# Developing Bacillus subtilis as a versatile bioproduct platform for agricultural and pharmaceutical applications

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# Chapter 1

## Introduction and scope of this thesis

## Introduction

*Bacillus subtilis*, also known as hay bacillus or grass bacillus, is a rod-shaped and Gram-positive bacterium, which is able to form protective endospores, permitting it to withstand extremely unfavorable environmental conditions.[1, 2] In scientific research, *B. subtilis* has become the best well-studied Gram-positive bacterium, serving as a model microorganism to investigate cell differentiation and bacterial chromosome replication.[3-5] In industries, the various applications of *B. subtilis* range from food and beverage processing, feed additive and pesticide in agriculture, aquaculture, waste treatment, and pharmaceuticals.[6, 7] This largely attributes not only to its generally regarded as safe status (GRAS), but also the high protein-secretion capacity, fast-growth rate in simple media, absence of codon bias, ease of genetic modifications and excellent fermentation properties.[8, 9]

*B. subtilis* is considered as one of the bacterial champions in producing secreted enzymes, such as proteases and α-amylases. Lacking an outer membrane gives *B. subtilis* the advantage of secreting target proteins directly to the culture medium, which does significantly simplify the downstream process without cell lysis or complex chemical-based procedures of purification. *B. subtilis* possesses multiple protein secretion pathways, including the general secretion (Sec) pathway which transports proteins in unfolded status, the twin-arginine translocation (Tat) system which releases proteins in folded conformation, ATP-binding cassette (ABC) transporters for exporting some proteins and the recently reported non-classical secretion pathway.[10-13] The majority of the bacterial proteins pass the cell membrane through the Sec transport system either co-translationally or post-translationally.

## Signal peptide

Secretory proteins via both Sec- and Tat- dependent pathways contain signal peptides (SPs) at the N-terminus of the proteins to direct the proteins targeting the secretory apparatus. Both the Sec- and Tat-dependent SPs consist of three distinct domains known as the positively charged N-region, the hydrophobic H-region and the C-region with the recognition and cleavage site (Ala-x-Ala) for signal peptidases (SPases). Tat SPs (about 30 residues) are generally longer than Sec SPs (about 17 to 24 residues).[14] The Tat-dependent SPs are characterized by the canonical twin-arginine motif (RR) at the

N-region, and are normally less hydrophobic than the Sec-dependent SPs. Considering the hydrophobicity of the SPs plays a crucial role during the early stage of protein secretion, the signal peptide prediction tool SignalP has been developed to facilitate choosing the suitable SPs for efficient protein secretion. Also, the SPs libraries that were constructed to optimize protein secretion system are associated with corresponding high-throughput screening methods.[15, 16] Recently, researchers also demonstrated that the artificial SPs generated by Transformer model are functional, and lead to secreted enzyme activity competitive with SPs used in industry.[17]

**Sec secretion pathway in *B. subtilis***

In the first stage of recognition and targeting of the Sec secretion pathway, the SPs are recognized by the signal recognition particle (SRP), which consists of a scRNA, two histone-like proteins (HBsu) and Ffh.[10] Subsequently, the protein complex is docked to the SRP membrane receptor FtsY. Both Ffh and FtsY belong to the extensively conserved SRP-GTPase family and the hydrolysis of GTP at FtsY and SRP can influence the release of the ribosome-nascent chain complex (including the nascent peptide chain, tRNA and the ribosome) from SRP to the Sec translocation channel. Meanwhile, the cytoplasmic chaperones facilitate the preproteins at the state of translocation competence. Two major intracellular chaperones in *B. subtilis* include GroE and DnaK series, which are regulated by the *groE* operon (*groES–groEL*) and the *dnaK* operon (*hrcA-grpE-dnaK-dnaJ-yqeT-yqeU-yqeV*), respectively.[18, 19] GroEL-GroES components provide an isolated and "folding-friendly" environment for proper protein folding, whereas DnaK chaperones are responsible for binding and stabilizing of the unfolded or partially folded protein regions.[20] The molecular chaperones not only participate in protein folding, but are also closely involved in aggregation prevention, protein quality control, chaperone-assisted protein degradation and the heat shock response.[21]

Then in the following transferring and passing stage, the cargo proteins are transported across the channel of SecYEG translocase accompanied by the accessory protein SecDF, by consuming the energy provided by SecA through hydrolyzing ATP. The overexpression of the artificial *secYEG* operon could enhance the secretion of α-amylase by increasing the number of translocons.[22] SecDF, a proton-driven motor for protein export, has been demonstrated to be one of the key non-essential determinants for protein secretion. Since the deficiency of secDF could remarkably decrease the extracellular levels

of multiple enzymes (amylases from *B. subtilis* and *Bacillus licheniformis*, and the serine protease from *Bacillus amyloliquefaciens*) in *B. subtilis*.[23] Overexpression of secDF could enhance the secretion of different amylases in *B. subtilis*.[24]

Later in the stage of removal and degradation, after the protein is delivered to the desired destination, the SPs will be immediately cleaved and removed by corresponding SPases. Then signal peptide peptidases (SPPases) including SppA and TepA are employed to degrade the cleaved signal peptides to avoid them being inhibitors to other proteins' translocation.[10] Among the five chromosomally encoded SPases (SipS, SipT, SipU, SipV and SipW), SipS and SipT play a major role in precursor processing. Two other SPases (designated SipP) were identified present on two cryptic plasmids pTA105 and pTA104 in some *B. subtilis* (natto) strains. And the plasmid-encoded SipP could functionally replace SipS and SipT.[25]

In the last modification and maturation stage, multiple factors are involved including chaperones, thiol-disulfide oxidoreductases BdbBCD, metal ions, as well as the quality control system.[10] The major extra cytoplasmic foldase in *B. subtilis* is the peptidyl-prolyl cis/trans isomerase PrsA, which is a membrane-anchored lipoprotein. PrsA plays an important role in the folding of penicillin-binding proteins which is critical for the synthesis of cell wall, thus is essential for cell viability.[26] In addition, PrsA serves as an efficient folding catalyst for exported proteins. Overexpression of the substrate-specific chaperone PrsA could dramatically increase amylase production when there was a good PrsA-amylase pairing to lower the secretion stress response of *B. subtilis*.[24, 27] BdbBCD catalyze the formation of disulfide bonds and are required for the stability and activity of multiple exo-proteins. Overexpression of BdbBCD could improve the secretion amounts of disulfide bonds-containing proteins, but not at significant levels.[28] The protein quality control proteases (like HtrA, HtrB and WprA) in *B. subtilis* remove misfolded or incompletely synthesized proteins. Expression of the two membrane-anchored proteases HtrA and HtrB is under the control of CssRS in response to the stress induction caused by overexpression of secretory proteins.[29] The cytoplasmic insoluble aggregates can be decreased by ClpCP, ClpXP, and ClpEP.[30]

**Tat secretion pathway in *B. subtilis***

The Tat secretion pathway stands out by specifically exporting fully folded proteins usually in association with their respective cofactors.[14] Until now, only four proteins have been identified to be substrates of the Tat pathway, including Rieske iron-sulfur protein QcrA, the metallophosphoesterase YkuE, the hemoprotein EfeB (previously named as YwbN) and the phosphodiesterase PhoD.[31-34] The Tat-mediated secretion mechanisms are highly conserved in Gram-positive bacteria, and the basic secretion procedures are as follows. Before translocation, pre-proteins fold in the cytoplasm assisted by cofactors. Then pre-proteins are transported through the Tat translocase with the aid of a pH gradient across the cytosolic membrane. After translocation, SPases cleave signal peptides and the remaining proteins are secreted into the medium.[35]

**Strategies to improve protein production in *B. subtilis***

To sufficiently make use of *B. subtilis* as protein expression and secretion platform strains, strategies are mainly focused on two aspects, the protein synthesis and the secretion capacity. To enhance protein synthesis, either constitutive or inducible strong promoters are extensively employed to increase gene transcription. Numerous expression systems have been established and are widely used to improve the production levels of different proteins.[36, 37] Also, suitable RBSs are optimized to enhance protein translations thus increasing protein synthesis. To enlarge protein secretion yields, a common strategy is recruiting strains with deficiency of non-essential extracellular proteases, to avoid secretory proteins in the culture medium being degraded. Multiple protease-deficiency strains have been constructed and progress has been well reviewed by Westers *et al.*[38] Take the construction of strain WB800 as an example, when seven extracellular proteases were deleted (encoded by *nprE, nprB, aprE, epr, mpr, bpf,* and *vpr*) in strain WB700, there still approximately 0.15% of the wild-type extracellular protease activity being detected after 24 h cultivation in rich medium. The disruption of the eighth extracellular protease WprA, which is constitutively expressed in the exponential phase and up-regulated during the post-exponential phase, lead to even higher protein production and no degradation of the target protein was observed after 48 h fermentation.[39] In addition, it's crucial to utilize suitable and efficient signal peptides to guarantee the efficient secretion of target proteins. SP libraries and SP prediction tools undoubtedly provide potential options to accelerate this process.[40] Moreover, some components of secretion machinery have been identified to be the bottlenecks and hinder the secretion and release

of high level of proteins. Overexpression of these factors has been demonstrated to be useful approaches to increase the final secretion production of target proteins.[24, 41, 42] In general, the current engineering approaches remain to address the secretion bottlenecks at the single-molecule level. To maximize protein productions, it's also important to explore the protein interaction networks, the dynamic transcriptome, proteome and metabolome conditions and even whole-cell physiology to balance the cargo protein production and the available cellular resources.

### *B. subtilis* as terpenoid cell factory

Except being well-known protein expression platform, in recent decades, *B. subtilis* has been discovered to be a promising host for terpenoid production.[43] Terpenoids, also known as isoprenoids, are a large group of naturally occurring organic chemicals, comprising around 60% of known natural products. Structurally, they are composed of different numbers of isoprene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), without or with different modifications. According to the number of carbon units, they can be divided into hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40) and polyterpenoids.[44] Functionally, they have been extensively used in numerous fields of daily life, including food, energy, cosmetics and pharmaceuticals, since plenty of them have been reported to be antibacterial, antioxidant, anti-inflammatory and anticancer.[45] For instance, the antimalarial drug artemisinin, the anticancer Paclitaxel (Taxol), the anti-oxidant carotenoids, and the nutrient and anticancer ginsenosides.[46-50] Terpenoids are widespread in plants but many are present at only low levels, extraction from plants thus suffering from low yields yet high costs. Chemical synthesis is often hindered by the complex structures and stereochemistry of terpenoids, and is also environmentally unfriendly. Therefore, to satisfy the increasing demands of important terpenoids, biosynthesis of these promising natural products by microbes becomes an important sustainable alternative.

Compared with *Escherichia coli* and yeast, *B. subtilis* possesses its own advantages as a promising potential platform for terpenoids, for instance, the GRAS status, the relatively fast growth rate and the theoretically powerful 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprene precursor biosynthesis.[51] In *B. subtilis*, the inherent MEP pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to form

1-deoxy-D-xylulose-5-phosphate (DXP) catalyzed by Dxs.[52] Then DXP is reduced and isomerized to produce MEP through a putative two-step reaction by 1-deoxy-D-xylulose 5-phosphate reductoisomerase IspC.[53] These two steps are recognized to be critical and rate-limiting steps.[54] Subsequently, 4-diphosphocytidyl-2-C-methylerythritol synthase (IspD) adds the cytidine monophosphate (CMP) moiety to MEP resulting 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME). In the following step, IspE catalyzes the generating of 4-diphosphocytidyl-2-C-methyl-d-erythritol 2-phosphate (CDP-ME2P) with the addition of extra phosphate group to CDP-ME. Immediately, the cytidyl moiety of CDP-MEP2P is removed and is cyclized to develop methyl erythritol cyclic diphosphate (MEcDP), with the IspF conducted catalysis.[55] In the last two reductive steps, IspG and IspH are responsible for the conversion of MEcDP to 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBDP) and dihydroxylation/ Isomerization of HMBDP to either IPP or DMAPP, respectively.[56] Further, prenyltransferases, terpene synthases, terpene cyclase, and P450s mediate the generation of diverse terpenoids and derivatives.[57]

Recent years have witnessed significant progress of *B. subtilis* producing terpenoids, such as, isoprene, carotenoids, amorphadiene, taxadiene and Menaquinone-7. [43, 58-62]Also, some studies focus on investigating the functions and contributions of MEP pathway aiming at discovering and resealing the rate-limiting factors. However, not all of the crystal structures and catalytic parameters of these enzymes are available. In addition, biosynthesis of large numbers of cyclic triterpenoids has not been explored in *B. subtilis*, neither with approaches to improve their final production levels. Moreover, the predominant strategy to elevate terpenoids production remains to be overexpression of terpene synthases and MEP pathway enzymes by plasmids. Hence, simple, efficient and scar-less genetic modification of *B. subtilis* at genome level are urgently needed to sufficiently make full advantages of *B. subtilis* as terpenoid cell factories.

**Scope of this thesis**

The aim of this thesis represents two main aspects. The first part involves the construction of *B. subtilis* protein expression system for high production and efficient secretion of β-mannanase. The second part focuses on the biosynthesis of terpenoid and development of genetic tool based on clustered regularly interspaced short palindromic repeats (CRISPR) and application of this strategy to improve terpenoids' production in *B. subtilis*.

In **chapter 1**, we describe the outline and the main goal of this thesis. Specifically, an overview of *B. subtilis* as a prominent host to express and secrete enzymes is provided. Firstly, we introduce the different protein secretion pathways in *B. subtilis*. Furthermore, we discuss the basic procedures of Sec and Tat secretion pathway, as well as the knowledge of involved components of the secretory machinery. Finally, the strategies to improve protein secretion levels and future directions are summarized. In addition a prelude on the use of *B. subtilis* as terpenoid cell factory is provided.

In **chapter 2**, we explore the potential of *B. subtilis* as an industrial strain for the expression and secretion of β-mannanase, an important enzyme involved in hydrolyzing hemicellulose which has been evaluated as the second most abundant polysaccharide in nature. Initially, four Sec-dependent and two Tat-dependent signal peptides are individually fused at the N-terminus of β-mannanase to facilitate its efficient secretion. Subsequently, the strain with the highest secretion amount of β-mannanase lead by $SP_{lipA}$ was further optimized by overexpression of different secretion components, chaperones as well as signal peptidase in the genome. In addition, three constitutive promoters and two inducible promoters were separately employed and tested, in order to increase β-mannanase synthesis in strains with efficient signal peptides and secretion machinery components. Consequently, the enzyme activity of extracellular β-mannanase secreted by the final strain significantly reached 2207 U/L after 72 h of flask fermentation.

In **chapter 3**, we review the current knowledge of *B. subtilis* as terpenoid cell factory. Firstly, the up-to-date information of each endogenous MEP pathway enzyme is provided in detail along with the enzymes responsible for further isomerization and condensation of C5 isoprene precursors. Secondly, productions of several terpenoids in *B. subtilis* are summarized and the applied engineering approaches for the improvements are also discussed. Thirdly, we outline the present genetic engineering tools and propose promising strategies to improve terpenoids production in *B. subtilis* for further studies.

In **chapter 4**, we investigate *B. subtilis* to produce squalene, the common precursor of triterpenoids, by introducing multiples squalene synthases (SQSs) from bacteria, fungi and plants to *B. subtilis*. Furthermore, the expression vector, cultivation temperature and rate-limiting enzymes within the MEP pathway were systematically explored to enhance squalene production. A finally 29-fold increase of squalene titer is achieved by overexpressing SQS from *Bacillus megaterium* (BmSQS) and MEP pathway enzymes

compared with the starting strain. This represents the first trial of squalene synthesis and improvement in *B. subtilis*.

In **chapter** 5, we develop CRISPR-Cas9 approach in *B. subtilis* and applied it to increase the production of C15 amorphadiene, the critical precursor of anti-malarial drug artemisinin. By using this efficient scarless genome editing tool, the branch pathways are depressed by knockout of unessential genes and knockdown of essential genes. In addition, the amorphadiene synthase (ADS) is engineered by introducing single or double mutations as well as introducing an extra copy of ADS at the genome. Moreover, the effects of TCA enzymes on amorphadiene production are explored by replacing the inherent promoters with strong and weak promoters. Finally, these steps are combined to improve the production of amorphadiene, and a 43% increase of amorphadiene is obtained.



In **chapter 6**, we focus specially reviewing on the development and application of CRISPR based genetic tools in *Bacillus* species. The principle of CRISPR system and its mechanism in genetic engineering is first introduced. Further, the three important components of CRISPR-Cas9 tools and their effects on genetic editing efficiency in *B. subtilis* are discussed along with the different types of CRISPR-cas systems. Moreover, we describe the prospect and progress of CRISPR-Cas9 in gene editing (gene knockout, gene knock-in, large fragment deletion and site specific mutation) and gene regulation (CRISPRa and CRISPRi) in *B. subtilis*. Besides, an overview of the development of CRISPR-based tools in other *Bacillus* species is also provided, including *Bacillus licheniformis*, *Bacillus methanolicus*, *Bacillus anthracis*, *Bacillus cereus* and *Bacillus smithii*.

**Chapter 7** summarizes the studies in this thesis, and provides some future perspectives.

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