EXPRESSION AND ACTIVITY ANALYSES OF INDUSTRIALLY IMPORTANT EXTRACELLULAR ENZYMES PRODUCED BY A BACILYSIN KNOCK-OUT MUTANT OF *BACILLUS SUBTILIS*

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ABSTRACT

EXPRESSION AND ACTIVITY ANALYSES OF INDUSTRIALLY IMPORTANT EXTRACELLULAR ENZYMES PRODUCED BY A BACILYSIN KNOCK-OUT MUTANT OF *BACILLUS SUBTILIS*

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Bacilysin, the smallest peptide antibiotic known to date, is produced non-ribosomally by *Bacillus subtilis* by the collective actions of seven proteins transcribed from *bacABCDEF* operon and *bacG* gene. Bacilysin is a two-amino acid peptide composed of L-alanine and a modified amino acid, L-anticapsin. Bacilysin biosynthesis was shown to be strongly regulated by quorum sensing through the actions of global regulator proteins including Spo0K, Spo0H, Spo0A, ComQ/ComX, ComP/ComA as well as several Phr proteins, OppA, CodY, SrfA and AbrB. Involvement of large numbers of regulatory proteins in bacilysin biosynthesis raises the question of whether the bacilysin itself affects physiological processes its own producer cells. Comparative secretome analysis between *B. subtilis* PY79 and its *bac* operon-silenced derivative OGU1, recently performed by our group, has identified more than 200 proteins differentially expressed in the mutant strain. Since *B. subtilis* is one of the most important cell-factories with a capacity to produce a wide range of extracellular enzymes, of biotechnological interest was a significant increment in levels of the industrially-important extracellular enzymes upon the disruption of *bac* operon. These enzymes included arabinanase, chitosanase, levanase, phytase, lipase, bacillopeptidase F, minor extracellular protease and endonuclease. This study aims to compare the

expression profiles of the respective *abn2, csn, sacC, phy, estA, bpr, vpr and YhcR* genes as well as the enzymatic activities of their products between two strains. Both approaches showed a significant increase of these eight selected proteins in the OGU1 strain, suggesting a possible direct or indirect effect of the bacilysin on their biosynthesis.

Keywords: *B. subtilis*, bacilysin, gene expression, hydrolytic enzyme activity, extracellular enzymes

ÖZ

BASİLİSİN BİYOSENTEZİ SUSTURULMUŞ *BACILLUS SUBTILIS* MUTANTI TARAFINDAN ÜRETİLEN ENDÜSTRİYEL ÖNEME SAHİP ENZİMLERİN GEN İFADESİ VE AKTİVİTE ANALİZLERİ

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Bilinen en küçük peptit antibiyotiği olan basilisin, *Bacillus subtilis* tarafından *bacABCDEF* operonu ve *bacG* geni tarafından üretilen yedi proteinin ortak çalışması ile sentezlenir. Basilisin L-alanin ve L-antikapsinden oluşan bir dipeptitdir. Basilisin biyosentezinin Spo0K, Spo0H, Spo0A, ComQ/ComX ve ComP/ComA proteinlerinin yanı sıra çeşitli Phr proteinleri, OppA, CodY, SrfA ve AbrB gibi regülatör proteinler vasıtasıyla çoğunluğu algılama mekanizması ile kontrol edildiği grubumuz tarafından gösterilmiştir. Bu denli çok regülatör proteinin basilisin biyosentezine etki edişi, basilisinin kendi üretici organizmanın fizyolojik aktiviteleri üzerindeki etkilerinin ne derecede olduğu sorusunu doğurmaktadır. Grubumuzca gerçekleştirilen karşılaştırmalı sekretom analizi çalışmaları *B. subtilis* PY79 ve onun *bac*-operonu susturulmuş türevi OGU1 arasında 200 kadar proteinin değişken ifade edildiğini göstermiştir. *B. subtilis*'in yüksek miktarlarda enzim üretme yeteneğine sahip en önemli organizmalardan biri oluşu göz önüne alındığında, *bac* operonunun bozulmasının endüstriyel öneme sahip hücre dışı enzimler üzerinde anlamlı bir artışa sebep olması oldukça önemlidir. Mutant suşta anlamlı artış gösteren bu enzimler arabinanaz, kitosanaz, levanaz, fitaz, lipaz, basilopeptidaz F, minör ekstraselüler proteaz ve endonukleaz şeklinde sıralanabilir. Bu çalışmanın

amacı, söz konusu enzimleri üreten *abn2, csn, sacC, phy, lipA, bpr, vpr* ve *yhcR* genlerinin önce ifade değerlerinin, ardındansa ortaya çıkan proteinlerin enzimatik aktivitelerinin *B. subtilis* PY79 ve OGU1 suşları arasında karşılaştırılmasıdır. Her iki yaklaşım OGU1 suşunda bu sekiz enzimin anlamlı olarak arttığını göstermiş olup, bu durum basilisinin doğrudan ya da dolaylı olarak bu proteinlerin biyosentezine etki ettiğini göstermektedir.

Keywords: *B. subtilis*, basilisin, gen ifadesi, hidrolitik enzim aktivitesi, ekstraselüler enzimler

To Vincent and Salvador,

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TABLE OF CONTENTS

ABSTRACT v
ÖZ vii
TABLE OF CONTENTS xii
LIST OF TABLES xv
LIST OF FIGURES xvi
CHAPTERS 1
1. INTRODUCTION 1
1.1 General Properties of *Bacillus subtilis* 1
1.1.1 *Bacillus subtilis* as a Model Organism 1
1.1.2 Nonribosomal Peptide Synthesis in *Bacillus subtilis* 3
1.1.3 Bioactive Peptides Synthesized by the Genus *Bacillus* 5
1.1.4 Global Regulation of Gene Expression in *Bacillus subtilis* 7
1.1.5 Sporulation in *Bacillus subtilis* 12
1.1.6 Swarming and Biofilm Formation in *Bacillus subtilis* 16
1.1.7 Secretion System in *Bacillus subtilis* 16
1.2 Dipeptide Antibiotic Bacilysin 19
1.2.1 Biosynthesis of Bacilysin 19
1.2.2 Regulation of Bacilysin Biosynthesis 21
1.2.3 Comparative Proteome Analysis between Wild Type *Bacillus subtilis* and Its Bacilysin Non-producer Strain 23
1.3 Extracellular Enzymes of *Bacillus subtilis* 26
1.3.1 Endoarabinanase 26
1.3.2 Chitosanase 29
1.3.3 Levanase 31
1.3.4 Phytase 33
1.3.5 Lipase 37
1.3.6 Protease 38

1.3.7 Endonuclease 40
1.4 Quantification of the Gene Expression by qRT-PCR 40
1.5 The Aim of the Present Study 43
2. MATERIALS AND METHODS 45
2.1 Bacterial Strains and Their Growth and Maintenance 45
2.2 Culture Media 46
2.3 Buffers and Solutions 46
2.4 Chemicals and Materials 46
2.5 Enzyme Activity Assays 46
2.5.1 Arabinanase Activity Assay 46
2.5.2 Chitosanase Activity Assay 47
2.5.3 Levanase Activity Assay 48
2.5.4 Phytase Activity Assay 49
2.5.5 Lipase Activity Assay 50
2.5.6 Protease Activity Assay 51
2.5.7 Endonuclease Assay 52
2.5.8 Statistical Analysis of Enzyme Activities 52
2.6 RNA Procedures 53
2.6.1 Isolation of RNA Samples 53
2.6.2 cDNA synthesis by Reverse Transcription PCR 53
2.6.3 Real Time Quantitative PCR 54
2.6.4 RT-qPCR Analysis 55
2.6.5 Statistical analysis 55
3. RESULTS AND DISCUSSION 57
3.1 Results of Enzymatic Activity Assays 57
3.1.1 Endoarabinanase activities of *Bacillus subtilis* PY79 and OGU1 ... 58
3.1.2 Chitosanase activities of *Bacillus subtilis* PY79 and OGU1 strains 61
3.1.3 Levanase Activities of *Bacillus subtilis* PY79 and OGU1 63
3.1.4 Phytase activities of *Bacillus subtilis* PY79 and OGU1 strains 67
3.1.5 Lipase activities of *Bacillus subtilis* PY79 and OGU1 71

3.1.6 Protease activities of *Bacillus subtilis* PY79 and OGU1 74
3.1.7 Endonuclease activities of *Bacillus subtilis* PY79 and OGU1 76
3.2 Comparison of the expression profiles of eight genes between *Bacillus subtilis* PY79 and OGU1 strains 78
3.2.1 Amplification efficiency calculation on the basis of standard curve formation 78
3.2.2. Expression analysis of the selected genes by quantitative PCR..... 80
3.2.3 Quantification of the RT-qPCR Data 84
4. CONCLUSION 89
REFERENCES 91
APPENDICES 119
A. COMPOSITIONS OF THE CULTURE MEDIA 119
B. BUFFERS AND SOLUTIONS 121
C. CHEMICALS AND MATERIALS 125

LIST OF TABLES

TABLES

Table 1. 1 Non-ribosomally synthesized antibiotics in *Bacillus* species 6

Table 1. 2 *Bacillus subtilis* lantibiotics 7

Table 1. 3 List of extracellular enzymes found to be overrepresented in OGU1 strain. 25

Table 2. 1 Primers designed for RT-qPCR analysis..... 55

Table 3. 1 Amplification efficiencies of the each gene of interest and the reference gene *rpoB* 78

Table 3. 2 C_q and T_m values of the amplification plots and melting curves..... 80

LIST OF FIGURES

FIGURES

Figure 1. 1 Regulatory pathways controlling the biosynthesis of *B. subtilis* antibiotics……9
Figure 1. 2 Transcriptional regulatory network of the sporulation process in *B. subtilis*……15
Figure 1. 3 Organisation of *bacABCDEF* polycistronic operon and *bacG* monocistronic gene……20
Figure 1. 4 Detailed presentation of the recently hypothesized bacilysin biosynthetic pathway……20
Figure 1. 5 Schematic structure of maize bran heteroxylan……..27
Figure 1. 6 Primary structure of arabinan……27
Figure 1. 7 General structure of chitin and chitosan……30
Figure 1. 8 General structure of levan and inulin……..32
Figure 1. 9 Enzymatic hydrolysis of myo-inositol-hexaphosphate (phytate) by the phytase activity……35
Figure 2. 1 Calibration curve for endoarabinanase assay……..47
Figure 2. 2 Calibration curve for chitosanase assay……..48
Figure 2. 3 Calibration curve for levanase assay……...49
Figure 2. 4 Calibration curve for phytase assay……50
Figure 2. 5 Calibration curve for lipase assay……...51
Figure 2. 6 Calibration curve for protease assay……...52
Figure 3. 1 Endoarabinanase activities of *B. subtilis* PY79 and OGU1 culture supernatants……...58
Figure 3. 2 Chitosanase activities of *B. subtilis* PY79 and OGU1 culture supernatants……...61
Figure 3. 3 Levanase activities of *B. subtilis* PY79 and OGU1 culture supernatants……...63
Figure 3. 4 Phytase activities of *B. subtilis* PY79 and OGU1 culture supernatants……...67

Figure 3. 5 Lipolytic activities of *B. subtilis* PY79 and OGU1 culture supernatants. 71
Figure 3. 6 Proteolytic activities of *B. subtilis* PY79 and OGU1 culture supernatants……...74
Figure 3. 7 Endonuclease activities of *B. subtilis* PY79 and OGU1 culture supernatants……...76
Figure 3. 8 Standard curves for genes of interest *abn2, csn, sacC, phy, lipA, bpr, vpr, yhcR* and the reference gene *rpoB* 80
Figure 3. 9 Amplification plots and melting curves of a) *abn2*, b) *csn* and c) *sacC*. 81
Figure 3. 10 Amplification plots and melting curves of d) *phy*, e) *lipA* and f) *bpr*. 82
Figure 3. 11 Amplification plots and melting curves of g) *vpr*, h) *yhcR* and the reference gene i) *rpoB*. 83
Figure 3. 12 Quantification of *abn2, csn, sacC, phy, lipA, bpr, vpr* and *yhcR* gene expressions 84

CHAPTER 1

INTRODUCTION

1.1 General Properties of *Bacillus subtilis*

1.1.1 *Bacillus subtilis* as a Model Organism

Bacillus subtilis is the model organism for gram-positive bacteria and it is extensively studied for bacterial regulation, differentiation, metabolic pathways, as well as quorum sensing and sporulation analysis. It has been already proved to be one of the best cellular factories with a capability to produce large amounts of antibiotics, secondary metabolites, and industrial enzymes (Sonenshein et al., 2002). Although it is well accepted that *Escherichia coli* is the best established model organism for molecular studies, *B. subtilis* can be more advantageous in several perspectives. Unlike *E. coli*, in which the secondary metabolites and hydrophobic products aggregate quickly to form inclusion bodies inside the cell, *B. subtilis* secretes most of the cloned gene products and secondary metabolites directly to the cell culture supernatant (Zweers et al., 2008). This important feature makes *B. subtilis* a perfect organism to be used in the purification processes in biotechnological and industrial applications.

The natural habitat of *B. subtilis* is soil and water, where it is mostly found associated with plants. It is additionally found in the human gut microbiota and considered to be one of the important probiotics. *B. subtilis* is a chemoorganotroph bacterium which requires the oxidation of organic material for its growth. *B. subtilis* can form regular sized colonies within 16 hours when supplemented with necessary nutrients and sufficient aeration at 37ºC.

Nevertheless, *B. subtilis* is a facultative anaerobe organism and it can also grow in the absence of oxygen. Another striking and industrially important feature of *B. subtilis* is its ability to display different metabolic activities depending on the availability of oxygen or alternative electron acceptors. This alteration is carried out by the two component signal transduction system called ResDE, which is composed of one response regulator, ResD, and its sensor kinase, ResE. Under the shortage of oxygen, ResDE induces the expression of *fnr* gene, whose product, FNR, is a transcriptional activator which, in turn, induces the expression of anaerobic respiration genes. One important operon induced by FNR is *narGHJI*, which encodes a nitrate reductase, which is the main enzyme of nitrate respiration in the absence of oxygen. It should also be noted that *B. subtilis* uses the pyruvate dehydrogenase enzyme to metabolize pyruvate, therefore, it does not require external electron acceptors. On the other hand, many other anaerobic bacteria use another enzyme, called pyruvate formate lyase, which requires electron acceptor input for its action (Nakano and Zuber, 1998). By making use of the fact that *B. subtilis* alters its metabolic pathways with the environmental conditions, it is possible to choose the right conditions for a wide variety of industrial applications in order to get the metabolic product of interest with the maximum efficiency.

In the case of nutritional starvation, *B. subtilis* cease growing and employs different metabolic strategies to cope with the situation; these include the production of antibiotics to eliminate other competitors around, the production

of proteases and hydrolases to increase nutrient uptake and the induction of motility and chemotaxis to find a better environment for survival. If neither of these responses can help, one of the most striking features of *Bacillus subtilis* takes place - induction of sporulation. During sporulation, single cell *B. subtilis* compartmentalize into mother and forespore cells, each with the entire copy of genomic material but different regulation and expression profiles. These differences result in the engulfment of the forespore and programmed death of the mother cell, with the subsequent release of the mature spore (Stragier and Losick, 1996).

The whole genome sequence of *B. subtilis* was published in 1997 (Kunst et al., 1997) and shown to contain 4106 protein-coding genes as well as 86 tRNA, 30 rRNA, and 3 small RNA genes (Ando, 2002; Kobayashi et al., 2003). The genome of *B. subtilis* was found to be 4.2 Mb (Franguel et al., 1999) and it encodes 275 functional genes to be able to grow in rich medium. *B. subtilis* genome also contains 17 sigma factors and more than 250 transcriptional regulators (Kobayashi et al., 2003).

B. subtilis 168 is a tryptophan-requiring autotrophic strain and one of the most commonly used laboratory organisms for molecular biology studies (Harwood et al., 1990). The original strain used in this thesis is *B. subtilis* PY79, a prototrophic wild-type derivative of *B. subtilis* 168. Whole-genome sequencing of *B. subtilis* PY79 has also been completed and revealed 4278 features with 4140 coding regions, 86 tRNA genes and 30 rRNA genes (Schroeder & Simmons, 2013).

1.1.2 Nonribosomal Peptide Synthesis in *Bacillus subtilis*

These bioactive peptide compounds are mainly produced by two distinct cellular mechanisms. Ribosomal synthesis accounts for the production of

multicyclic lantibiotics. In this mechanism, gene-encoded peptide precursors are translated ribosomally and modified, if necessary, by posttranslational processes (Schnell et al., 1988; Zuber et al, 1992). Non-ribosomal synthesis, on the other hand, accounts for the production of a large number of cyclic and linear peptides, which are important for therapeutic or industrial applications (Weber and Marahiel, 2001). In this mechanism, the synthesis is protein-template directed and it is independent of nucleic acids and ribosomes. Pharmaceutically useful peptides like penicillin, vancomycin, cyclosporine A, epothilone as well as other antiviral and antitumor compounds are mostly produced as secondary antibiotics via large multifunctional enzymes (Mootz and Marahiel, 1999; Schwarzer et al., 2002; Sieber et al., 2002).

Secondary metabolites can vary in their structure and modifications. Their peptide chains may be linear, cyclic or branched and they can undergo modifications such as fatty acid attachment to N-terminus, methylation, formylation, glycosylation, and phosphorylation. Furthermore, nonribosomally synthesized compounds may contain many building blocks other than common amino acids, demonstrating their difference from those generated through the ribosomal pathway (Grünewald and Marahiel, 2006).

In the nonribosomal synthesis model, the amino acid substrates are first converted to acyl adenylate on a multienzyme called peptide synthetase (NRPS) by ATP hydrolysis. This intermediate is then transferred to an enzyme bound 4'-phosphobirşey cofactor, where the elongation of amino acid sequence takes place. NRPS is composed of repetitive units called modules, the number of which matches the number of amino acids in the growing peptide chain, thus making these modules protein templates (Kallow et al., 2002).

Among these modules, three have been shown to be essential; an adenylation domain is required for the activation of aminoacyl by ATP hydrolysis, a

peptidyl carrier domain is required for the binding of 4-PP cofactor, on which the acyl adenylate is covalently attached and lastly, a condensation domain is required to catalyse the formation of peptide bond between each newly added amino acid and the previous one (Konz and Marahiel, 1999; Schwarzer and Marahiel, 2001).

1.1.3 Bioactive Peptides Synthesized by the Genus *Bacillus*

Among the biologically important compounds, microbially produced peptides constitute one of the largest and most important groups for their applications in bio-industry and molecular biology research. Particularly, microbial antibiotics are broadly used in a wide range of applications as immunosuppressors, antimicrobials, antitumor agents, cytostatic drugs and as promoters of animal and plant growth (Devine, 1995; Boman, 1995; Boman, 1996; Gill et al., 1996).

B. subtilis is one of the most important antibiotic-producing organisms with the ability to synthesize at least two dozen antimicrobial peptides. These antibiotics stand out with their hydrophobic rigid structures with unusual D-amino acid constituents, their ability to withstand peptidase/protease hydrolysis and their insensitivity to oxidation, due to disulfide residues and intramolecular C-S (thioether) linkages. Antibiotics and antimicrobial agents produced by *B. subtilis* can be synthesized both ribosomally (lantibiotics) and non-ribosomally. These agents have already been shown to have distinct properties beyond sole antimicrobial activity. Depending on their structure and chemical properties, *B. subtilis* antibiotics can function in programmed cell death, swarming motility, quorum sensing, and biofilm formation (Stein, 2005).

Table 1. 1 Non-ribosomally synthesized antibiotics in *Bacillus* species (Mannanov and Sattarova, 2001)

Peptide	Organism	Structure
Bacilysin	*B. subtilis*	Linear
Edeine	*B. brevis Vm4*	Linear
Gramicidin	*B. brevis*	Linear
Iturin	*B. subtilis*	Cyclopeptide
Gramicidin S	*B. braves* ATCC 9999	Cyclopeptide
Tyrocidine	*B. brevis* ATCC 8185	Cyclopeptide
Mycobacillin	*B. subtilis*	Cyclopeptide
Surfactin	*B. subtilis*	Lacton
Polymyxin	*B. polymyxa*	Polypeptide
Bacitracin	*B. licheniformis*	Polypeptide
Bacilysocin	*B. polymyxa*	Phospholipid

Lantibiotics (***lant***hathionine containing antib***iotics***) contain characteristic lanthionine or methyllanthionine, which are polycyclic thioether amino acids with antimicrobial functions or pheromone-like properties (Sahl and Bierbaum, 1998). These molecules can exert their functions through the formation of pores (mercasidin, nisin), the inhibition of phospholipases, the prevention of peptidoglycan biosynthesis (cinnamycin) as well as by eliciting other secondary effects on the susceptible cell (Klein et al., 1993; Engelke et al., 1994; Altena et al., 2000). For example, lantibiotics can increase autolytic enzyme production, cause cell wall degradation or inhibit spore outgrowth (Liu and Hansen, 1993; Chan et al., 1996). Together with their antibiotic actions, lantibiotics can also serve as pheromones, which are signal molecules that contribute to cell-cell communications, especially during quorum sensing.

Table 1. 2 *Bacillus subtilis* lantibiotics (Mannanov and Sattarova, 2001)

Lantibiotic	MW (Da)	# of aminoacids	Total Charges	Properties
Pep 5	3488	34	7	Elongated, helical
Nisin	3353	34	3	cationic, amphiphilic
Subtilin	3317	32	2	energy-dependent
Epidermin	2164	32	3	membrane
Gallidermin	2164	22	3	pore formers
Mersacidin	1825	20	0	Amphiphilic
Astagardin	1890	19	-1	hydrophobic

1.1.4 Global Regulation of Gene Expression in *Bacillus subtilis*

Gene expression and development in *B. subtilis* is mostly mediated by cell to cell signalling. One of these signaling pathways is called quorum sensing, a regulatory response that is induced by a high density of surrounding cells (Fugua et al., 1994). Almost all of the characteristic features of *B. subtilis* - including the virulence, genetic material exchange, competence, cellular death and production of bioactive components like antibiotics - are regulated by this mechanism (Solomon et al., 1996). The activators and regulators of quorum sensing come with a large variety: large and small peptides, single amino acids, acyl homoserine lactones, and chemicals.

Although quorum sensing regulatory pathways differ significantly from organism to organism, they can be categorized into two main paradigms. The first type of quorum sensing is the LuxI/LuxR system, in which bacteria respond by producing type I autoinducers or N-acyl-L-homoserine lactone inducers; this system generally takes part in the regulation of Gram-negative bacteria. The second mechanism used by quorum sensing is a two

component/oligopeptide circuit; is mostly seen in gram-positive bacteria and it results in autoinducer peptide production (Lazazzera et al., 1997; Perego, 1997; Fuqua and Greenberg, 2002).

The transduction of the signal in two-component quorum sensing is mainly composed of one histidine protein kinase that will bind to a specific signal to phosphorylate itself on a histidine residue and one response regulator to which this phosphate group is transferred. Upon activation by this phosphorylation, the response regulator is able to exert its function - mostly as a transcriptional regulator. Up- and downregulation of specific genes, in turn, allows cells to respond to specific signals and help their adaption to the changing environmental conditions (Hoch and Silhavy, 1995).

Three known peptide signals (or pheromones) - ComX, Subtilin and Phr peptides - take place in the initiation of quorum sensing in *B. subtilis*. ComX is a 5 to 10 amino acid modified peptide that binds its receptor extracellularly. Subtilin and similar lantibiotic peptides also interact with their receptors extracellularly (Magnuson et al., 1994; Piazza et al., 1999; Tortosa et al., 2001). Phr peptides are encoded as pro-Phr and exported from the cell to be cleaved outside and form mature pentapeptides. Internalization of Phr peptides occurs via oligopeptide permease (Opp) complex which is an ATP-binding cassette transporter (Perego et al., 1991; Rudner et al., 1991). These peptides interact and inhibit their corresponding Rap proteins after they are internalized by the cell (Lazazzera, 2001; Perego and Brannigan, 2001; Stein, 2005). Phr peptides are transcribed from an operon which also contains their target Rap-encoding gene; each Phr peptide is responsible for the activity inhibition of their respective and co-transcribed Rap proteins (Perego and Hoch, 1996; Jiang et al., 2000; Ogura et al., 2003; Hayashi, 2006). As an exception to this rule, competence and sporulation stimulating factor (CSF, also known as PhrC) is also able to inhibit the activity of RapB protein even though it is not coupled to

the CSF encoding operon (Perego, 1997). There are 11 Rap proteins and 8 Phr peptides identified in *B. subtilis* to date.

CSF (PhrC) peptide and ComX pheromone are important representatives of intracellular and extracellular signaling mechanisms, respectively. These peptide pheromones accumulate during exponential phase and are involved in the regulation of genetic competence, production of antibiotics and degradative enzymes, as well as several other important processes (Lazazzera and Grossman, 1998).

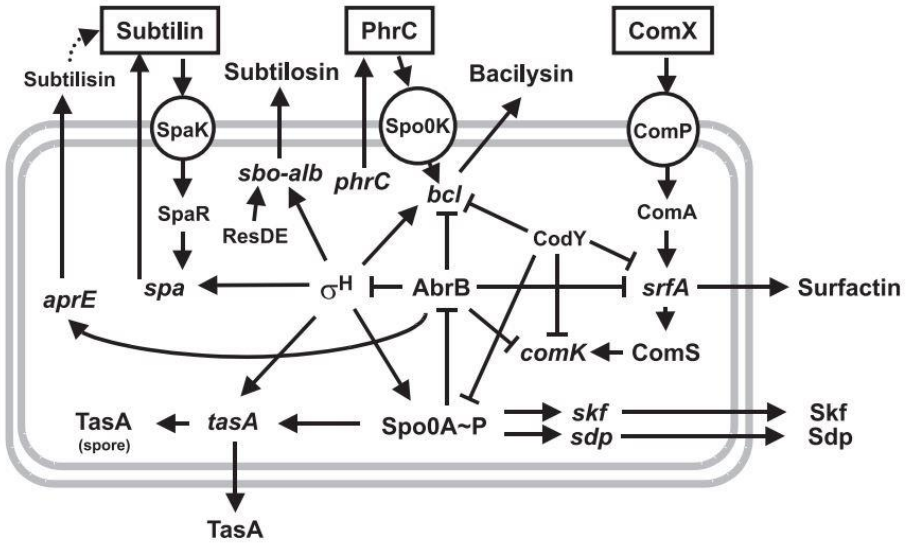


Figure 1. 1 Regulatory pathways controlling the biosynthesis of *B. subtilis* antibiotics. Regulation of the synthesis of TasA, Sdp, Skf, surfactin, subtilosin, subtilin and bacilysin is illustrated (Stein, 2005). Arrows and T-bars represent the positive and negative regulations, respectively.

ComX is an example of tryptophanmodified peptides with 10 amino acid residues and this pheromone requires the activity of both *comQ* and *comX* genes (Magnuson et al., 1994). ComQ, produced from the *comQ* gene located just upstream of the *comX* gene, is thought to take part in processing/modification steps to produce the active ComX peptide, as suggested by the fact that *comQ* null mutants cannot produce mature ComX. On the other hand, the 55 amino acid ComX precursor is encoded by *comX* gene; and its function is to increase the activity of ComP, which is a histidine kinase receptor bound to the membrane (Lazazzera et al., 1999). The modification of ComX is important to increase its hydrophobicity and its local concentration at the membrane, where it can act as a ligand and bind to ComP. As previously mentioned for histidine kinases, ComP activates itself by autophosphorylation and transfers this phosphate group to its conjugate response regulator, ComA. Activated ComA, in turn, turns on the expression of important genes, e.g. *comS* (*srfA*) whose product is necessary for the development of competence (Lazazera and Grossman, 1998; Core and Perego, 2003). The activity of ComA is also increased indirectly by the 5-amino acid unmodified peptide PhrC (CSF) through the inhibition of RapC activity (Solomon et al., 1996). Not only PhrC but also PhrF and PhrK peptides have been shown to be required for full expression of ComA regulated genes. Just like other Phr peptides, PhrF and PhrK also function as the inhibitors of their respective co-transcribed proteins RapF and RapK (Bongiorni et al., 2005; Auchtung et al., 2006).

Compared to ComX, the regulatory function of CSF is more complex and concentration-dependent. When the concentration of CSF outside of the cell is relatively low (1-5 nM), it stimulates ComA activity through the inhibition of RapC (Solomon et al., 1996). On the other hand, when its concentration reaches a certain level (>20 nM), CSF is shown to inhibit the activity of ComP histidine kinase, which in turn results in decreased activity of ComA

(Lazazzera et al., 1997). Besides the ComA regulatory functions, CSF, when in high concentrations, also takes part in the stimulation of sporulation through the inhibition of another aspartyl-phosphate phosphatase, RapB (Pereggo, 1997). RapB is normally responsible for the dephosphorylation of Spo0F-P, one of the most important components of the phosphotransfer pathway, in which the phosphate group is transferred to Spo0A for the initiation of sporulation (Grossman, 1995; Hoch, 1993). Moreover, CSF has also been shown to stimulate competence during mid-exponential phase, while inhibiting the expression of competence genes during stationary phase by the inhibition of ComS activity (Mirel et al., 2000; Stephens, 1998).

Spo0H and Spo0A require more attention as they encode response regulators of alternative sigma factor, σ^H, and multicomponent phosphorelay, respectively (Dubnau et al., 1988; Burbulys et al., 1991). Two response regulators, Spo0F and Spo0B (also called phosphorelay proteins) and five histidine kinases (KinA to KinE) take part in multicomponent phosphorelay signal transduction system that initiates the sporulation (Perego and Hoch, 2002). This phosphorelay pathway ends with the phosphorylation of Spo0A, the key transcriptional regulator of sporulation (Sonenshein, 2000). Phosphorylated Spo0A increases the expression of its own gene, *spo0A*, but the transcription of this gene is also activated by *sigH* expression. The latter encodes the sigma factor H that directs its conjugate RNA polymerase to recognize an alternative promoter upstream of *spo0A* and also activates the transcription of *kinA* and *spo0F*, whose products are involved in the phosphorylation of *spo0A* (Predich et al., 1992). Furthermore, σ^H also activates the expression of *spoIIA* operon that encodes another sporulation-specific sigma factor, σ^F.

Activated Spo0A activates or represses the expression of target genes by binding a specific DNA sequence called 0A box (Strauch et al., 1992). Although it exerts its role mainly in the onset of sporulation, Spo0A directly or

indirectly affects the expression of many other stationary phase genes. There are more than 520 genes influenced by Spo0A activity; this illustrates the importance of this protein in the global regulatory system of *B. subtilis* (Fawcett et al., 2000; Liu et al., 2003). 121 out of 520 genes are directly controlled by Spo0A and most of these proteins are themselves transcriptional regulators (Molle et al., 2003).

Another role of Spo0A which is worth mentioning is its ability to repress the transcription of the *abrB* gene (Robertson et al., 1989). This gene normally encodes the AbrB transcriptional regulator, which decreases the expression of several stationary phase genes, including *abrB* itself, *kinA* and *sigH*, keeping Spo0A dephosphorylated. Spo0A alleviates this AbrB-related gene expression when cells enter stationary phase; *sigH* and *kinA* expression increases and the phosphorylation of more Spo0A is achieved following the activation of spore-related genes and initiation of sporulation (Strauch, 1995).

Even though the phosphorelay signaling pathway of sporulation is by far one of the most important regulatory pathways, *B. subtilis* is characterized with more than 35 two-component regulatory systems.

1.1.5 Sporulation in *Bacillus subtilis*

B. subtilis sporulation is a multiple stage developmental process responsible for the transformation of the vegetative cell into a dormant cell called endospore (Stragier and Losick, 1996; Piggot and Losick, 2002). The cell that started sporulation is called sporangium and it is divided into two different sized chambers by a septum that is positioned asymmetrically. Forespore is the smaller chamber and it is the forespore destined to form mature endospore, while the mother cell is the larger chamber responsible for the nurture of the forespore. These two new cells have very distinct fates and regulations.

In later stages of sporulation, the septum moves toward the forespore and engulf this smaller compartment. This structure with two membrane layers is called protoplast and it is surrounded entirely by the mother cell. The cortex and the germ cell wall, which are specialized peptidoglycan structures, assemble in the space between two membranes of protoplast. These structures are eventually surrounded by the protective shell of the endospore, called coat layer. After the formation of the coat shell, the mother cell lyses and the mature spore is released to the environment.

The coat shell in *Bacillus subtilis* is composed of over 20 different polypeptides with sizes ranging from 6 to 69 kDa. The arrangement of these so-called coat proteins gives three distinct layers within the coat itself: an electron dense thick outer coat, a lightly stained laminated inner coat and a diffuse undercoat. At least 18 coat protein-encoding genes (*cot* genes) take part in the coat structure, while a large number of other genes are required for the guidance and full assembly of these coat proteins (Serrano et al., 1999). TasA is one of the structural proteins important for the assembly of the final spore coat. It is also the major component of the extracellular biofilm matrix with a wide spectrum of antimicrobial properties (Branda et al., 2006). It is useful for *B. subtilis* to inhibit the growth of surrounding competitor microorganisms during the sporulation process and after the germination (Stöver and Dricks, 1999).

The sporulation process in *B. subtilis* is regulated both spatially and temporarily (Fig. 1. 2). It is important for transcription factors to be activated sequentially at the right time and in the right compartment. As previously introduced, Spo0A is the master regulator for the initiation of sporulation (Hoch, 1993). It is a response regulator transcription factor and it mediates global gene expression pattern by controlling genes that encode important regulatory factors. Among these, 121 genes organized into 24 operons and 30

single gene loci are shown to be under the direct regulation of Spo0A; the expression of 40 of these is increased by Spo0A, while the remaining 81 genes are negatively controlled by Spo0A (Molle et al., 2003).

Genes that encode efflux pumps and metabolic enzymes are mostly stimulated by the activity of Spo0A. On the other hand, the transcription of genes that encode components of flagellum biosynthesis, chemotaxis and DNA replication machinery is mostly inhibited by Spo0A activity. Another important activity of Spo0A is the regulation of sporulation killing factor (SkfA)-encoding operon called *skf*. Spo0A stimulates the production of more SkfA, which is excreted from the cell to induce the lysis of surrounding *B. subtilis* cells that have not initiated sporulation at that moment. Using this mechanism, sporulating *B. subtilis* provides more nutrients for itself to support necessary differentiation processes (Fawcett et al., 2000; Molle et al., 2003).

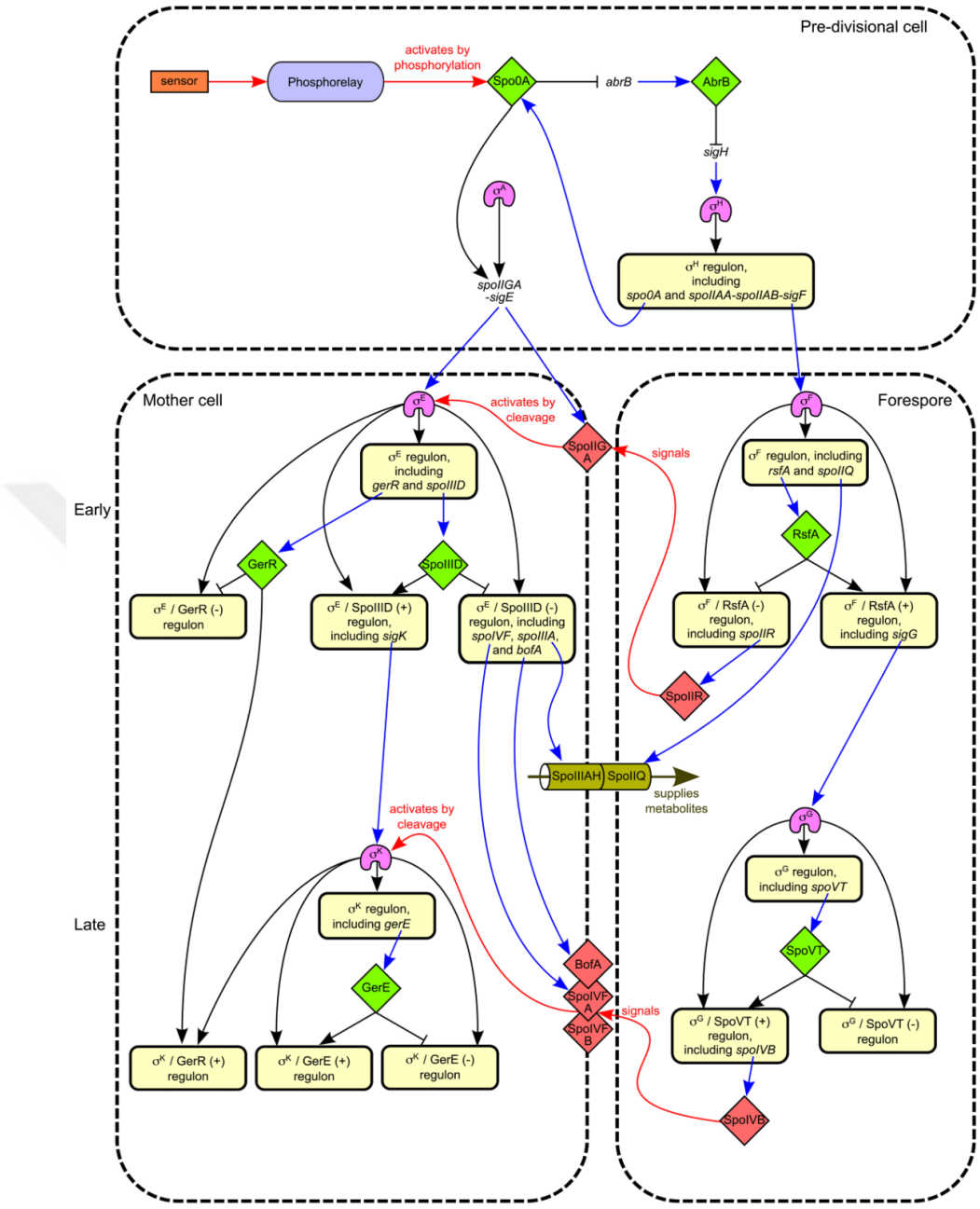


Figure 1. 2 Transcriptional regulatory network of the sporulation process in *B. subtilis* (De Hoon et al., 2010)

1.1.6 Swarming and Biofilm Formation in *Bacillus subtilis*

In the non-agitated liquid culture, *B. subtilis* shows swarming motility, which is required for the subsequent formation of floating biofilms called pellicles (Branda et al., 2001). Biofilms are, generally, microbial cell communities that are densely packed on various surfaces. These communities produce a vast variety of secreted polymers to surround themselves and to attach to the surface and to other cells. The mechanism of biofilm formation is complex and can show diversity among organisms. This physiological complexity of biofilms together with the cooperative and coordinated actions of cell communities in biofilms is one of the most striking similarities of microorganisms to their multicellular and eukaryotic cousins (Nadell et al., 2009). The rapid colonization of microbial cells on surfaces to form biofilm structures mostly requires swarming motility beforehand.

During swarming and biofilm formation, in order to carry out specific functions, bacteria undergo differentiation to form different types of subpopulations. For example, certain cells produce surfactants, which have been shown to be required for coordinated movement (Nagorska et al., 2010), whereas other cells produce extracellular protease Epr, which are required for swarming motility (Connelly et al., 2004).

Both swarming motility and biofilm formations have been receiving great attention lately, since these mechanisms have shown to be directly related to the production of virulence factors and the resistance against antibiotics (Connelly et al., 2004).

1.1.7 Secretion System in *Bacillus subtilis*

There are four major pathways for protein export in *Bacillus subtilis*: ATP-binding cassette transporter, the Sec Pathway, the pseudopilin export pathway

and the twin-arginine translocation (Tat) pathway (Harwood and Cranenburg, 2008; Du Plessis et al., 2011). Signal peptides of the secretory proteins are mostly found as N-terminal hydrophobic extensions. Proteins to be exported either by Sec or Tat pathways share common features such as a net positively charged N-terminus, a hydrophobic core-region and a polar C-terminus with the signal peptidase recognition site (Harwood and Cranenburg, 2007; Anne et al., 2016). More specifically, signal sequences can be one of four types: type I (classical), type II (lipoprotein), type III (Prepilin-type) or type IV (twin-arginine). On the other hand, signal peptidases fall into two major groups; type I peptidases cleave the majority of secretory proteins, including the proteins associated with the cell wall, which are subsequently released into culture medium. Although topologically similar to type I group, type II peptidases include shorter N- and H-regions and they share a different consensus cleavage site called Lipobox, which is exclusively found in lipoproteins.

Sec pathway is the main secretory pathway in *B. subtilis* and it contains five type I peptidases, namely SipS, SipT, SipU, SipV, and SipW. Signal peptidases have three important functions: blocking of the folding of preproteins to retain their translocation competence, interacting with the secretion machinery components to direct the translocation process and, lastly, acting as a topological determinant for preproteins in the membrane (Park and Schumann, 2015).

Sec pathway translocates the secretory protein in an unfolded state. This pathway is composed of three components; SecA motor protein, the SecYEG translocon and a heterotrimeric complex called SecDF-YrbF. Additionally, the CsaA chaperon protein is found to interact with SecA motor and to bind to the preprotein to be secreted. There are two SecA proteins in *B. subtilis*. SecA1 is essential for almost all secretory proteins in normal circumstances via the canonical Sec pathway. SecA2, on the other hand, translocates specific proteins

that are produced under certain conditions. Although SecA2 is similar to SecA1, it contains several deletion mutations which are thought to give SecA2 several advantages such as effectiveness in ATP hydrolysis and better interaction with Sec apparatus (Green and Mecsas, 2016).

Tat pathway, on the other hand, translocates proteins in their folded states. Compared to Sec SPases, Tat peptidases are generally less hydrophobic and longer. Signal sequences of proteins to be secreted through the Tat system contain twin-arginine as S-R-R motifs in their N-terminus, which is cleaved once they reach extracellular space. Unlike Sec, Tat translocon is a single complex with three subunits TatA, TatB and TatC. However, TatB is not found in B. subtilis species. There are only four substrates of the Tat system in this organism: a phosphodiesterase (PhoD), a cell wall bound metallo-prhosphoesterase (YukE), the newly identified YwbN and an extracellular lipase LipA.

Two other minor transportation systems in *B. subtilis* are ATP-binding cassette and the pseudopilin export system. The former is composed of two hydrophobic membrane-spanning domains and two hydrophilic domains called nucleotide binding domain. The energy input for the translocation step comes directly from the ATP hydrolysis. The most well-known examples of proteins secreted through this system are the lantibiotics subtilin and sublancin, which are transported through SpaT and SunT ATP-transporters, respectively (Fu et al., 2007). The pseudopilin export pathway has a 33 amino acid long recognition signal sequence which allows its secretory proteins to bypass both Sec and Tat pathways. Proteins related to cell competence such as ComGC, ComGD, ComGE, and ComGG are the main substrates of this pathway (Tjalsma et al., 2004).

1.2 Dipeptide Antibiotic Bacilysin

Bacilysin is the smallest antibiotic known to date, with a dipeptide structure that contains L-alanine at the N-terminus and L-anticapsin at the C-terminus (Rogers et al., 1965). Its chemical structure is L-alanyl-(2,3-epoxycyclohexanane-4)-L-alanine, described as $C_{12}H_{18}N_2O_5$ and it has a molecular mass of 279 Da (Walker and Abraham, 1970). Bacilysin's antimicrobial activity against several bacteria and fungi is exerted by L-anticapsin, which is a non-proteinogenic amino acid that is cleaved and released by peptidases when secreted bacilysin is taken up by target cells (Neuss et al., 1970; Shah et al., 1970). The antimicrobial activity of this intracellular free anticapsin is the inhibition of glucosamine synthetase, which is required for bacterial peptidoglycan and fungal mannoprotein synthesis. The blockage of glucosamine synthetase activity results in the protoplast formation and it is followed by the lysis of the affected cell (Walton and Ricker, 1962).

1.2.1 Biosynthesis of Bacilysin

Even though the participating genes in bacilysin biosynthesis pathway had already been known before, the underlying mechanism of dipeptide formation of bacilysin has only recently been found, opening ways for new enginnering strategies to be developed for obtaining bacilysin derivatives with novel functions or totally different dipeptides.

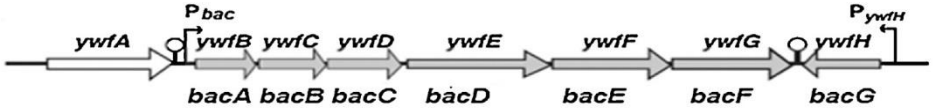


Figure 1. 3 Organisation of *bacABCDEF* polycistronic operon and *bacG* monocistronic gene (Steinborn et al., 2005)

Bacilysin is produced and secreted by the collective activities of proteins encoded by one polycistronic operon containing six genes (*bacA, bacB, bacC, bacD, bacE* and *bacF*) and a monocistronic gene (*bacG)* at the opposite strand adjacent to *bacABCDEF* operon (Figure 1.3) (Steinborg et al., 2005; Mahlstedt and Walsh, 2010). Among these seven genes, *bacE* is the only one which encodes a protein that is not involved in the biosynthetic pathway of bacilysin. Rather, BacE is responsible for the resistance of bacilysin-producer *B. subtilis* by pumping the bacilysin outside of the cell (Steinborg et al., 2005).

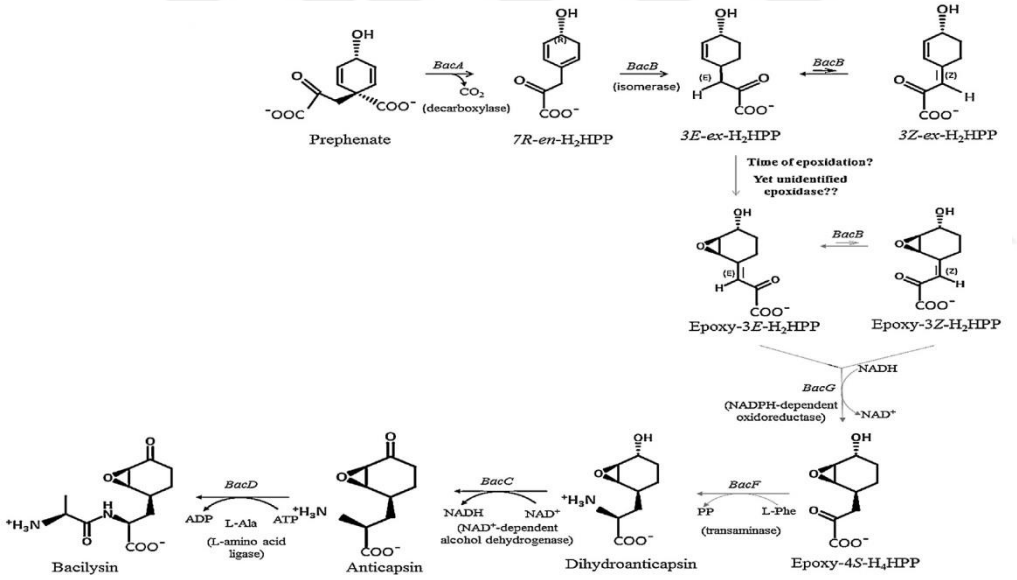


Figure 1. 4 Detailed presentation of the recently hypothesized bacilysin biosynthetic pathway (Parker and Walsh, 2013).

As illustrated in Figure 1.4, the first step of nonribosomal bacilysin biosynthesis is the decarboxylation of propionate and isomerization of pro-R double bond by BacA enzyme to form 7R-endocyclic-

dihydrohydroxyphenylpyruvate (H_2HPP) (Mahlstedt et al., 2010). The resulting endocyclic dienyl product is isomerized to 7R-exocyclic-H_2PP by the activity of BacB oxidase enzyme, which transfers the double bond to the adjacent 2-keto moiety (Rajavel et al., 2009). The products, 3Eand 3Z geometric isomers, are reduced by NADH-dependent BacG reductase to form 4R and 4S-diastereomers of tetra-(H_4HPP). These are converted to corresponding H_4Tyr diastereomers by the subsequent action of BacF aminotransferase, which catalyses the amination of 2-keto group by transferring an amino group from candidate L-phenylalanine, yielding so-called dihydroanticapsin (Parker and Walsh, 2012). The next step is the oxidation of C7-hydroxyl group by the action of NAD^+-dependent BacC dehydrogenase; giving the final form of the anticapsin. Dipeptide bond formation between the final anticapsin and L-alanine is carried out by the action of BacD ligase, in which L-alanine is attached to the amino group of anticapsin, completing the bacilysin formation (Tabata et al., 2005).

1.2.2 Regulation of Bacilysin Biosynthesis

Bacilysin biosynthesis is found to be active when *B. subtilis* cells grow in synthetic medium. Certain factors such as glucose, ammonium and casamino acid represses or inhibits the bacilysin production, while temperatures above 30°C result in a decreased bacilysin activity (Özcengiz et al., 1990; Ozcengiz and Alaeddinoglu, 1991). It is proposed by Inaoka et al. (2003) that the expression of the *bacABCDEF* operon is positively regulated by the presence of guanosine tetra and penta phosphate and negatively regulated by high intracellular amounts of GTP, of which the latter acts as a signal via the CodY-dependent repression pathway.

Both stringent response and feedback mechanisms seem to be effective on the bacilysin production (Ozcengiz and Alaeddnoglu, 1991; Inaoka et al., 2003).

Most recently and more importantly, bacilysin is shown to a part of the quorum sensing mechanism that is mentioned above.

As demonstrated by previous members of our group, bacilysin biosynthesis is strongly controlled by quorum sensing mechanism through the actions of Spo0K (Opp), PhrC (CSF), ComQ/ComX and ComP/ComA (Yazgan et al., 2001; Karataş et al., 2003)

It has been shown that in *phrC*$^{-}$ and *comA*$^{-}$ mutants, bacilysin production is abolished, while *phrA*$^{-}$ strains showed no significant change in the bacilysin biosynthesis (Yazgan et al., 2001). Another study showed a significant decrease in the bacilysin production in *comP* inactive mutants, and complete absence of bacilysin in *comQ* inactive mutants (Karataş et al., 2003). In the same study, it was also revealed that inactivation of the *srfA* operon, which encodes a lipopeptide antibiotic surfactin, ceases the production of bacilysin, while the inactivation of the *abrB* gene significantly increases its production. This increase is lost when Spo0A is also inactivated in *abrB*$^{-}$ strains, suggesting that the bacilysin operon is under the negative control of AbrB regulator. Another pioneering study, in which the *bacA* gene is silenced by fusing it to an insertional vector containing lacZ gene to create *B. subtilis* PY70 bacA:lacZ:erm strain called OGU1, further explored the relationship of bacilysin and the quorum sensing pathways (Köroğlu et al., 2011). In this study, genes expressing important proteins, namely *srfA, ooppA, comA, phrC, phrF, phrK, comQ (comX), comP, spo0H, spo0A, abrB,* and *codY,* are blocked individually or in certain combinations to see the effects that the absence of their products have on bacilysin production. This was evaluated by monitoring the activity of β-galactosidase produced from *lacZ* gene inserted in *bacA*, where *lacZ* gene is still expressed from the promoter of bacilysin biosynthetic operon. The results clearly showed that the expression of bacilysin operon is significantly decreased in the *spo0H, spo0A, comP, comQ, phrF, phrK, phrC,*

comA, *oppA* and *srfA* silenced mutants as well as in *codY*⁻ mutants during stationary phase, whereas it is almost doubled in *abrB* inactive mutant during exponential phase. According to electrophoretic mobility shift analysis (EMSA) studies, ComA, Spo0A, AbrB and CodY proteins are shown to directly bind to and regulate the bacilysin biosynthetic operon.

Similarly, in *B. amyloliquefaciens*, another important two-component system, DegS/DegU, has been shown to positively regulate both the bacilysin operon and *bacG* gene at the transcriptional level (Mariappan et al., 2012). On the other hand, ScoC (Hpr), a negative regulator of protease production and sporulation, has been shown to directly bind to the promoter of the *bacABCDEF* operon and suppresses its expression (Inaoka et al., 2009). LutR, a GntR type transcription factor encoded by *lutR*, is also one of the important regulator proteins that take part in a wide range of physiological processes and it is found to be essential for the production of bacilysin in *B. subtilis* (Köroğlu et al., 2008; İrigül Sönmez et al., 2014).

1.2.3 Comparative Proteome Analysis between Wild Type *Bacillus subtilis* and Its Bacilysin Non-producer Strain

In order to study the effects of the absence of bacilysin on other *B. subtilis* proteins, comparative proteome analysis between wild-type strains PY79 and its bacilysin-nonproducer derivative OGU1 have been carried out in our laboratory recently. In the study carried out by Taşkın (2010; manuscript submitted), the separation of more than 1900 proteins was achieved by the 2DE approach and the proteins were subsequently identified by using MALDI-TOF mass spectrometry. Over 250 proteins were found to be differentially expressed between PY79 and OGU1 strains. The functions of 159 proteins could be identified, which corresponded to 121 distinct ORFs. It was found that 63

proteins were downregulated, 39 were upregulated, 20 proteins were absent and 1 protein was newly induced in bacilysin negative strain.

Another comparative proteome analysis, this time by LC MS/MS method, was carried out by Demir (2013; manuscript submitted) has identified more than 1200 cytoplasmic proteins in PY79 and OGU1 strains. Among 76 proteins differentially expressed between two strains, 50 were absent in OGU1, 7 proteins were underrepresented and 19 were found only in the mutant strain.

Most of the differentially expressed proteins were determined to belong to two categories, designated as "Metabolism" and "Lifestyle". The subcategory "Sporulation and Germination" under the "Lifestyle" constituted as much as 58% of all missing and downregulated proteins in OGU1 strain. Another important subcategory was "Coping with Stress", representing 12-18% of all differentiated proteins.

Most recently, another member of our laboratory carried out dynamic (time-dependent) comparative secretome analysis between PY79 and OGU1 strains using both 2DE MALDI-TOF and LC-MS/MS techniques (İşlerel, 2017; manuscript submitted). Proteins were isolated and purified from cell culture supernatants at three different time points: 12^{th}, 16^{th} and 24^{th} hours of incubation. By using LC-MS/MS technique, 414 proteins were identified in PY79 and 694 proteins were identified in OGU1 at the 12^{th} hour; among these, 386 proteins were overlapping between two strains. For 16^{th} hour samples, these numbers were 458 and 489 for PY79 and OGU1, respectively, with 407 overlapping proteins. Lastly, 743 proteins of PY79 and 687 proteins of OGU1 secretome were identified at the 24^{th} hour samples, among which 615 proteins belonged to each strain. As most of the differentiated secretome proteins were found in 12^{th}-hour samples, bacilysin can be proposed to exert a more profound

effect on cellular physiology at this stage, during which the producer organism actively commits itself to sporulation.

Among 108 differentially expressed proteins found in 12th-hour samples, 60 were cytoplasmic, 18 were extracellular and the remaining proteins were either cell wall/membrane-bound or unknown proteins. 8 of 18 extracellular proteins were catalytic enzymes with great importance in medical and industrial biotechnology, as well as in molecular biology studies. Fold changes in the levels these enzymes can be found in Table 1. 3.

Table 1. 3 List of extracellular enzymes found to be overrepresented in OGU1 strain.

Locus Name	Protein	Gene	Log2FC	Fold Change	P Value	Functional Category Codes	Function	Mass (Da)	pI
BSU39330	Extracellular endo-alpha-(1->5)-L-arabinanase 2	*abn2*	1.585	3	0.0006	SW 2.2	Arabinan degradation	52607	7.37
BSU27030	Levanase	*sacC*	2.202	4.6	0.0023	SW 2.2	Degradation of levan to fructose	75951	6.76
BSU26890	Chitosanase	*csn*	2.323	5.01	00005	SW 2.2	Chitin degradation	31497	8.89
BSU19800	3-phytase	*phy*	1.054	2.08	0.0467	SW 2.6	Utilization of phytate	41946	4,94
BSU02700	Lipase LipA	*estA*	1.876	3.67	0.0001	SW 2.4	Lipid degradation	22791	10
BSU09190	Endonuclease YhcR	*yhcR*	2.381	5.21	0.0014	SW 3.2	Utilizations of nucleic acids	132686	4.69
BSU15300	Bacillopeptidase F	*bpr*	2.042	4.12	0.0041	SW 3.3	Protein Degradation	154578	4.98
BSU38090	Minor extracellular protease Vpr	*vpr*	1.648	3.13	0.0013	SW 3.3	Protein Degradation	85608	5.77

1.3 Extracellular Enzymes of *Bacillus subtilis*

1.3.1 Endoarabinanase

Plant cell walls are composed of lignocelluloses, which include lignin, pectin, cellulose, and hemicellulose. Out of these, the most abundant renewable biomass polymer is cellulose, followed by hemicelluloses which account for up to 35% biomass of lignocelluloses (Ward and Moo-Young, 1989). Hemicelluloses are complex polysaccharides, which may contain sugar acids, hexoses (mannose, glucose, galactose) and pentoses (xylose, arabinose). Hemicelluloses, in contrast to celluloses, are not chemically homogeneous structures and their composition may change greatly depending on their source. For example, hemicelluloses of softwood are mostly composed of glucomannans, while those of hardwood are composed mainly of xylans (McMillan, 1994). While xylans are heteropolysaccharides, their backbone composes of homopolymeric chains of 1,4- linked D-xylopyranose. Almost 80% of this chain is largely substituted with both monomeric arabinoses and oligomeric arabinan chains linked to O-2 and/or O-3 of xylose units.

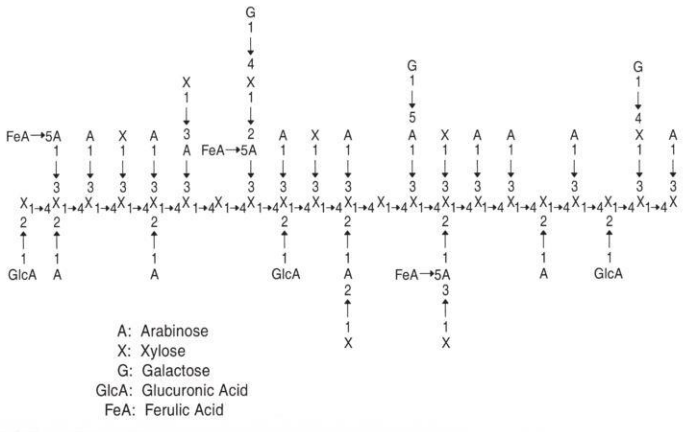


Figure 1. 5 Schematic structure of maize bran heteroxylan (Saulnier et al., 1995).

Arabinans, on the other hand, are homopolysaccharides composed of a long linear chain of 1,5-linked arabinofuranosyl residues, substituted with other short arabinofuranosyl residues. Arabinans are found in several plant tissues, most abundantly in pectin. The polymer backbone of pectin contains scattered rhamnose units carrying neutral sugar chains. These sugar chains mostly contain galactose and arabinose which form galactans, arabinogalactans, and arabinans (Catoire et al., 1998; Habibi et al., 2004).

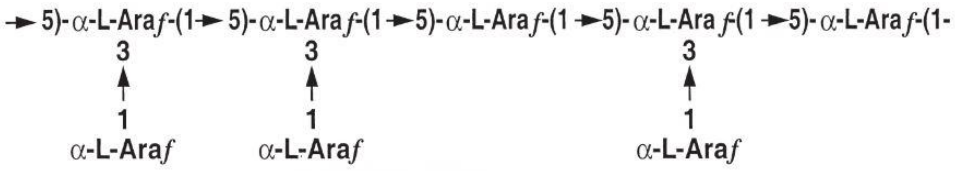


Figure 1. 6 Primary structure of arabinan (Saha, 2000).

The breakdown of lignocelluloses is central to numerous industrial processes such as paper pulp delignification, fuel and chemical production, enhancement of the digestibility of animal feedstock, juice clarification and beer consistency (Campbell and Bedford, 1992; Vikari et al., 1993; Wong et al., 1988; Zeikus et al., 1991). However, due to the excessive side chains found both in hemicelluloses and pectins, it is harder to subject these lignocelluloses to simple enzymatic digestion. Therefore, they represent an obstacle in several important bioindustrial processes (Saha, 2000). The only way to achieve the total breakdown of hemicelluloses and pectins is by the collective action of several enzymes, among which the α-L-arabinofuranosidases (AFs) and endo-

1,5-α-L-arabinanases (arabinanases) are important for the cleavage of side chains and subsequent exposure of the backbone to the rest of the degradative enzymes (Dodd and Cann, 2009). These enzymes are the most effective catalysts known to date with the ability to increase the rate of the hydrolysis of glycosidic bonds found in lignocelluloses by more than 1017-fold (Rye and Withers, 2000; Shallom et al., 2002).

AFs are able to hydrolyze 1→3 and 1→5 arabinosyl linkages at terminal nonreducing residues and take part in the degradation of hemicelluloses. Arabinanases, on the other hand, are endo-fashion enzymes which hydrolyze the interior 1→5 arabinosyl linkages of arabinan chains, hence take part mostly in the hydrolysis of pectin and other arabinan containing structures (Saha, 2000). Among the microorganisms screened for the expression of these enzymes, several *Bacillus* species were found to be the most active producers (Karimi and Ward, 1989).

B. subtilis produces at least five different enzymes to hydrolyze arabinosyl linkages: two intracellular arabinofuranosidases, AbfA and Abf2, one extracellular arabinoxylan arabinofuranohydrolase XynD, one extracellular endoarabinanase AbnA and also a recently characterized extracellular endoarabinanase, Abn2 (Kaji and Saheki, 1975; Weinstein and Albersheim, 1979; Kaneko et al., 1994; Raposo et al., 2004; Icacio and Sa-Nogueira, 2008). Abn2 from *B. subtilis* 168 has only 27% sequence homology to AbnA from the same organism. The analysis of substrate specificity showed that Abn2 is active towards linear α-1,5-L-arabinan, sugar beet arabinan and apple pectin. The optimal activity of Abn2 was found to be around 50°C, which is lower than that of AbnA, making Abn2 a better candidate for industrial applications at moderate temperatures. Abn2 was found to be transcribed from both σ^H-dependent and σ^A-dependent promoters, and also be stimulated by the presence of arabinan and pectin. Unlike AbnA, AraB regulation was not involved in the

abn2 expression, and arabinose itself was also not found to increase the transcription of *abn2* gene. However, Abn2 production was found to be subjected to catabolite repression in the presence of glucose in which the expression of *abn2* gene is repressed by CcpA regulator (Icacio and Sa-Nogueira, 2008).

For *B. subtilis*, following model was proposed for arabinan degradation: two major GH43 family endoarabinanases first attack to the extracellular homopolysaccharide to release arabinose oligomers and monomers, which are then transported into the cell by specific permease transport system called AraE together with an ABC-type transporter called AraNPQ. Once inside the cell, two GH51 family α-L-arabinofuranosidases AbfA and Abf2 further hydrolyze the remaining arabinose oligosaccharides and arabinosyl side chains (Icacio and Sa-Nogueira, 2008).

1.3.2 Chitosanase

Chitosans are linear 1,4-β-linked D-glucosamine polysaccharides substituted by various degrees of N-acetyl groups. Chitosan is significantly found in the cell walls of many fungi and algae, a group of phytoplankton called *Rhizopus*, *Zygomycetes* and opportunistic human pathogen *Mucor* being the most studied ones (Davis & Eveleigh, 1984). Chitosans comprise up to 30% of the dry weight of these organisms. Their degradation requires the action of chitosanases which hydrolyzes the glycosidic bonds between D-glucosamine residues to release various lengths of chitooligomers with diverse properties such as wound-healing, blood anticoagulants, hemostatic material, moisturizing agents, wastewater treatment, seed coating and food additives (Sandorf, 1989; Hirano, 1996).

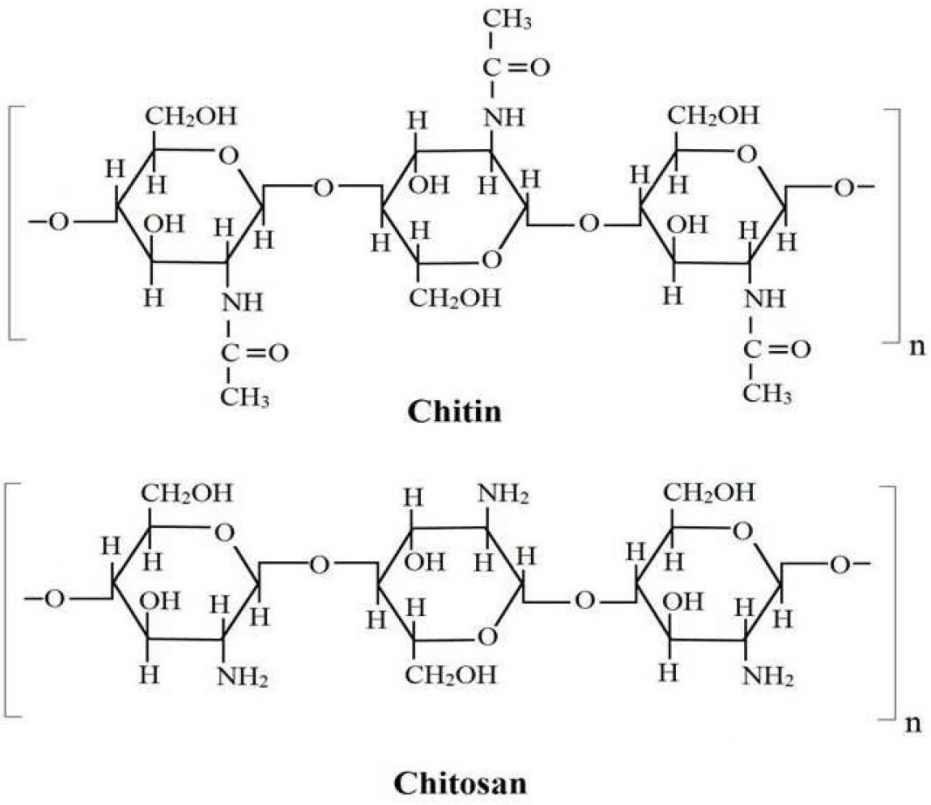


Figure 1. 7 General structure of chitin and chitosan (Carneiro et al., 2014).

Chitosan degrading enzymes are already characterized in many microorganisms, plants (Somashekar & Joseph, 1996) and even in some viruses like Chlorella PBCV-1 and CVK2 (Lu et al., 1996; Yamada et al., 1997). Among these, *B. subtilis* 168 chitosanase Csn has an estimated molecular weight of 27.4 kDa with 242 amino acid mature length (Rivas et al., 2000). Sequence analysis of Csn showed a significant homology with chitosanases from actinomycetes than those from other *Bacillus* species. Csn was found to be negatively regulated by AbrB global regulator, but not regulated by catabolite repression as there is no CRE element found on *csn*. Csn of *B. subtilis* 168 is considered as a temporarily regulated enzyme as its transcription of *csn* peaks at the transition to stationary phase. It has an optimal pH of 5.7 and an optimal temperature around 60°C, which is similar to

other chitosanases characterized. The stability of this enzyme decreases significantly above 60°C. Additionally, the activity of the enzyme is not affected by the presence of Mg^{2+} or Ca^{2+}, but it is almost completely inhibited by Cu^{2+} or Fe^{3+}, showing that it has a strong tendency to form stable complexes with ions (Rivas et al., 2000).

1.3.3 Levanase

The polymer unit attached to the starter glucose can range from two fructose units to hundreds of it linked through (β-2,1) or (β-2,6) fructosyl bonding. Many plants contain both levan and inulin as their fructan reserves, while most microorganisms, including *B. subtilis* contain only levan (Jensen et al., 2016). In plants, these fructan reserves function as both short and long term carbon and energy storages to be used for the survival under cold stress, by employing them as membrane stabilizers or osmolytes (Livingston et al., 2009; Valluru & Van den Ende, 2008). It is also known that fructans play important roles during oxidative stress in the producer cell (Van den Ende & Valluru, 2009). Additionally, levan is specifically used by microorganisms as exopolysaccharide (EPS) agents to help in biofilm formation (Smeekens et al., 1999). Levan is already found in commercial products such as wheat, rye, oat, and barley (Pollock & Cairns, 1991). Their degradation is important for the release of a large number of fructose monomers for adsorption in the digestive tract for subsequent use in a diverse range of metabolic pathways.

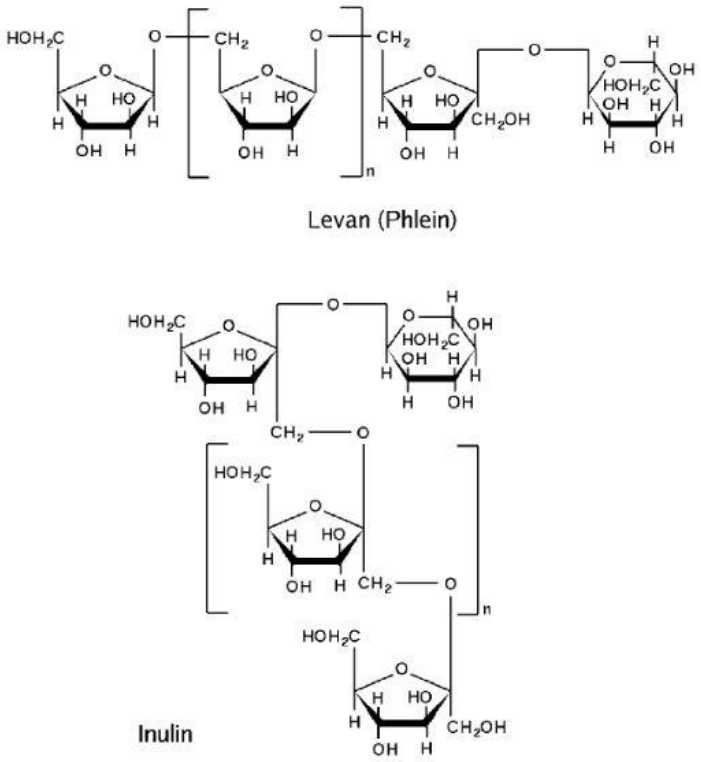


Figure 1. 8 General structure of levan and inulin (Meier and Reid, 1982; Butler and Bailey, 1973).

Fructans are degraded by a family of enzymes called fructofuranosidases which are further categorized with respect to their specific substrates including levanases (hydrolyse levan), inulinases (hydrolyse inulin), sucrases and invertases (hydrolyse disaccharide sucrose) (Wanker et al., 1995). Despite this classification, many of these enzymes are fully capable of degrading other kinds of fructans as secondary substrates. Majority of fructofuranosidases are found in microorganisms as extracellular enzymes secreted outside of the bacteria to degrade the surrounding fructans. The action of these exoenzymes is to attack the inulin or levan molecules from the fructose end, and to release the fructose monomer units as the sole products. Although melezitoses are also trisaccharides with the same terminal configuration with inulin, they are hydrolysed neither by inulinases nor by levanases (Snyder & Phaff, 1962).

In *B. subtilis*, there are three enzymes with saccharolytic activities: an intracellular sucrase (Lepesant et al., 1972), an extracellular levansucrase (Lepesant et al., 1972), and an extracellular levanase SacC (Kunst et al., 1977). These enzymes are classified as beta-D-fructofuranose transferases and they all are capable of hydrolysing sucrose. However, the levanase SacC, in addition to its specific substrate levan as its name suggests, can also hydrolyse inulin as its distinctive feature (Martin et al., 1987). Unlike levansucrase and sucrase genes, the expression of *sacC* is not induced by the presence of sucrose (in fact, no inducer has been found to date); yet, it has been shown to be subjected to catabolite repression through CcpA by several carbon sources like glycerol and glucose. On the other hand, it was shown to be positively regulated by LevR, which is the transcriptional activator of *levDEFG-sacC* operon. Rather than the sucrase or levansucrase genes of *B. subtilis* itself, *sacC* gene sequence showed strong homology with that of intervase from the yeast. There was indeed no homology at all between levanase and levansucrase genes of *B. subtilis*, illustrating the difference between the mechanisms of action of these two proteins. Enzymatic activity studies demonstrated that the SacC has an optimal pH value between 5 and 6.5 and an optimal temperature between 47 and 55°C (Martin et al., 1987).

1.3.4 Phytase

Phytic acid is the major component of all plant seeds and accounts for almost 90% of total phosphorus (Graf, 1983). Its chemical structure is myo-inositol 1,2,3,4,5,6,-hexakis-dihydrogen phosphate and it is constituted 1-3 % by weight by common cereals. Phytic acid accumulates together with other storage materials like starch and lipids during the ripening process of grains and seeds. It accumulates in aleurone particles in cereals and in globoid crystals in legumes (Reddy et al., 1982; Tyagi & Verma, 1998). As the main

phosphorus storage unit, phytic acid helps the initiation of dormancy and acts as a source of energy, cations, and myo-inositols (Singh et al. 2011).

Phytic acid is generally accepted as an antinutrient also a strong chelator of important mineral ions such as Fe^{+2}, Ca^{+2}, Zn^{+2}, and Mg^{+2}, preventing their accessibility for absorption in the monogastric animals' intestines, including humans, poultry, swine, and fish (Greiner & Konietzny, 2006). Not only to minerals but these phytic acid salts (phytates) can also bind to amino acids, proteins, and starch, thus preventing the assimilation of essential organic materials by the digestive system (Noureddini & Dang, 2009). Especially the deficiency of any of the minerals that phytic acid binds can result in serious metabolic disorders, particularly in the regions where the diet depends mostly on cereals and legumes (Lopez et al., 2002).

Another result of undigested phytic acid is the eutrophication, the gradual increase of phosphorus in soil and water. Unabsorbed phytate gets excreted through the urinary system of the organism and accumulates in the environment. The use of seeds and other feedstock rich in phytic acid for poultry, swine and fish industry make the excessive accumulation of phosphorus in earth and water bodies (Singh et al., 2013). Help comes from the introduction of a class of enzymes called phytases. Currently, phytases are being studied extensively for their high potential of liberating phosphorus residues in animal and human feedstock industry.

Phytases sequentially hydrolyze the inositol hexakisphosphate to myo-inositol derivatives with lower substitutions of phosphorus, and release inorganic phosphate as a result (Reddy et al., 2015). By decreasing the antinutritional properties of the phytic acid, phytases allow the absorption of phosphorus in the digestive system of animals with an inadequate level of phytase production and prevents environmental eutrophication (Yoon et al., 2011). Although

phytases have also been found in plants and several animals tissues, phytate degrading activities of these are negligible compared to bacteria and fungi.

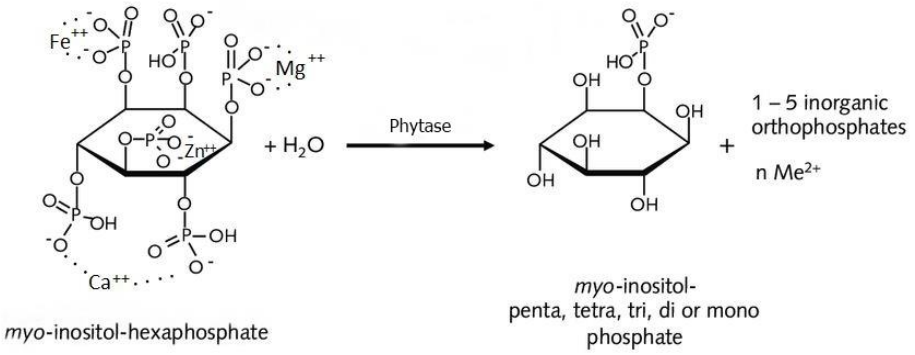


Figure 1. 9 Enzymatic hydrolysis of myo-inositol-hexaphosphate (phytate) by the phytase activity (Troesch et al., 2013).

There are two main types of phytases studied to date: Myo-inositol hexakisphosphate 3-phosphohydrolase, or shortly 3-phytases hydrolyze the ester bond at 3 positions of phytic acid first and are found mostly in microorganisms (Jonson & Tate, 1969). Myo-inositol hexakisphosphate 6-phosphohydrolases or shortly 6-phytases, on the other hand, catalyze the hydrolysis of the 6-position ester bond first, and reported mainly in plants (Cosgrove, 1970). Yet, for a better classification, spatial structure and mechanism of the phytase can be considered. Accordingly, there are four classes of phytate-degrading enzymes: purple acid phosphatases (PAPs), cysteine phytases, β-propeller phytases (BPPs) and histidine acid phosphatases (HAPs) (Mullaney & Ullah, 2003). Lastly, phytate-degrading enzymes can also be categorized as alkaline phytases and acid phytases with respect to their optimal pH of activity (Kaur et al. 2007; Singh et al. 2011). Most widely used among phytases in the market are HAPs produced by fungal *Aspergillus* species. *Aspergillus* phytases are acid phytases with an optimal pH of 4.5-5.5,

and they can only hydrolyze metal-free phytic acid, but not phytate salts. Another feature of *Aspergillus* HAPs that made them unfavorable is the broad specificity and low thermostability of these enzymes, which make them mostly unacceptable in the industrial processes.

Bacillus sp., on the other hand, produces alkaline phytases with an optimal pH in the neutral range. These phytases have high thermostability, high specificity for phytic acid salts, resistant to proteolysis and are able to degrade metal-bound phytate, making them attractive with a great application potential in the industry (Fu et al., 2008). These phytases are able to withstand high temperatures during pelleting processes and are still able to function in the neutral environment of the digestive tract of animals (Kim et al., 1999). Genome-wide binding profile studies AbrB global regulator showed that phytase biosynthesis is negatively regulated by the action of AbrB.

Several *Bacillus subtilis* phytases are already characterized and found to be strictly specific for calcium-phytate complexes with no activity on any other phosphate esters. At the first step of the reaction, P_3-Ca^{2+}-P_4 bidentate of $InsP_6$ binds to the two phosphate binding sites on the enzyme and the phosphate group at d-3 position is hydrolyzed, releasing $InsP_5$ as the first product. Next, the enzyme binds to P_1-Ca^{2+}-P_2 bidentate and hydrolyzes the cleavage of the second phosphate group at d-1 position. Lastly, P_5-Ca^{2+}-P_4 bidentate of $InsP_4$ attaches on the enzyme and the phosphate group at d-5 position is released, leaving myo-inositol-2,4,6-P_3 as the final product (Kim et al., 1998).

1.3.5 Lipase

Lipolytic enzymes with lipases on the front line represent enormous attention due to their irreplaceable applications in biotechnology and the industrial market. These enzymes catalyze both synthesis and hydrolysis reactions with high enantio- and region-selectivity. Therefore, lipolytic enzymes have also become one of the most widely used enzyme classes in the synthetic organic chemistry (Bornscheuer & Kazlauskas, 2006).

B. subtilis produces different enzymes with lipolytic activities including a phospholipase, a lipase LipA, and an esterase LipB. Among these, LipA and LipB are both synthesized in the rich medium while LipB is not detectable in the minimal medium, when glucose is used as the only carbon source. LipA and LipB are the smallest lipases with a molecular weight of only 19.3 kDa and 22.2 kDa, respectively. Although there is no difference with respect to specific activities of LipA and LipB against p-nitrophenyl esters, LipA shows higher specific activity towards substrates with long chain lengths when compared to LipB. Both of these enzymes show lipase activities while LipB is classified as an esterase as it does not take part in the degradation of other typical lipase substrates such as tung oil triacyglycerol, seed kernel fluorescent triacylglycerol, and triolein. Neither of these enzymes showed hydrolytic activity against phospholipids (Eggert et al., 2001a; Eggert et al., 2001b; Eggert et al., 2003).

Remarkably, LipA lacks the conserved Gly-X-Ser-X-Gly pentapeptide conserved sequence found in and required for catalytic activity in most lipases. Apart from being one of the smallest lipases, LipA also shows a very high pI value and optimal pH around 10. It exhibits high stability in alkaline solutions but the activity sharply decreases below 6.5 and above 10 pH values. The optimal temperature of LipA was determined as found to be 35°C and its

activity is mostly lost above 40°C (Lesuisse et al., 1993). Expression of *lipA* has also been shown to be negatively regulated by AbrB global regulator (Banse et al., 2008).

1.3.6 Protease

Proteases are categorized into different families and further subfamilies among which endopeptidases (proteinases) and exopeptidases (or just peptidases) are the major interest in the bioindustry. These two proteases can further be grouped into four subclasses namely metalloproteases, cysteine proteinases, serine-proteinases and aspartic-, carboxyl or acidic-proteinases. *B. subtilis* is already known to produce large numbers of serine proteases, cysteine proteases, and to a smaller extent metalloproteases. These *Bacillus* proteases are mostly alkaline and have an optimal activity above pH 7.0 (Contesini et al., 2018).

Being as one of the most important microbial proteases, serine proteases have a hydrolysis mechanism of two-step reaction. First, the peptide chain is broken into two by the covalent attachment of one part of the enzyme to the oxygen of the serine found in the catalytic site of the enzyme, freeing the rest of the protein. After this acylation step, a water molecule exerts nucleophilic attach on the enzyme-peptide intermediate and the result is the release of the peptide sequence attached to the serine residue in the first step. Binding of the protein to the S1 pocket of enzyme occurs very specifically. If the specific recognition site still exists inside one or two of the broken pieces of original protein, serine proteinase can continue to hydrolyze the peptide into smaller pieces (Fastrez & Fersht, 1973).

Bacillus subtilis genome project revealed at least 11 different extracellular proteases. The corresponding genes have already been identified and the

products of these enzymes have been characterized. Two of these extracellular proteases which are responsible for more than 90% of total extracellular protein degrading activity, are subtilisin and a neutral protease encoded by *apr* and *npr* genes, respectively. The remaining protein degrading activity is mostly distributed to three minor extracellular proteases namely bacillopeptidase F, Epr, and Mpr. Both bacillopeptidase F and Epr are serine proteases encoded by *bpr* and *epr* genes, respectively. Mpr, encoded by *mpr* gene, on the other hand, is a minor metalloprotease (Kho et al., 2005). Bacillopeptidase F, with a molecular weight of 47 kDa and pI between 4.4 and 4.7, shows also esterase activity against a wide range of p-nitrophenyl esters. Although it shows strong sequence homology to the remaining four *Bacillus* serine proteases, BpF shows remarkable features such as its (i) ability to free single amino acid units from intact casein, (ii) larger size as compared to most of the other proteases, (iii) acidic isoelectric point, (iv) high ratio of esterolytic to proteolytic activity and (v) broad range of specificity. The large C-terminus of BpF is thought to be involved in the anchoring of enzyme to the cell wall or membrane of *B. subtilis*, which is also supported by the sequence analysis programs showing that BpF is probably a cell envelope attached protein (Mäntsälä & Zalkin, 1980). Moreover, Bpr was found to be positively regulated by DegU, one of the most important two-component regulatory response proteins (Tsukahara & Ogura, 2008).

The observation that the *B. subtilis* strains with null mutations on the genes coding for all five different proteases mentioned above still retain detectable extracellular protein hydrolysis activity resulted in the characterization of another minor extracellular serine protease called Vpr, encoded by *vpr*. Vpr is produced as an 85 kDa pre-protein and it has a predicted mature molecular mass of 68 kDa. Although Vpr activity has been shown in the supernatants of *B. subtilis*, its high sequence homology to *Lactococcus lactis* serine proteases suggests that Vpr can actually be bound to the membrane. Another study

revealed that Vpr undergoes auto-processing or lysis in the presence of other proteolytic enzymes (Kho et al., 2005). Surprisingly, Vpr is regulated by at least four different regulators, its transcription is activated by phosphate metabolism regulator PhoP and is repressed by the global regulator CodY, the SOS regulon repressor LexA and the replication initiation protein DnaA.

1.3.7 Endonuclease

Another enzyme of interest in this study is an extracellular endonuclease (YhcR) produced by *B. subtilis*. YhcR is a high MW, non-specific nuclease with a maximal activity at pH 9.0; its activity is inhibited by high concentrations of NaCl and Mg^{2+} but increased by Ca^{2+} and Mn^{+2}. YhcR is active against both DNA and RNA, which it cleaves endonucleolytically and thus generates 3' monophosphate nucleosides. YhcR is also found to be subjected to strong proteolysis in *B. subtilis* culture supernatant and the presence of the putative anchor sequence suggests that YhcR is attached to the cell wall through by the action of sortase YhcS (Oussenko et al., 2004).

1.4 Quantification of the Gene Expression by qRT-PCR

Quantitative reverse transcriptase PCR (qRT-PCR) is an enhanced PCR technique which employs fluorescence emission for the amplification and the real time detection of DNA techniques, even in the presence of only a trace amount of starter DNA template, which is generally not possible by conventional PCR techniques. Applications of qRT-PCR range from diagnostics to clinical studies, from pathogen detection to forensics as well as from food technology to functional genomics (Bustin, 2005; Yuan et al., 2008). The main use of qRT-PCR is to track or to show spatial and temporal gene expression profiles and their simultaneous quantification. Its high sensitivity, accuracy and simplicity allowed qRT-PCR to replace the traditional tools with

same purposes, such as Northern blotting and nuclease protection assay. Nevertheless, it should be noted that the equipment and the reagents of qRT-PCR is more expensive than those of traditional methods. The accuracy of the obtained data depends on several factors including the quality and the quantity of RNA samples, the efficiency of reverse transcription step, the detection methods used, the strategy used to quantify amplification data and efficiency, normalization of data and the statictical approach used (Bustin, 2000; Pfaffl and Hageleit, 2001; Pfaffly, 2005; Derveaux, 2010).

There are four main phases of qRT-PCR protocol. The initial phase is constituted by the first 3-15 cycle where the fluorescence emission starts to increase slightly above a certain baseline level, which indicates the first detection of increasing amount of the PCR product. In the second stage, fluorescent emission exponentially increases above the baseline level and eventually reaches to a pre-determined threshold level. The number of the cycle of this cross point is called C_t (threshold cycle) or C_q (quantification cycle) and the values of these cycles are directly proportional to the initial amount of the template with the region to be amplified. Higher initial DNA results in a shorter time of fluorescent emission increase, hence a smaller value of C_t. If the efficiency is assumed as 100%, each cycle corresponds to a doubling of the PCR product in the optimal conditions. The efficiency of amplification depends on several factors such as the quality of template, primers, PCR conditions and the length of amplicon (Valesek and Repa, 2005; Yuan et al., 2006). In the third phase of qRT-PCR, fluorescent emission continues to increase linearly as the number of amplified segments increases more and more, and then it reaches a plateau stage which corresponds to the last phase of qRT-PCR where the reaction components are exhausted and fluorescent emission increase is not obtained any more (Wong and Medrano, 2005).

qRT-PCR can be carried out as either one-step or two-step reactions. In one step qRT-PCR, cDNA synthesis from RNA and its subsequent amplification are performed in a single tube whereas in two step qRT-PCR, RNA samples are first converted to cDNA and then the cDNA is amplified in a different PCR reaction employing different reagents and a different protocol. When SYBR green is to be used as the fluorescent detection method, two-step qRT-PCR is more preferred as it reduces the possibility of primer dimer formation and it is readily reproducible. Yet, two-step qRT-PCR has a higher possibility of contamination as the required work is almost doubled.

The gene expression in qRT-PCR technique can be quantified either as absolute or relative, in both of which the C_t value is used for quantification. In absolute quantification, the gene with the unknown expression profile is quantified based on a standard curve of a gene with known expression profile, assuming expression of both genes have similar efficiency. In relative quantification, on the other hand, the changes of the steady state level of the gene of interest is quantified relative to a reference gene, which is generally a housekeeping gene that is assumed to be continuously (constitutively) expressed in the cell. In this method, result is given as the expression ratio of gene of interest/reference gene. As there is no need to construct a standard curve, the relative quantification approach is used more frequently. For relative quantification, the normalized mean expression level can be determined by different mathematical approaches, among which the most preferred two are the employment of comparative C_t values and the employment of efficiency corrected C_t model of Pfaffl (2001). The former model assumes the amplification efficiencies of both the target and the reference genes are same.

However, most of the time, this is neither biologically nor experimentally possible. In such cases where the efficiencies are different, the efficiency corrected C_t model has to be used in which the amplification efficiency of both

the gene of interest and of the reference gene are incorporated to the formula (Pfaffl, 2001).

1.5 The Aim of the Present Study

In a recent work in our laboratory, eight different industrially important extracellular enzymes produced by *B. subtilis* were found to be significantly increased in bacilysin-negative OGU1 strain when compared to its parental strain PY79, as determined by comparative secretome analysis between the two strains. With the aim of further supporting these findings of secretome analysis, the transcript analyses of their corresponding genes as well as the relevant enzyme assays were performed in a comparative fashion.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Their Growth and Maintenance

The strains of *B. subtilis* used in this study were prototropic wild-type strain PY79 and *bacA::lacz::erm* strain OGU1, the latter being bacilysin non-producer derivative of PY79 which was constructed by our group (Köroğlu et al, 2011). The strains were maintained on Luria Bertani (LB) agar plates and inoculated into the liquid Perry and Abraham (PA) media. When growing OGU1, the media was supplemented with lincomycin and erythromycin at final concentrations of 25 μg/mL and 1 μg/mL, respectively, for the selectivity. Initial growth was performed in 10 mL of medium in 50 mL Falcon tubes incubated at 37^{o}C overnight. Then 0.5 to 1.0 mL of this culture was inoculated into 100 mL of PA medium contained in a 250 mL flask to an initial optical density of 0.1 at 595 nm. After inoculation, the cultures were incubated at 37°C (200 rpm) and culture supernatants were collected at 12th h by centrifugation at 16,000 g for 15 min and the supernatants were subjected to enzyme activity determinations.

2.2 Culture Media

Composition and preparation of PA media are given in Appendix A.

2.3 Buffers and Solutions

Composition and preparation of buffers and solutions are given in Appendix B.

2.4 Chemicals and Materials

The chemicals are given in Appendix C.

2.5 Enzyme Activity Assays

2.5.1 Arabinanase Activity Assay

The 3,5-dinitrosalicylic acid method by Miller et al. (1959) was applied for the determination of hydrolytic activities of arabinanase, chitosanase and levanase enzymes. For arabinanase assay, 100 µl of the culture supernatants was incubated with 600 µl of 1% (w/v) sugar beet red arabinan (Sigma Chemicals Co.) in 50 mM sodium acetate buffer (pH 5.6) at 50°C for 15 min. 700 µl of DNS reagent was added to the reaction mixture. After 5 min incubation in boiling water, the mixture is cooled down to room temperature and the quantity of liberated non-reducing sugars was measured by measuring the absorbance at 540 nm. One unit of arabinanase enzyme activity was defined as the amount of enzyme required to liberate 1 µmol L-arabinose per min under optimal assay conditions. The calibration curve shown in Figure 2.1 was used to determine the activity of endoarabinanases produced by parental and bacilysin-negative strains.

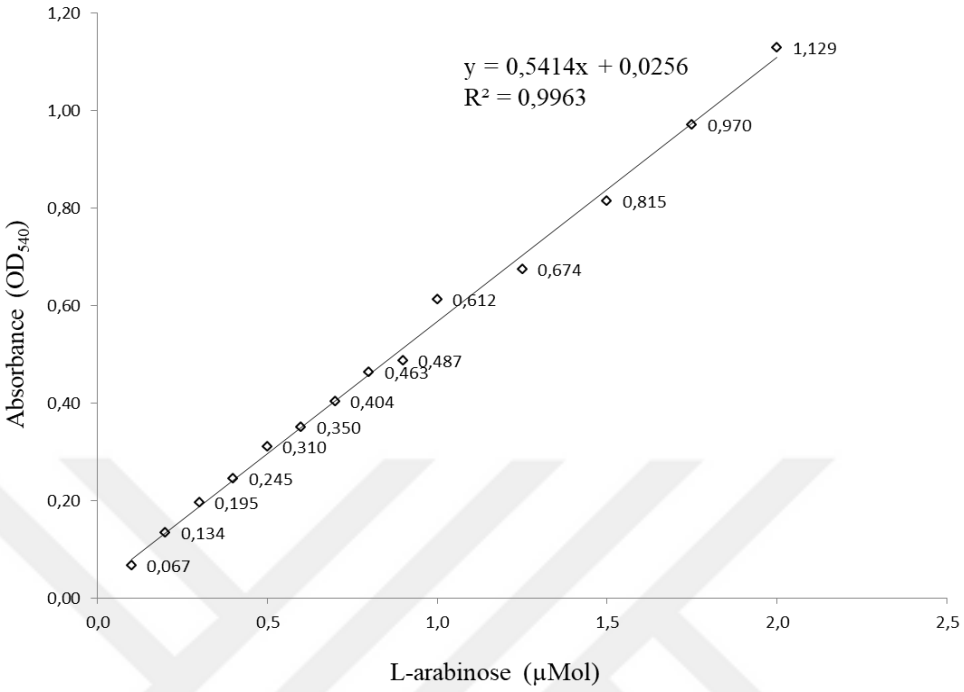


Figure 2. 1 Calibration curve for endoarabinanase assay

2.5.2 Chitosanase Activity Assay

For the chitosanase activity assay, 100 µl of the culture supernatants (pH 5.5) was incubated with 500 µl of 0.5% (w/v) low molecular weight chitosan (Sigma) in 200 mM sodium acetate buffer (pH 5.6) at 37°C for 30 min. After the addition of 600 µl of DNS reagent followed by the boiling and cooling steps, the absorbance at 540 nm was measured. One unit of chitosanase activity was defined as the amount of enzyme required to liberate 1 µmol of D-glucosamine per min under optimal assay conditions. The calibration curve shown in Figure 2.2 was used to determine the activity of chitosanase produced by parental and bacilysin-negative strains.

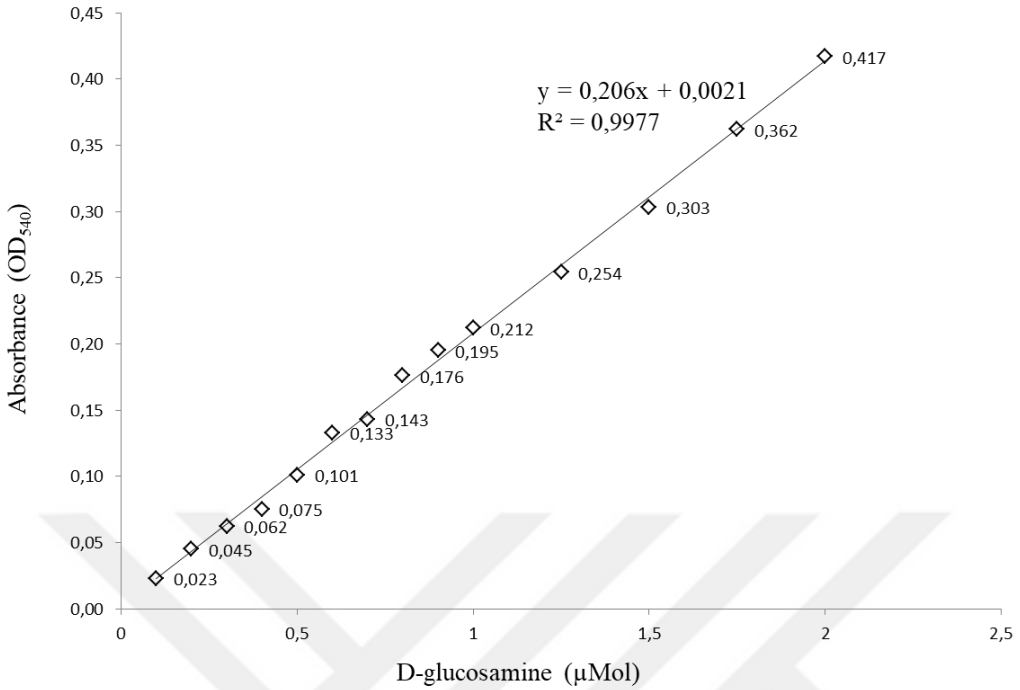


Figure 2. 2 Calibration curve for chitosanase assay

2.5.3 Levanase Activity Assay

For levanase activity assay, 100 µl of the culture supernatants (pH 5.5) was incubated with 400 µl of 5% (w/v) inulin in 0.1 M sodium acetate buffer (pH 5.6) at 55°C for 30 min. 500 µl of DNS reagent was added to the reaction mixture, kept in boiling water for 5 min and then cooled down to room temperature before the absorbance measurement at 540 nm. One unit of levanase activity was defined as the amount of enzyme required for the liberation of 1 µmol of fructose per min under optimal assay conditions. The calibration curve shown in Figure 2.3 was used to determine the activity of levanase produced by parental and bacilysin-negative strains.

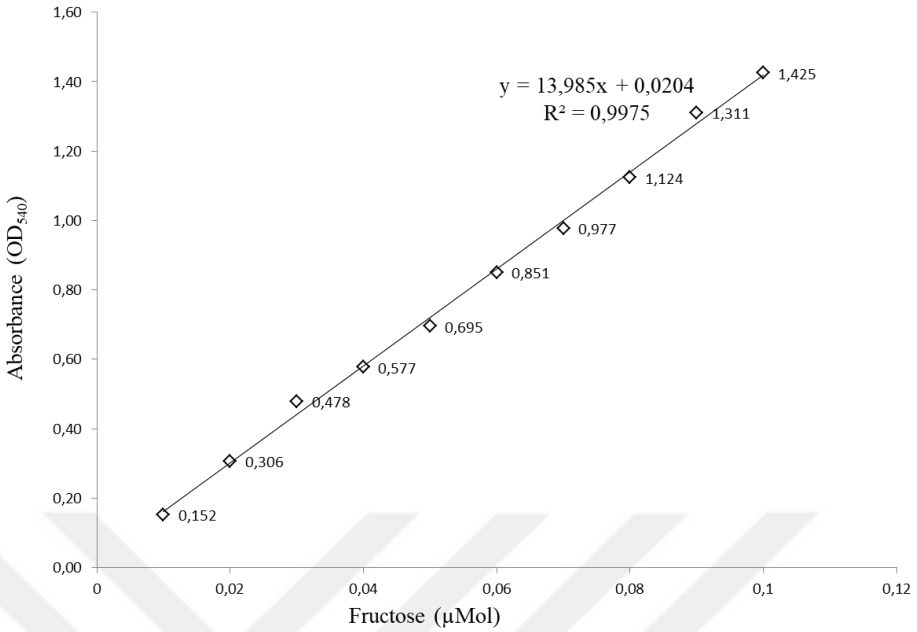


Figure 2. 3 Calibration curve for levanase assay

2.5.4 Phytase Activity Assay

For the quantitative phytase assay, 100 µl of the culture supernatants (pH 5.5) of OGU1 and PY79 strains was incubated with 500 µl of 0.2% (w/v) phytic acid sodium salt (Sigma) in 0.1 M sodium acetate buffer (pH 5) at 55°C for 2 h. The reaction was stopped by the addition of 600 µl of 5% (w/v) trichloroacetic acid. For the development of colour, 600 µl of colour reagent (Yanke et al., 1998) containing 1 volume of 2.7% ferrous sulphate solution and 4 volumes of 1.5 % (w/v) ammonium molybdate solution in 5.5% (v/v) sulphuric acid was added. After 5 min incubation for complete development of colour, liberated inorganic phosphate was measured at 700 nm. One unit of phytase activity was defined as the amount of enzyme required or the liberation of 1 µmol of Pi under the optimal assay conditions. The calibration curve shown in Figure 2.4 was used to determine the activity of phytase produced by parental and bacilysin-negative strains.

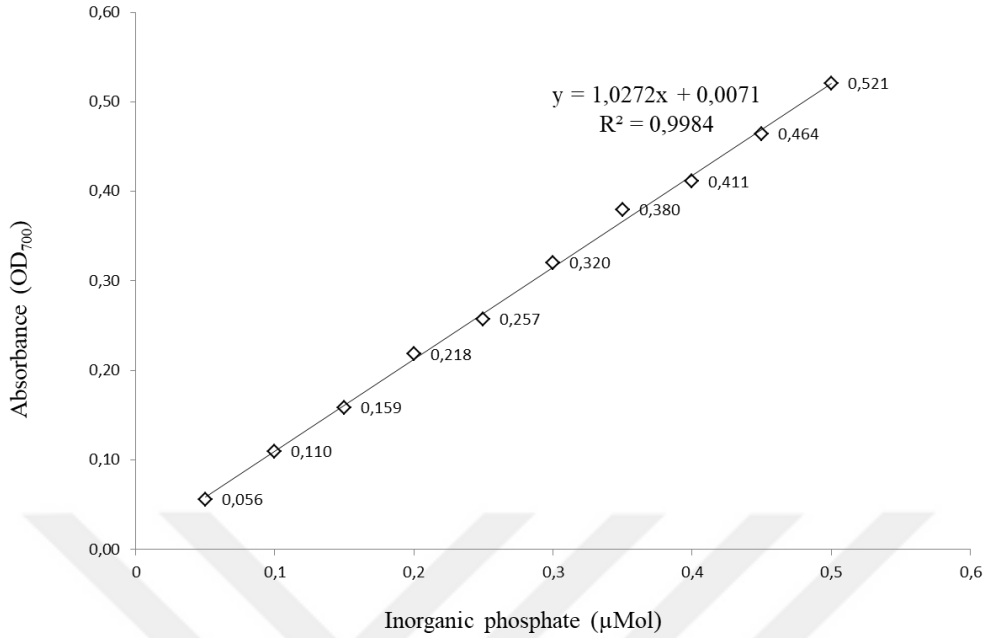


Figure 2. 4 Calibration curve for phytase assay

2.5.5 Lipase Activity Assay

Extracellular lipase activity was estimated by using the method described by Prim et al. (2000) Briefly, 500 µl of p-nitrophenyl palmitate solution were added to 50 µl of culture supernatants (pH 10) of OGU1 and PY79 strains which were then incubated at 37°C for 1 h. pNPP stock solution was prepared by the addition of 1 volume of 0.15% (w/v) pNPP in isopropanol to 9 volumes of glycine-NaOH buffer (pH 10) containing 0.1% (w/v) gum Arabic and %0.4 (v/v) Triton X-100. 50 µl of 0.1 M sodium carbonate was used to terminate the reaction. Liberated p-nitrophenol (pNP) is measured by the absorbance at 410 nm. One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of pNP per minute under the optimal assay conditions. The calibration curve shown in Figure 2.5 was used to determine the activity of lipases produced by parental and bacilysin-negative strains.

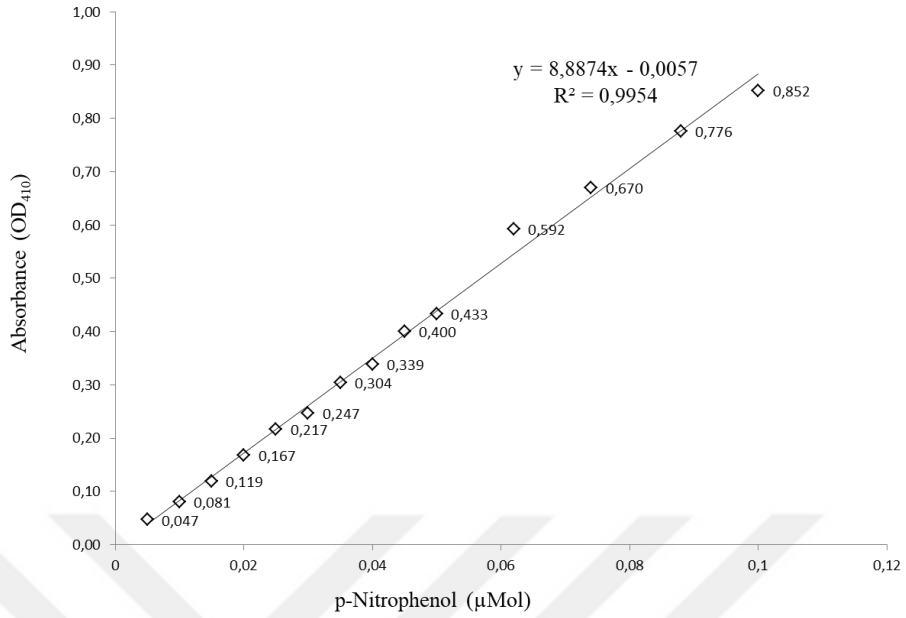


Figure 2. 5 Calibration curve for lipase assay

2.5.6 Protease Activity Assay

For the activities of both bacillopeptidase F and minor extracellular protease Vpr, 2 ml of 0.65% (w/v) casein in 50 mM potassium phosphate buffer (pH 7.5) was incubated with 1 ml of the culture supernatants of OGU1 and PY79 at 37°C for 10 min. Subsequently, the procedure outlined by Cupp & Enyard (2008) was followed. Shortly, 2 ml of TCA was added to the reaction mixture and incubated 30 min for non-digested proteins to aggregate. 2 mL of the clear part of the reaction mixture is filtered through 45 nm syringe filters. 5 ml of 0.5 M sodium carbonate and 1 ml of 0.5 M Folin & Ciocalteu's Reagent (Sigma) were then added. The mixture was incubated for an additional 30 min for the formation of bluish colour and the absorbance was measured at 660 nm. One unit of proteolytic activity was defined as the amount of enzyme required to release 1 µmol of tyrosine per min under the optimal assay conditions. The calibration curve shown in Figure 2.6 was used to determine the activity of proteases produced by parental and bacilysin-negative strains.

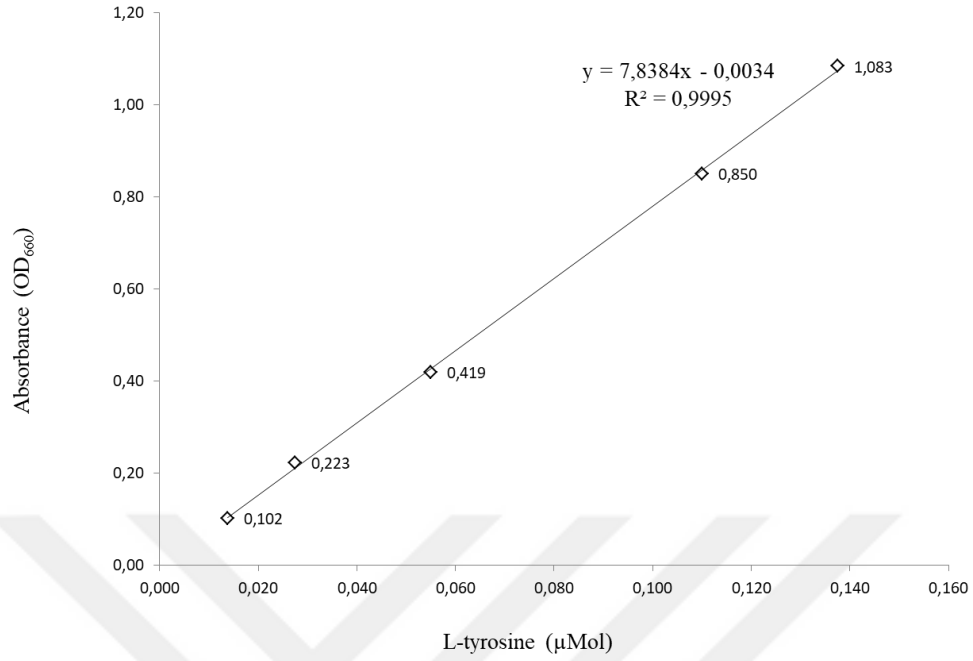


Figure 2. 6 Calibration curve for protease assay

2.5.7 Endonuclease Assay

Endonuclease activity of culture supernatants was measured by following the method of Mossakowska et al. (1989). Shortly, 100 µl of culture supernatants (pH 9) were incubated with 900 µl 0.2% (w/v) yeast RNA in pre-warmed Tris/HCL buffer (pH 8.5) at 37°C for 10 minutes. RNA hydrolysis activities of the strains were compared by the decrease in absorbance at 298 nm.

2.5.8 Statistical Analysis of Enzyme Activities

Three biological replicates (supernatants obtained from three parallel cultures) with three technical replicates were carried out for each of the seven different enzyme activity measurements. Student's unpaired parametric t-test was used to compare the enzymatic activities of two strains. The application of this test

and the plotting of the results were carried out by using the GraphPad Prism® 7.04 program. Statistical significance of results were as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in the graphs.

2.6 RNA Procedures

2.6.1 Isolation of RNA Samples

For the isolation of total RNA, 100 ml of 12^{th} h cultures of *B. subtilis* PY79 and OGU1 were aliquoted to 2 mL Eppendorf tubes and centrifuged at 3000 rpm for 10 min to collect the cells. To prevent any physiological changes, the pellets were stored in -80°C until further used for the isolation of RNA samples. Macherey-Nagel Nucleopsin® RNA isolation kit was used following the manufacturer's instructions with two minor modifications: (i) the use of 450 µL RA1 lysis buffer and (ii) the use of 450 µL 70% (v/v) ethanol. To prevent any possible RNase contamination, the working area was cleaned by using RNase inhibitor solution (RNase AWAY®, Sigma-Aldrich) and all chemicals were prepared by using nuclease-free sterilized distilled water containing diethyl pyrocarbonate (DEPC).

2.6.2 cDNA synthesis by Reverse Transcription PCR

For the construction of cDNA library from the isolated RNA samples, BioRad iScript® cDNA kit containing both oligo(dT) and random hexamer primers was used following instructions in the user's manual. From each RNA sample, a volume of 20 µL reaction mixture contained 4 µL of 5X iScript reaction mixture, 1 µL of reverse transcriptase, 1 µL of isolated RNA and an adequate amount of nuclease-free distilled water to complete the mixture to 20 µL was used. The PCR conditions were as follows: 5 min at 25°C for the annealing of oligo(dT) and random primers; 30 min at 46 °C for the cDNA synthesis to occur and lastly, 5 min at 95°C for the inactivation of the reverse transcriptase

activity. Resulting cDNA samples were kept in -20°C until to be used in qPCR reaction.

2.6.3 Real Time Quantitative PCR

For determination of Real Time (RT) quantitative mRNA expression profiles of *B. subtilis* PY79 and OGU1 samples, SYBR Green chemical fluorescent technique was employed using SYBR Green Supermix (BioRad) by following the manufacturer's instructions. The list of primers is given in Table 2.1. To determine the optimized primer concentration that yields none or the least primer-dimer formation, several primer concentrations ranging from 50 to 300 nM were tested. Among these, 200 nM final primer concentration was found to be the most optimal for the amplification of the cDNA samples. The formation of primer-dimers was checked through the melting curve analysis in which a single melting curve corresponds to the amplification of only the gene of interest. The reaction mixture (10 µl) contained 5 µl of SYBR Green Supermix, 2 µl of forward and reverse primers, 1 µl of cDNA (100 ng final concentration) template. For no-template control, 1 µl of RNase- free distilled water was used instead of cDNA. The PCR conditions were as follows: initial denaturation step for 30 sec at 95°C followed by 40 cycles of denaturation step for 5 sec at 95°C and amplification step with 1 min at 62°C. For melting curve analysis, a measurement from 65 to 95°C at 0.5°C intervals was carried out. Standard curves were constructed by using 100, 10, 1 and 0,1 ng concentrations of cDNA samples. Both amplification and efficiency runs were carried out in Bio-Rad CFX Connect^{TM} Real-Time PCR Detection System. Each qPCR run included two biological replicates with three technical replicates, yielding a total of 6 C_q values for *B. subtilis* PY79 and 6 C_q values for *B. subtilis* OGU1 cDNA templates.

Table 2. 1 Primers designed for RT-qPCR analysis.

Gene Name	Primer	Primer Length	GC content (%)	Self-dimer energy	Heterodimer energy
abn2	5' CCACAACTCCGCATACTATGAT 3'	22	45,5	-4,39	-4,64
(BSU39330)	5' GGACTCTGACTTCGTGTTCTTC 3'	22	50	-3,61	
csn	5' GCCGCTCAAGACAAAGTAAATG 3'	22	45,5	-3,61	-3,61
(BSU26890)	5' ACCATCGCCATGCTGAATAA 3'	20	45	-5,38	
sacC	5' CTGGCTGGACGACTGTAAAT 3'	20	50	-3,61	-3,61
(BSU27030)	5' CCGGATGCTGAAGACAAGAT 3'	20	50	-9,75	
phy	5' ACAGATCCGAACCATCCTATTTC 3'	23	43,5	-4,62	-4,89
(BSU19800)	5' CCCTTGTTTGCCTGTCACTA 3'	20	50	-3,14	
lipA (estA)	5' ATCTGGACGGCGGAAATAAAG 3'	21	47,6	-3,61	-3,61
(BSU02700)	5' TATCGGCACTGCTGTAAATGG 3'	21	47,6	-5,09	
bpr	5' GGAGAGAAAGAAGCAGGAGAAA 3'	22	45,5	-3,14	-4,64
(BSU15300)	5' GTTGTTCCGTTGTCAGTTGATG 3'	22	45,5	-3,16	
vpr	5' TCTTGCTTATCGTGTGTTAGGG 3'	22	45,5	-3,61	-5,25
(BSU38090)	5' CGAGAGACAGGTTCATCACATC 3'	22	50	-3,61	
yhcR	5' AGCTCTCCAGTTTCCTCTCT 3'	20	50	-6,34	-3,89
(BSU0190)	5' CATCCGTGCCTTCATCAAATTC 3'	22	45,5	-5,36	
rpoB	5' TGAACATCGGGCAGGTATTG 3'	20	50	-3.61	-6.14
(BSU01070)	5' GTTTCCCAGACATCCTCTTCTC 3'	22	50	-3.17	

*All primers were designed with a T_m value of 62.

2.6.4 RT-qPCR Analysis

Relative expression in folds for the eight genes of interest was normalized to the expression fold of *rpoB* using the formula:

$$R = (E_{gene})^{\Delta C_{t\ gene}(\text{MEAN PY79} - \text{MEAN OGU1})} / (E_{rpoB})^{\Delta C_{t\ rpoB}(\text{MEAN PY79} - \text{MEAN OGU1})}$$

2.6.5 Statistical analysis

Student's unpaired parametric t-test was used for statistical analysis of RT-qPCR fold change data obtained with the formula given above. The application of this test and the plotting of the results were carried out by using the GraphPad Prism® 7.04 program. Statistical significance of results were as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in the graphs.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results of Enzymatic Activity Assays

Main difference between the proteomic analysis and the enzymatic activity assay was the use of purified proteins in the former but the use of whole culture supernatants in the latter, which eventually may result in a significant difference between two assays. The reason behind the use of culture supernatants in this study was to prove the presence of more enzymatic activity (resulting by the presence of more enzymes) in OGU1 strain when compared to those of PY79 strain in the *same volume* of extracellular fluid produced by the *same density* of cells, determined by the OD_{600} absorbance values. In accordance with this purpose, the present study was a success considering that all enzyme activities were shown to be higher in OGU1 strain to different extents, even though there was no direct correlation to the results of proteomic analysis.

3.1.1 Endoarabinanase activities of *Bacillus subtilis* PY79 and OGU1

The average results of endoarabinanase activity assays are given in Figure 3.1.

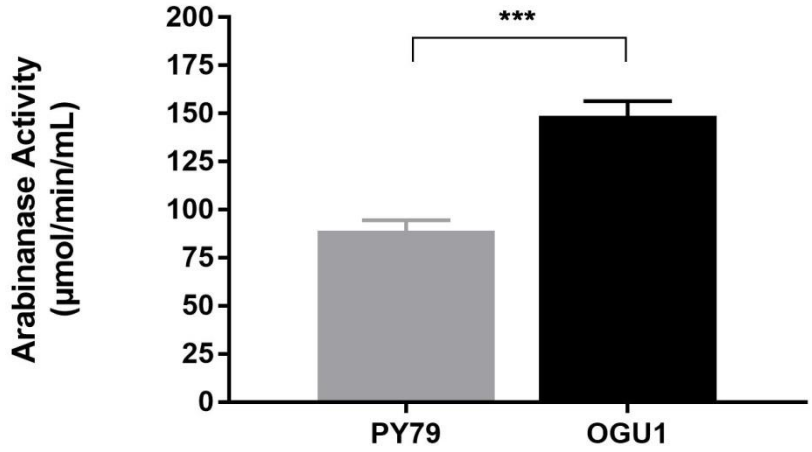


Figure 3. 1 Endoarabinanase activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of L-arabinose released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

Average endoarabinanase activities for *B. subtilis* OGU1 and PY79 were found to be 148.7 ± 7.637 and 88.98 ± 5.591 U/min/mL, respectively. Bacilysin non-producer strain OGU1 has an average of 1.78-fold increase in its endoarabinanase activity against substrate arabinan, measured by DNS method. This result was consistent with that of secretome analysis, supporting the expectation that not only the amount of protein but also its activity should indeed be higher in the absence of bacilysin, and suggesting bacilysin's possible interaction with one of the components of arabinanase biosynthetic pathways either directly or indirectly.

However, the ratio of OGU1/PY79 arabinanase activity was lower than the OGU1/PY79 Abn2 ratio found in proteomic data.

Although the proteomic analysis is capable of showing an increase in the level of Abn2 directly, the enzymatic activity for arabinan degradation was most probably not due to Abn2 alone, but also due to AbnA endoarabinanase found in the culture supernatant (Leal & de Sá-Nogueira, 2004). The presence of this second enzyme with the same substrate specificity and catalytic action may be one of the reasons why the difference with respect to arabinanase activity between the two strains was less than expected.

L-arabinose is recently gaining a growing interest for its possible use as a food additive, due to its sweet taste, its low uptake/absorption by the human body and its ability to inhibit sucrase (Matuso et al., 2000). Intestinal sucrase is competitively inhibited by the presence of arabinose; therefore the glycaemic response after sucrose ingestion is reduced. *In vivo* experiments have already shown that the increase in blood glucose level after sucrose ingestion is suppressed by arabinose supplementation in a dose-dependent manner (Sanai et al., 1997). Due to this feature, arabinose is considered to be one of the biomedical agents for the prevention of hyperglycaemia, especially in diabetic patients. Endoarabinanases, due to their ability to release a large number of arabinoses from arabinan polysaccharides, are the main enzymes to be used in this pharmaceutical industry.

Arabinanases are also receiving attention for their use in the fruit juice industry. Pectinolytic enzyme preparations utilized during the making of fruit juice contain significant amounts of α-L-arabinofuranosidases (AFs), which specifically remove 1,3-side chains and expose arabinan backbone chains. The remaining polysaccharide is responsible for the precipitation (haze) formation in fruit juices. Endoarabinanases hydrolyze long chains of arabinan and release

arabinose monomers, which results in a decrease of the level of haze formation as well as in sweeter taste (Romboust et al., 1988).

Another major area of arabinanase use is the production of bioethanol from lignocellulosic materials for both industrial and academic research. The most critical step in the bioethanol production from biomasses (such as sugarcane, sugar beet, corn or wheat) is the efficient and preferably rapid conversion of hemicelluloses and celluloses to sugar monomers (Balat et al., 2008). Both AFs and endoarabinanases represent an important factor in the hydrolysis of arabinose side chains and long arabinosyl residues found in arabinan. They expose the remaining hemicellulose structure for other hydrolytic enzymes to be used in the complete degradation to monomers. However, as these polymer structures in recalcitrant biomasses are only accessible to enzymatic and chemical degradation in high temperatures, the thermostability of the enzymes is the main limiting factor during the process. Up to date, the reported number of AFs and endoarabinanases is low compared to that of other commercial and industrial enzymes, and still very little is known about their catalytic activity and thermostability (Shallom & Shoham, 2003; Wong et al., 2008).

3.1.2 Chitosanase activities of *Bacillus subtilis* PY79 and OGU1 strains

The average results of chitosanase activity assays are given in Figure 3.2.

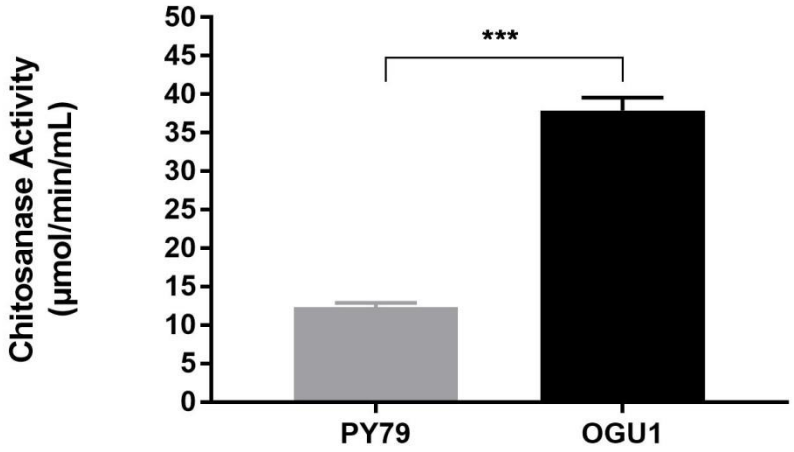


Figure 3. 2 Chitosanase activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of D-glucosamine released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

As can be seen in Figure 3.2, the chitosanase activity against low molecular weight chitosan was higher in OGU1 culture supernatants when compared to PY79, with the specific enzymatic activities of 37.89 ± 1.668 and 12.3 ± 0.5853 U/min/mL, respectively. Even though it was likewise measured by the DNS method, the chitosanase activity in the OGU1 strain was found to be 3.09-fold higher, being thus the closest to the proteomic analysis result. One possible explanation for this is that chitosanase is the major extracellular enzyme produced by *B. subtilis* and it releases large amounts of D-glucosamine by the hydrolytic action (Voigt et al., 2009). Therefore, it may be easier to see the difference between two strains, since other non-reducing end sugars already

found in the supernatant are in comparatively lower amounts. Still, the ratio of OGU1/PY79 chitosanase activity was slightly lower than the ratio of OGU1/PY79 Csn (5.0) found in the proteome analysis. This small difference may, again, be possibly attributed to the difference between the use of purified proteins and culture supernatants and the composition of supernatant itself.

Microbial chitosanases come with different applications, on the front burner of which are the environmental use of these enzymes and their role in the production of biologically important agents, called chitooligosaccharides.

During food processing, the seafood industry generates a huge amount of chitinous by-products and biowaste all over the world. Chitosanases, together with chitinases are responsible for the degradation and subsequent recycling of enormous amounts of crustacean shell accumulation in nature (Suresh, 2012). Moreover, chitosanases also have an important role in the bioconversion of these marine crustacean accumulations for the production of bioactive molecules with antioxidant and enzymatic activities (Wang et al., 2009; Wang et al., 2011). Furthermore, these enzymes are also important for the prevention of Zygomycete propagation, as the major structural component of the cell wall of these harmful fungi is chitosan polymers. Plants have already been found to produce chitosanases against Zygomycetes as a defence mechanism to phytopathogenesis (Hsu et al., 2012).

From the molecular biology perspective, the most important application of chitosanases is to produce chitooligosaccharides (COS) which are low molecular weight polysaccharides with less than 10 kDa and which, unlike chitosan, these are readily soluble in water, due to their free amino groups on glucosamine residues and their relatively short length (Kim & Rajapakse, 2005; Liu et al., 2009). COSs exhibits more biological functions than chitosans, including antioxidant, antimicrobial and anti-tumor actions, lowering

of blood cholesterol, protection against infections, controlling of arthritis as well as induction of callose, formation of root nodules and inhibition of the growth of pathogens in plants (Kim & Rajapakse, 2005). These properties have resulted in a growing demand for COS production in recent years, especially in biomedical and food industry.

Due to their chitosan rich cell wall hydrolysis activities, chitosanases have also been used for fungal protoplast generation, which is generally employed in genetic studies for somatic hybridization, and in strain engineering for industrial and biotechnological applications. Fungal protoplasts are also important for ongoing mycological studies (Price & Storck, 1975; Fenton et al., 1978).

3.1.3 Levanase Activities of *Bacillus subtilis* PY79 and OGU1

The average results of levanase activity assays are given in Figure 3.3.

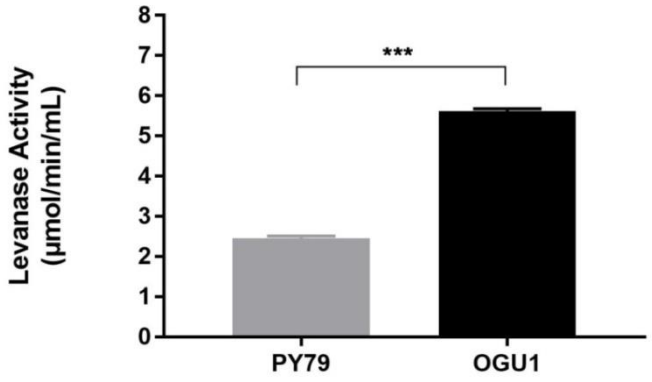


Figure 3. 3 Levanase activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of fructose released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

The inulinase acitivites of OGU1 and PY79 levanases were found to be 5.618 ± 0.05577 and 2.453 ± 0.05423 U/min/mL respectively. This activity of inulinase was found to be 2.30-fold more in OGU1 (Fig. 3.3). Although it was lower than the ratio found in proteomic data (4.6-fold), inulinase activity assay clearly showed that the amount of levanases in supernatant was increased at least more than 100% in the bacilysin non-producer strain. The reason why the difference between two strains with respect to their levanase activities is less than the difference with respect to protein levels of SacC can be the fact that levanase SacC hydrolyze not only inulin, but also levan and sucrose (Martin et al., 1987). *B. subtilis* itself is able to produce and secrete levan polysaccharites (Méndez-Lorenzo et al., 2015) and sucrose is already found in the PA medium used to grow *B. subtilis* cells, hence some of the measured fructose could have been released from the hydrolysis of levan and sucrose.

The major component of dental plaque is shown to be *Actinomyces viscous* and it plays an important role in the development of plague-related oral diseases. Extracellular levan produced by *A. viscous* is one of the components of the dental plaque, and it is utilized by plaque flora as a carbohydrate source, resulting in acid production. Moreover, levan was showed to be mitogenic for B-lymphocytes and to activate the alternative complement system. Hence, levan present in plaque lesions is thought to be involved in the induction of periodontal diseases. Levan degrading enzymes are potential agents both to eliminate dental plaque formation and to induce immune system for periodontal diseases by reducing the levan level in the oral cavity (Igarashi et al., 1987).

Yet, the main interest in levanase is its use in the degradation of biological slime and biofilms, especially in the paper industry. Levanase catalyzes the dissolution of levan, the major component of these slime polysaccharides, which normally act as a barrier to the action of biocides by preventing their

penetration deep inside the slime. By the action of levanases, biocides can come in contact with the surface layers of slime producing microorganisms, can enter the cells and exert their mechanisms of action to prevent further growth and production (Chaudhary et al., 1995).

Other applications of fructan degrading enzymes such as levanases and inulinases are the production of high fructose inulin syrups by the enzymatic hydrolysis of inulin, and the direct fermentation of fructans to produce several important chemical products including acetone-butanol and ethanol. As an important example, inulin can be converted to 2,3- butanediols (BDs), which are the starting materials for the production of bulk chemicals such as 1,3-butadiene and methyl ethyl ketone (Huang & Ouyang, 2011; Syu, 2001). Not only this, but 2,3-BDs can replace ethanol and methanol as fuel additives and/or fuel, thanks to their high heating value of 27,200 J/g, which makes them important potential candidates as energy sources, especially considering that the crude oil reserves of the earth are gradually being exhausted (Celińska & Grajek, 2009). Hence, there is an ongoing interest in finding ways to produce large concentrations of 2,3-BDs from non-food, cheap and renewable sources. For this, levan and inulin represents two strong candidates. These fructans are not just found in a wide range of plant species but they are also the second most abundant non-structural polysaccharides units of these plants. Out of these fructans, inulin stands out as a large storage component of Gramineae and Compositae family plants, in which it accumulates in tubers and underground roots (Gupta & Kaur, 1997). Although many levanases and inulinases have already been shown to be effective tools for the production of 2,3-BDs, the only commercial inulinases, which are obtained from *Aspergillus niger*, are expensive, and there is no commercial levanase at all for the moment (Li et al., 2014).

Fructan degrading enzymes are also responsible for the production of important oligosaccharides to be used in the probiotic industry. Fructooligosaccharides (FOSs) are fructans with a fructose polymerization degree of 10 or lower, and they can be classified either as inulin-type or levan-type FOSs. Inulo- and levanooligosaccharides have already been shown to have a wide range of important functions as energy sources, in the protection from aging, the differentiation in plants, for promoting the growth of several enteric bacteria and for the stimulation of the immune system (Lim et al., 1997). These oligosaccharides are further hydrolyzed by *Bifidobacterium*, which is found predominantly in the digestive tract of humans, and they exert probiotic properties by selectively inhibiting the proliferation of harmful bacteria inside the host (Hidaka et al., 1986). Furthermore, these oligosaccharides are potential non-caloric sweeteners as mentioned above. Especially inulooligosaccharides, together with GOS (galactooligosacharides), are now the two most important components of industrial probiotic foods. These commercial inulin FOSs are produced either by using fungal frucosyltransferases, using sucrose as the substrates, or by microbial inulinases, by using chicory inulin as the substrate (Kolida & Gibson, 2007).

3.1.4 Phytase activities of *Bacillus subtilis* PY79 and OGU1 strains

The average results of phytase activity assays are given in Figure 3.4.

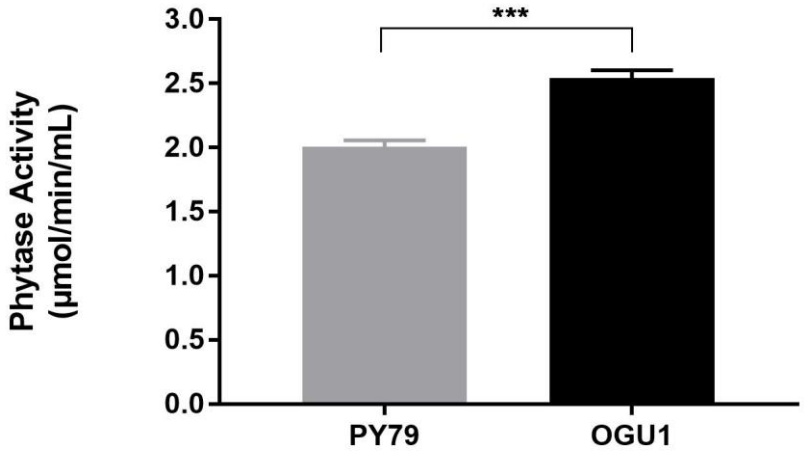


Figure 3. 4 Phytase activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of inorganic phosphate released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

As can be seen in figure 3.4, the specific phytase activities of *B. subtilis* OGU1 and PY79 strains were 2.54 ± 0.0636 and 2.007 ± 0.04914 U/min/mL, respectively. Although the comparative secretome data revealed a 2.08-fold increase and the expression data confirmed this with a 3.05-fold increase (see Fig. 3. 12), the enzymatic activity difference was the lowest among the eight proteins, with an increase in phosphate liberated by 3-Phy activity of only 1.27-fold in OGU1 strain. As the enzyme is not purified but used as it is in the culture media, the presence of phosphate groups that were not liberated specifically by this enzyme could have resulted in the decrease of the difference between the two compared strains. However, the main reason of this

small difference is possibly because of the use of phytate salt as the substrate. *B. subtilis* phytase is well known to have strict substrate specificity against phytate-Ca^{2+} complexes (Kerovuo et al., 1998). Although there is Ca^{2+} found in PA medium, incubation time might have been insufficient for most of the phytate molecules to form stable complexes with calcium ions. As a result, the available phytate in the medium would not be an appropriate substrate for the phytase enzyme, leading to the unexpectedly low activity difference.

Phosphorus has important roles in the regulation of enzymes, the biosynthesis of nucleic acids and the construction of cell membranes. Deficiency of available phosphorus in soil and water is one of the major problems, especially for the agricultural industry. Being the major storage of phosphorus, phytates are not readily available for plants, as they form complexes with cations, metals, amino acids, proteins and several other soil components (Noureddini & Dang, 2008). These complexes are insoluble and not nutritionally available for the absorption from human and animal digestion system, which lacks adequate phytate-degrading enzyme activity. Considering that the diet of a large population of humans depends on plant staple foods, ingesting high levels of phytate results especially in iron and zinc deficiency (Lopez et al., 2002). Moreover, many populations lack the adequate supplement of minerals in their diet. The concern grows when considering the mineral uptake lowering impact of phytate on these vulnerable segments of the populations that has to survive almost solely on grain and legume staple foods. While removal of plant tissues containing phytic acid may help to solve this problem, most of the mineral deposits of these grains are also removed during the milling and polishing processes. To overcome this problem, phytases can be used in breadmaking and animal feedstock industry, and desirably in the environment of gastrointestinal tracts of monogastric organisms, due to the fact that they are exceptionally strong improvers of available phosphorus and minerals. For their large-scale use in industrial applications, phytases should be capable of

remaining active in high temperatures and should be highly specific for phytate even in a wide range of temperature and pH values (Greiner & Konietzny, 2006). Hence, research is still ongoing in an attempt to find the ideal phytase with more desirable features or to engineer phytases to optimize their stability and catalytic properties.

Commercial phytases were first launched into the industry in 1991, and today they compromise one-third of the market of feed enzymes, with an estimated annual sale of 350 million US dollars (Greiner et al., 2006; Reddy et al., 2015). Up to date, phytase degrading enzymes have mostly, if not solely, been used as feed additives for feedstock for swine, poultry, and fish. Especially for the fish industry, large amounts of phytate, hence phosphorus, are discarded into the water as fish, unlike ruminants and some of the monogastric animals, are incapable of digesting the phytic acid. The quality of the water bodies is directly determined by the particulate form of phosphorus excreted by fish as undigested phytic acid. This particulate phosphorus accumulates in the sludge and becomes available for phytoplankton. Use of microbial phytases in the fish industry can overcome this problem, by making chelated phosphorus readily available for fish and resulting in less fecal excretion (Sajjadi & Carter 2004 ; Singh & Satyanarayana, 2006; Singh et al., 2011).

Similarly, animals such as humans, pigs or chickens cannot produce an adequate number of phytases and therefore an enormous amount of undigested phytate is excreted through manure each day. Soil microorganisms eventually hydrolyze these phytates and the resulting phosphorus is transported to rivers, lakes, and sea, causing eutrophication and subsequent oxygen depletion (Murugesan et al., 2005; Pillai et al., 2009).

Recent *in vivo* experiments have moved the scope into the regulations and impacts of phytases on the metabolic, biochemical and molecular perspective.

Firstly, phytase comes to the front as one of the most important enzymes to produce inositol derivatives. Inositol phosphates have diverse roles in the cellular regulation, communication, and signalling. Among these, the most important ones are the mobilization of calcium from ER reserves and the stabilization of enzymes (Billington, 1993; Siren, 1986). Inositol derivatives can be used as enzymes substrates or enzymes inhibitors, the latter being an important feature for these molecules to be used in drug development (Laumen & Ghisalba, 1994). However, the application of phytase in medicine and human health represents a brand new avenue. An experiment with piglets has clearly shown that the expression of Na-dependent glucose transporter 1 in small intestines is decreased in the presence of phytic acid; this decrease was revealed by the addition of dietary phytase (Woyengo et al., 2011). In broilers fed with rapeseed-rich diet, phytase supplementation was shown to improve liver insulin receptor sensitivity and the growth rate (Jozefiak et al., 2010). Another experiment has shown that high dietary phytase supplementation improves the development of bones in piglets, thus suggesting that phytase can be a possible preventive and treatment agent for osteoporosis (Hakimov et al., 2009). Finally, a recent experiment with human trials demonstrated a potential role of phytase in increasing the effectiveness of botulinum toxin on rhytids, hemifacial spasm, and benign essential blepharospasm (Koshy et al., 2012).

Due to these diverse applications of phytases, researchers have taken an interest in finding more economical ways to produce this enzyme in bacterial systems. Although many characterization studies are carried on several fungi, yeast and bacteria, commercial production of phytases was solely from *Aspergillus niger* at the beginning and has expanded only to a few microorganisms since then. However, these enzymes were shown to not be thermostable at the elevated temperatures needed for feedstock making processes, and they exhibited broad range of substrate specificity. *Bacillus subtilis* phytases step forward as an ideal substitute, due to their high

thermostability, their substrate specificity for calcium-phytate complexes which inevitably form in neutral pH and their strong resistance against the proteolysis which can occur during industrial processes and/or in the digestive tract of animals (Elkhalil et al., 2007).

3.1.5 Lipase activities of *Bacillus subtilis* PY79 and OGU1

The average results of lipase activity assays are given in Figure 3.5.

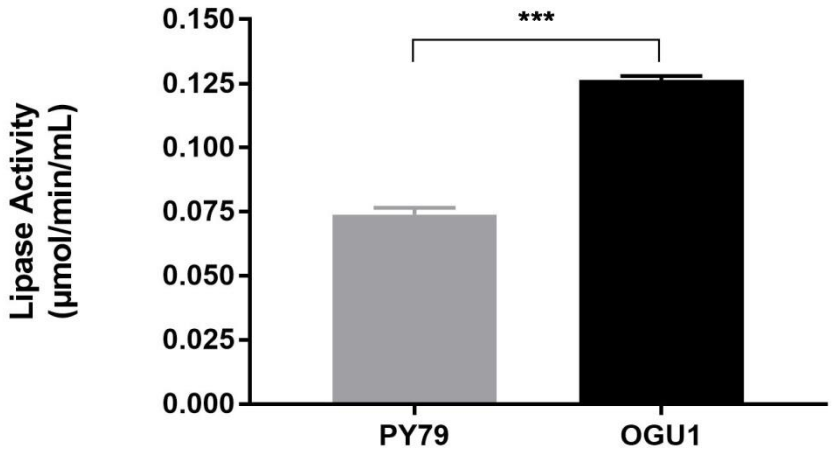


Figure 3. 5 Lipolytic activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of p-nitrophenol released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

The total lipase activities of both strains were measured against p-nitrophenyl palmitate (pNpp) as the substrate and were found to be 0.1263 ± 0.001667 and 0.074 ± 0.002539 U/min/mL for *B. subtilis* PY79 and OGU1, respectively. Although enzymatic activity (1.72-fold) clearly showed the presence of more lipolytic protein in OGU1 strain, the difference between two strains was not

high with respect to the liberated pNP. This was an expected result, considering there is at least one other extracellular lipolytic enzyme, LipB (Eggert et al., 2001), with the ability to hydrolyze triaclyglycerides and nitrophenyl esters, hence it was not possible to clearly track the difference with respect to LipA activity only, within the scope of this study. Still, it was possible to obtain 70% increase in the lipolytic activity in the OGU1 strain when compared to PY79, suggesting a possible role of bacilysin in the biosynthesis of LipA protein.

Lipases account for more than one-third of the total sales in the industry. Because of their strict specificities against a diverse range of substrates and their other desirable enzymatic properties, microbial lipases constitute the majority of the industrially and biotechnologically commercial enzymes.

Applications of lipases, just like that of proteases, are distributed throughout most of the sectors of the industry. Microbial lipases are widely used in fat and oil processing, paper manufacturing, food processing, as well as in the formulation of detergents and degreasing agents, production of a diverse range of pharmaceuticals and chemicals, production of a great majority of cosmetics, degradation of fat-containing environmental waste and the production of synthetic plastics like polyurethane (Aravindan et al., 2007; Gunasekaran & Das, 2005; Hasan et al., 2006; Gupta et al., 2004; Sharma et al., 2001). The lipase-mediated modification has significant importance in the production of lipids in the desired structures, as lipases show high specificity and work under a wide range of reaction conditions. Another application of microbial lipases is in the food industry, in which they modify flavor through the selective degradation of triglycerides to release free fatty acids, which in turn serve as specific flavors or at least as flavor precursors (Macedo et al., 2003). It is also common to use lipases for removing fatty tissues from meat and fish products during their processing for the market (Kazlauskas & Bornscheuer, 1998). Yet another emerging industrial application of microbial lipases is the

transesterification of organic solvents, including the production of the equivalent of cocoa butter and human milk, the synthesis of polyunsaturated fatty acid and the production of biodiesel from vegetable oils (Nakajima et al., 2000). Aromatic polyesters can also be produced by the action of lipase catalysis.

The most commercially significant field for microbial lipases, however, is the detergent industry. These lipases are selected specifically for their low substrate specificity to degrade different types of fatty stains, their ability to withstand washing conditions with high pH and temperate, and their resistance to damaging surfactants and other enzymes present in commercial detergents (Andualema & Gessesse, 2012).

Lipases are also one of the most used enzymes in the pulp and paper industry, where waxes and triglycerides are associated with major problems in paper mills. Lipases remove these pitches and hydrolyze triglycerides to liberate glycerol and monoglycerides, which are much less sticky and readily more hydrophilic (Jaeger & Reetz, 1998).

Highly specific lipases are also used in leather industry, where they are the preferred enzymes to remove fat tissues during the degreasing processes, as they do not harm the rest of the skin and replace costly and time-consuming mechanical degreasing processes (Andualema & Gessesse, 2012).

3.1.6 Protease activities of *Bacillus subtilis* PY79 and OGU1

The average results of protease activity assays are given in Figure 3.6.

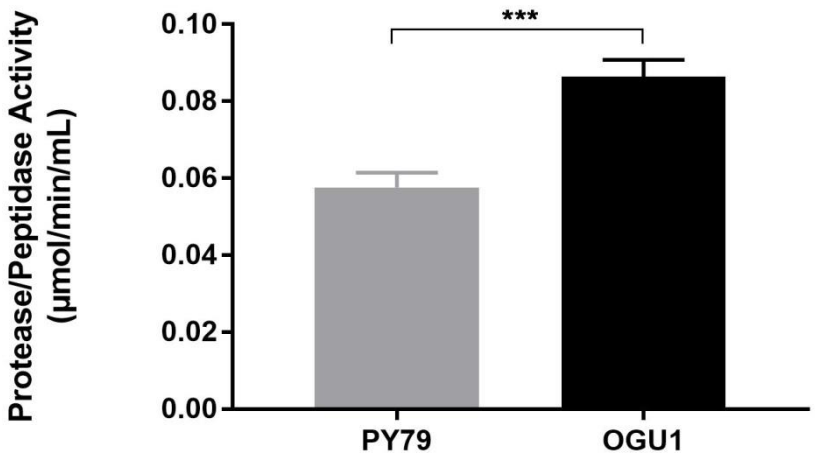


Figure 3. 6 Proteolytic activities of *B. subtilis* PY79 and OGU1 culture supernatants. Enzyme units are defined as the amount of L-tyrosine released per min per mL of supernatant. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

Two more enzymes overshadowed by the presence of similar proteins with same substrate specificities were bacillopeptidase F and the minor extracellular protease Vpr. These enzymes are already responsible for a very small percentage of extracellular proteolytic activity of *B. subtilis* (Kho et al., 2004). Furthermore, the BpF and Vpr mature proteins might be attached to the membrane or cell wall of *B. subtilis* cells and therefore they might not be able to act on the substrate. There is also evidence that these two proteins are targets for proteolysis and auto-lysis in the presence of major extracellular proteases of *B. subtilis* (Mäntsälä & Zalkin, 1980; Kho et al., 2004). Nevertheless, it was still possible to obtain more than 50% increase (1.52-fold) in the total protease

activity and a significant expression ratio (1.88 for *bpr* and 2.42 for *vpr*), which strongly supports the proteomic data. The total proteolytic activities of PY79 and OGU1 strains were 0.08638 ± 0.004446 and 0.0576 ± 0.00387 U/min/mL, respectively, as determined by measurement of the released tyrosine units after the hydrolysis of casein.

Bacteria are the most important group of protease producers in industrial scale, with the most prominent source being the genus *Bacillus*, due to their capacity to secrete as much as 20 g of protease per liter (Harwood & Cranenburg, 2008). Besides, many species of *Bacillus* synthesize proteases that optimally work in neutral and alkaline conditions, which is a desirable feature for their use in the industrial applications. *Bacillus* proteases account for almost 60% of total worldwide enzyme market, due to their broad pH and temperature activity range and their strong stability. These features, together with their ability to avoid inactivation by oxidants and surfactants, make them perfect candidates for the detergent industry (Annamalai et al., 2013). The use of these proteases to remove protein-rich stains like blood allows for shorter agitation time (just a preliminary period of soaking) and for washing in much lower temperatures.

Bacillus proteases and peptidases are also used in the food industry to produce bioactive peptides and to process different human and animal foods (Bougatef et al., 2012; Özcan & Kurdal, 2012). Yet another important property of *Bacillus* proteases is their stability also in organic solvents, which allows their application in organic syntheses (Caille et al., 2002).

Proteases and peptidases are used to hydrolyze proteins in a limited-fashion to release protein hydrolysates (specific peptides), which have a diverse range of biological functions such as immunostimulating, antioxidant, antihypertensive and antimicrobial agents (Danquah & Agyei, 2012). Such peptides can easily be obtained by the use of these proteases/peptidases on common proteins like

casein (milk), albumin (egg) or soy (Corhonen & Pihlanto, 2006). For example, Daroit and Correa (2012) used *Bacillus sp*. P45 protease to show that different reaction times of casein hydrolysis yielded peptides of different degrees of antioxidant and antimicrobial functions. One hour incubation with this protease resulted in ovine sodium caseinates with an ability to inhibit the growth of different screened fungi and bacteria, including *S. enteritidis, E. coli C. fimi,* and *L. monocytogene*.

3.1.7 Endonuclease activities of *Bacillus subtilis* PY79 and OGU1

The average results of endonuclease activity assays are given in Figure 3.7.

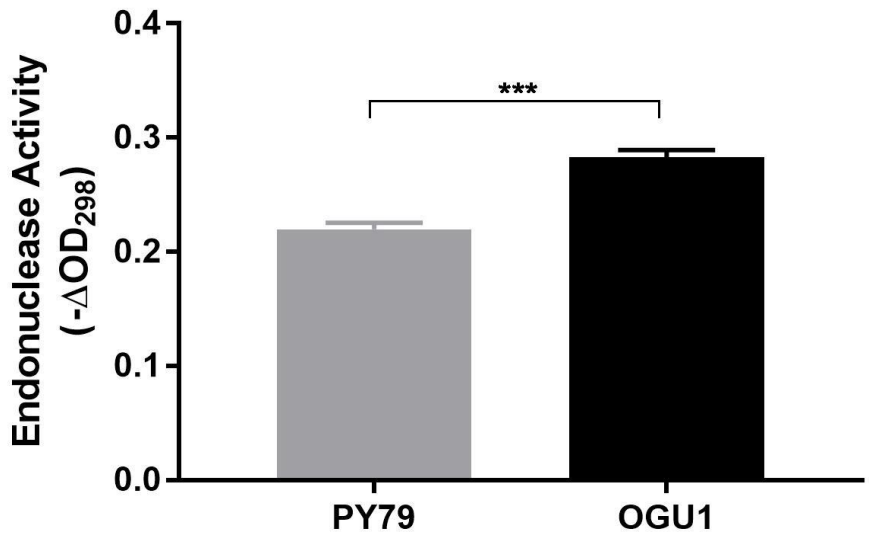


Figure 3. 7 Endonuclease activities of *B. subtilis* PY79 and OGU1 culture supernatants. Endonuclease activities are defined as the decrease of absorbance in 298.5 nm. Student's t-test was used as the statistical analysis to compare the activities between two strains. p value smaller than 0.001 is indicated by the three asterisks above the graph.

The last of the eight enzymes showed differential expression and protein levels between two strains was an extracellular endonuclease, YhcR. The total nucleolytic activity was measured by the decrease of absorbance value at 298.5 nm and it is found to be 0.283 ± 0.006429 for *B. subtilis* OGU1 and -0.220 ± 0.005439 for PY79, which corresponds to an average OGU1/PY79 fold of 1.29. This value supports the results of secretome analysis which clearly shows there is more endonuclease YhcR in OGU1 supernatants when compared to those of PY79. Still, the increase in the activity was largely lower than the increase in protein amount, which can be explained by several factors. Firstly, characterization studies have showed that the presence of Mg^{+2} inhibits the endonuclease activity of YhcR (Oussenko et al., 2004). Mg^{+2} already found in PA medium may result in a significant decrease in the endonuclease activity in both *B. subtilis* OGU1 and PY79 cell culture supernatants. Secondly, YhcR proteins isolated from *Bacillus* supernatants have been shown to be degraded possibly by the activities of extracellular proteases, which were supported by the cloning studies in not only *B. subtilis* but also *E. coli* (Oussenko et al., 2004). Although it is still not clear whether these multiple forms of extracellular YhcR can still retain their endonuclease activities or not, their proteolysis is expected to be at different levels in OGU1 and PY79 strains as both the presence of proteolytic enzymes and the activity or proteases were shown to be greater in OGU1 strain. Another reason why the endonuclease activity was different between two strains may be the presence of a putative anchor sequence found on YhcR mature protein, which may be responsible for its attachment to *B. subtilis* cell wall (Oussenko et al., 2004). Cell wall proteins are well known to present also in the culture supernatants, yet these are cleaved forms of the mature proteins and most of time these proteins lose their catalytic activities. Depending on how much of the YhcR were found on the cell wall, hence washed away by the discard of pellets after initial centrifuge step, the endonuclease activity found in activity assays may decrease significantly. Still, the most important factor on the non-correlated result of enzymatic activity and

secretome analysis should be, just like the cases of Abn2, LipA, Brp and Vpr, the presence of many other extracellular nucleases found in *B. subtilis* strain, including Bs-10, Bs-IA, Bs-IB and Bs-II as well as PNPase, RNase R and YhaM (Kanamori et al., 1974; Mitra et al., 1996; Oussenko & Bechhofer, 2000; Oussenko et al., 2002).

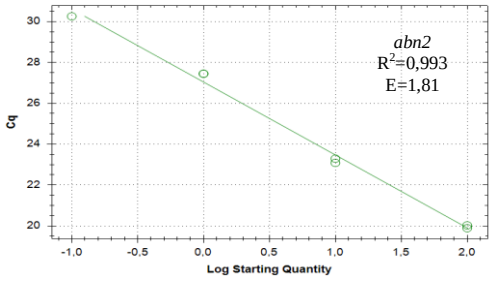
3.2 Comparison of the expression profiles of eight genes between *Bacillus subtilis* PY79 and OGU1 strains

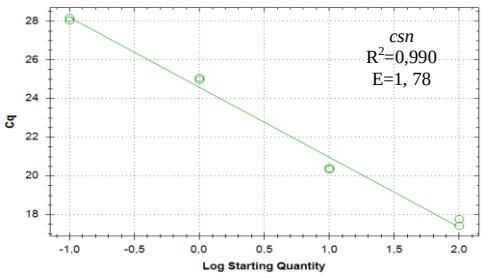
3.2.1 Amplification efficiency calculation on the basis of standard curve formation

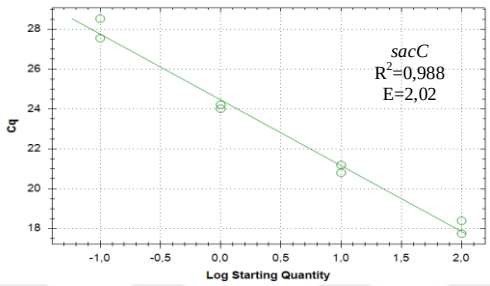
The cDNA sample of *B. subtilis* PY79 was used as a template to plot standard curves of each gene of interest and the reference gene. Amplification efficiency was calculated by the formula $E=10^{[-1/slope]}$ (Pffafl, 2001) by using the slope of the standard curves given in Figure 3.8, obtained from serially diluted cDNA samples with concentrations of 100, 10, 1 and 0.1 ng. Efficiency values over 2 are given in the Table 3.1.

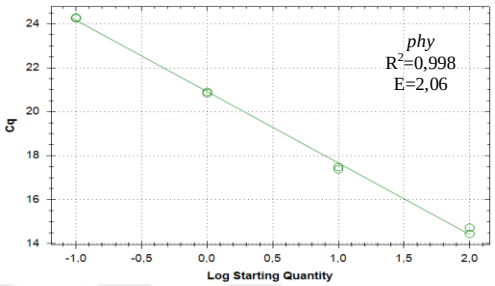
Table 3. 1 Amplification efficiencies of the each gene of interest and the reference gene *rpoB*

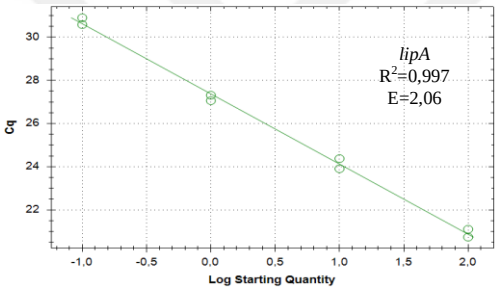
Gene Name	Amplification Efficiency
abn2	1.81
csn	1.78
sacC	2.02
phy	2.06
lipA (estA)	2.06
bpr	1.81
vpr	1.73
yhcR	1.79
rpoB	1.86

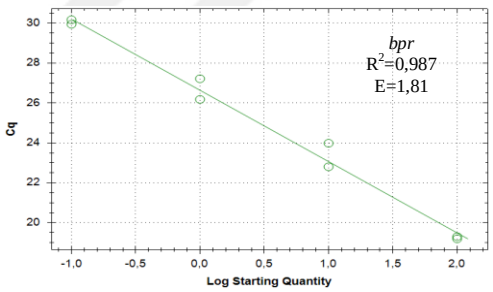

abn2
R2=0,993
E=1,81
Cq
Log Starting Quantity

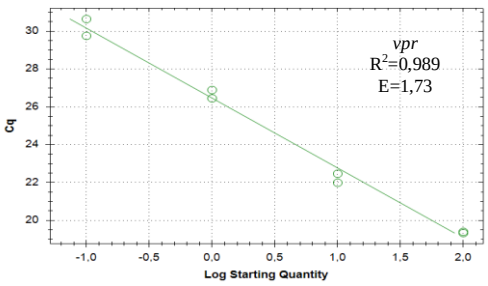

csn
R2=0,990
E=1, 78
Cq
Log Starting Quantity

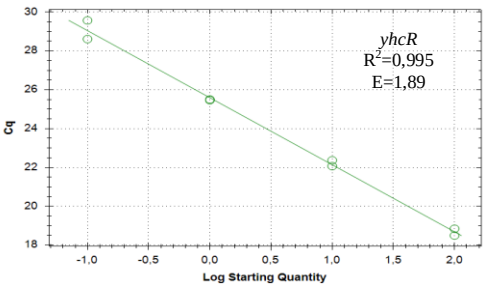

sacC
R2=0,988
E=2,02
Cq
Log Starting Quantity


phy
R2=0,998
E=2,06
Cq
Log Starting Quantity


lipA
R2=0,997
E=2,06
Cq
Log Starting Quantity


bpr
R2=0,987
E=1,81
Cq
Log Starting Quantity


vpr
R2=0,989
E=1,73
Cq
Log Starting Quantity


yhcR
R2=0,995
E=1,89
Cq
Log Starting Quantity

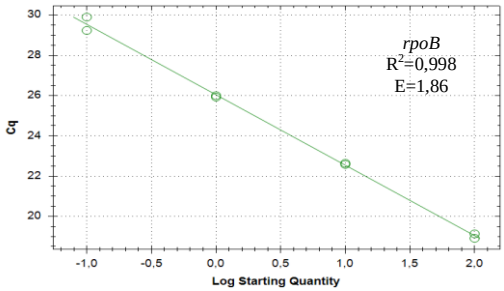

rpoB
R2=0,998
E=1,86
Cq
Log Starting Quantity

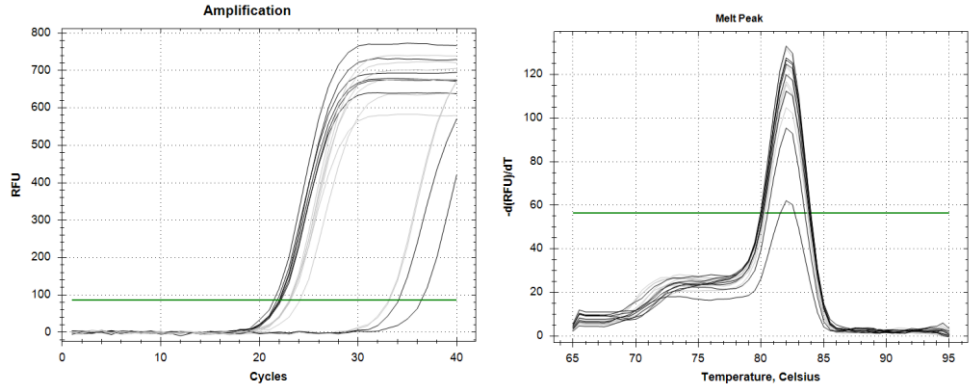
Figure 3. 8 Standard curves for genes of interest *abn2, csn, sacC, phy, lipA, bpr, vpr, yhcR* and the reference gene *rpoB*, with the corresponding efficiencies.

3.2.2. Expression analysis of the selected genes by quantitative PCR

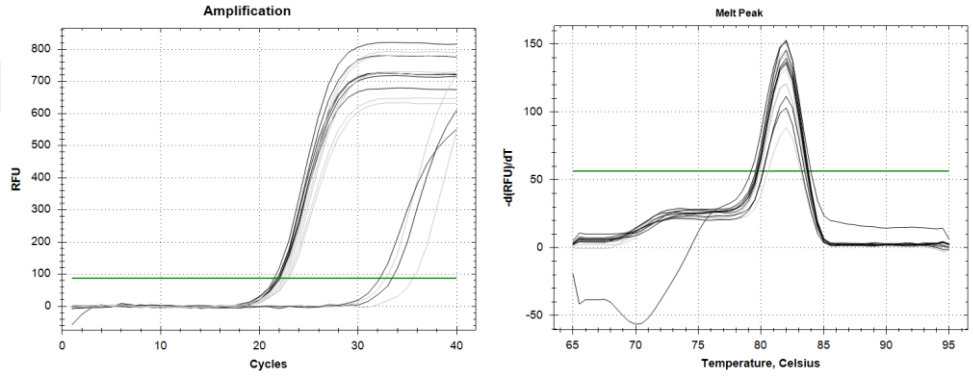
A housekeeping gene *rpoB*, which codes for the beta subunit of RNA polymerase in *Bacillus subtilis*, was chosen to be used as a reference gene in qPCR analysis to find the relative expression changes of the selected genes between *B. subtilis* PY79 and OGU1 strains. After different concentrations of primer sets have been tested, 200 nM was found to be the most favourable primer concentration for amplification of the eight genes and the reference gene. 62 celcius annealing temperature and 40 cycle number were chosen as the parameters in qPCR analysis. Amplification plots and melting curves for each gene are given in Figure 3.9 to 3.11 and C_q and T_m values of these were tabulated in Table 3.2.

Table 3. 2 C_q and T_m values of the amplification plots and melting curves

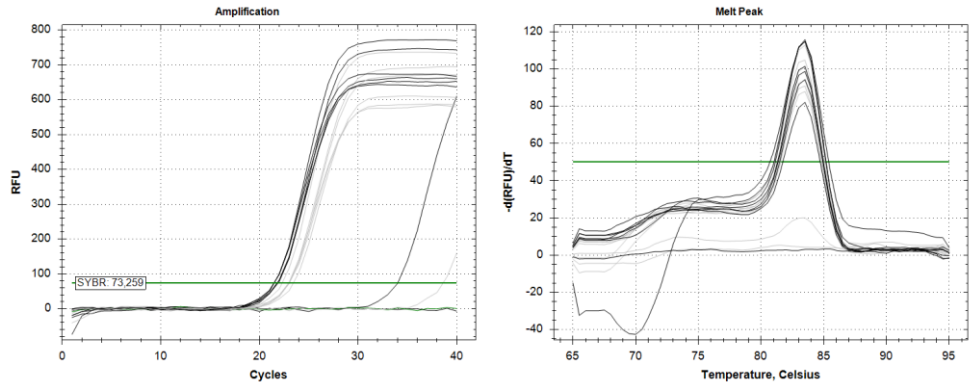
	C_q			T_m	
	PY79	OGU1	NTC	PY79	OGU1
abn2	23,27	21,95	34,21	82,00	82,00
csn	22,47	21,81	33,50	82,00	82,00
sacC	22,53	21,23	36,31	83,50	83,50
phy	23,60	22,12	33,75	79,50	79,50
lipA(estA)	23,13	21,87	32,95	85,50	85,67
bpr	23,33	22,32	33,80	83,83	83,75
vpr	23,56	21,90	34,92	87,00	87,00
yhcR	22,97	21,86	34,54	81,50	81,50
rpoB	21,63	21,67	37,75	83,00	83,00



a) abn2

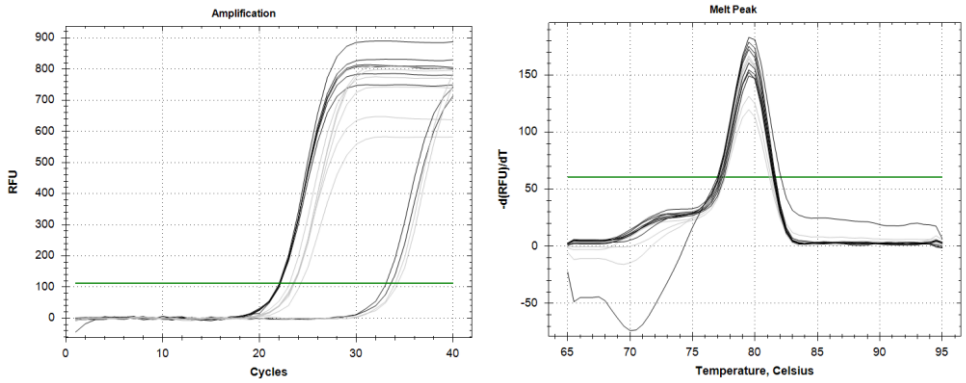


b) csn

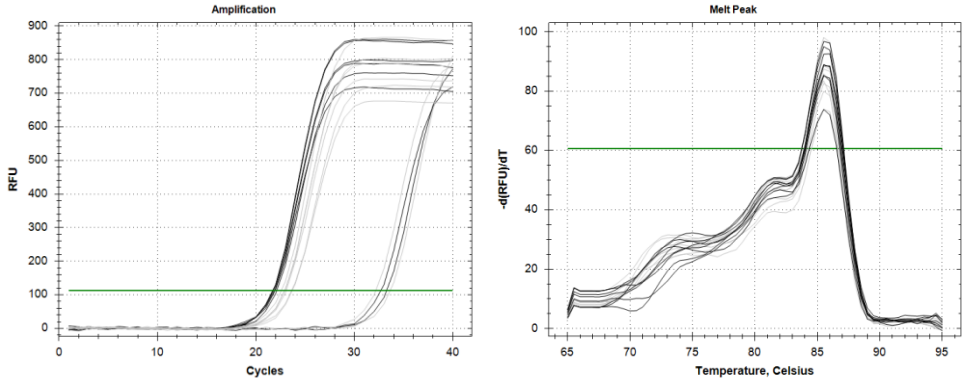


c) sacC

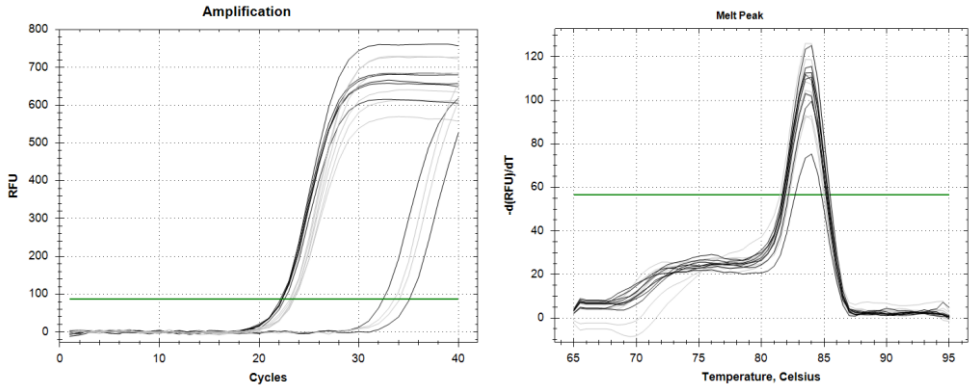
Figure 3. 9 Amplification plots and melting curves of a) *abn2*, b) *csn* and c) *sacC*. Black lines represent *B. subtilis* OGU1 genes and grey lines represent *B. subtilis* PY79 genes.



d) phy

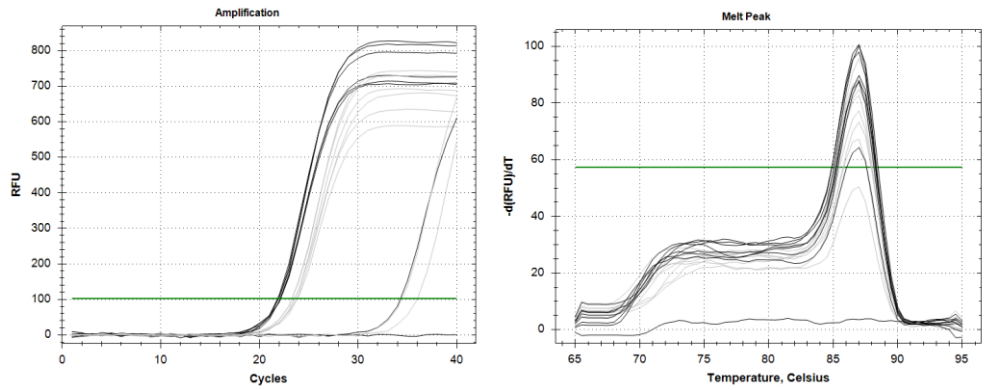


e) lipA

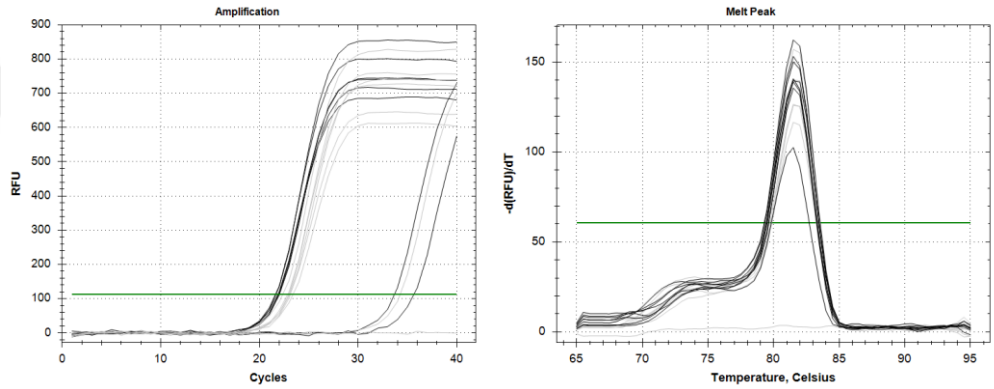


f) bpr

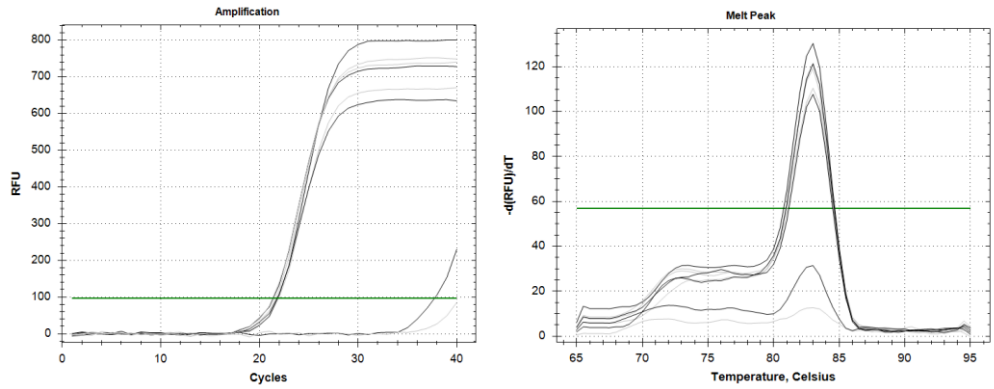
Figure 3. 10 Amplification plots and melting curves of d) *phy*, e) *lipA* and f) *bpr*. Black lines represent *B. subtilis* OGU1 genes and grey lines represent *B. subtilis* PY79 genes.



g) vpr



h) yhcR



i) rpoB

Figure 3. 11 Amplification plots and melting curves of g) *vpr*, h) *yhcR* and the reference gene i) *rpoB*. Black lines represent *B. subtilis* OGU1 genes and grey lines represent *B. subtilis* PY79 genes.

3.2.3 Quantification of the RT-qPCR Data

The OGU1/PY79 expression fold of *rpoB* was found to be 0.98, indicating almost exactly same expression profile of this housekeeping gene in both of the strains. The expression value of *rpoB* was assumed to be 1 for subsequent expression fold change analysis of other eight genes to be normalized with respect to *rpoB*. All genes showed a significant expression increase in bacilysin non-producer strain OGU1 when compared to wild type PY79 strain (Figure 3.12).

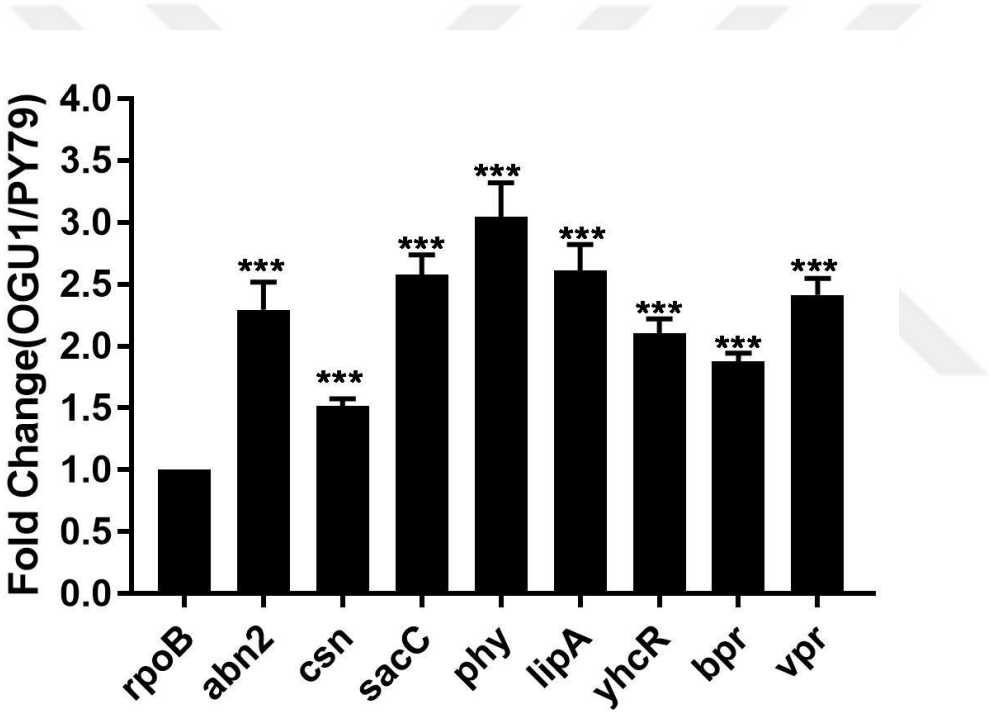


Figure 3. 12 Quantification of *abn2, csn, sacC, phy, lipA, bpr, vpr* and *yhcR* gene expressions in the wild type *B. subtilis* PY79 and its bacilysin non-producer derivative OGU1. Each qPCR run included two biological replicas with three technical replicas for each sample. Student's unpaired parametric t-test was used for statistical analysis; with three asterisks indicate $p < 0.001$.

Expression profiles of all the selected genes were found to be significantly increased in bacilysin non-producer OGU1 strain when compared to *B. subtilis* PY79. Although there is not a direct correlation, this result strongly supports the enzymatic activity measurements and also the comparative secretome data given in Table 1.3. The reason of the difference between fold changes found by the secretome analysis and by the RT-qPCR detection can be attributed to the fact that the mRNA half-time is much smaller than their corresponding proteins in prokaryotes (Maier et al., 2009). RT-qPCR data gives the abundance of mRNAs at 12 h while the secretome analysis inevitable reveals not only the proteins produced at the 12 h but also the proteins that are produced and secreted before this time point.

Comparative secretome analysis between two strains revealed 18 extracellular proteins to be differentially expressed in OGU1, and all of these proteins, except for FlgK, were found to be increased in this bacilysin negative strain (Tekin İşlerel, 2007; manuscript submitted).

Interestingly, among 17 differentially expressed proteins, 9 proteins are known to be negatively regulated by AbrB. 3 out of 8 extracellular enzymes studied in this thesis which are repressed by AbrB are namely Csn, Phy and LipA. Increase of all proteins that are normally repressed by AbrB in the secretome of *B. Subtilis* OGU1 suggests that the absence of bacilysin may cause a shift in the level of AbrB in a way that either the expression of AbrB is repressed directly by the action of Spo0A or it is inactivated by forming a complex with AbbA, whose transcription is positively regulated by also Spo0A. Considering that AbrB is a global regulator and it represses hundreds of proteins in normal circumstances, the producer OGU1 cell may interpret the absence of bacilysin as a stress condition, hence decrease the intracellular amount of AbrB to keep expressing the genes whose products may have important roles during the stress conditions, including chitosanase, phytase and lipase.

Another note-worthy key regulator is CcpA, which binds to catabolite repression elements in the target genes to be repressed in the case of catabolite repression. Among the 17 extracellular proteins found to be overrepresented in the secretome data, two proteins which are known to be regulated by catabolite repression through the action of CcpA were found, namely Abn2 and SacC. Significant increase of the level of these two proteins, their expression and also their activities in the OGU1 strain may be explained by the decrease of intracellular CcpA level. The level of CcpA is regulated by Hpr, which in turn is activated by Hpr phosphorylase/kinase. Similarly to the above scenario, the absence of bacilysin may be perceived as a stress condition in which the extracellular levels of catalytic enzymes are elevated through the repression and/or inhibition of general catabolite repressor CcpA.

Regardless of the secretome data, the expression of *bpr* was already expected to be increased in OGU1 strain as the regulator of *bpr* transcription, DegU, was found to be significantly increased in bacilysin non-producer strain, as revealed both by 2DE MALDI-TOF and LC MS/MS comparative proteome analysis carried out in our laboratory previously. Although it is still possible for bacilysin to directly regulate the expression of *bpr* gene, its effect is most probably indirect through the repression/inhibition of DegU regulator.

Comparative proteome analysis between *B. subtilis* PY79 and OGU1 has also clearly showed that there is a significant decrease in the level of proteins regulated by CodY in bacilysin-negative strain. This suggests a possible direct or indirect inhibitory role of the bacilysin on CodY regulatory pathway. Vpr is one of the proteins negatively regulated by the activity of CodY. Hence, it was unexpected to find both the amount of Vpr, its enzymatic activity and its expression profile was significantly higher in OGU1 strain. This unexpected result can be explained by the presence of other regulatory proteins acting on *vpr* transcription. Although it could not be confirmed by either of proteome or

secretome analysis, a decrease in LexA and DnaA or an increase in PhoP proteins may resulted in an increase in the expression level of *vpr*.

Unfortunately, it is not possible to speculate about the significant increase of YhcR endonuclease as there is no known regulator of this protein yet. However, the secretome analysis, activity measurement and expression assay all showed an increase in the presence of YhcR in the bacilysin non-producer strain OGU1, suggesting that bacilysin may have a possible repressor role in the biosynthesis of YhcR.

CHAPTER 4

CONCLUSION

To understand the effects of bacilysin, eight selected extracellular proteins, which had previously been shown to be overrepresented in the secretome analysis, were examined in the level of transcription and enzymatic activities. For each of these eight enzymes, both the enzymatic activity and the expression profile differences were found to be consistent with the results of the comparative secretome analysis. The bacilysin-negative OGU1 strain showed significant increase in both gene expression and enzyme activity assays, as compared to the wild type PY79 strain. Besides strongly supporting the proteomic data, these results suggest a possible role of bacilysin in the biosynthetic pathway of these eight proteins. Further studies are needed in order to elucidate either the direct role, if any, of bacilysin or indirect effects involving theinteraction of bacilysin with the global or specific regulators and other components influencing the biosynthesis of these enzymes.

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APPENDIX A

COMPOSITIONS OF THE CULTURE MEDIA

Perry and Abraham (PA) Medium (pH 7.4)

Sucrose*	10 g/L
Glutamate.Na.H_2O	4 g/L
KH_2PO_4	1 g/L
$MgSO_4.7H_2O$*	0.5 g/L
KCl	0.2 g/L
Ferric citrate**	0.15 g/L
Trace Elements**	
$MnCl_2.4H_2O$	0.001 g/L
$CoCl_2.6H_2O$	0.0001 g/L
$ZnSO_4.7H_2O$	0.0001 g/L
Ammonium molybdate	0.0001 g/L
$CuSO_4.5H_2O$	0.00001 g/L

*Autoclave separately

**Filter sterilization

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
$NaCl_2$	5 g/L
Agar	15 g/L

APPENDIX B

BUFFERS AND SOLUTIONS

Sodium-Acetate Buffer (1 M)

Sodium-acetate	8.2 g
dH_2O	90 mL

pH is adjusted with acetic acid (3 M) to appropriate values to be used

dH_2O is added to 100 mL

1 M buffer solution is diluted with dH_2O to appropriate concentrations

Glycine-NaOH Buffer (0.08 M)

Glycine	3.75 g
NaOH	1.28 g
dH2O	900 mL

pH is adjusted to 10 with HCl

dH_2O is added to 1 L

Tris-HCl Buffer (0.1 M)

Tris base	1.21 g
dH_2O	90 mL

pH is adjusted to 8.5 with HCl

dH_2O is added to 100 mL

DNS Reagent

Potassium sodium tartrate (Rochelle)	45 g
NaOH	2 M
3,5-Dinitrosalicylic acid	1.5 g

Dissolve Rochelle salt in 75 mL of dH_2O*

Dissolve DNS in 30 mL of NaOH**

Dissolve DNS solution** in Rochelle salt solution*

Add dH_2O to 150 mL, stir in 60 celcius, prepare freshly

Arabinanase Substrate Solution

Sodium-acetate buffer (pH 5.6)	50 mM
Sugar beet red arabinan	0.1 gr

Dissolve arabinan in 10 mL of sodium acetate buffer

Levanase Substrate Solution

Sodium-acetate buffer (pH 5.5)	100 mM
Inulin	0.5 gr

Dissolve inulin in 10 mL of sodium acetate buffer

Phytase Substrate Solution

Sodium acetate buffer (pH 5.0)	100 mM
Phytic acid sodium hydrate	0.2 g

Dissolve phytate in 100 mL of sodium acetate buffer

Phytase Colour Reagent

Sulfuric acid	2.2 mL
Ammonium molybdate	0.6 g
Ferrous sulfate	2.7% (w/v)

Dissolve in 50 mL of dH_2O

Lipase Substrate Solution

Isopropanol (?? %)	
p-Nitrophenyl palmitate (pNPP)	0.15 g
Glycine-NaOH buffer (pH 10)	80 mM
Gum Arabic	0.1 g
Triton X-100	400 uL

Dissolve pNPP in 100 mL isopropanol*

Dissolve Gum Arabic and Triton X-100 in 100 mL of Glycine-NaOH buffer (pH 10)**

Dissolve 1 volume of pNPP solution* in 9 volume of Gum Arabic/Triton X-100 solution**

Endonuclease Substrate Solution

Tris-HCl buffer (pH 8.5)	100 mM
Yeast RNA	0.2 g

Dissolve yeast RNA in 100 mL of Tris-HCl buffer

APPENDIX C

CHEMICALS AND MATERIALS

Chemicals and Materials	Supplier
3,5-Dinitrosalicylic acid	Sigma
4-Nitopheyl palmitate	Sigma
4-Nitrophenol	Sigma-Aldrich
Acetic acid	Merck
Agar	Merck
Ammonium molybdate	Sigma
$CoCl_2.6H_2O$	Merck
Ferric citrate	Sigma
Ferrous sulfate	Sigma
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich
$Glutamate.Na.H_2O$	Sigma
Glycine	Merck
Gum Arabic	Sigma
HCl	Merck
Inulin	Difco
Isopropanol	Merck
KCl	Merck
KH_2PO_4	Merck
Low molecular weight chitosan	Aldrich
$MgSO_4.7H_2O$	Merck
$MnCl_2.4H_2O$	Merck

$NaCl_2$	Sigma-Aldrich
NaOH	Merck
Phytic acid sodium salt hydrate	Sigma
Potassium sodium tartrate	Merck
Ribonuclease T1	Sigma
Sodium-acetate	Merck
Sucrose	Merck
Sugar beet red arabinan	Sigma
Sulfuric acid	Merck
Triton X-100	Sigma
Tryptone	Oxoid
Yeast Extract	Merck
Yeast RNA	Roche
$ZnSO_4.7H_2O$	Sigma
L-Arabinose	Merck
L-Tyrosine	Sigma