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Spore-Based Designer Enzyme Cascade Biocatalysts

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Despite with high efficiency and intrinsic specificity, industrial applications of biocatalysts are still restrained due to relatively high cost of production, limited stability and reusability, and expensive co-factor regeneration. This dissertation aims to develop a *Bacillus subtilis* spore-based biocatalysis platform to address these issues. Different from all previous spore display studies, anchoring scaffoldins based on cellulolosome were designed and constructed to display cascade enzymes in stoichiometrically controllable manner, assemble multimeric enzyme complex, and recruit large number of synergic enzymes. We found among *B. subtilis* outer coat proteins, CotG mediated a high expression level of cohesin domains. Functional display of dockerin tagged xylose reductase and phosphite dehydrogenase on spore surface at optimized stoichiometry achieved efficient regeneration of NADP(H). Because coat proteins do not need to translocate across
membrane during sporulation, co-expression of spore surface anchoring scaffoldins carrying multiple cohesin domains and dockerin tagged β-galactosidase (β-gal) in the mother cell compartment, resulted in self-assembly of tetrameric β-gal in its active form with a high display density of > $3 \times 10^4$ per spore. Spore display dramatically increased transgalactosylation yields in water/organic emulsions, enhanced thermostability and reusability, and exhibited long-term storage stability at ambient temperature for more than 60 days. Overall this dissertation suggests that spore-based biocatalysts incorporating multiple synergic enzymes hold a great promise in a wide range of biocatalysis applications.
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Chapter 1. Introduction

Enzymes are polypeptide biocatalysts, and their catalysis is usually carried out under moderate conditions (e.g. room temperature and atmospheric pressure). They are also capable of generating chiral products with high specificity. In sharp contrast, chemical synthesis of value-added compounds often requires harsh conditions, and is generally accompanied with expensive catalysts based on rare metals and low stereoselectivity. More importantly, the undesired by-products generated via chemical synthesis make purification challenging and increase the total production cost. Because of this, industries heavily rely on enzymes to produce commercial perfumes, antibiotics, and pharmaceuticals, etc.

Nonetheless, enzymes have their own inherent limits. Naturally occurring enzymes usually have insufficient levels of reusability and stability, increasing the cost of industrial applications. An ideal enzyme should meet the following requirements: high activity, strong stability, multiple reuses, and easy manipulation of processes such as production and purification. The first two requirements can be met through protein engineering, primarily through directed-evolution or rational designs. However, the rest of the requirements cannot be met using conventional protein engineering methods. This causes a major issue because, reusability and easy manipulation are the most important factors when considering industrial costs. Therefore, this thesis is focused on addressing the current challenges associated with enzymes. We specifically worked to develop a facile and robust platform for industrial applications.
1.1 Multi-enzyme Complex Can Improve Overall Reaction Rate

Several studies have provided evidence showing that many \textit{in vivo} cellular reactions of metabolic pathways are performed by multienzyme complexes instead of freely floating enzymes (Agapakis et al., 2012; Lee et al., 2012), \textit{e.g.} polyketide synthase and fatty acid synthase (Menzella et al., 2005; Jenni et al., 2007). The non-covalently bound enzyme networks accelerate overall reaction rates by avoiding distribution of the intermediary products (Figure 1.1), allowing for the improvement of the production yield and specificity via “substrate channeling”. The term substrate channeling refers to the process of the direct transfer of the product of one enzyme to another nearby enzyme or cell without equilibration with the bulk phase.

\textbf{Figure 1.1} Multi-enzyme complex enhances overall reaction rate. (A) Free disconnected enzymes generate intermediates that will diffuse to subsequent enzyme active site for consequent reaction. (B) Connected multi-enzyme complex generate intermediates that will immediately access the subsequent enzyme active site for consequent reaction. Multi-enzyme complex reduces intermediates transportation distance and therefore accelerates the overall reaction rate. Modified with permission from ref (Pröschel et al., 2015). Copyright (2015) Front Bioeng Biotechnol.
1.1.1 Synergy Effects in Cellulosomes

Substrate channeling has been well utilized by natural anaerobic cellulolytic bacteria, forming an intricate multi-enzyme complex termed cellulosome. The cellulosome hydrolyses crystalline cellulose much more efficiently than free enzymes (Doi and Kosugi, 2004; Fontes and Gilbert, 2010). Cellulosomes of all cellulolytic bacteria are non-covalently connected via a strong interaction between two modules – scaffoldin comprised of repeating cohesin domains, and corresponding dockerin domain which is tagged at C termini of cellulases. Another useful motif existing in the scaffoldin polypeptide is carbohydrate binding modules (CBMs), which help the cellulosome bind to cellulose substrates.

The cellulosome of Clostridium thermocellum represents one of the most extensively characterized extracellular multi-cellulase complexes, containing at least 14 different species of catalytic subunits (Lamed et al., 1983; Bayer et al., 1985). C. thermocellum expresses two types of scaffoldin: primary and secondary scaffoldins. CipA, a primary scaffoldin, contains 9 repeated type I cohesins specifically recognizing type I dockerins and a C-terminal type II dockerin which does not recognize CipA cohesins. The type II dockerin recognizes type II cohesion, the repeat of which composes secondary scaffoldin (Fontes & Gilbert, 2010). The most common secondary scaffoldin is the Cthe_0736, which contains 7 repeated type II cohesin domains. In total the cellulosomes assembled by CipA and Cthe_0736 could potentially contain up to 63 catalytic subunits (Figure 1.2), generating multi-enzyme complexes larger than 1 million Da.
Many researchers believe that cellulosomes facilitate synergistic cellulose degradation, but so far in vitro disassociation and reconstitution of cellulosomes to verify this ideology have been challenging (Currie et al., 2013). Instead, researchers have sought to build cellulolytic “minicellulosomes” using genetically recombinant proteins (Bomble et al., 2011; Fierobe et al., 2002; Merino & Cherry, 2007; Morag et al., 1996). Conclusions have been drawn from the exogenously expressed and assembled polypeptide scaffoldins containing limited cohesin domains and limited species of cellulolytic subunits. The binding of a dockerin-tagged cellulase to a single cohesin domain does not enhance its...
cellulolytic activity as expected (Karuss et al., 2012), but this interaction reportedly improves cellulase stability for a wide range of pH and temperatures (Kouassi et al., 2005). In addition, the connection of CBM to the scaffoldin, either N-terminal position or central position, has been shown to stimulate cellulolytic activity by 2-fold (Karuss et al., 2012; Caspi et al., 2009). Synergistic cellulolytic activities have been investigated by varying the copies of cohesin domains. A synergy factor of 1.7 was observed when cellulases were docked onto a two-cohesin polypeptide towards crystalline substrates (Karuss et al., 2012), while further elongation of cohesin-containing scaffoldins resulted in diminishingly small synergistic actions (Karuss et al., 2012; Caspi et al., 2009; Cho et al., 2004; Fierobe et al., 2002; Fierobe et al., 2005).

1.1.2 Substrate Channeling in Artificial Enzyme Complexes

The ubiquitous presence of substrate channeling within natural multi-enzyme complex inspires people to create other artificial enzyme complexes besides minicellulosomes. The most direct way of constructing a multi-enzyme complex is to express recombinant fusion proteins from metabolic pathways (Iturrate et al., 2009). Unfortunately, this method provides a limited degree of substrate channeling (Pettersson & Pettersson, 2001), as well as compromised protein solubility (Hoelsch & Weuster-Botz, 2010). Chemical cross-linking is also a promising approach to establish enzyme complexes. Cross-linked mitochondrial proteins of Saccharomyces cerevisiae have demonstrated significantly higher enzymatic fuel cell power output (Aman et al., 1985; Moehlenbrock et al., 2010; Wu & Minteer, 2015). However, treatment with cross-linking agent may potentially impair enzyme activity, thus compromising the practical utility of
Another enzyme complex construction method uses DNA scaffold-mediated protein assembly (Wilner et al., 2009; Sun & Chen, 2016). This highly operable method has mediated a 20-30 fold reaction enhancement by tethering glucose oxidase and horseradish peroxidase (Wilner et al., 2009), but the high cost of the process considerably restricts its large-scale applications in industry.

Proteinaceous modules provide following advantage for the construction of enzyme complexes: ability to be genetically encoded and thus produced by cells, relatively inexpensive cost, minimum effect on tagged catalytic subunits, and relatively small size (C. thermocellum Coh and Doc are around 14 and 7 kDa, respectively). For example, a cascade enzyme complex containing triosephosphate isomerase, aldolase, and fructose 1,6-bisphosphate using three cohesin-dockerin pairs derived from distinguished bacterial species has mediated an impressive 33-fold enhancement in terms of catalytic efficiency ($k_{cat}/K_M$) (You et al., 2012) (Figure 1.3). Dueber et al. built synthetic protein scaffolds that can spatially recruit three mevalonate biosynthetic enzymes using modular peptides (GBD, SH3, and PDZ) other than the cohesin-dockerin, which resulted in a 77-fold increase in mevalonate production, the most astonishing reaction rate improvement brought by substrate channeling so far (Dueber et al., 2009). Taking it a step further, researchers have displayed multi-enzyme cascades on microbial surfaces (Tsai et al., 2009; Tsai et al., 2010; Wen et al., 2010). Multi-enzyme complex mediated substrate channeling has the potential to accelerate overall enzymatic catalysis reactions, and thus fits the current needs of industry applications.
1.2 Spore Display is a Promising Biocatalyst Platform

In rare cases, chemical compounds are enzymatically produced by purified enzymes. Various methods have been established to simplify the industrial application and reduce the associated costs. For example, whole cell cytoplasmic protein expression has been used for target compound synthesis. This technique is called metabolic engineering. Other techniques primarily display enzymes on microbe surface or immobilize enzymes on beads. In the following parts, the advantages and disadvantages of these techniques will be discussed and extensively compared with spore enzyme display.

1.2.1 Current Methods of Biocatalysis

Although purified free enzymes have the least interferences of other enzymatic reactions, the high cost of purification severely restrict their wide application in industry except when the value of the product well outweighs the associated costs. Several other
techniques have been developed to omit enzyme purification or to enhance enzyme reusability. Whole cell-based biocatalysis does not require a protein purification procedure, and the biocatalysts can then easily be prepared after protein induction, and proceed to fermentation. However, whole cell-based biocatalysis faces severe challenges including the risk of substrates interfering with host cell’s native metabolism, difficult transfer of the large substrates due to cell membrane or cell wall barriers, and the possibility of undesired branched reaction pathways. Another feasible method is to anchor enzymes on solid beads via chemical cross-linking. Although this method allows enzyme display with a high surface density, it still requires a protein purification procedure. Another issue is that the chemical agents used to cross-link the enzymes severely deteriorate enzyme activities.

To overcome the limitations mentioned above, surface display on microbes, particularly *Saccharomyces cerevisiae* and *Escherichia coli*, has been developed. Typically, heterologous proteins are linked to the extracellular surface through direct fusion with a surface protein. The platforms of microbial surface display are attractive for three reasons: 1) the substrates get free access to the enzymes without having to cross membranes or cell wall barriers, 2) immobilized enzymes usually exhibit improved protein stability compared with free enzymes, and 3) microbial particles are readily applied in a continuous reactor. This platform has already been employed into a vast sphere of applications, including enhanced biodegradation of pesticides and xenobiotics (Richins et al., 1997; Shimazu et al., 2004), and bio-accumulation of inorganic contaminants (Bae et al., 2000; Bae et al., 2003). Despite the superior performances of conventional microbial surface display, it has one significant challenge that limits the active display of multimeric
enzymes. In conventional microbial surface display, proteins get expressed and folded in the cytoplasm followed by their transportation across cell membrane to extracellular surface. However, multimeric proteins often fail to display their active formats after the trans-membrane process. For example, β-galactosidase (MW 116 kDa per unit and only active as a tetramer) could not be actively displayed on the surface of *E. coli* due to toxicity of membrane jamming (Wu et al., 2008).

### 1.2.2 Mechanism of Sporulation Enables Enzyme Self-assembly on Spore Surface

When *B. subtilis* is provided with sufficient nutrients and proper growth conditions it remains in its vegetative stage, growing and dividing like other bacteria. However, under adverse conditions such as starvation or UV irradiation, the vegetative *B. subtilis* cell undergoes uneven division into mother cell and forespore parts, around the second hour of sporulation. The mother cell compartment serves to nurture the forespore part until it fully develops into a mature spore. Mother cell nurtures the forespore by engulfing it, synthesizing an inner peptidoglycan germ cell wall and an outer cortex, later depositing them onto the forespore (Figure 1.4). Subsequently, the amorphous undercoat and the inner and outer coat proteins synthesized in the mother cell are formed and assembled outside of cortex. This occurs during fifth hour of sporulation. After all the morphological changes, the mother cell is lysed and fully matured spores are released. Once initiated, the total sporulation process requires approximately eight hours to complete. Upon adding 1% fresh *B. subtilis* LB culture into 2 × SG medium, it takes about 24 h for most of the spores to be formed (Driks, 1999).
A total of 121 genes are involved in the programmed morphological sporulation process. The extremely complex process is directed by a strict sequence of sigma factor activations occurring at the right time and space. The main sporulation associated sigma factors acting sequentially are: $\sigma^H$, which acts in the early sporulation stages and plays early roles in the appearance of sporulation septum; $\sigma^F$ and $\sigma^E$, which function in the forespore and mother cell respectively and program the engulfment event; $\sigma^G$ and $\sigma^K$, which control the synthesis of the coat proteins and cortex.

![Diagram of B. subtilis sporulation process](image)

**Figure 1.4 B. subtilis sporulation process** (Adapted from Driks, 1999). Reprinted with permission from Ref (Driks, 1999). Copyright (1999) ASM.

*B. subtilis* sporulation is a highly complex and coordinated successive morphological alteration process. An intriguing property of the sporulation process is that coat protein assembly on nascent spores does not require transmembrane procedure, and
thus it provides potential solution to display multimeric proteins. Moreover, several outer coat proteins, e.g. CotB, CotC, and CotG, have been identified, facilitating the establishment of a spore-based platform.

1.2.3 Advantage of Spore Display for Biocatalysis

_Bacillus subtilis_ spore display, one of the microbial surface display platforms, can potentially address all the issues mentioned above. _B. subtilis_ sporulation is an intriguing process that provides an opportunity for multimeric protein display. The details of this process are reviewed in Section 1.2.1. The most important part of the process is the division of the bacterial cell into two compartments, mother cell and forespore, upon sporulation. All required components of spore maturation are synthesized and folded by mother cell. Hence, the _B. subtilis_ spore is an ideal platform for multimeric protein display.

In this thesis, we primarily focused on development of a facile self-assembly spore-based biocatalyst platform, which in theory has advantages compared to other biocatalysis methods. Spore-based biocatalysts are easy to prepare from a single-step purification. Since the enzyme is on the outer surface of spore, the enzymatic catalysis is thus free from interference by host metabolism, and substrates get free access to the displayed enzymes. Another intriguing property of _B. subtilis_ spore is its robustness against various harsh treatment, such as UV, heat, or extreme pHs. Therefore, the solid support of spore surface is supposed to enhance enzyme thermostability and storage stability. Moreover, because sporulation is free from transmembrane process, spore surface display can potentially display large multimeric proteins. In addition, utilizing dense spore coat proteins on the
surface, diverse enzyme species with synergistic functions can be displayed, thus facilitating substrate channeling.

1.2.4 The Current Stage of Spore Display

Spore surface display is a promising platform for biocatalysis because, proteins anchored on spore surface have shown dramatically elevated enzyme stability in acidic pH and high temperatures (Sirec et al., 2012). The high spore display capacity of the proteins – ranging between $10^3$ and $10^4$ protein molecules per spore, allows for a higher catalytic efficiency (Isticato et al., 2001; Mauriello et al., 2004; Duc et al., 2007; Kwon et al., 2007; Potot et al., 2010). Spores have a formidable resistance to extreme temperatures (up to 90 °C), pH, organic solvents, humidity and even UV and gamma radiation and lysozyme allowing them to be used in more versatile situations than other microbial display platforms (Ricca and Cutting, 2003; Knecht et al., 2011; Barak et al., 2005; Kim and Schumann, 2009).

Two approaches are generally adopted to display proteins on spore surface: non-specific adsorption and direct fusion with a “carrier” coat protein. Heterologously expressed β-galactosidase in *E. coli* was attempted to adsorb onto spore surfaces (Sirec et al., 2012). However, the adsorption capacities of spore varied from strain to strain, primarily because the varying spore surface charge and protein molecular behavior, that determine the interaction between spore and adsorbed proteins, were unique to each strain. Another adopted spore display method fuses the target protein with a spore outer surface protein. Emerging outer surface coat proteins, *e.g.* CotB, CotC, CotG, and OxdD (Sacco et al., 1995; Driks, 1999; Baccigalupi et al., 2004; Potot et al., 2010; Kwon et al., 2007; Hinc
et al., 2010; Wang et al., 2011), have been utilized as fusion partners for the display of heterogeneous proteins, such as vaccines, GFP, or streptavidin (Cutting et al., 2009; Maurielloa et al., 2004; Istitato et al., 2001; Duc et al., 2007; Duc et al., 2003; Kim et al., 2005; Park et al., 2004; Kim et al., 2007; Thompson & Stewart, 2008; Du et al., 2005). A method to quantitatively compare and identify the most potent fusion partner and surface display of enzyme for biocatalysis purposes is desperately needed.

1.3 Scope of this thesis

In the thesis, I mainly intended to provide supplemental solutions to three related issues. In Chapter 2, regeneration of expensive pyridine nucleotide cofactor, such as NADPH, was essential to reduce the industrial production cost. We coupled xylose reductase and phosphite dehydrogenase on spore surface in a controllable stoichiometry for efficient production of xylitol as well as NADPH regeneration, with phosphite as a sacrificial agent. In Chapter 3, we took advantage of B. subtilis sporulation process and co-expressed scaffoldin and Doc-tagged enzyme in the mother cell compartment. Therefore, after the autolysis of mother cell, mature spore carrying enzyme via Coh-Doc interaction is generated. This method allows active display of multimeric enzyme.

1.3.1 Spore Based Biocatalyst for Cofactor Regeneration

Many value-added compounds in an expanding area of industrial and medical importance are enzymatically synthesized. Oxidoreductases constitute over a quarter of those enzymes, including reductases and dehydrogenases which are both conventionally employed in industry. Most reductases and dehydrogenases require the conversion of the expensive co-enzyme, NAD(P)H, to NAD(P)^+ for catalytic electron transfer purpose.
Considering the high cost of these co-factors (~$1000/mol), a feasible way to make large-scale application affordable is to regenerate the NAD(P)H. Regeneration of nicotinamide cofactors can be accomplished using several methods, namely chemical, electrochemical, photochemical or enzymatic.

Photocatalytic and electrochemical reactions are most commonly used chemical methods for the regenerations of NAD(P)(H). The photocatalytic regeneration of NAD(P)H requires the presence of light-harvesting chromophores that transfer electrons during the catalytic process. Two types of light-harvesters are generally used: semiconductors and macromolecular heavy metal complexes (Dibenedetto et al., 2011). Conductive sol-gel derived matrices encapsulating functional enzymes have been utilized extensively as electron transporters (Gill, 2001; Jin & Brennan, 2002; Rolison & Dunn, 2001). However, there are many obstacles that inhibit the large-scale application of chemical regeneration of NAD(P)(H) (Hollmann et al., 2010; van der Donk & Zhao, 2003; Manica et al., 2003, Leonida, 2001a; Leonida et al., 2001b; Manjon et al., 2002; Suye et al., 2002), and the main one is the low TTN (total turnover number) (< 1,000) (Leonida, 2001; Faber, 1997). Conversely, enzymatic reactions are considered the most promising methods for the regeneration of nicotaminade cofactors. Different from conventional chemical reactions, enzymes are renowned to undergo reactions with high specificity and selectivity in relatively mild conditions. A reaction selectivity of 99.99993% must be met to reach a TTN of 10⁶, which is readily achieved by enzymatic catalysis, further emphasizing the significance of enzymes (Chenault & Whitesides 1987, Chenault et al. 1988).
Generally, a second enzyme, such as formate dehydrogenase, glucose dehydrogenase, alcohol dehydrogenase, or glucose-6-phosphate dehydrogenase, is coupled to regenerate the cofactor using a cheap co-substrate. Currently one of the most prevalent approaches for the production of regenerative NADH is to utilize formate dehydrogenase isolated from *Candida boidinii* oxidizing formate to carbon dioxide (Wichmann et al. 1981). A good example of an industrial large-scale application of formate dehydrogenase-regenerated NADH is the production of L-tert-leucine (Bommarius et al. 1998). Large-scale industrial applications primarily utilize ultrafiltration membrane reactors to retain macromolecules-tethered NAD(P)(H) that are larger than the molecular weight cut-off of the ultrafiltration membrane while generated products flow out (van der Donk & Zhao, 2003; Wecbecker et al., 2010). Some advantages of utilizing formate dehydrogenase include the availability of inexpensive formate and the automatic elimination of generated CO₂. However, it also has major drawbacks, including relatively low specific activity (~ 6 U mg⁻¹, Slusarczyk et al., 2000) and rapid deactivation by organic solvents.

NADP-dependent enzymes are less common than NAD-dependent enzymes, and therefore the options for NADPH regeneration are limited (Faber, 2000). Several conventionally employed regenerative enzymes include glucose-6-phosphate dehydrogenase, *Thermoanaerobium brockii* alcohol dehydrogenase, or mutated formate dehydrogenase that has preference to NADP⁺ instead of NAD⁺ (Tishkov et al., 1999). Recently, a particularly interesting enzymatic NAD(P)H regeneration method using PTDH (phosphate dehydrogenase) has been developed. Wild type PTDH catalyzes the irreversible oxidation of phosphite to phosphate assisted by NAD⁺ with low thermostability. But,
PTDH mutants exhibiting either relaxed specificity towards both nicotinamide cofactors (Woodyer et al., 2003) or improved thermostability (Johannes et al., 2005) have been screened with a 96-well plate format method. The former mutants mentioned have comparable turnover rates towards both nicotinamide cofactors similar to FDH. While the latter thermostable mutant has a 2.4-fold greater half-life at 50 °C than FDH. This regenerative enzyme is promising because it has some of the same advantages as FDH, such as using a low-cost substrate and the ready elimination of by-products.

In my thesis, I was particularly interested in finding a way to continuously produce xylitol by coupling xylose reductase and phosphite dehydrogenase while using NADP+ as a cofactor, and we aimed to achieve recycled NADPH participation in xylose reduction. We operated the whole process on the spore surface, as it provides an inert and stable environment. The platform of *B. subtilis* spore further confers two more compelling advantages for NADPH regeneration, i.e. enzyme stability enhancement due to the support of a solid surface and faster reaction rate due to substrate channeling.

1.3.2 *Spore Based Biocatalyst for Organic Solvent Reactions*

Enzymes for value-added compounds synthesis are advantageous compared with other chemical catalysts, because enzymes do not require harsh reaction conditions but own high specificity and reactivity. During industrial production, the problem of insoluble substrates or products is frequently encountered, and organic solvents are usually employed to enhance their solubility or change the kinetic equilibrium, thus enhancing productivity (Khmelnitsky & Rich, 1999; Angelova & Schmauder, 1999). Unfortunately, organic solvents rapidly deactivate free enzymes. Protein-anchoring system was developed in
response to this issue, and it is held that the solid surface tends to protect protein from being denatured. The first protein-anchoring technique was developed using virus, and the displayed protein size for this system was rather limited (Smith, 1985). Following, microbial cell surface display enabled the display of larger proteins (Charbit et al., 1986; Freudl et al., 1986). The microbial surface display brings attractive properties, such as stability enhancements in high temperatures and organic solvents. For example, a lipase anchored on the surface of \textit{P. putida} did not show any activity weakening after incubating at 37 °C for 12 hours, while the free lipase treated under the same condition retained only 70% of original activity (Jung et al., 2006). Organic solvent resistance can also be dramatically enhanced by using surface display technique. \(\beta\)-Galactosidase displayed on the surface of \textit{B. subtilis} spore exhibited 20-100 fold activity enhancement in the emulsions of water/toluene and water/ethyl acetate compared with free \(\beta\)-galactosidase (Kwon et al., 2007).

Figure 1.5 Determining the localization of spores at the interfacial surfaces of microemulsions with confocal fluorescence microscopy of microemulsion of fluorescently labeled spore of \textit{B. subtilis} WB700 displaying \(\beta\)-Gal using primary rabbit anti-\(\beta\)-Gal antibodies and secondary fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G antibodies. Scale bars = 10 µm. Reprinted with permission from Ref (Kwon et al., 2007). Copyright (2007) ASM.
A typical chassis where organic solvent is used is the transgalactosylation reaction of lactose and alcohols. Alkyl glycosides are a family of natural and environmental friendly surfactants. Compared with conventional surfactants, they have the superior properties of low foaming, non-irritation to the skin and eyes, and ready biodegradation (Yang et al., 2017). The most conventional way for generation of alkyl glycosides is through β-galactosidase enzymatic catalysis. This transgalactosylation requires lactose and alcohols, and produced alkyl glycosides are insoluble in water. To increase the solubility of the products, non-polar organic solvents (such as ethyl ether) are usually added. However, as expected, free β-galactosidase had short life in the presence of organic solvents (Kwon et al., 2007). Displaying β-galactosidase on the surface of an organic solvent resistant microbe is an ideal option.

In this part, I focused on the bi-phasic transglactosylation reaction of β-galactosidase displayed on B. subtilis spore surface. In addition to the abovementioned stability enhancements, B. subtilis spore platform also attractively exhibits another property, i.e. the interfacial localization between aqueous and organic phases (Figure 1.5). This feature allows enzyme’s access to substrates in both phases, and generates substrates with maximum efficiency. My aim in this part is to develop a self-assembly B. subtilis spore platform by co-expressing CotG-Coh fusion protein and Doc-tagged β-galactosidase within mother cell during sporulation process. I also compared my self-assembly platform with passive adsorption and direct fusion methods, and concluded that this self-assembly platform was an efficient way of generating biocatalysts.
References


Chapter 2. Spore-Displayed Enzyme Cascade with Tunable Stoichiometry

2.1 Abstract

Taking the advantages of inert and stable nature of endospores, we developed a biocatalysis platform for multiple enzyme immobilization on *Bacillus subtilis* spore surface. Among *B. subtilis* outer coat proteins, CotG mediated a high expression level of *Clostridium thermocellum* cohesin (CtCoh) and a functional display capability of $10^4$ molecules per spore of xylose reductase *C. termocellum* dockerin fusion protein (XR-CtDoc). By co-immobilization of phosphite dehydrogenase (PTDH) on spore surface via *Ruminococcus flavefaciens* cohesin-dockerin modules, regeneration of NADP(H) was successfully achieved. Both XR and PTDH exhibited stability enhancement upon immobilization. More importantly, by altering the copy numbers of CtCoh and RfCoh fused with CotG, the molar ratio between immobilized enzymes was adjusted in a controllable manner. Optimization of spore-displayed XR/PTDH stoichiometry resulted in increased xylitol production. In conclusion, endospore surface display presents a novel approach for enzyme cascade immobilization with improved stability and tunable stoichiometry.

2.2 Introduction

Enzymes catalyze specific reactions at environmentally benign conditions and often generate chiral products, therefore holding great promises for a broad range of chemical conversions (Bommarius, 2015; Choi et al., 2015). However, employing enzymes...
for large-scale industrial applications is in general limited by high cost of purification and low stability of the biocatalysts. Using whole cells that carry the enzymes of interest can avoid protein purification, but substrate uptake and product excretion are restrained by cell membrane and cell wall barriers. In addition, native metabolisms of the host cells often interfere with the target reaction and generate byproducts or even adverse cellular effects. To overcome these issues, display of enzymes on microbial cell surface has been developed for chemical synthesis, protein engineering and environmental applications (Schüürmann et al., 2014; Tanaka & Kondo, 2015; Tozakidis et al., 2015; Smith et al., 2015; Liu et al., 2014). Taking the advantages of stable and inert nature of endospores, this study aims to develop a platform technology of biocatalysis based on Bacillus subtilis endospore surface display. When starved or under extreme conditions, e.g. temperatures, radiation, pH, and harmful chemical agents, B. subtilis cells undergo asymmetrical division to form two compartments called mother cell and forespore (Cutting & Vander Horn, 1990). During sporulation, mother cell engulfs forespore, then a variety of cortex and coat proteins are produced in the mother cell cytoplasm and assembled on the surface of nascent spore. Finally mother cells lysate to release matured spores. Because coat protein expression and assembly on endospore do not require translocation across membrane (Pan et al., 2014; Kim & Schumann, 2009), this approach is attractive to display complex multimeric enzymes and enzyme cascades.

Pioneered studies of spore surface display have mainly focused on development of a robust vaccine delivery method (Knecht et al., 2011; Duc le et al., 2003; Duc le et al., 2007; Cutting et al., 2009) and system establishment using model proteins such as GFP or
streptavidin (Kim et al., 2005; Kim et al., 2007). Further researches include the study of adoption β-galactosidase on wild type and mutated B. subtilis spores, which exhibited dramatic improvements of enzyme stability at acidic pH and high temperatures (Sirec et al., 2012). Presumably the capacity to retain certain proteins on spore surface via passive adsorption is however largely dependent on the nature and properties of the target enzymes due to the nonspecific interactions between spore and adsorbed proteins. Several coat proteins (e.g. CotB, CotC, CotG and OxdD) present on outer surface of spores have been identified (Donovan et al., 1987; Driks, 1999) and employed as fusion partners for display of heterologous proteins (Isticato et al., 2001; Isticato et al., 2007; Costa et al., 2004), but display of enzyme cascades has not been reported. Herein we study spore surface display of multiple enzymes in a stoichiometrically controllable manner by recruiting cohesin (Coh) and dockerin (Doc) modules derived from cellulosomes (Doi & Kosugi, 2004; Bayer et al., 2004).

2.3 Materials and Methods

Construction of Spore Surface Display Plasmids and Transformation of B. Subtilis.

The gene encoding Clostridium thermocellum (ATCC# 27405) cohesin (CtCoh) was PCR amplified from its genomic DNA with a C-terminal FLAG tag (DYKDDDDK), and cloned between HindIII/EcoRI sites on a Bacillus chromosome integration vector pDG364 to yield pDG364-CtCoh. Genomic DNA of B. subtilis PY79 was isolated, and the genes of spore coat proteins CotB, CotC, and CotG including their promoter regions were amplified and cloned into the BamHI/HindIII sites on pDG364-CtCoh, resulting in pDG364-CotX-CtCoh (CotX=CotB/C/G) (Figure 2.2A). Similarly, 1, 2 or 3 repeats of Ruminococcus
flavefaciens cohesin (RfCoh) genes were PCR amplified from pET20b-cbm-scaf3 (You & Zhang, 2014) and cloned to the HindIII site of pDG364-CotG-CtCoh, resulting in pDG364-Scaf11, pDG364-Scaf12, and pDG364-Scaf13 respectively (Figure 2.3A). All constructed plasmids were confirmed by restriction digestion and DNA sequencing. For chromosomal integration at α-amylase locus, the resulting vectors were linearized by XhoI digestion, and transformed into B. subtilis competent cells (Zhang et al., 2013) for selection on agar plates supplemented with 5 μg/mL chloramphenicol.

Cloning, Expression and Purification of XR-CtDoc and PTDH-RfDoc. The gene encoding a type I C. thermocellum dockerin (CtDoc) was PCR amplified from its genomic DNA with a c-Myc tag on its C-terminus, and cloned into EcoRI/XhoI sites on pET28b(+) to give pET28b-CtDoc. Neurospora crassa D-xylose reductase gene was amplified from pTrcXR (Nair & Zhao, 2010) and inserted in NdeI/EcoRI sites on pET28b-CtDoc, resulting in pET28b-XR-CtDoc. Similarly, PTDH gene of Pseudomonas stutzeri WM88 (Relyea & van der Donk, 2005) was cloned to generate pET28b-PTDH, and Ruminococcus flavefaciens dockrin (RfDoc) was PCR amplified from pET20b-FBP-RfDoc (You & Zhang, 2014) to generate pET28b-PTDH-RfDoc. Constructed plasmids were confirmed by DNA sequencing, and transformed into E. coli BL21(DE3) for production of XR-CtDoc and PTDH-RfDoc. When OD_{600} reached 0.8, 0.4 mM IPTG was added to induce protein expression at room temperature overnight. Both XR-CtDoc and PTDH-RfDoc were purified with Ni-NTA agarose and prepared in 50 mM phosphate (pH 7.0). The concentrations of protein were determined using a BioTek spectrophotometer with
calculated extinction coefficients $\varepsilon_{280} = 63830 \text{ M}^{-1} \text{ cm}^{-1}$ for XR-CtDoc and $33460 \text{ M}^{-1} \text{ cm}^{-1}$ for PTDH-RfDoc.

**Spore Production and Coat Protein Extraction.** *B. subtilis* cells were cultured in 2×SG medium at 37 °C for 24-28 hours (Cutting & Vander Horn, 1990), and the optimal culture time for spore harvest was determined by OD measurements and microscopic imaging (Figure 2.1). Spores were collected by centrifugation at 4,000 ×g for 6 min and washed with 1.5 M KCl and 0.5 M NaCl. Unsporulated mother cells were lysed by incubation with 50 µg/mL egg white lysozyme (Sigma-Aldrich, St. Louis, MO) in 50 mM Tris-HCl (pH 7.2) at 37 °C for 1 hour, and spores was separated from cell debris by centrifugation. Obtained spores were then washed with 1 M NaCl, water, 0.05% SDS and water, and finally resuspended in 50 mM phosphate buffer (pH 7.0). For immunofluorescence microscopy, 5 OD spores collected at different culture times were washed four times with TBS (50 mM Tris-HCl with 150 mM NaCl, pH 7.5) and incubated with 1 µL anti-FLAG-FITC (Sigma-Aldrich, St. Louis, MO) in 500 µL TBS for 30 min under gentle rotation. After washing with TBS, spore samples were imaged by an immunofluorescence microscope (Olympus, Cypress, CA). For biocatalysis assays, excess amounts of dockerin-fused enzymes were mixed with prepared spore suspension for 5 min, then washed with phosphate buffer twice to remove nonspecifically bound proteins. To release coat proteins and immobilized enzymes, spores were incubated with 1% SDS and 50 mM dithiothreitol (DTT) at 70 °C for 30 min, and centrifuged at 10,000 ×g for 10 min. The supernatants were loaded for SDS-PAGE, and Western blotting signals were developed using anti-FLAG-HRP (Bethyl Laboratories Inc., Montgomery, TX) or anti-C-Myc-HRP (Santa Cruz
Biotechnology Inc., Santa Cruz, CA) with supplement of chemiluminescent substrates (Thermo Scientific Inc., Rockford, IL). In order to estimate the capacity of spore surface display, dot blotting was performed as a densitometry method. Both purified and extracted XR-CtDoc/PTDH-RfDoc were serially diluted on PVDF membrane, and the signals were developed using anti-C-Myc-HRP.

**Enzymatic Reaction Assays and Spore Display Capacity Estimation.** The optimal pHs for XR and PTDH are 5.5 and 8, respectively (Woodyer et al., 2005; Costas et al., 2001). At pH 7, both enzymes retained over 50% of their individual enzymes. Therefore, the activities of enzymes in solution were measured in 50 mM phosphate (pH 7.0) with 0.2 mM NADP(H) and 0-200 mM D-xylose or 0-10 mM phosphite. The rates of NADPH consumption (for XR-CtDoc) or generation (for PTDH-RfDoc) were measured by monitoring absorbance at 340 nm ($\varepsilon = 6300 \text{ M}^{-1} \text{ cm}^{-1}$). When both enzymes were displayed, 5 mM phosphite, 200 mM D-xylose, and 2 mM NADP$^+$ was added to spore suspension. Produced xylitol was quantified by an Agilent HPLC, equipped with an Aminex HPX-87H ion exchange column and a refractive index detector. Filtered 5 mM H$_2$SO$_4$ was employed as the mobile phase. In order to semi-quantitatively determine the capacity of spore surface immobilization, XR-CtDoc was incubated with spores displaying CotG-CtCoh. After washing to remove excess enzymes, 0.2 mM NADPH and 200 mM D-xylose were added into the spore suspension to monitor NADPH consumption rates. The quantities of enzyme molecules per spore were calculated based on $k_{cat}$ of XR-CtDoc and the correlation between spore numbers and optical density, *i.e.* $1.5 \times 10^8$ CFU/ml at an optical density at 600 nm (OD$_{600}$).
2.4 Results

**Sporulation Process Optimization.** To maximize spore yields for biocatalysis applications, sporulation process of *B. subtilis* strain PY79 was studied by measuring OD\textsubscript{600} and observing the morphology under a microscope at different time points when cultured in sporulation medium. Collected culture samples were also treated with lysozyme to lyse vegetative cells followed by repeated centrifugation and washing to remove cell debris. Thus, the OD\textsubscript{600} values of post-lysozyme treatment were associated with formed spores. As expected *B. subtilis* cells quickly grew to OD ~2.5 in 6 hours after inoculation without detectable forespore or spore formation (Figure 2.1). Microscope images taken at 12 h showed forespores appeared in a small fraction of cells (indicated by arrows in Figure 2.1B), which correlated with a slight increase of OD\textsubscript{600} associated with spores, while OD\textsubscript{600} of the cell sample without lysozyme treatment increased to ~3.0. As significant amounts of nutrients were consumed between 12 and 20 h, OD\textsubscript{600} associated with spores rapidly increased by 1.8 units, while the OD\textsubscript{600} of cell samples without lysozyme treatment only increased by 0.9, indicating more vegetative cells developed to spores during the period. At 20 h, the culture sample was predominated with mature endospores. After 20 h, the OD\textsubscript{600} associated with spores was relatively constant at ~1.8, but the OD\textsubscript{600} for culture mixture without lysozyme treatment started to decrease at 26 h, suggesting cell death.
Figure 2.1 Sporulation process of *B. subtilis*. (A) OD$_{600}$ curves of *B. subtilis* cultured in 2×SG medium before lysozyme treatment (including vegetative cells, spores, and debris of autolysed mother cells) and after lysozyme treatment and wash/centrifugation cycles (associated with spores only). (B) Microscopic images at different culture time. Arrows indicate the appearance of forespores at 12h. Mature spores predominated the 24h culture sample. Bars = 5 µm.

**CotG Mediated High Display Level of Scaffoldins.** Within the > 50 polypeptides involved in coat assembly, the major components of the outer coat (the most outside layer of endospores) and their associated genes have been identified by biochemical and genomic means (Donovan et al., 1987; Driks, 1999). Since these discoveries, several proteins have been displayed on the surface of spores by fusing the target proteins to the C-terminus of one of the major outer coat proteins such as CotB, CotC, CotG and OxdD (Isticato et al., 2001; Isticato et al., 2007; Costa et al., 2004). Different from all previous spore display studies, we intend to immobilize multiple enzymes on spore surface with controllable stoichiometry. We hypothesized that using the dockerin and cohesin modules derived from
cellulosomes can achieve this goal (Doi & Kosugi, 2004; Bayer et al., 2004). To identify the most suitable *B. subtilis* coat proteins for enzyme immobilization via cohesin-dockerin, *C. thermocellum* cohesin (CtCoh) was cloned to the C-termini of CotB/CotC/CotG, which were regulated by their native promoters (Figure 2.2A). In addition, a FLAG tag was introduced at the C-termini of CotX-CtCoh for detection. After chromosome integration, spores carrying CotX-CtCoh were harvested after 24 h, which was the optimal culture time for sporulation (Figure 2.1). Collected spores were then labeled with anti-FLAG-FITC and analyzed by fluorescence microscopy. As shown in Figure 2.2B, the spores obtained from CotB/C/G-CtCoh strains all exhibited strong fluorescence, whereas PY79 host without transformation did not show detectable signal, indicating successful display of scaffoldins on spore surface. CotB and CotG fusions exhibited higher fluorescence signals than CotC. Furthermore, the outer coat fractions were extracted from spores by SDS-DTT treatment to visualize the expression levels of cohesins by Western blotting. The results showed single bands with expected MWs for CotB-CtCoh (49.8 kDa), CotC-CtCoh (33.4 kDa), and CotG-CtCoh (43.2 kDa) (Figure 2.2C). It also demonstrated that CotG-CtCoh was expressed at least 5-fold more than CotB/C-CtCoh.
Figure 2.2. Spore surface display via cohesin-dockerin interaction. (A) Construction of gene cassettes for display of coat protein-cohesin (CotX-Coh, CotX=CotB/C/G) by chromosomal integration to achieve enzyme immobilization on spore surface. Purified spores were (B) labeled with anti-FLAG-FITC and examined with fluorescent microscopy, or (C) treated with SDS/DTT solution and examined by Western blotting using anti-FLAG-HRP.

Xylose Reductase Immobilization via Cohesin-Dockerin Interaction. Enzymatic redox reactions catalyzed by dehydrogenases and reductases are widely applied for the synthesis of specialty chemicals, such as single-isomer alcohols, chiral amino acids and other enantiomeric pure compounds (Musa & Phillips, 2011; Chen et al., 2012). Among ketoreductases, xylose reductase (XR, EC1.1.1.307) has gained interests not only because the native reaction it catalyzes, xylose to xylitol, is the first step to utilize C5 for biofuel production, but also it accepts many non-natural substrates for chiral conversions (Kratzer & Nidetzky, 2007). Particularly, XR from xylose assimilating fungus Neurospora crassa can utilize both NADH and NADPH and catalyzes reduction of a variety of sugar substrates over a wide pH range (Woodyer et al., 2005). To display N. crassa XR on spore surface
via anchored scaffoldins, its gene was cloned to the N-terminus of *C. thermocellum* dockerin (CtDoc), and XR-CtDoc chimeric protein was heterologously expressed in *E. coli*. Cell lysate containing XR-CtDoc was then incubated with the suspension of prepared scaffoldin-displaying spores allowing self-assembly governed by cohesin-dockerin interaction. After washes to remove nonspecifically bound enzymes, the resulted spores were tested for XR activity. Because of the long and flexible linkers between coat protein and cohesin and between XR and dockerin, it is expected that XR immobilized on the spore surface has a similar specific activity as that of free XR. Results indicated that spores displaying XR-CtDoc via CotG-CtCoh exhibited a rapid NADPH consumption, at a rate of 22 ± 1.8 μM/min per OD spores (standard deviation included). Given that an OD$_{600}$ is equivalent to 1.5×$10^8$ spores/ml and the measured turnover rate of XR is 42 sec$^{-1}$, the display capacity was estimated to be approximate $10^4$ XR-CtDoc molecules per spore carrying CotG-CtCoh.

**Xylitol Production by Multiple Enzyme Display.** Most oxidoreductases involved in specialty chemical synthesis, including XR, utilize pyridine nucleotides, NAD(P)$^+$ and NAD(P)H, as cofactors for catalysis. Because these cofactors are expensive (~$1000/mole) and are consumed in stoichiometric quantity, their recycling is essential for the *in vitro* reactions to be cost-effective. Furthermore, cofactor regeneration also helps drive the thermodynamically unfavorable reaction towards product formation and thereby improves the reaction yields (van der Donk & Zhao, 2003; Weckbecker et al., 2010; Abu & Woodley, 2015). We therefore further investigated the feasibility of simultaneously immobilizing a second enzyme on spore surface for NAD(P)H regeneration. Phosphite dehydrogenase
(PTDH) catalyzes the conversion of phosphite to phosphate, which is coupled with reduction of NADP\(^+\) to NADPH (Relyea & van der Donk, 2005). Because this irreversible reaction utilizes an inexpensive inorganic as the co-substrate, PTDH has been recognized as an ideal enzyme for cofactor regeneration (Johannes et al., 2007). However, PTDH has a relatively low specific activity with a turnover rate of 2.3 sec\(^{-1}\). Inspired by the substrate channeling phenomena seen in natural and synthetic multi-enzyme cascades, we aimed to improve the efficiency of cofactor regeneration not by engineering of PTDH itself but by providing a close proximity with XR for an efficient NADPH-NADP\(^+\) recycling through their spatial organization on the surface of spores.

Figure 2.3. Varying ratios of XR/PTDH on spore surface. (A) Constructions of scaffold proteins Scaf11 (CtCoh/RfCoh = 1:1), Scaf12 (CtCoh/RfCoh = 1:2), and Scaf13 (CtCoh/RfCoh = 1:3) for chromosomal integration. (B) Analysis of XR-CtDoc and PTDH-RfDoc immobilized on spores. Spores displaying scaffolds were harvested and incubated with excess amounts of XR-CtDoc and PTDH-RfDoc. After washing, immobilized enzymes were eluted by SDS/DTT for Western blotting. (C) Xylitol production via NADPH regeneration and the yield improvement by stoichiometry optimization. Assays were performed with XR-CtDoc and PTDH-RfDoc either immobilized on spores (black) or in solution (red), in the presence of phosphite, D-xylose and NADP\(^+\). Produced xylitol was quantified with HPLC, and experiments were repeated in triplicate.
To independently control the numbers of PTDH and XR immobilized on spores, *Ruminococcus flavefaciens* cohesin-dockerin modules were utilized for recruitment of *Pseudomonas stutzeri* PTDH. PTDH Gene was cloned to the N-terminus of *R. flavefaciens* dockerin (RfDoc), and PTDH-RfDoc chimeric protein was produced in *E. coli*. *R. flavefaciens* cohesin (RfCoh) was cloned between CotG and CtCoh on the *B. subtilis* chromosomal integration plasmid, to encode a tripartite fusion protein CotG-RfCoh-CtCoh (Figure 2.3A). For detection, a c-Myc tag was incorporated at the C-termini of XR-CtDoc and PTDH-RfDoc. Similar to XR-displaying spores, the spores carrying both XR and PTDH were tested by Western blotting with anti-c-Myc-HRP. Results indicated that XR-CtDoc and PTDH-RfDoc were present in the spore coat extraction samples with their expected MWs at approximately equal molar amounts (Figure 2.3B). The spores displaying both enzymes were then subjected to catalysis assays with substrates D-xylose and phosphite. Because the cofactor was provided at its oxidized form as NADP⁺, xylitol can only be produced when both enzymes are functional. After overnight reaction, 74.2 μM xylitol was produced with an initial rate of 15.0 μM xylitol produced per OD spores per hour (Figure 2.3C). Using the same amounts of enzymes, catalysis assays were also performed in solutions to compare their specific activities. The results suggested that the enhancement by spore display is negligible, presumably because the spatial distance between XR and PTDH on spores via the scaffodin containing one CtCoh and one RfCoh, was not proximal enough to drive significant substrate channeling.
Figure 2.4. Quantification of immobilized XR-CtDoc (A) and PTDH-RfDoc (B). 10 OD spores displaying scaffolds were harvested and incubated with excess amounts of XR-CtDoc or PTDH-RfDoc. After washing, immobilized enzymes were eluted by SDS/DTT. Both purified and extracted enzymes were serially diluted, and 2μL were used for dot blotting analysis. The lanes of × 1, × 2, and × 3 denote extracted enzymes from Scaf11, Scaf12, and Scaf13, respectively.

Improved Product Yields by Controlling Enzyme Stoichiometry. It is not uncommon that the kinetics of coupled enzymes in either natural or artificial metabolic cascades are not in balance. This imbalance usually results in the accumulation of intermediates and sub-optimal product yields. Optimizing the ratios of enzymes within a cascade reaction is a practical strategy to improve the rate of overall conversion. To achieve tunable stoichiometry, the ratio between XR and PTDH was manipulated by altering the repeat numbers of the associated cohesins on the anchoring scaffoldins (Figure 2.3A). Since PTDH has significant lower specific activity than that of XR, higher copy numbers of PTDH is highly desirable. Two more scaffoldin structures were constructed by inserting either one or two more RfCoh domains at the N-terminus of CtCoh in the previously constructed CotG-RfCoh-CtCoh expression cassette, generating additional XR/PTDH stoichiometry of 1:2 and 1:3 respectively. The DNA fragments encoding these multi-cohesin fusions were prepared by overlapping-extension PCRs, and the resulting constructs
were nominated as Scaf11, Scaf12, and Scaf13 reflecting there were one *C. thermocellum* cohesin domain and one, two or three *R. flavefaciens* cohesin domain(s) on the scaffoldins. As abovementioned, display of Scaf11, Scaf12 and Scaf13 on the spore surface were achieved by cell culture in sporulation-inducing media 2×SG for 24 h. The spores carrying various scaffoldins were directly incubated with lysates of cells expressing XR-CtDoc and PTDH-RfDoc to assemble multi-enzyme complexes on spore surface. The compositions of these obtained enzyme complexes were biochemically characterized by coat protein extraction and Western blotting. Results confirmed that increased amounts of PTDH were incorporated into the spore-displayed complexes when more RfCoh modules were utilized, while the amounts of XR stayed unchanged (Figure 2.3B). Detailed analysis of the degree of darkness for each single band in Figure 2.3B also showed that the ratios of PTDH-RfDoc:XR-CtDoc on spore surface were roughly 0.9 ± 0.1, 1.8 ± 0.3, and 2.6 ± 0.2 for Scaf11, Scaf12, and Scaf13 respectively, close to preset stoichiometry. Moreover, dot blot was conducted to more accurately quantify the immobilized enzyme density (Figure 2.4). By careful comparison of serially diluted purified and extracted enzymes, the display capacity of XR-CtDoc was determined to be $1.5 \times 10^4$ molecules per spore. In terms of PTDH-RfDoc, the immobilized enzyme amounts were $1.4 \times 10^4$, $2.5 \times 10^4$, and $3.6 \times 10^4$ molecules per spore for Scaf11, Scaf12, and Scaf13, respectively. These results indicate that the molar ratios of XR/PTDH was successfully altered on spore surface in the indicated manner. In enzymatic assays supplied with D-xylose, phosphite and NADP+, spores carrying Scaf12 and Scaf13, which mediated a display ratio of XR-CtDoc:PTDH-RfDoc at 1:2 and 1:3, produced 57% and 1.2 folds more xylitol compared to that of Scaf11. And
initial specific activities of spores were 18.0, 29.3, and 41.8 μM/h for Scaf11, Scaf12 and Scaf13 respectively. In comparison with the activities of the same amounts of enzymes in solutions, the enhancements of spore surface display were 10-30% for Scaf12 and Scaf13.

**Spore Surface Immobilization Enhanced Enzymes Stability.** After incubation for extended periods, activities of XR and PTDH in solution and on spore surface were measured. The half-life of free XR-CtDoc was calculated to be 3.6 h, while the spore surface immobilized XR-CtDoc had a half-life of 11.6 h (**Figure 2.5**). After 10 h incubation, immobilized XR-CtDoc still had 45.4% activity left, while free XR-CtDoc only retained 16.4% of its activity. A similar stability improvement was observed for PTDH-RfDoc as well, where after incubation for 10 h, immobilization helped increase residual activity from 10.3% to 24.1%.

![Figure 2.5. Stability enhancement of enzymes immobilized on spores.](image)

**Figure 2.5. Stability enhancement of enzymes immobilized on spores.** Relative activities of XR-CtDoc and PTDH-RfDoc were tested on spore surface (black) or in solution (red). Experiments were repeated in triplicate.
2.4 Discussions

As an extreme survival strategy, certain Gram-positive bacteria such as *B. subtilis* undergo a series of developmental processes to form endospores when starved or under adverse conditions. *B. subtilis* sporulation involves more than 120 gene expressions and is associated with several morphological stages: asymmetric cell division, engulfment, cortex and coat assembly, and eventually formation of the matured spore for future germination upon appropriate environments (Cutting & Vander Horn, 1990). To generate reliable enzyme immobilization, sporulation process of *B. subtilis* was monitored by OD$_{600}$ measurements and microscopic imaging the cells before and after lysozyme treatment. Results confirmed that in 2×SG media, sporulation started at 12 h and forespore formation and mature endospores dominated at 24 h, which was therefore determined as the optimal time to harvest *B. subtilis* spores (Figure 2.1).

Most previous studies of protein immobilization on spore surface utilized one of the following two strategies: passive adsorption (Pan et al., 2014; Sirec et al., 2012) or direct fusion with a coat protein (Driks, 1999). The amounts of proteins that can be immobilized by these methods primarily depend on the properties of target proteins including charge, size and hydrophobicity, and thus varied for different target enzymes. Here, we developed a general method of spore surface immobilization by employing the strong and specific interaction between cohesin and dockerin derived from cellulosomes of thermophilic bacteria. This approach is presumably less dependent on properties of immobilized enzymes, and therefore is suitable for a broad range of enzymes for biocatalysis applications. Via Coh-Doc, the amount of enzymes displayed on spore surface, $\sim10^4$ per
spore, was significantly higher than direct coat protein-enzyme fusion approach (Isticato et al., 2001; Isticato et al., 2007), likely because the cohesion-dockerin modules acted as a spacer to extend enzymes away from the spore surface thereby reducing steric hindrance and making enzymes more accessible to their substrates. Moreover, the display capacity on spore surface in this study was approximately equivalent to other platforms. For example, surface density of $10^4$ molecules per cell has been reported for *Staphylococcus carnosus* (Andreoni et al., 1997), while baker yeast has the display capacity of $10^4$-$10^5$ molecules per cell (Doerner et al., 2014; Hoogenboom, 2005; Pepper et al., 2008). Considering the size of baker yeast cell, which has a diameter of 3-4 times of *B. subtilis* spore, the display capacities per unit surface were roughly the same.

After verifying the functions of XR and PTDH individually on spore surface, we further coupled the two enzymes on spores for enzymatic assays with the presence of merely xylose, phosphite, and NADP$^+$. The regeneration of cofactor and the production of xylitol confirmed the simultaneous displays of both XR and PTDH in their functional formats on spore surface.

Fine-tuning enzyme ratios is difficult to achieve in whole-cell biocatalysts, because protein expression is a multi-step process, in which the transcript amounts, mRNA stability, translation rate, and folding speed and efficiency of produced polypeptides are all difficult to be controlled precisely. Free enzyme complexes are able to regulate enzyme ratios but suffer from low stability and high purification costs. Coupling the spore display and specific Coh-Doc interaction, this study addresses these problems by developing a designer biocatalyst system that facilitates the manipulation of enzyme stoichiometry. We
demonstrated that the ratio of enzymes, \( i.e. \) XR/PTDH in this current study, could be accurately adjusted through recruiting different copy numbers of their associative cohesin modules on spore surface. More importantly, it showed that with higher ratio of PTDH over XR, the production yields of xylitol were significantly increased.

Another advantage of enzyme immobilization on spores is stability enhancement. Our results proved that by displaying on spore surface, retained activities of XR and PTDH were dramatically improved 2.8-fold and 2.3-fold respectively after 10 h incubation (Figure 2.5). The spore surface, like many other solid supports, likely prevents protein denaturation (Wang et al., 2011; Yim et al., 2009). This stability improvement could be particularly beneficial when biocatalysis is performed in organic solvents (Jia et al., 2014; Kwon et al., 2007).

2.5 Conclusions

In summary, via cohesin-dockerin interaction, two enzymes were immobilized on \( B. \) subtilis endospore surface in a stoichiometrically controllable manner. Functional co-immobilization of both XR and PTDH resulted in cofactor regeneration and production of xylitol. With increasing amounts of PTDH on spore surface by manipulating the copy numbers of the associated cohesins, a higher product generation rate was achieved. One future development could be the co-expression of both scaffolding and target enzymes in the mother cell compartment for self-assembly during sporulation process.
References


Chapter 3. Highly Active Spore Biocatalyst by Self-assembly of Co-expressed Anchoring Scaffoldin and Enzyme

3.1 Abstract

We report a spore-based biocatalysis platform capable of producing and self-assembling active multimeric enzymes on spore surface. This was achieved by co-expressing both a spore surface anchoring scaffoldin protein containing multiple cohesin domains and a dockerin-tagged enzyme of interest in the mother cell compartment during *Bacillus subtilis* sporulation. The feature that endospore formation is a transmembrane free process allows functional display of multimeric enzymes on nascent spores, and subsequent mother cell autolysis releases mature spores carrying target enzyme. Using this method, active tetrameric β-galactosidase was successfully displayed on spore surface with a high loading density of $3.6 \times 10^4$ enzymes per spore particle. The resulting spore biocatalysts exhibited increased transgalactosylation yields in water/organic emulsions, enhanced thermostability, superior reusability, and long-term storage stability at ambient temperature for more than 60 days, and thus holding a great potential in a wide range of biocatalysis applications.

3.2 Introduction

Microbial surface display, *e.g.* on *Escherichia coli* and *Saccharomyces cerevisiae* (Boder & Wittrup, 1997; Becker et al., 2005; Lattemann et al., 2000; Kim et al., 2000; Kondo & Ueda, 2004; Wen et al., 2010; Gai & Wittrup, 2007; Pepper et al., 2008), has been developed for chemical synthesis to overcome the challenges associated with free
enzymes, *i.e.* high purification cost and low stability, and the challenges associated with whole cell based biocatalysts, *i.e.* substrate and product transport limit, and interference with host native metabolism (Ajikumar et al., 2010; Neoigt, 2008; Hollinshead et al., 2014; Ostergaard et al., 2000). To localize on cell surface, enzymes of interest produced in cytoplasm need to translocate across cell membrane, which could be problematic for many enzymes possessing multiple domains or subunits. For example, β-galactosidase (β-gal), a large enzyme with a monomer MW of 116 kDa and only active as a tetramer, could not be functionally displayed on surface of *E. coli* due to the toxicity of membrane jamming (Wu et al., 2008).

Utilizing the mechanism of *Bacillus subtilis* endospore formation, this study aims to develop a facile display technique bypassing transmembrane process and self-assembling multimeric enzymes on surface of spores with a high loading density. During sporulation, *B. subtilis* cells undertake several morphological changes: asymmetric cell division to form a large compartment (mother cell) and a smaller one (forespore), engulfment of forespore into mother cell, cortex formation, coat protein expression and assembly, and lysis of mother cell and release of the matured spore (Sonenshein et al., 2001; Kroos and Yu, 2000). Because no transmembrane is required during the process of coat proteins assembly on spore surface (Driks, 1999; Driks, 2002), this approach provides the potential to display complex multimeric enzymes in their active formats.

To date, spore surface display usually employs the following two methods. Passive adsorption utilizes the weak interaction between proteinaceous spore surface and the target protein (Sirect et al., 2012; Donadio et al., 2016; Pan et al., 2014), but unfortunately this
non-specific affinity leads to uncontrollable and in general relatively low amounts of protein display. Alternatively, several coat proteins present at the outer surface of spores including CotB, CotC, and CotG (Driks, 1999; Driks, 2002) can be directly fused with target enzymes for display (Kwon et al., 2007; Isticato et al., 2001; Isticato et al., 2007). This method generates a covalent bond between the spore anchoring protein and target enzyme, but large portion of immobilized enzymes are buried within the thick layer of spore coat proteins resulting in nonfunctionality and/or inaccessibility by substrates (Chen et al., 2017).

To improve display level of functional enzymes, in this study, we co-expressed two recombinant proteins in the mother cell compartment of sporulating cells: a spore anchoring scaffoldin by fusing a coat protein with cellulosomic cohesin domain (Coh), and a dockerin (Doc)-tagged enzyme (Figure 3.1). We hypothesized that: (1) Via Coh-Doc interaction, active multimeric enzymes can self-assemble with the scaffoldins anchored on the surface of nascent spores; (2) The Coh-Doc modules and scaffoldin proteins can act as a spacer to extend enzymes away from the spore surface thereby reducing steric hindrance and making enzymes more accessible to their substrates; (3) The copy number of Coh domains on scaffoldin protein can be manipulated to increase the display level; (4) Following mother cell autolysis, mature spores carrying large amounts of active enzyme can be easily harvested. We demonstrated the feasibility of our strategy using β-galactosidase, and compared its display level with passive adsorption and direct fusion. Stability, reaction efficiency in organic solvents and water/organic emulsion, reusability,
and long-term storage were also tested, showing the superior performance of the biocatalysis platform described here.

Figure 3.1 Self-assembly of multiple copies of enzymes on spore surface by co-expression. Anchoring scaffoldin (coat protein-cohesin fusion, orange) and dockerin-tagged enzymes (blue) are produced in mother cell compartment of sporulation cells. Following mother cell autolysis, spores with superior catalysis performances are generated.

3.3 Material and Methods

Materials. *B. subtilis* KO7 and integration plasmid pDG1729 were from *Bacillus* Genetic Stock Center (BGSC). Oligonucleotides were synthesized by IDT (Coralville, IA). DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). LB medium and Difco nutrient broth were produced by BD Difco (Franklin Lakes, New Jersey). Potassium chloride (KCl), magnesium sulfate heptahydrate
(MgSO$_4$•7H$_2$O), calcium nitrate (Ca(NO$_3$)$_2$), manganese chloride (MnCl$_2$), ferrous sulfate (FeSO$_4$), and glucose were from Fisher Scientific. 2×SG medium was made with following recipe: 16 g/L Difco nutrient broth, 2 g/L KCl, 0.5 g/L MgSO$_4$•7H$_2$O, 1 mM Ca(NO$_3$)$_2$, 0.1 mM MnCl$_2$, 1µM FeSO$_4$, and 0.1% (w/v) glucose. Kanamycin, spectinomycin, chloramphenicol, and β-D-1-thiogalactopyranoside (IPTG) were purchased from Fisher Scientific. O-Nitrophenyl-β-D-galactopyranoside (ONPG), lactose, hexanol, octanol, hexyl- and octyl-galactoside, ethyl acetate, ethyl ether, toluene, hexane, egg white lysozyme and anti-FLAG-HRP were purchased from Sigma-Aldrich (St. Louis, MO). Anti-β-gal-HRP was from abcam (Cambridge, MA). PVDF membrane was from Merck Millipore (Darmstadt, Germany). All other chemicals were purchased from Fisher.

**DNA construction and *B. Subtilis* transformation.** Spore display plasmid pDG364-CotG-Coh encoding anchoring scaffoldin of one *Clostridium thermocellum* type I cohesion domain (Coh) was constructed previously (Chen et al., 2017). The scaffoldin gene encoding three copies of Coh was PCR amplified from pET28b-CBM-3TypeI-DocII (unpublished) and cloned into pDG364-CotG (Chen et al., 2017) resulting in pDG364-CotG-(Coh)$_3$. Both spore anchoring scaffoldins CotG-Coh and CotG-(Coh)$_3$ have a FLAG tag at their C-termini for detection in Western blots. β-galactosidase (β-gal) gene was amplified from pDG1729 and cloned into pDG364-CotG resulting in pDG364-CotG-β-Gal. A multiple cloning site of BamHI/HindIII/EcoRI was introduced to a *Bacillus* integration plasmid pDG1729 via PCR. *C. thermocellum* type I dockerin gene was amplified from its genomic DNA and linked with β-gal gene by overlap PCR. The generated β-gal-Doc fragment was cloned into HindIII/EcoRI sites of pDG1729 resulting
in pDG1729-β-gal-Doc. Segments of IPTG-inducible promoter P_{grac} and GerE-dependent promoter P_{CotG} were assembled by synthesized oligonucleotides and inserted into BamHI/HindIII sites of pDG1729-β-gal-Doc, resulting in pDG1729-P_{grac}-β-gal-Doc and pDG1729-P_{CotG}-β-gal-Doc respectively. For chromosome integration, competent cells of *B. subtilis* KO7, a multiple proteases deficient strain, were transformed with *ScaI* linearized pDG1729-P_{grac}-β-gal-Doc or pDG1729-P_{CotG}-β-gal-Doc, and selected on LB-agar plates supplemented with 100 µg/mL spectinomycin. Obtained cells were then transformed with *XhoI* linearized pDG364-CotG-Coh or pDG364-CotG-(Coh)₃ and selected on 5 µg/mL chloramphenicol plates, resulting in clones able to express both β-gal-Doc and spore anchoring scaffoldins.

**Spore production and storage.** *B. subtilis* spores were produced as described previously (Chen et al., 2017). Briefly, cells were cultured in 2×SG medium at 37 °C for 24 h. For β-gal-Doc expression under P_{grac}, 0.4 mM IPTG was added. Spores were collected by centrifugation at 4,000 ×g for 6 min and washed with 1.5 M KCl and 0.5 M NaCl. Unsporulated cells (typically less than 10% in sporulation products) were removed by treatment with 50 µg/mL egg white lysozyme in 50 mM Tris-HCl (pH 7.2) at 37 °C for 1 hour, and spores were separated from cell debris by centrifugation. Obtained spores were further washed and resuspended in 50 mM phosphate buffer (pH 7.0) for enzymatic activity, or Western/dot blotting, etc. For long-term storage, the produced spores were lyophilized, aliquoted, sealed with parafilm and stored at room temperature for up to two months, during which β-gal hydrolysis activities were measured weekly.
Western blot and dot blot. Coat proteins were released from spore surface with SDT (1% SDS and 50 mM dithiothreitol) treatment at 70 °C for 30 min, and clarified by centrifugation at 10,000 ×g for 10 min. 40 µL supernatants were subjected to SDS-PAGE and then transferred to PVDF membranes. Western blot signals were developed with anti-FLAG-HRP or anti-β-gal-HRP. Dot blot experiments were performed using Bio-Dot microfiltration apparatus (Bio-Rad). 20 µL serially diluted β-gal standards or coat protein elutions were added into wells, and the liquids were drained by gravity. The module was then disassembled, and PVDF membranes were applied for Western blotting with anti-β-gal-HRP to develop the signals. The images were taken by a ChemDoc MP imager (Bio-Rad) and the densitometry analysis was performed with ImageJ.

β-galactosidase activity assay. β-gal activity was measured using 96-well plates with 200 µL 0.1 M phosphate buffer (pH 7.5) supplemented with 1 mM MgCl₂ and 50 mM β-mercaptoethanol (stabilizer for β-gal) (Pelisek et al., 2000; Moses & Sharp, 1970). Reactions were initiated by addition of 3 mM colorimetric substrate ONPG (o-nitrophenyl-β-D-galactopyranoside) and incubated with shaking at room temperature. Produced o-nitrophenol was monitored in real-time with absorption at 420 nm. One unit of β-gal activity was defined as the amount of enzyme hydrolyzing 1 µmol ONPG in 1 min. Typically 500 ng free β-gal or 0.01 OD₆₀₀ purified B. subtilis spores were used for the assays.

Bi-phasic transgalactosylation. 100 mM lactose in 0.3 mL aqueous buffer solution (0.1 M phosphate buffer, pH 7.5) and 100 mM alkyl alcohol (hexanol or octanol) in 0.7 mL organic solvent (ethyl ether or n-hexane) were mixed, and the reactions were started with
addition of either 10 U free β-gal or 10 U (equivalent to around 14 OD) spores co-expressing CotG-(Coh)₃ and β-gal-Doc. The reaction vials were sealed to avoid evaporation. Transgalactosylation reactions were carried out at room temperature with vigorous shaking to maintain stable emulsions. After centrifugation and filtration, organic phase samples were analyzed with Agilent HPLC equipped with an octadecyl silica column (Eclipse XDB-C18, 5 µm, 4.6 × 150 mm) and a DAD detector at 190 nm to measure produced alkyl galactosides. The mobile phases were methanol-water (3:2, v/v) for hexyl galactoside, and acetonitrile-water (1:1, v/v) for octyl galactoside. 0-50 mM of both alkyl galactosides were used as standards to calibrate the reaction products.

**Figure 3.2** Spore-based biocatalyst formation by co-expression and self-assembly of anchoring scaffoldins and dockerin-tagged enzymes. Gene cassettes encoding coat protein CotG fused with one or three copies of Coh domains (CotG-Coh and CotG-(Coh)₃) and β-gal-Doc were constructed and integrated into B. subtilis chromosome at amyE and thrC loci. Scaffoldins were under P_CotG promoter and enzymes were under either P_CotG or P_grac (IPTG-inducible) promoter. During sporulation scaffoldins and enzymes were produced in mother cell compartment and immobilized on nascent spores.
3.4 Results

**Self-assembly of β-gal on spore surface via anchored scaffoldins.** To increase functional display efficiency of tetrameric β-gal, we designed to graft β-gal-*C. thermocellum* type I dockerin fusion protein (β-gal-Doc) onto spore anchoring scaffoldins that carried type I cohesin (Coh) domains (**Figure 3.2**). We hypothesized that Coh-Doc modules and scaffoldin backbones could act as a spacer to extend tetrameric β-gal away from the spore surface thereby reducing steric hindrance and exposing β-gal to its substrates. The gene cassette encoding a *B. subtilis* major coat protein CotG (Sacco et al., 1995) with its native promoter region (PCotG, GerE-dependent, Driks, 1999; Sacco et al., 1995) was fused with one or three copies of Coh domains to encode spore anchoring scaffoldins CotG-Coh and CotG-(Coh)3. A FLAG tag was introduced at C-termini of both scaffoldins for detection (**Figure 3.2**). After chromosome integration at *amyE* locus, cells cultured in sporulation medium (2×SG) for 24 h were harvested and treated with SDS-DTT solution to release total coat proteins for Western blotting analysis (**Figure 3.3**). Results showed that both scaffoldins were displayed on spore surface, and CotG-Coh was present at a much higher level than CotG-(Coh)3, presumably due to its smaller MW.

We next tested whether dockerin-tagged β-gal could self-assemble with scaffoldins anchored on spores. β-gal-Doc gene was cloned at the downstream of PCotG, allowing expression at the time of outer coat formation during the Stage V of sporulation (Errington, 1993). The constructed expression cassette was inserted into an integration plasmid targeting at *thrC* locus on *B. subtilis* chromosome (**Figure 3.2**). After transformation into *B. subtilis* KO7 carrying scaffoldin genes, cells were cultured in 2×SG for 24 h and coat
protein preparation was subjected for Western blotting analysis using anti-β-gal-HRP (Figure 3.4a). Results indicated that β-gal-Doc was present on spore surface, and significantly higher display amount was associated with the scaffoldin protein encoding three copies of Coh, i.e. CoG-(Coh)₃. In a control clone that no scaffoldin gene was transformed, only a trace amount of β-gal-Doc was detected, suggesting that (1) passive adsorption led to a dramatically low display capacity; and (2) immobilization of β-gal-Doc on spore was indeed mediated by the anchoring scaffoldins. Direct fusion of β-gal with CotG (by the cassette of P_CotG-CotG-β-gal) was also tested, and results indicated this construct exhibited a higher display amount than that via anchored scaffoldins (Figure 3.4a).

Figure 3.3 Western blotting of anchored scaffoldin proteins eluted from spore surface. CotG-Coh and CotG-(Coh)₃ under P_CotG promoters were expressed in mother cells and assembled on spores during sporulation. Harvested spores were treated with SDS-DTT to release total coat proteins for Western blotting. MWs of CotG-Coh and CotG-(Coh)₃ were 42 and 104 kDa respectively. Signals were developed by anti-FLAG-HRP.

Considering β-gal is a large protein with a MW of 116 kDa for monomer, we hypothesized that its expression could be the factor limiting display amounts. To improve its expression, β-gal-Doc was cloned at the downstream of a hybrid promoter P_grac that
consists *B. subtilis* groE promoter, lac operator and gsiB ribosome binding site (Phan et al., 2006). The expression cassette was integrated into chromosome of *B. subtilis* carrying scaffoldin genes CoG-(Coh)₁/₃, and transformed cells were cultured in 2×SG supplemented with 0.4 mM IPTG for 24 h. As shown in Figure 3.4b, Western blotting analysis of released spore surface proteins indicated that β-gal-Doc expressed under P_grac self-assembled on spore anchoring CoG-(Coh)₁/₃, while spores without scaffoldins co-expression resulted in a dramatically lower display level. Compared with direct fusion (CotG-β-gal), co-expression of β-gal-Doc under P_grac and CoG-(Coh)₃ under P_CotG exhibited an over 3-fold increase of display amount, presumably due to high expression level driven by the strong IPTG-inducible promoter P_grac.

![Figure 3.4 Western blotting of β-gal-Doc immobilized on the spore surface via anchoring scaffoldins CotG-Coh and CotG-(Coh)₃.](image)

**Figure 3.4 Western blotting of β-gal-Doc immobilized on the spore surface via anchoring scaffoldins CotG-Coh and CotG-(Coh)₃.** β-gal-Doc (124 kDa) was under the control of either (a) a GerE-dependent CotG promoter P_CotG or (b) a strong IPTG-inducible promoter P_grac. Passive adsorption in the absence of anchoring scaffoldin and direct fusion with CotG (CotG-β-gal, 140 kDa) were also tested. Signals were developed by using anti-β-gal-HRP.

**High display density of β-gal on spore surface quantified by dot blotting.** Using *B. subtilis* strains carrying both scaffoldin and β-gal-Doc genes, coat protein solutions were prepared from ~ 0.2 OD spores and dotted on PVDF membranes for analysis with anti-β-gal-HRP. For quantification, 1.3-40 ng purified β-gal-Doc aliquots were applied as the
standards, and a strong linear relation between enzyme amounts and dot intensities (with a $R^2$ of 0.9) was established. Assuming one unit of OD$_{600}$ is equivalent to $1.5 \times 10^8$ spores per ml (Paidhungat et al., 2002), the absolute display amounts (in the unit of number of enzyme molecules per spore particle) were subsequently calculated with the calibration curve (Figure 3.5). When β-gal-Doc was controlled under $P_{\text{CotG}}$, scaffoldin CotG-Coh mediated an average display level of $5.7 \times 10^3$ enzymes per spore. This number was increased to be $7.4 \times 10^3$ with CotG-(Coh)$_3$, consistent with Western blotting results in Figure 3.4a. However, the increase was not simply three folds likely because of the lower expression level of CotG-(Coh)$_3$ (Figure 3.3). Without scaffoldin co-expression, the display amount of β-gal-Doc was below the detection limit of our dot blotting experiments (data not shown). Direct fusion (CotG-β-gal) gave a calculated average display density of $9.6 \times 10^3$ enzymes per spore, higher than CotG-(Coh)$_{1/3}$ mediated level when β-gal-Doc was at downstream of $P_{\text{CotG}}$, in good agreement with Western blotting results (Figure 3.4a).

<table>
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<th>Dot blotting</th>
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**Figure 3.5 Display density quantification by dot blotting.** Spores of five recombinant *B. subtilis* strains displaying β-gal were subjected to spore surface protein extraction, and dot blotting with anti-β-gal-HRP. 1.3-40 ng purified β-gal served as the standards. The display density (in the unit of number of enzymes per spore particle) was calculated based on the assumption that one mL of spore solution with one OD$_{600}$ is equivalent to $1.5 \times 10^8$ spores (Paidhungat et al., 2002).
With a strong IPTG inducible promoter \( P_{\text{grac}} \), on average \( 1.3 \times 10^4 \) and \( 3.6 \times 10^4 \) \( \beta \)-gal-Doc molecules were displayed per spore via anchoring scaffoldins CotG-Coh and CotG-(Coh)\(^3\) respectively. This represented a 1.7-fold increase, consistent with Western blotting results (Figure 3.4b). However, the display capacity difference between these two scaffoldins was 1.3-fold for \( P_{\text{CotG}} \) expressed \( \beta \)-gal-Doc, suggesting the \( \beta \)-gal-Doc expression level was indeed the limiting factor with \( P_{\text{CotG}} \), and therefore the strains with \( P_{\text{grac}} \)-controlled \( \beta \)-gal-Doc expression were further investigated for enzymatic activities. Considering spore diameter is \( \sim 0.94 \) µm (Mamane & Linden, 2006) and the size of monomeric \( \beta \)-gal is \( 8.54 \) nm \( \times \) 6.38 nm (Bartesaghi et al., 2015), each spore particle at most carries \( 5.1 \times 10^4 \) \( \beta \)-gal molecules if the enzymes are closely arranged into a monolayer. As a result, the spore surface coverage efficiency is determined to be \( \sim 71\% \), implying efficient biocatalyst generation.

**\( \beta \)-gal activity of spore biocatalysts.** 0.01 OD\(_{600}\) of prepared \( B. \) subtilis spores were tested for \( \beta \)-gal hydrolysis activity in 200 µL reaction buffer containing 3 mM substrate o-nitrophenyl-\( \beta \)-D-galactopyranoside (ONPG). Produced colorimetric o-nitrophenol was measured by monitoring absorbance at 420 nm. As shown in Figure 3.6, scaffoldin CotG-Coh mediated \( \beta \)-gal-Doc display exhibited 0.32 U per OD spores. By increasing the copy number of Coh domains on spore anchoring scaffoldin from one to three, \( i.e. \) using CotG-(Coh)\(^3\), the specific activity improved 2.1-fold to be 0.68 U per OD spores. In contrast, passive adsorption by expressing \( \beta \)-gal-Doc without anchoring scaffoldin only exhibited activity of 0.049 U per OD spores, consistent with Western blotting and dot blotting.
(Figure 3.4b, Figure 3.5), suggesting that the scaffoldin proteins were critical for high display capacity and activity. As a comparison, activity of spores displaying CotG-β-gal fusion was determined as 0.18 U per OD spores, 80% less than that with scaffoldin CotG-Coh. Interestingly, results of quantitative dot blotting indicated a 35% improvement of CotG-Coh compared to direct fusion (Figure 3.5). The activity improvement was higher than the increase associated with display amounts, presumably because some displayed CotG-β-gal fusion was buried within the thick layer coat proteins and thus not active, while scaffoldin mediated display reduced this steric hindrance allowing more β-gal being active.

![Figure 3.6 β-gal hydrolysis activity of produced spore biocatalysts](image)

**Figure 3.6 β-gal hydrolysis activity of produced spore biocatalysts.** β-gal-Doc was expressed under P_{grac} with spore anchoring scaffoldins of CotG-Coh or CotG-(Coh)₃ or without scaffoldin (passive adsorption). Direct fusion with CotG (CotG-β-gal) was also tested for comparison. One unit of β-gal activity is defined as the amount of enzyme hydrolyzes 1 µmol ONPG in 1 min. One OD spores are equivalent to 1 ml spore solution with OD₆₀₀ absorbance of 1.

**Improved stability, long-term storage, and reusability.** Taking advantage of *B. subtilis* endospore’s robustness and inert nature, we next tested the effects of spore surface display on stability of β-gal. Free β-gal and spores carrying P_{grac}-β-gal-Doc and CotG-(Coh)₃ constructs were applied for the assays. After incubation at 37°C for 3 h, the remaining
activity of β-gal in solution was 56%, and spore surface display improved that to 78% (Figure 3.7). At 40°C, the half-live of free β-gal was 2.1 h, and spore surface displayed exhibited an increased half-live of 3.2 h. These results were in good agreements with previous studies (Han et al., 2009; Li et al., 2014; Jung et al., 2006; Peng, 2013; Bielen et al., 2009; Abramic et al., 1999; Lescic et al., 2001), suggesting spore surface display can significantly improve stability of immobilized enzymes.

![Figure 3.7 Stability enhancement by spore display.](image)

**Figure 3.7 Stability enhancement by spore display.** Free β-gal and spores carrying P_gnac-β-gal-Doc/P_CotG-CotG-(Coh)3 were applied. Samples were incubated at 37 or 40°C for 0-3 h and tested for enzymatic activity.

After verification of improved stability for short incubation periods, we next tested activities of the spore biocatalyst after long-term storage. Purified β-gal protein and spores displaying β-gal-Doc via scaffoldin CotG-(Coh)3 were freeze-dried, and lyophilized aliquots were stored at ambient temperature in a sealed container with the presence of sufficient desiccant for more than 60 days. Weekly hydrolysis activity tests of stored samples indicated that free β-gal rapidly dropped 31% activity after one week storage, however, when displayed on spores, only 21% activity lost after storage for 3 weeks
(Figure 3.8). At week 5, free β-gal remained less than 40% of its original activity, but it took spore-based biocatalyst more than 9 weeks to reach the same loss. Overall the half-lives of free β-gal and spores displaying the same enzyme were determined to be 21 and 43 days respectively, suggesting the superior storage stability of spore-based biocatalyst.

Due to relatively high costs of biocatalysts in general, the abilities to recycle and reuse are often desired features for economic feasibility of any industrial biocatalysis process. As an inert and stable carrier, spore particles can be easily separated from reaction solution by either centrifugation or filtration. To test reusability, spores were recovered from solution after each round of reaction by centrifugation at 10,000 ×g for 3 min, and extensively washed with 0.1 M phosphate buffer (pH 7.5) before the subsequent repeated reaction. As shown in Figure 3.8b, averagely only 3.5% of hydrolysis activity was lost for each round, and 87% activity retained after four successive uses, demonstrating our spore display system had excellent reusability.

![Figure 3.8 Long-term storage stability (a) and reusability (b) of spore-based biocatalysts. (a) Spores (P_{grac}-β-gal-Doc/CotG-(Coh)₃) were lyophilized and stored in dry form at ambient temperature. (b) For each round, spores (P_{grac}-β-gal-Doc/CotG-(Coh)₃) were purified and washed before reuse.](image)
**Bi-phasic transgalactosylation reaction.** For many biocatalysis utilizing hydrophobic substrates, non-polar organic solvents are often required to improve solubility of the substrate, which otherwise is not soluble in aqueous solutions. Unfortunately, the presence of organic solvents rapidly deactivates free enzymes in general. In sharp contrast, surface displayed enzymes are renowned to be resistant against organic solvents (Jung et al., 2006). We incubated free β-gal and spores displaying β-gal in the emulsions of aqueous/organic solvents (1:1, v/v), and monitored their hydrolysis activities. In the emulsions containing ethyl acetate, ethyl ether, toluene, or n-hexane as the organic phase, residual activities of spores displaying β-gal were 29%, 55%, 76%, and 78%, while the residual activities of free β-gal were considerably less in all tested emulsions (11%, 46%, 28%, and 74% respectively) (**Table 3.1**). Particularly in water/ethyl acetate emulsion, spore biocatalyst reserved 2.6-fold more hydrolysis activity than free β-gal. Moreover, it was found that the residual hydrolysis activities of spores displaying β-gal were roughly correlated with the solvent hydrophobicities, *i.e.* higher hydrophobicity resulted in higher residual activity.

**Table 3.1 Fraction of residual activity of free β-gal and spore display in four aqueous/organic solvent emulsions (1:1, v/v).**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Log P value of solvent</th>
<th>Spore display</th>
<th>Free β-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>n.a.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.68</td>
<td>0.29 ± 0.04</td>
<td>0.11 ± 0.003</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>0.8</td>
<td>0.55 ± 0.03</td>
<td>0.46 ± 0.10</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.5</td>
<td>0.76 ± 0.16</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>3.5</td>
<td>0.78 ± 0.30</td>
<td>0.74 ± 0.36</td>
</tr>
</tbody>
</table>
An important industrial application of β-gal is the enzymatic synthesis of alkyl galactosides, a family of environmentally friendly and dermatologically superior surfactants (Yang et al., 2017). The substrates for enzymatic transgalactosylation are lactose as galactosyl donor in aqueous phase and fatty alcohol as alkyl donor in organic phase (Figure 3.9a). Spore surface display is an attractive approach for bi-phasic reaction, because of stability enhancement in the water/solvent emulsion and interfacially localized spores allow maximum access of surface bound enzymes to substrates in both phases (Kwon et al., 2007). Based on these advantages, we carried out the bi-phasic transgalactosylation reactions with ethyl ether or n-hexane as organic solvent, and hexanol or octanol as alkyl donors. Water/organic solvent emulsions (3:7, v/v) containing 10 U/mL free β-gal or spores displaying β-gal sealed in Eppendorf tubes were constantly shaken at room temperature, and generated alkyl galactosides in organic phase were separated by centrifugation and analyzed with HPLC (Figure 3.9b). When 100 mM n-hexanol was used as the alkyl donor, spore display yielded 2.9 mM and 25.2 mM hexyl galactoside in emulsions of ethyl ether and n-hexane respectively, whereas free β-gal yielded 1.7 mM and 11.8 mM hexyl galactoside in corresponding organic emulsions. When 100 mM octanol, a fatty alcohol with a longer hydrocarbon chain was used, octyl galactoside yields by spore display were 4.2 mM and 9.5 mM in ethyl ether and n-hexane respectively, while the yields by free β-gal were 1.9 mM in both emulsions. Consistent with literatures (Yang et al., 2017), higher transgalactosylation efficiencies were observed with the shorter fatty alcohol. Moreover, n-hexane mediated 2.2- to 8.6-fold higher transgalactosylation efficiencies than ethyl ether, suggesting n-hexane was a better organic solvent for bi-phasic transgalactosylation.
Overall, spore surface display achieved a conversion rate of 25% for hexanol in emulsion of water/n-hexane.

Aiming to improve fatty alcohol conversion rate, lactose concentration in aqueous phase was increased to thermodynamically drive the transgalactosylation reaction. Emulsions of 100, 200, and 300 mM lactose in aqueous phase and 100 mM hexanol in n-hexane were carried out for transgalactosylation with 10 U spores displaying β-gal (Figure 3.10). In the initial 4 hours, the reaction rate with 100 mM lactose was 2.0 mM hexyl galactoside per hour, and with 200 or 300 mM lactose, the reaction rate was increased to 2.6-2.8 mM/h. After overnight reaction, 100, 200 and 300 mM lactose led to the generation of 25.2, 32.5, and 35.5 mM hexyl glycoside respectively. A larger improvement of conversion from 100 mM to 200 mM than from 200 mM to 300 mM indicates the diminishingly weakened role of lactose in driving transgalactosylation.

Figure 3.9 Transgalactosylation of produced spore biocatalyst in water/organic emulsions. (a) Bi-phasic reaction mediated by interfacially localized spores (P_{grac-β-gal-Doc/CotG-(Coh)}). (b) Transgalactosylation reaction results. Alcohol donor was either octanol or hexanol, and organic solvent was either ethyl ether or n-hexane.
Figure 3.10 Time course of transgalactosylation with 10 U spores (P\textsubscript{grac-β-gal-Doc/CotG-(Coh)}). 100, 200, or 300 mM lactose was used as glycosyl donor in aqueous phase, and 100 mM hexanol was used in n-hexane organic phase. The emulsion was water/n-hexane (3:7, v/v).

### 3.5 Discussions

Compared with other microbes, e.g. *E. coli*, *Saccharomyces cerevisiae*, and phage, *B. subtilis* spores offer many distinguished advantages for surface display of enzymes. At first, active display of a large functional multimeric enzyme is possible because the protein does not have to cross a membrane and all the nutrients and required machineries, e.g. ATP-dependent chaperons, for oligomerization and correct folding of the fusion protein and assembly of spores, are available in the mother cell compartment. Secondly, the robust immobilization matrix endows the carried enzymes with high reusability and stability at harsh reaction conditions, strong resistance against organic solvents, and preeminent long-term storage stability. Enzymes purification and immobilization steps are not required as the expressed enzymes are self-assembled onto surface of spores during sporulation and assembled spores released by autolysis of mother cells can be recovered from the culture broth by centrifugation or filtration. Thirdly, biocatalysis is not interfered by the host cell metabolism as the spore is a dormant/inert carrier, which also in turn facilitates product recovery.
Up to now, *B. subtilis* spore surface display research has mainly focused on two strategies, *i.e.* passive adsorption and direct fusion. However, capacity of passive adsorption is significantly influenced by pH and spore surface charge etc (Sirec et al., 2012), and direct fusion has the incompetence of displaying enzyme cascades in a controllable manner (Chen et al., 2017). The programmed successive *B. subtilis* sporulation process undergoes several morphological stages (Cutting & Vander Horn, 1990), among which the most intriguing is that cortex and coat syntheses are within the mother cell compartment, and their assemblies on spore surface do not require transmembrane process. This feature allows the spore surface display of multimeric proteins, that have posed severe challenges to conventional bacterial surface display. We therefore attempted to co-express scaffodins (coat protein-cohesin fusions) and dockerin-tagged enzyme (β-gal-Doc fusion) during *B. subtilis* sporulation process, so that released matured spore would automatically carry self-assembled active multimeric β-gal (*Figure 3.1*).

As a first step, we co-expressed CotG fused with a single copy of Coh and β-gal-Doc, both of which were under the control of $P_{\text{CotG}}$ promoter. The resulting construct was determined to carry $5.7 \times 10^3$ β-gal molecules per spore (same unit as below, *Table 3.1*). We then attempted to increase self-assembled β-gal amount through adding two more copies of Coh domains, resulting in CotG-(Coh)$_3$. Despite the lower expression of CotG-(Coh)$_3$ compared with CotG-Coh (*Figure 3.3*), β-gal display amount increased to $7.4 \times 10^3$, presumably because out-extended repeated Coh domains away from spore surface gained more accessibility of enzymes. We further increased the amount of spore displayed β-gal by placing β-gal-Doc under a strong IPTG-induible $P_{\text{grac}}$ promoter. Using $P_{\text{grac}}$, spore
display capacity further increased to $1.3 \times 10^4$ and $3.6 \times 10^4$ for CotG-Coh and CotG-(Coh)$_3$ respectively, both were higher than that of direct fusion CotG-β-gal ($9.6 \times 10^3$, Figure 3.5). We also estimated spore surface β-gal loading amounts based on their hydrolysis activities. Based on that approximately 0.5 µg free β-gal has the equal hydrolysis activity of 1 OD ($1.5 \times 10^8$ CFU) spores of $P_{grac}$-β-gal-Doc/CotG-(Coh)$_3$, the display capacity was $\sim 1.4 \times 10^4$ enzyme molecules per spore. The display amount determined via enzymatic activity calculation was slightly lower than the dot blotting calculation, presumably because during dot blotting both active and inactive β-gal were eluted while only active β-gal could be identified through enzymatic activity estimation.

Reusability and storage stability of enzymes are critical features for industrial applications. The separation of free enzymes from other chemicals is not readily available, and thus inexpensive reusing free enzymes is highly challenging. However, enzymes on spore surface can be easily collected via centrifugation, and consequent multiple reuses can therefore be achieved. We found 87% of original spore hydrolysis activity was retained after four rounds of reuse. Stable storage for long period is another critical feature, as it reduces storage costs and longer the shelf time. Lyophilized spores retained 37% of original hydrolysis activity even after 63 days of storage at ambient temperature, with a half-life of 43 days. In contrast, free β-gal only had a half-life of 21 days. In agreement with previous studies (Han et al., 2009; Li et al., 2014; Jung et al., 2006; Peng, 2013; Bielen et al., 2009; Abramic et al., 1999; Lescic et al., 2001), spore surface acts as solid support to prevent enzyme denaturation, and this beneficial protection particularly improved enzyme stability.
The industrial utilization of β-gal is always centered on its transgalactosylation activity. Solubility of generated alkyl galactosides is usually improved by adding organic solvents, which unfortunately rapidly deactivate free β-gal. We took advantage of enhanced β-gal stability on spore surface, and achieved a 35% hexanol conversion rate spores. Moreover, our platform is advantageous because 1) *B. subtilis* is generally regarded as safe (GRAS) and has minimum potential harm to human, and 2) the genomic integration of heterologous genes stabilizes the gene of interest and attenuates the dependence of antibiotics to maintain plasmids, thus further reducing associated cost.

### 3.6 Conclusions

In this study, we developed a *B. subtilis* spore based self-assembly platform via co-expressing the spore coat protein-cohesin fusion scaffoldins and dockerin-tagged enzyme in the mother cell compartment during sporulation process. This platform enabled functional display of tetrameric β-gal, which is a challenge for other conventional bacterial surface display. Moreover, we improved surface display amount by increasing associated Coh copy number, and replacing GerE-dependent P_CotG promoter with a strong IPTG-inducible P_grac promoter. Both dot blotting and activity measurements verified the high β-gal display amounts with spore carrying P_grac-β-gal-Doc/CotG-(Coh)$_3$ construct. Compared with free β-gal, the self-assembled spore biocatalysts owned superior thermostability, reusability, and storage stability. Additionally, we applied our spore biocatalyst in biphasic transgalactosylation in water/organic emulsions, and our platform again exhibited enhanced alkyl galactosides generation compared with free β-gal. The spore biocatalyst mediated generation of 35.5 mM hexyl galactoside (a 35% conversion rate) in *n*-hexane.
emulsion. Our self-assembly platform was thus concluded to be promising in industrial applications.
References


Chapter 4. Conclusions

Surface display and substrate channeling are of great interests to researchers, owing to their superior performance. In my thesis, I thus focused on two topics that are intercorrelated: 1) using \textit{B. subtilis} spore to develop a versatile self-assembly surface display platform; and 2) constructing scaffolds containing varying copies of cohesin domains to verify the synergistic actions of immediate proximity brought by multi-enzyme complex.

Industrially utilized enzymes are in either of following formats: 1) purified free enzymes; 2) microbial surface displayed enzymes; and 3) beads anchored enzymes. The applications of these platforms are restricted by their own inherent limitations, of which typical drawbacks are additional purification cost and impotency of displaying multimeric enzymes. To meet the standards that address abovementioned limitations, it is highly desired to establish a surface display platform that can achieve self-assembly of multimeric enzymes. Under this setting, enzyme display on spore surface was proposed. We aimed to realize this goal with two steps. At first, we tested the viability of spore display. Two enzymes, \textit{i.e.} xylose reductase and phosphate dehydrogenase, were heterologously expressed in \textit{E. coli}. Either sonicated cell lysates or further purified enzymes were loaded on spore surface displaying cohesin domain fused to spore coat protein. We successfully observed xylitol production accompanied by NADP(H) regeneration. Additionally, we increased the copy number of PTDH-specified cohesin domains as specific activity of PTDH was lower than XR. Optimized xylitol yield was obtained after fine tuning of PTDH:XR ratios. We next delved into the possibility of creating a spore-based self-
assembly platform. An intriguing property of morphological alterations during sporulation is that the cortex and coat surrounding the nascent endospore are essentially synthesized within the mother cell compartment. We hence utilized the transmembrane free sporulation process to co-express scaffoldins and dockerin-tagged enzyme within the mother cell compartment. After auto-lysis of mother cell, released maturated spore was verified to have superior activity compared with direct fusion method, while stability enhancement and reusability robustness were not compromised.

Based on the outcomes of this thesis, the future research may include construction of secondary structured enzyme on spore surface using co-expression method. This fashion enables higher display capacities. A potential pitfall is that scaffoldins containing multiple copies of cohesin domains expressed in *B. subtilis* may be difficult, even though multiple protease-deficient strains, such as WB800, has been created. Nonetheless, this problem can be possibly addressed through protein engineering, or avoiding repeated nucleotide sequence by utilizing cohesins derived from distinguished bacterial species.