-1.2

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name (Bacillus equivalent)	Product name		LogFC	
			t_{15min}	t_{30min}	T_{120min}
2503218035	BSU29790|murC|NP_390857.1|GeneID:937311	UDP-N-acetylmuramate--alanine ligase (EC:6.3.2.8)	-1.1	1.3	-1.8
2503218830	BSU15200|murD|NP_389403.1|GeneID:935950	UDP-N-acetylmuramoylalanine--D-glutamate ligase (EC:6.3.2.9)	1.2	-1.3	1.1
2503218833	BSU15180|murE|NP_389401.1|GeneID:937567	UDP-N-acetylmuramyl-tripeptide synthetases (EC:6.3.2.13)	-1.0	1.2	1.3
2503218832	BSU04570|murF|NP_388338.1|GeneID:939947	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (EC:6.3.2.10)	1.0	1.2	1.5
2503218831	BSU15190|mraY|NP_389402.2|GeneID:939856	phospho-N-acetylmuramoyl-pentapeptide-transferase (EC:2.7.8.13)	1.2	2.4	4.0
2503217061	BSU22320|ponA|NP_390113.1|GeneID:939044	penicillin-binding protein, 1A family (EC:2.4.1.- ,EC:3.4.-)	1.3	-1.3	-1.1
2503218565	BSU22320|ponA|NP_390113.1|GeneID:939044	penicillin-binding protein, 1A family (EC:3.4.- ,EC:2.4.1.-)	1.2	1.3	-1.2
2503216337	BSU00100|dacA|NP_387891.1|GeneID:940000	D-alanyl-D-alanine carboxypeptidase (EC:3.4.16.4)	1.2	-1.5	2.6
2503216808	BSU04560|ddl|NP_388337.1|GeneID:938224	D-alanine--D-alanine ligase (EC:6.3.2.4)	1.4	1.0	-1.1
	Fatty acid				
2503218093	BSU29200|accA|NP_390798.1|GeneID:936367	acetyl-CoA carboxylase, carboxyl transferase, alpha subunit (EC:6.4.1.2)	1.4	1.0	1.8
2503218092	BSU29210|accD|NP_390799.2|GeneID:936186	acetyl-CoA carboxylase, carboxyl transferase, beta subunit (EC:6.4.1.2)	-1.4	1.1	3.3
2503218777	BSU15900|fabD|NP_389472.1|GeneID:938488	malonyl CoA-acyl carrier protein transacylase (EC:2.3.1.39)	1.3	-1.1	1.8
2503217428	BSU11340|fabF|NP_389016.1|GeneID:939803	3-oxoacyl-[acyl-carrier-protein] synthase 2 (EC:2.3.1.179)	1.3	1.1	1.9
2503217427	BSU11330|fabHA|NP_389015.1|GeneID:936392	3-oxoacyl-(acyl-carrier-protein) synthase III (EC:2.3.1.180)	-1.2	1.1	1.1
2503218776	BSU15910|fabG|NP_389473.1|GeneID:938113	3-oxoacyl-(acyl-carrier-protein) reductase (EC:1.1.1.100)	-1.1	1.6	3.8
2503218957	BSU18500|fabG|NP_389732.1|GeneID:940090	Short-chain alcohol dehydrogenase of unknown specificity	-1.9	1.4	1.2

Table 1S The gene transcripts of *Virgibacillus* sp. SK37 associated with NaCl stress condition at 15% NaCl (Continued).

Genes	Gene name	Product name		LogFC	
	(Bacillus equivalent)		t_{15min}	t_{30min}	T_{120min}
2503219799	BSU36370|fabZ|NP_391518.2|GeneID:936918	beta-hydroxyacyl-[acyl carrier protein] dehydratase FabZ (EC:4.2.1.-)	1.1	1.1	-1.3
2503216557	BSU11720|fabI|NP_389054.2|GeneID:939379	Enoyl-[acyl-carrier-protein] reductase (NADH) (EC:1.3.1.9)	1.2	-1.6	1.2
2503218778	BSU15890|plsX|NP_389471.1|GeneID:938066	fatty acid/phospholipid synthesis protein PlsX (EC:2.3.1.15)	-1.1	-1.8	1.5
2503218626	BSU18070|yneS|NP_389689.1|GeneID:938037	acyl-phosphate glycerol-3-phosphate acyltransferase (EC:2.3.1.15)	1.2	-1.4	1.2
2503218513	BSU09540|plsC|NP_388835.1|GeneID:936269	1-acyl-sn-glycerol-3-phosphate acyltransferases (EC:2.3.1.51)	1.0	-1.2	1.0
2503218707	BSU16540|cdsA|NP_389536.1|GeneID:939607	CDP-diglyceride synthetase (EC:2.7.7.41)	1.1	-1.1	1.6
2503218667	BSU16920|pgsA|NP_389574.2|GeneID:939675	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase (EC:2.7.8.5)	1.1	1.3	1.3
2503219916	BSU36590|clsA|NP_391540.1|GeneID:936957	Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthases and related enzymes (EC:2.7.8.-)	1.0	1.5	-1.2

CURRICULUM VITAE

Sornchai Sinsuwan was born in November 19, 1979 in Phichit, Thailand. In 1998, he got his high school diploma (M. 6) from Phichitpitayakom School. In 2002, he received Bachelor's degree in Food Technology from Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. In 2002-2003, he worked as a research assistant at the School of Food Technology, SUT. He has co-published the article entitled "Aggregation and conformational changes of tilapia actomyosin as affected by calcium ion during setting" in Food Hydrocolloids (2007, Vol. 21, P. 359-367). Then, he received scholarship from the National Center for Genetic Engineering and Biotechnology (BIOTEC), to study for a Master degree at SUT. In 2006, he graduated with the Master of Science (Food technology). In 2008, he received the Strategic Scholarships for Frontier Research Network from the Office of the Higher Education Commission to study Ph.D. in Food Technology at SUT.