Received: 11 August 2017 | Revised: 7 October 2017 | Accepted: 6 November 2017
DOI: 10.1002/bit.26492

ARTICLE

WILEY BIOTECHNOLOGY BIOENGINEERING

Highly active spore biocatalyst by self-assembly of co-expressed anchoring scaffoldin and multimeric enzyme

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Funding information
Division of Chemical, Bioengineering, Environmental, and Transport Systems, Grant number: 1265044; National Science Foundation

Abstract

We report a spore-based biocatalysis platform capable of producing and self-assembling active multimeric enzymes on a spore surface with a high loading density. 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. Using this method, tetrameric β-galactosidase was successfully displayed on the spore surface with a loading density of 1.4×10^4 active enzymes per spore particle. The resulting spore biocatalysts exhibited high conversion rates of transgalactosylation in water/organic emulsions. With easy manufacture, enhanced thermostability, excellent reusability, and long-term storage stability at ambient temperature, this approach holds a great potential in a wide range of biocatalysis applications especially involving organic phases.

KEYWORDS
biphasic reaction, self-assembly, spore surface display, transgalactosylation

1 | INTRODUCTION

Microbial surface display, for example, on bacteria and yeast (Becker et al., 2005; Liu, Zhang, Lian, Wang, & Wright, 2014; Schüürmann, Quehl, Festel, & Jose, 2014; Smith, Khera, & Wen, 2015; Tanaka & Kondo, 2015), has been developed for chemical synthesis to overcome challenges associated with free enzymes, that is, expensive purification and low stability, and the ones associated with whole-cell biocatalysts, that is, substrate and product transportation, and interference with host native metabolism (Bommarius, 2015; Choi, Han, & Kim, 2015). To localize on cell surface, enzymes of interest produced in cytoplasm need to translocate across the cell membrane, which could be problematic for enzymes possessing multiple domains or subunits. For example, β-galactosidase (β-gal), an enzyme important for synthesis of alkyl galactosides (Yang et al., 2017), having a monomer MW of 116 kDa and only active as a tetramer, could not be functionally displayed on surface of *Escherichia coli* due to the toxicity of membrane jamming (Shuman & Silhavy, 2003).

Exploiting the mechanism of *Bacillus subtilis* endospore formation, this study aims to develop a facile display technique bypassing transmembrane process via self-assembling multimeric enzymes on the surface of spores with a high loading density. During sporulation, *B. subtilis* cells undertake several morphological changes in the following order: 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, lysis of mother cell, and release of the matured spore (Driks, 2002; Errington, 1993; Foster & Popham, 2002). Because no transmembrane translocation is required during the process of coat proteins assembly on spore surface (Driks, 1999; Kroos & Yu, 2000), this approach provides a means to display complex multimeric enzymes in their active form.

To date, spore surface display usually employs the following two methods: passive adsorption and direct fusion. Passive adsorption utilizes the weak interactions between the proteinaceous spore surface and the target proteins (Donadio, Lanzilli, Sirec, Ricca, & Isticato, 2016; Pan, Choi, Jung, & Kim, 2014; Sirec et al., 2012). 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 have been directly fused with target enzymes for display (Isticato et al., 2001; Isticato, Di Mase, Mauriello, De Felice, & Ricca, 2007). This method generates a covalent bond between the spore anchoring protein and target enzyme, but a large portion of immobilized enzymes are buried within the thick layer of spore coat proteins which results in impaired functionality and/or inaccessibility by substrates (Chen, Mulchandani, & Ge, 2017).

In this study, we co-expressed two recombinant proteins in the mother cell compartment of sporulating cells: a spore surface-anchoring scaffoldin by fusing a coat protein with cellulosomic cohesin domain (Coh), and a dockerin (Doc)-tagged enzyme (Figure 1). We hypothesized that: (1) active multimeric enzymes can self-assemble with the scaffoldins anchored on the surface of nascent spores via high affinity Coh-Doc interaction; (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 the enzymes more accessible to their substrates; (3) the copy number of Coh domains on scaffoldin protein can be manipulated to increase the display level; and (4) following mother cell autolysis, mature spores carrying active enzymes can be easily harvested. We demonstrated the feasibility of our method for spore display using β-galactosidase, and compared the display level from our method with the display levels from passive adsorption and direct fusion. Stability, reaction efficiency in organic solvents and water/organic emulsion, reusability, and long-term storage were also tested.

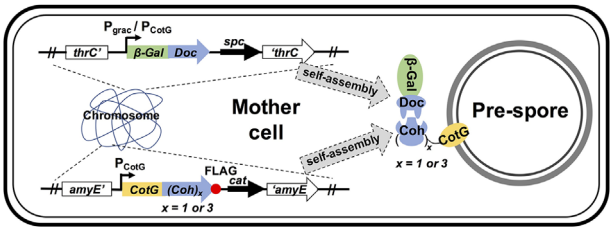

FIGURE 1 Spore-based biocatalyst formation by co-expression and self-assembly of anchoring scaffoldins and dockerin-tagged enzymes. Gene cassettes encoding coat protein CotG fused with one or three copies of Coh domains (CotG-Coh and CotG-$(Coh)_3$) and β-gal-Doc were constructed and integrated into *B. subtilis* chromosome at *amyE* and *thrC* loci. Scaffoldins were under P_{CotG} promoter and enzymes were under either P_{CotG} or P_{grac} promoter. During sporulation scaffoldins and enzymes were produced in mother cell compartment and immobilized on nascent spores

2 | MATERIAL AND METHODS

2.1 | Materials

B. subtilis multiple proteases deficient strain KO7 (Zeigler DR, unpublished) 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 from BD Difco (Franklin Lakes, NJ). 2 × SG medium was made with following recipe: 16 g/L Difco nutrient broth, 2 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, 1 μM $FeSO_4$, and 0.1% (w/v) glucose. 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 from Sigma–Aldrich (St. Louis, MO). Anti-β-gal-HRP was from Abcam (Cambridge, MA). PVDF membrane was from Millipore (Billerica, MA). Kanamycin, spectinomycin, chloramphenicol, isopropyl β-D-1-thiogalactopyranoside (IPTG), and all other chemicals were purchased from Fisher Scientific (Hampton, NH).

2.2 | DNA construction and *B. Subtilis* transformation

Plasmid pDG364-CotG-Coh encoding CotG fused with *Clostridium thermocellum* type I cohesion (Coh) was constructed in a previous study (Chen et al., 2017). A gene encoding three copies of Coh was PCR amplified from pET28b-CBM-3TypeI-DocII and cloned into pDG364-CotG (Chen et al., 2017) resulting in pDG364-CotG-$(Coh)_3$. FLAG tags were fused at C-termini of both spore-anchoring scaffoldins CotG-Coh and CotG-$(Coh)_3$ for Western blotting. The β-galactosidase (β-gal) gene was amplified from pDG1729 and cloned into pDG364-CotG resulting in pDG364-CotG-β-gal. The *C. thermocellum* type I dockerin gene was amplified from its genomic DNA and linked to the C-terminal of β-gal by overlap PCR, and the fragment β-gal-Doc was cloned into 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 in front of β-gal, resulting in pDG1729-P_{grac}-β-gal-Doc and pDG1729-P_{CotG}-β-gal-Doc, respectively. For chromosome integration, competent cells of KO7 were transformed with linearized pDG1729-P_{grac}-β-gal-Doc or pDG1729-P_{CotG}-β-gal-Doc, and selected on 100 μg/ml spectinomycin LB-agar plates. Obtained cells were then transformed with linearized pDG364-CotG-Coh or pDG364-CotG-$(Coh)_3$ and selected with 5 μg/ml chloramphenicol, resulting in clones able to express both β-gal-Doc and spore anchoring scaffoldins.

2.3 | Spore production and storage

B. subtilis spores were produced by culturing in 2 × SG medium at 37°C for 24 hr (Foster & Popham, 2002). To induce β-gal-Doc expression under P_{grac}, 0.4 mM IPTG was added 6 hr after inoculation (Nguyen & Schumann, 2014). Spores were collected by centrifugation

at 4,000*g* for 6 min and washed with 1.5 M KCl and 0.5 M NaCl. Trace amounts of unsporulated cells (typically <10% of 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 hr. The spores were then separated from the cell debris by centrifugation. The spores were further washed and suspended in 50 mM phosphate buffer (pH 7.0) for enzymatic assays and Western/dot blotting. For long-term storage, purified β-gal and the spores were lyophilized, aliquoted, and stored at ambient temperature in a sealed container with the presence of sufficient desiccant for more than two months, during which β-gal hydrolysis activities were measured weekly.

2.4 | Western and dot blot

The coat proteins were released from the spore surface by treatment with 1% SDS and 50 mM dithiothreitol (SDS-DTT) at 70°C for 30 min. The eluents were then clarified by centrifugation at 10,000*g* for 10 min for Western blotting, in which signals were developed with anti-FLAG-HRP or anti-β-gal-HRP. Twenty microliter serially diluted β-gal standards (1.3–40 ng) or coat protein elution from 0.2 OD_{600} spores were subjected to dot blotting using Bio-Dot microfiltration apparatus (Bio-Rad) and anti-β-gal-HRP. Blotting images were taken by a ChemiDoc MP (Bio-Rad), and the densitometry analysis was performed with ImageJ for quantification. Absolute surface display amounts were calculated in the unit of number of enzyme molecules per spore particle.

2.5 | β-galactosidase hydrolysis activity assay

The β-gal activity was measured in 0.1 M phosphate buffer (pH 7.5) supplemented with 1 mM $MgCl_2$ and 50 mM β-mercaptoethanol (a stabilizer for β-gal; Moses & Sharp, 1970). Typically, 5 ng β-gal or 0.01 OD_{600} prepared spores were used for 200 μl assays. Reactions were initiated by addition of 3 mM ONPG and incubated with moderate shaking at room temperature. Produced colorimetric o-nitrophenol was monitored in real-time at 420 nm, and the production rate was calculated using the results from the first minute. One unit of β-gal activity was defined as the amount of enzyme hydrolyzing 1 μmol ONPG in 1 min. For reusability tests, after each round of the reaction with 3 mM ONPG at room temperature for 30 min, spores were recovered from solution by centrifugation at 10,000*g* for 3 min and extensively washed with 0.1 M phosphate buffer (pH 7.5) before repeating the reaction. When tested in aqueous/organic emulsions, either ethyl acetate, ethyl ether, toluene, or *n*-hexane was 1:1 (v/v) mixed with 0.1 M phosphate buffer.

2.6 | Biphasic transgalactosylation

A total of 0.3 ml 100–300 mM lactose in 0.1 M phosphate buffer (pH 7.5) was mixed with 0.7 ml 0.1 M alkyl alcohol (hexanol or octanol) in organic solvent (ethyl ether or *n*-hexane). The reactions were started with the addition of either 10 U free β-gal (equivalent to ~7 μg) or spores (CotG-$(Coh)_3$/β-gal-Doc) exhibiting 10 U activity (equivalent to ~14 OD_{600}). The reaction vials were sealed to avoid evaporation, and transgalactosylations were carried out at room temperature with vigorous shaking to maintain stable emulsions. After centrifugation and filtration, the 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 the amount of alkyl galactosides produced. 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 the alkyl galactosides were used as standards for calibration. For reusability tests, 10 U of spores (P_{grac}-β-gal-Doc/CotG-$(Coh)_3$) were incubated with 100 mM lactose and 100 mM hexanol in phosphate buffer/hexane (3:7) biphasic emulsion for 24 hr. After each round of reaction, spore biocatalysts were recovered by centrifugation, gently washed three times, and applied for next round of biphasic reaction.

2.7 | Microscopic imaging

One OD_{600} spores were added into 1 ml phosphate buffer/ethyl ether solution (3:7, v/v). Emulsions were generated by vigorous vortex for 10 min, then applied for microscopic observations using Olympus BX51 under 1,000 × amplification oil lens. Software cellSens was used to analyze the biphasic emulsions and the localization of the spores.

3 | RESULTS

3.1 | Self-assembly of β-gal on spore surface via anchored scaffoldins

B. subtilis major coat protein CotG, along with its native promoter (P_{CotG}, GerE-dependent; Sacco, Ricca, Losick, & Cutting, 1995) were cloned to the N-termini of one or three copies of Coh domains to encode spore-anchoring scaffoldins CotG-Coh and CotG-$(Coh)_3$. After integrating the scaffoldin gene into *amyE* locus on the chromosome of *B. subtilis* multiple proteases deficient strain KO7 (Figure 1), the spores were prepared and treated with SDS-DTT to release coat proteins. Western blotting results indicated that both scaffoldins were displayed on the spore surface, and the densitometry analysis showed that CotG-Coh was present 2.5-fold more than CotG-$(Coh)_3$ (Figure S1). Next, we tested whether co-expressed dockerin-tagged β-gal could self-assemble on the displayed scaffoldins. The β-gal-Doc gene was cloned at the downstream of P_{CotG}, allowing it to be produced at Stage V of sporulation (Errington, 1993) (Figure 1). After gene integration into *thrC* locus on the KO7 chromosome, Western blot analysis of the coat proteins using anti-β-gal-HRP indicated that β-gal-Doc was immobilized on the spore surface and that CoG-$(Coh)_3$ mediated a significantly higher display amount than CoG-Coh. However, the display amount was lower than that of direct fusion (P_{CotG}-CotG-β-gal) (Figure S2). To improve the display amount, β-gal-Doc was cloned at the downstream of P_{grac}, an IPTG-inducible strong promoter that consists *B. subtilis* groE promoter, lac operator, and gsiB ribosome binding site (Phan, Nguyen, & Schumann, 2006). The expression cassette P_{grac}-β-gal-Doc was chromosomally integrated, and spores were produced with 0.4 mM IPTG. Western blotting results indicated that P_{grac} mediated

significantly higher display amounts compared to P_{CotG}. Particularly, co-expression of P_{grac}-β-gal-Doc and CoG-$(Coh)_3$ led to three-fold higher display amounts compared to direct fusion (CotG-β-gal) (Figure 2a). In the control strains where no scaffoldin genes were transformed, only trace amounts of β-gal-Doc were detected in coat protein fraction (Figures S2 and 2a), suggesting that (1) passive adsorption led to a dramatically low display capacity, and (2) anchoring scaffoldins indeed facilitated immobilization of β-gal-Doc on the spore surface.

3.2 | High display density of β-gal on spore surface quantified by dot blotting

Based on a strong linear relationship between 1.3 and 40 ng β-gal and its dot intensities ($R^2 = 0.99$), and the assumption that one unit of OD_{600} is equivalent to 1.5×10^8 spores per ml (Paidhungat et al., 2002), the absolute display amounts were determined by dot blotting the coat protein solutions (Figure 3). When β-gal-Doc was controlled under P_{CotG}, scaffoldin CotG-Coh mediated an average display level of 5.7×10^3 enzymes per spore. This number increased to 7.4×10^3 with CotG-$(Coh)_3$. However, the increase was less than three-fold, likely because of the lower expression level of CotG-$(Coh)_3$ compared to that of CotG-Coh (Figure S1). Without scaffoldin co-expression, the display amount of β-gal-Doc was below the detection limit in our dot blotting experiments (data not shown), indicating a low display level from passive adsorption. Direct fusion (CotG-β-gal) yielded a calculated average display capacity of 9.6×10^3 enzymes per spore. With the strong IPTG-inducible promoter P_{grac}, an average of 1.3×10^4 and 3.6×10^4 β-gal-Doc molecules were displayed per spore via anchoring scaffoldins CotG-Coh and CotG-$(Coh)_3$, respectively (Figure 3). The display capacity with CotG-$(Coh)_3$ was significantly higher than those of direct fusion, consistent with above Western blotting results (Figure 2). Therefore, the strains with P_{grac}-controlled β-gal-Doc expression were further investigated for enzymatic activities.

3.3 | β-gal activity of spore biocatalysts

The hydrolysis activities of spore-displayed β-gal were tested using substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), and results are shown in Figure 2b. With scaffoldin CotG-Coh, β-gal-Doc exhibited 0.32 U per OD_{600} spores. By increasing the copy number of Coh domains on spore anchoring scaffoldin from one to three, that is, using CotG-$(Coh)_3$, the specific activity improved 2.1-fold to 0.68 U/OD_{600}. In contrast, passive adsorption by expressing β-gal-Doc without anchoring scaffoldin only had 0.049 U/OD_{600}, consistent with above Western and dot blotting results (Figures 2a and 3), suggesting that the scaffoldin proteins were critical for high display capacity and activity. Activity of spores displaying direct fusion (CotG-β-gal) was determined as 0.18 U per OD_{600} spores, representing P_{grac}-β-gal-Doc/CotG-Coh was 78% more active than direct fusion. However, semi-quantitative dot blotting results indicated a 35% increase of display amount comparing CotG-Coh to direct fusion (Figure 3). The improvement difference between displayed amount and apparent activity was presumably because a considerable portion of displayed direct fusion was buried within the thick layer coat proteins and thus may be enzymatically inactive, while scaffoldin mediated display reduced this steric hindrance allowing more displayed β-gal being functional.

We also estimated spore surface β-gal loading amounts based on their hydrolysis activities. Based on facts that (1) β-gal specific activity did not change when immobilized on spore surface (Figure S3) and (2) 1 OD_{600} (1.5×10^8 CFU) spores of P_{grac}-β-gal-Doc/CotG-$(Coh)_3$ had approximately equal hydrolysis activity to 0.5 μg free β-gal, the display capacity was calculated as 1.4×10^4 enzyme molecules per spore. This determined display amount was 39% of the value estimated via dot blotting (3.6×10^4), 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.

3.4 | Improved stability, long-term storage, and reusability

Taking advantage of the robustness of *B. subtilis* endospore as well as its inert nature, we tested the effects of spore surface display on enzyme stability and long-term storage. Free β-gal and spores carrying P_{grac}-β-gal-Doc and CotG-$(Coh)_3$ construct were applied for these

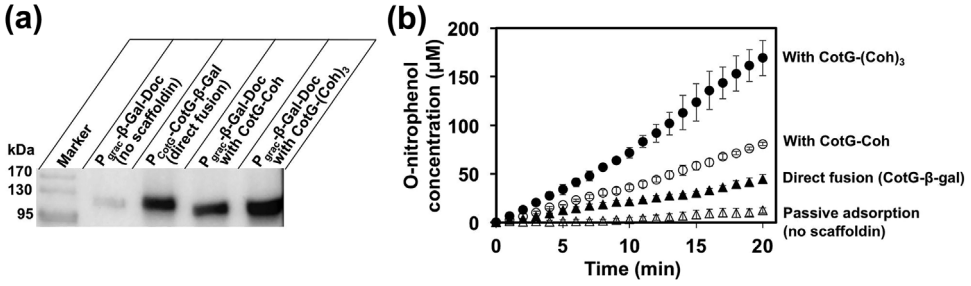

FIGURE 2 Western blot (a) and hydrolysis activity test (b) of β-gal-Doc immobilized on the spore surface via anchoring scaffoldins CotG-Coh and CotG-$(Coh)_3$. β-gal-Doc (124 kDa) was under the control of a strong IPTG-inducible promoter P_{grac}. Passive adsorption in the absence of anchoring scaffoldin and direct fusion with CotG (CotG-β-gal, 140 kDa) were also tested. Western blot signals were developed by using anti-β-gal-HRP, and hydrolysis activity was measured with ONPG

Samples			Dot blotting	β-gal (ng)	# of β-gal per spore (×10³)
Enzyme	Enzyme promoter	Scaffoldin			
CotG-β-gal	P_{CotG}	None		5.6	9.6
β-gal-Doc	P_{CotG}	CotG-Coh		3.3	5.7
		CotG-$(Coh)_3$		4.3	7.4
	P_{grac} (IPTG inducible)	CotG-Coh		7.4	13
		CotG-$(Coh)_3$		21	36

β-gal std 1.3 2.5 5 10 20 40 (ng)

FIGURE 3 Display density quantification by dot blot. Spores of five recombinant *B. subtilis* strains displaying β-gal were subjected to spore surface protein extraction, and dot blot with anti-β-gal-HRP. 1.3–40 ng purified β-gal served as the standards

assays. After incubation at 37°C for 3 hr, free β-gal retained 56% of its initial activity, and spore display improved that value to 81% ($p < 0.01$) (Figure S4). At 40°C, the half-lives of free and spore displayed β-gal were 2.1 and 4.4 hr ($p < 0.01$), respectively. In agreement with studies of others (Jia, Lee, & Farinas, 2014; Wang et al., 2011; Yim, Jung, Yun, & Pan, 2009), these results suggested that spore surface display significantly improved the stability of immobilized enzymes. Long-term storage stability was then tested using lyophilized biocatalyst samples stored at ambient temperature. Weekly hydrolysis activity tests indicated that the activity of free β-gal rapidly dropped by 31% of its initial activity after one week storage. However, when displayed on spores, 79% activity remained after 3 weeks (Figure 4a). At week 5, less than 40% of free β-gal's initial activity remained, but it took the spore-based biocatalyst more than 9 weeks to reach the same loss. Overall spore display significantly extended β-gal half-life from 21 days to 43 days. In reusability tests, each cycle of hydrolysis reaction was conducted in 0.1 M phosphate buffer with 3 mM ONPG for 30 min, and spores were recovered by centrifugation and extensively washed before subsequent rounds of reactions. On average, each round lost 3.5% hydrolysis activity, and 87% of the original activity remained after four successive uses, demonstrating excellent reusability of the spore-based biocatalysts (Figure 4b).

3.5 | Biphasic transgalactosylation

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 5a). To achieve efficient transgalactosylation, a stable emulsion and interfacial localization of the catalyst are desired. Microscopic imaging on spores in 0.1 M phosphate buffer/ethyl ether solution after vigorous shaking indicated that the generated emulsion was stable for more than two hours, while without spores the emulsion rapidly diminished in ~10 min. This suggested that the spores act as a stabilizer inhibiting droplet coalescence, similar phenomena were observed with other hydrophobic bacteria (Dorobantu, Yeung, Foght, & Gray, 2004; Honda et al., 2008; Wiencek, Klapes, & Foegeding, 1990). Moreover, a majority of spores were present at the biphasic interface, and emulsion particles had diameters of 5–10 μm providing large surface area and access of enzymes to substrates in both phases (Figure 6). Overall, our observations are consistent with literature suggesting that spore surface display can be an effective approach for biphasic reactions (Kwon, Jung, & Pan, 2007).

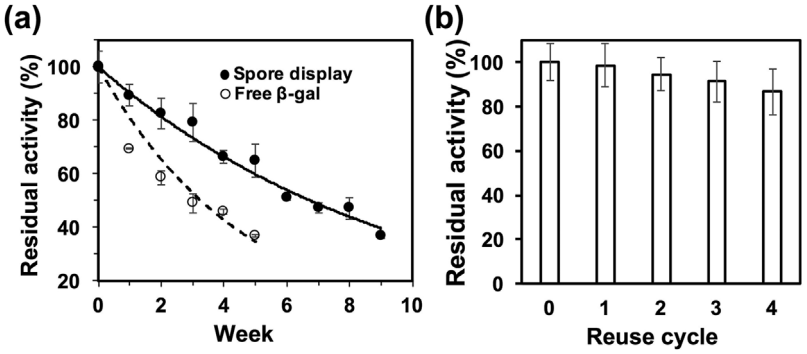

FIGURE 4 Long-term storage stability (a) and reusability (b) of spore-based biocatalysts (P_{grac}-β-gal-Doc/CotG-$(Coh)_3$). (a) Spores were lyophilized and stored in dry form at ambient temperature before assays. (b) For each round, the reaction was performed in 0.1 M phosphate buffer at room temperature with 3 mM ONPG for 30 min. Spores were isolated from the reaction mixture and washed before reuse

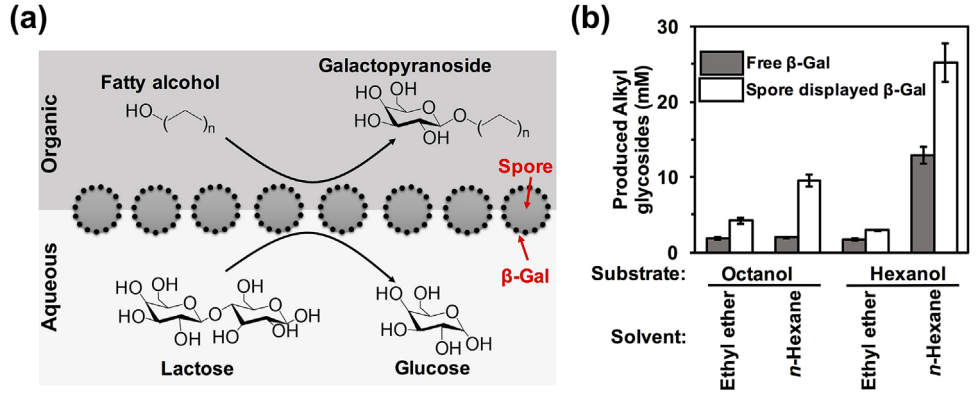

FIGURE 5 Transgalactosylation of produced spore biocatalyst in water/organic emulsions. (a) Biphasic reaction mediated by interfacially localized spores. (b) Transgalactosylation reaction results. Alkyl donor was either octanol or hexanol, and organic solvent was either ethyl ether or *n*-hexane

To choose the organic solvents best suitable for β-gal, spores displaying β-gal were incubated in a variety of aqueous/organic emulsions (1:1, v/v), and their activities on hydrolyzing ONPG were measured and compared with that of free β-gal. In emulsions containing either ethyl acetate, ethyl ether, toluene, or *n*-hexane as the organic phase, residual activities (the relative values over that in aqueous solution) of free β-gal were 11%, 46%, 28%, and 74%, respectively, while spore displayed β-gal maintained or considerably improved residual activities in all tested emulsions (29%, 55%, 76%, and 78% for above solvents, respectively) (Table S1). Particularly in phosphate buffer/ethyl acetate emulsion, spore biocatalyst was 1.6-fold more active than free β-gal. Moreover, it was found that the residual hydrolysis activities of spore displayed β-gal were positively correlated with the solvent hydrophobicities, likely due to the hydrophobic nature of spore surface (Wiencek et al., 1990).

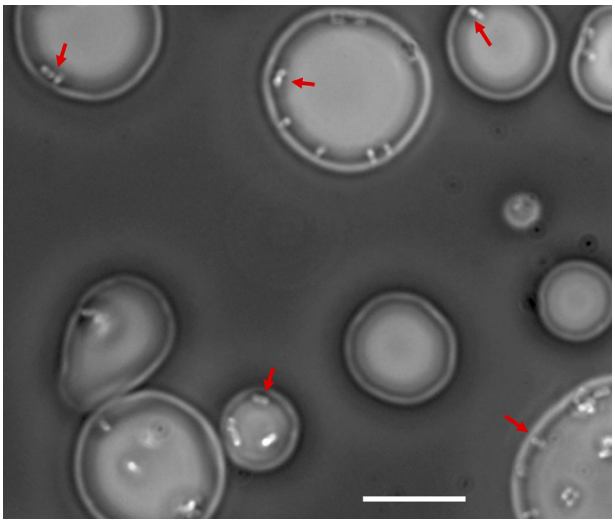

FIGURE 6 Interfacial localization of spores in emulsion. 1 OD_{600} spores were dispersed in 1 ml phosphate buffer/ethyl ether emulsions (3:7, v/v). Arrows indicate the spore particles. Scale bar = 10 μm

Because β-gal owned higher transgalactosylation activity with lower water content (Chen, Wei, & Hu, 2001) and minimum water content was necessary to dissolve lactose, a lower phosphate buffer/organic solvent (either ethyl ether or *n*-hexane) ratio of 3:7 was used in following biphasic reactions (Figure 5b). When 100 mM octanol was used as alkyl donor, free β-gal yielded 1.9 mM octyl galactoside in both emulsions, while the spores displaying β-gal of the same activity units yielded 4.2 and 9.5 mM octyl galactoside in ethyl ether and *n*-hexane, respectively, representing 1.2- and 4.0-fold of improvements. When 100 mM *n*-hexanol was used hexyl galactoside yields by free β-gal were 1.7 and 11.8 mM in ethyl ether and *n*-hexane, respectively, while spore display increased these values to 2.9 and 25.2 mM, suggesting higher transgalactosylation efficiencies were achieved with shorter fatty alcohols (Yang et al., 2017). To improve the conversion rate of fatty alcohol, lactose concentration in aqueous phase was increased from 100 to 200 and eventually 300 mM (Figure S5). During the first 4 hr, the average production rate with 100 mM lactose was 2.0 mM hexyl galactoside per hour. When the concentration of lactose was increased to 200 or 300 mM, the rate increased to 2.6–2.8 mM/hr. After 24 hr reaction, 100, 200, and 300 mM lactose generated 25.2, 32.5, and 35.5 mM hexyl glycoside, respectively. Overall, with the solvent and substrate concentration optimizations, spore display achieved a 35% conversion rate for hexanol.

4 | DISCUSSION

This study demonstrated functional display of multimeric enzymes on spore surface, which was achieved by co-expressing both the enzyme of interest and surface anchoring scaffoldin during *B. subtilis* sporulation (Figure 1). In this system, all the nutrients and required machineries for correct folding, for example, ATP-dependent chaperons, are available in the mother cell compartment, and spore formation does not need proteins to cross cell membranes. Although tetrameric β-galactosidase was exploited as the example, the platform technology developed here should be readily applied for other multimeric enzymes or enzyme complexes. Unlike current spore surface display techniques

via passive adsorption or direct fusion, spore surface anchoring scaffodins were designed in this study to serve two purposes: spacers to improve the functional display and mediators to increase the displayed amount by multiplying the copy number of cohesins on scaffodins (Figure 1). Both hydrolysis activity (Figure 2b) and quantitative dot blotting (Figure 3) showed that anchoring scaffodins significantly enhanced functional display, with a capacity of 1.4×10^4 active enzyme molecules per spore, when anchoring scaffoldins carrying three copies of cohesins were used. We speculate that with increased copy numbers of cohesin domains, higher display density can be achieved.

Due to the relatively high costs of biocatalysts in general, the ability to recycle and reuse them is often a desired feature for the economics of many industrial biocatalysis. This study suggested that spore particles can be easily separated from the reaction solution by centrifugation to achieve a high recovery rate for repeated reactions (Figure 4b). Recycle after 24 hr transgalactosylation reaction was also attempted, and results showed ~60% loss of activity on average for each round of biphasic reaction (Figure S6), primarily due to the denature effect of organic solvents on enzymes during extended incubation. The ability to be stored for long periods of time without refrigeration is another sought after feature, as it increases shelf life and reduces storage costs. In agreement with previous studies (Jia et al., 2014; Wang et al., 2011; Yim et al., 2009), our results showed that spore surface acts as a solid support to prevent enzyme denaturation (Figure S4), and this protection significantly improves enzyme storage (Figure 4a).

In addition, for many biocatatlysis involving hydrophobic substrates or products that have low solubility in aqueous solutions, non-polar organic solvents are often required to improve their solubilities (Lescic, Vukelic, Majeric-Elenkov, Saenger, & Abramic, 2001). Unfortunately, the presence of organic solvents in general severely deactivates enzymes. In sharp contrast, surface displayed enzymes are renowned to be resistant to organic solvents, as demonstrated by our study (Table S1 and Figure 5) and others (Jia et al., 2014; Jung, Kwon, & Pan, 2006; Kwon et al., 2007). Particularly, interfacial localization of spores and their role as emulsion stabilizer facilitated biphasic enzymatic reactions (Figure 6). From the biocatalysts manufacture viewpoint, our platform is also beneficial because (1) *B. subtilis* is a GRAS (generally regarded as safe) strain; (2) the genomic integration of heterologous genes stabilizes the gene of interest and attenuates the dependence of antibiotics to maintain plasmids; and (3) enzyme co-expression and self-assembly, and mature spore release by autolysis ease the manufacturing process, thus further reducing associated cost.

5 | CONCLUSION

In this study, we developed a *B. subtilis* spore based biocatalysis platform via co-expression and self-assembly of dockerin-tagged enzymes to scaffoldins of coat protein-cohesin fusions during sporulation process. This approach enabled functional display of tetrameric β-gal, which is a challenge for conventional microbial surface display. By manipulating the copy number of associated Coh domains and increasing β-gal expression in the mother cell compartment, a high display density of 1.4×10^4 active enzymes per spore particle was achieved, which was significantly higher than that of direct fusion. Compared with free β-gal, spore biocatalyst owned improved thermostability, reusability, and storage stability. In addition, spore surface display increased alkyl galactoside production in biphasic water/organic emulsions with a 35% conversion rate of trans-galactosylation. Overall, this spore biocatalysis platform was concluded to be promising in industrial applications for enzymatic reactions, especially the ones involving multimeric enzymes or enzyme complexes.

ACKNOWLEDGMENT

This study was supported by National Science Foundation (CBET 1265044).

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REFERENCES

Becker, S., Theile, S., Heppeler, N., Michalczyk, A., Wentzel, A., Wilhelm, S., Jaeger, K. E., & Kolmar, H. (2005). A generic system for the *Escherichia coli* cell-surface display of lipolytic enzymes. *FEBS Letters*, *579*, 1177–1182.

Bommarius, A. S. (2015). Biocatalysis: A status report. *Annual Review of Chemical and Biomolecular Engineering*, *6*, 319–345.

Chen, L., Mulchandani, A., & Ge, X. (2017). Spore-displayed enzyme cascade with tunable stoichiometry. *Biotechnology Progress*, *33*, 383–389.

Chen, S. X., Wei, D. Z., & Hu, Z. H. (2001). Synthesis of galacto-oligosaccharides in AOT/isooctane reverse micelles by β-galactosidase. *Journal of Molecular Catalysis B: Enzymatic*, *16*, 109–114.

Choi, J. M., Han, S. S., & Kim, H. S. (2015). Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnology Advances*, *33*, 1443–1454.

Donadio, G., Lanzilli, M., Sirec, T., Ricca, E., & Isticato, R. (2016). Localization of a red fluorescence protein adsorbed on wild type and mutant spores of *Bacillus subtilis*. *Microbial Cell Factories*, *15*, 153.

Dorobantu, L. S., Yeung, A. K., Foght, J. M., & Gray, M. R. (2004). Stabilization of oil-water emulsions by hydrophobic bacteria. *Applied Microbiology and Biotechnology*, *70*, 6333–6336.

Driks, A. (1999). Bacillus subtilis Spore Coat. *Microbiology and Molecular Biology Reviews*, *63*, 1–20.

Driks, A. (2002). Overview: Development in bacteria: Spore formation in *Bacillus subtilis*. *Cellular and Molecular Life Sciences*, *59*, 389–391.

Errington, J. E. (1993). *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. *Microbiological Reviews*, *57*, 1–33.

Foster, S., & Popham, D., (2002). Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules. In A. Sonenshein, R. Losick, & J. Hoch (Eds.), *Bacillus subtilis and its closest relatives*. Washington, DC: ASM Press.

Honda, K., Yamashita, S., Nakagawa, H., Sameshima, Y., Omasa, T., Kato, J., & Ohtake, H. (2008). Stabilization of water-in-oil emulsion by *Rhodococcus opacus* B-4 and its application to biotransformation. *Applied Microbiology and Biotechnology*, *78*, 767–773.

Isticato, R., Cangiano, G., Tran, H. T., Ciabattini, A., Medaglini, D., Oggioni, M. R., De Felice, M., Pozzi, G., & Ricca, E. (2001). Surface display of recombinant proteins on *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*, 6294–6301.

Isticato, R., Di Mase, D. S., Mauriello, E. M., De Felice, M., & Ricca, E. (2007). Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system. *Biotechniques*, *42*, 151–156.

Jia, H., Lee, F. S., & Farinas, E. T. (2014). *Bacillus subtilis* spore display of laccase for evolution under extreme conditions of high concentrations of organic solvent. *ACS Combinatorial Science*, *16*, 665–669.

Jung, H. C., Kwon, S. J., & Pan, J. G. (2006). Display of a thermostable lipase on the surface of a solvent-resistant bacterium, *Pseudomonas putida* GM730, and its applications in whole-cell biocatalysis. *BMC Biotechnology*, *6*, 23.

Kroos, L., & Yu, Y. T. (2000). Regulation of σ factor activity during *Bacillus subtilis* development. *Current Opinion in Microbiology*, *3*, 553–560.

Kwon, S. J., Jung, H. C., & Pan, J. G. (2007). Transgalactosylation in a water-solvent biphasic reaction system with beta-galactosidase displayed on the surface of *Bacillus subtilis* spores. *Applied and Environmental Microbiology*, *73*, 2251–2256.

Lescic, I., Vukelic, B., Majeric-Elenkov, M., Saenger, W., & Abramic, M. (2001). Substrate specificity and effects of water-miscible solvents on the activity and stability of extracellular lipase from *Streptomyces rimosus*. *Enz. Microb Tech*, *29*, 548–553.

Liu, Y., Zhang, R., Lian, Z., Wang, S., & Wright, A. T. (2014). Yeast cell surface display for lipase whole cell catalyst and its applications. *Journal of Molecular Catalysis B: Enzymatic*, *106*, 17–25.

Moses, V., & Sharp, P. (1970). Interactions between metabolic intermediates of beta-galactosidase from *Escherichia coli*. *Biochemical Journal*, *118*, 491–495.

Nguyen, Q. A., & Schumann, W. (2014). Use of IPTG-inducible promoters for anchoring recombinant proteins on the *Bacillus subtilis* spore surface. *Protein Expression and Purification*, *95*, 67–76.

Paidhungat, M., Setlow, B., Daniels, W. B., Hoover, D., Papafragkou, E., & Setlow, P. (2002). Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Applied and Environmental Microbiology*, *68*, 3172–3175.

Pan, J. G., Choi, S. K., Jung, H. C., & Kim, E. J. (2014). Display of native proteins on *Bacillus subtilis* spores. *FEMS Microbiology Letters*, *358*, 209–217.

Phan, T. T., Nguyen, H. D., & Schumann, W. (2006). Novel plasmid-based expression vectors for intra-and extracellular production of recombinant proteins in *Bacillus subtilis*. *Protein Expression and Purification*, *46*, 189–195.

Sacco, M., Ricca, E., Losick, R., & Cutting, S. (1995). An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. *Journal of Bacteriology*, *177*, 372–377.

Schüürmann, J., Quehl, P., Festel, G., & Jose, J. (2014). Bacterial whole-cell biocatalysts by surface display of enzymes: Toward industrial application. *Applied Microbiology and Biotechnology*, *98*, 8031–8046.

Shuman, H. A., & Silhavy, T. J. (2003). Microbial genetics: The art and design of genetic screens: *Escherichia coli*. *Nature Reviews Genetics*, *4*, 419–431.

Sirec, T., Strazzulli, A., Isticato, R., De Felice, M., Moracci, M., & Ricca, E. (2012). Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microbial Cell Factories*, *11*, 100.

Smith, M. R., Khera, E., & Wen, F. (2015). Engineering novel and improved biocatalysts by cell surface display. *Industrial & Engineering Chemistry Research*, *54*, 4021–4032.

Tanaka, T., & Kondo, A. (2015). Cell-surface display of enzymes by the yeast *Saccharomyces cerevisiae* for synthetic biology. *FEMS Yeast Research*, *15*, 1–9.

Wang, N., Chang, C., Yao, Q., Li, G., Qin, L., Chen, L., & Chen, K. (2011). Display of *Bombyx mori* alcohol dehydrogenases on the *Bacillus subtilis* spore surface to enhance enzymatic activity under adverse conditions. *PLoS ONE*, *6*, e21454.

Wiencek, K. M., Klapes, N. A., & Foegeding, P. M. (1990). Hydrophobicity of *Bacillus* and *Clostridium* spores. *Applied Microbiology and Biotechnology*, *56*, 2600–2605.

Yang, J., Perez, B., Anankanbil, S., Li, J., Gao, R., & Guo, Z. (2017). Enhanced synthesis of alkyl galactopyranoside by *Thermotoga naphthophila* β-galactosidase catalyzed transglycosylation: A kinetic insight of functionalized ionic liquid-mediated system. *ACS Sustainable Chemistry & Engineering*, *5*, 2006–2014.

Yim, S. K., Jung, H. C., Yun, C. H., & Pan, J. G. (2009). Functional expression in *Bacillus subtilis* of mammalian NADPH-cytochrome P450 oxidoreductase and its spore-display. *Protein Expression and Purification*, *63*, 5–11.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Chen L, Holmes M, Schaefer E, Mulchandani A, Ge X. Highly active spore biocatalyst by self-assembly of co-expressed anchoring scaffoldin and multimeric enzyme. *Biotechnology and Bioengineering*. 2017;1–8. https://doi.org/10.1002/bit.26492