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BIOCATALYSIS

Expression and display of a novel thermostable esterase from *Clostridium thermocellum* on the surface of *Bacillus subtilis* using the CotB anchor protein

Huayou Chen[1,2,3] · Tianxi Zhang[1] · Jinru Jia[1] · Ake Vastermark[3] · Rui Tian[1] · Zhong Ni[1] · Zhi Chen[1] · Keping Chen[1] · Shengli Yang[1]

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Abstract Esterases expressed in microbial hosts are commercially valuable, but their applications are limited due to high costs of production and harsh industrial processes involved. In this study, the esterase-DSM (from *Clostridium thermocellum*) was expressed and successfully displayed on the spore surface, and the spore-associated esterase was confirmed by western blot analysis and activity measurements. The optimal temperature and pH of spore surface-displayed DSM was 60 and 8.5 °C, respectively. It also demonstrates a broad temperature and pH optimum in the range of 50–70, 7–9.5 °C. The spore surface-displayed esterase-DSM retained 78, 68 % of its original activity after 5 h incubation at 60 and 70 °C, respectively, which was twofold greater activity than that of the purified DSM. The recombinant spores has high activity and stability in DMSO, which was 49 % higher than the retained activity of the purified DSM in DMSO (20 % v/v), and retained 65.2 % of activity after 7 h of incubation in DMSO (20 % v/v). However, the recombinant spores could retain 77 % activity after 3 rounds of recycling. These results suggest that enzyme displayed on the surface of the *Bacillus subtilis* spore could serve as an effective approach for enzyme immobilization.

✉ Huayou Chen
hyc@ujs.edu.cn

[1] Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, Jiangsu Province, China

[2] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 10090, China

[3] Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116, USA

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Introduction

Bacillus subtilis is a non-pathogenic bacterial species that has the ability to enter a complex differentiation process culminating in the formation of an extremely resistant spore under extreme nutrient deprivation [33]. This bacterial species is also was considered as GRAS (generally regarded as safe) by the US Food and Drug Administration. In addition, *B. subtilis* has an undisputed safety record based on the worldwide commercial use of its spores as probiotics for humans and animals and as the oral prophylaxis of gastrointestinal disorders [30]. For example, in animal nutrition, feed enzymes expressed on the surface of the spores can ensure in situ efficient enzymatic activity at a moderate cost [24]. In addition, it can be used as vaccine vehicles, carriers of active enzymes and antibodies, biosensors, whole-cell biocatalysts, and bioadsorbents [3, 13, 17, 32]. Simple and economic production of large quantities of spores is also ensured for industrial-scale production and commercialization of several spore-based products [21].

Bacillus subtilis spores are surrounded by a coat, which is composed of at least 20 polypeptides and two proteinaceous layers [2]. Some of these, like CotA, CotB, CotG, CotC, CotF, have been associated with the outer layer of the coat and are referred to as the outer coat proteins [25]. In this study, we used the abundantly present structural coat proteins-CotB, which is synthesized but not assembled into the coat [11] as an anchoring motif, for surface display on *B. subtilis* spores. CotB has a hydrophilic C-terminal, half of which is made up of three 27-amino acid repeats rich in serine, lysine, and glutamine residues [34]. Based on the analogy to the connective tissue proteins (collagen and elastin), the lysine residues in

Table 1 The primers used for construction of pET28a-DSM and pHS-CotB-DSM

Primers' name	Sequence of primer(5′–3′)	Restriction site
P*dsm*-F1	5′-CGC*GGATCC*ATGAACCCTTATAAAAGAATAGCTTTG-3′	*Bam*HI
P*dsm*-R1	5′-CCG*CTCGAG*TCATAGCAATTCCTCACTTTGC-3′	*Xho*I
P*pHS*-F	5′CCG*GAATTC*ACTGAGCGTCAGACCCCGTA 3′	*Eco*RI
P*pHS*-R	5′CCG*GAATTC*CTGCAGCCCGGGGGAT 3′	*Eco*RI
P*cotB*-F1	5′CGC*GGATCC*ACGGATTAGGCCGTTTGTCCTC-3′	*Bam*HI
P*cotB*-R1	5′CGATAAGAAAATATTCCCAAGTAGGATGATTGATAATCCGAAG-3′	none
P*dsm*-F2	5′TCGGATTATCAATCATCCGGAGGTGGGGGTTCGATGAACCCTTATAAAAGAATAGCTTG-3′	none
P*dsm*-R2	5′TGC*TCTAGA*TCATAGCAATTCCTCACTTTGCCC-3′	*Xba*I

the repeat area are considered to represent the sites of intra- or inter-molecular cross-linking [16]. CotB has been used to display the C-terminal fragment of the tetanus toxin (TTFC) [21], domains 1b-3 and 4 of the protective antigen (PA) of *Bacillus anthracis*, and the C-terminal part of the alpha toxin of *Clostridium perfringens* [19]. Therefore, CotB is an appropriate fusion partner for the display of functional proteins.

Esterases (EC3.1.1.1, carboxylester hydrolases) are a group of enzymes that catalyze the hydrolysis and synthesis of short-chain (carbon atoms <10) ester-containing molecules, are partly soluble in water [4], have a high regio- and stereo-specificity, and are usually stable and active in organic solvents. Thus, esterases are enzymes with considerable potential for the hydrolysis and synthesis of important ester compounds required for pharmaceutical, food, biochemical industries, and of biological interests, especially the thermostable esterase from thermophiles [15]. Therefore, esterase has an extraordinary significance in enhancing the stability of hydrolyzed compounds in organic solvents and other harsh conditions. Enzyme immobilization is the most crucial way to increase the stability of enzymes and reduce the cost by simple recovery and reutilization [45].

In this study, the esterase-*dsm* from *Clostridium thermocellum* was successfully immobilized on the surface of *B. subtilis* spores-CotB. The flexible linkers Gly–Gly–Gly–Gly–Ser can be used to separate the fused CotB protein and the esterase, which can be optimized for appropriate separation of the functional domains [6]. The result suggests that displaying enzymes on the *B. subtilis* spore surface is an effective approach for enzyme immobilization and has significant potential for its application in the biochemical industry.

Materials and methods

Chemicals and production of antiserum

The genomic DNA of *C. thermocellum* was preserved in the Institute of Applied Microbiology of Jiangsu University. The high-copy number shuttle vector pLJ was provided by Mingming Yan at Northwest A&F University. Restriction enzymes, PrimeSTAR HS DNA polymerase and DNA ligase were all purchased from TaKaRa Biotechnology (Dalian, China). The *p*-nitrophenyl esters were obtained from Sigma (St. Louis, USA). All chemicals were of analytical grade.

The *dsm* gene (gene ID: 12420791) was amplified using chromosomal DNA of *C. thermocellum* as template and primers P*dsm*-F1 and P*dsm*R1 (Table 1). The obtained PCR product of 936 bp was digested with *Bam*HI and *Xho*I, and cloned into the commercial vector pET28a (+). The recombinant plasmid was called pET28a-DSM. The resulting plasmid, pET28a-DSM, was verified by restriction analysis and nucleotide sequencing. PET28a-DSM was used to transform *Escherichia coli* strain BL21. Subsequently, expressed this esterase and purified it using Ni–NTA column (Genscript, Nanjing, China). Rat antiserum against recombinant DSM protein was prepared by immunization with the purified recombinant DSM protein following emulsification in Freund's adjuvant as per the Handbook of Animal Experiments (Sambrook and Fritsch 1989). The rat antiserum was prepared in the Laboratory Animal Centre of Jiangsu University, China.

Bacterial strains, plasmid, and transformation

The *B. subtilis* strains used in this study are listed in Table 2. *Escherichia coli* JM109 was used for cloning the recombinant plasmid, and DSM was displayed on the surface of *B. subtilis* DB403 spores. A high-copy number *E. coli–B. subtilis* shuttle vector pHS was amplified to remove the promoter of the maltose utilization operon (P*glv*-inframe) using pLJ as a template the plasmid pLJ with P*pHS*-F and P*pHS*-R (Table 1) as primers. The amplified sequence was digested with *Eco*RI and then self-looped with T4 DNA ligase. *Escherichia coli* JM109 was transformed with a recombinant plasmid of pHS-CotB-DSM and pET28a-DSM by the $CaCl_2$ method [35].

Table 2 List of strains used in this study

Strain	Relevant genotype	Source and reference
E. coli		
JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14− [F'traD36 proAB+ lacIq lacZΔM15] hsdR17(rK-mK+)*	Preserved in the lab
BL21(DE3)	*F− ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Preserved in the lab
B. subtilis		
168	*trpC2*	Zhejiang University, China
DB403	*His npr R2 npr E18 aprA*	Zhejiang University, China
DB403-pHS-CotB-DSM	*Cmr: His npr R2 npr E18 aprA*	This work

Construction of recombinant plasmid

The fusion gene of *cotB-dsm* linked by the flexible linker (Gly–Gly–Gly–Gly–Ser; inserted between the C terminus of *cotB* and the N terminus of the *dsm*) was constructed using the overlap PCR method. *cotB*, a 1184-bp fragment, containing the promoter region (P*cotB*) and the 927-bp DNA fragment coding for the first 304 amino acids of cotB (GenBank: CAB07789.1) was amplified from the chromosomal DNA of *B. subtilis* with P*cotB*-F1 and P*cotB*-R (Table 1). Further, *dsm* was amplified using the chromosomal DNA of *C. thermocellum* as the template using P*dsm*-F2 and P*dsm*-R2 (Table 1). The fusion gene was digested with *Bam*HI and *Xba*I and cloned into the vector pHS, to yield plasmid pHS-CotB-DSM.

Preparation of spores

Bacillus subtilis DB403 was transformed with pHS-cotB-DSM by the two-step (GM I, GM II) method [8]. Sporulation was induced by the exhaustion method in the DS (Difco-Sporulation) medium [31]. The cultures were harvested 30 h after the initiation of sporulation and purified using a lysozyme treatment to break residual sporangial cells, followed by washing in 1 M KCl and Tris–HCl (50 mM, pH 7.5), and resuspended in Tris–HCl (50 mM, pH 7.5).

Western blotting analyses

The expression of pHS-cotB-DSM was confirmed by western blot of a specific rat antiserum, which was obtained from immunized rats, and the specificity was determined using the prokaryotically expressed recombinant DSM protein as the antigen. Spore coat proteins were extracted from 50 μl of spore suspensions by treatment with a decoating extraction buffer at 65 °C for 10 min [29]. The western blot analysis was performed using standard procedures. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (TaKaRa, Dalian, China) was used for immune detection of the fusion protein. The enhanced HRP-DAB chromogenic substrate color kit (Tiangen, Beijing, China) was used for the color reaction according to the manufacturer's instructions.

Enzyme activity assays

Spectrophotometric determination of the optical density (OD) at 405 nm was conducted to calculate the activity of the enzyme and the immobilized enzyme with *p*-nitrophenyl esters as substrate. Unless otherwise noted, in the standard assay, enzyme activity was measured using a substrate mixture consisting of 2 mM *p*-nitrophenyl butyrate in 50 mM Tris–HCl buffer (pH 7.4). The purified spores (1.43×10^9 spores/ml) and the esterase-DSM (15.25 μg/ml) were pre-incubated for 2 min. Then the reaction was started after *p*-nitrophenyl butyrate (pNP-C4) added to a final concentration of 2 mM. After incubation for 2 min, the spore suspension reaction mixture was centrifuged at 10,000×*g* for 30 s at room temperature, and then cooled on ice to stop the reaction. Enzyme activity was measured using the UV1000 Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenyl per minute under the above conditions. The extinction coefficient (ε405) for *p*-nitrophenyl was 16540 M^{-1} cm^{-1}. The number of spores was calculated by counting directly on LB solid medium using the gradient dilution method, and the concentration of the esterase was determined by the Bradford method.

The optimum temperature was determined by enzyme activity assays at various temperatures ranging from 30 to 80 °C in 50 mM Tris–HCl buffer (pH 7.4) with pNP-C4 as substrate. The thermostability of the spore surface-displayed DSM and esterase-DSM was examined by incubation in 50 mM Tris–HCl buffer (pH 7.4) at three different temperatures (60, 70, and 80 °C) and (60, 65, and 70 °C) for different time intervals from 0 to 300 min. The optimum pH was determined by spectrophotometric

analysis with pNP-C4 as substrate at different pH values at the optimum temperature previously determined. The following buffers were used: Na_2HPO_4–citric acid buffer (pH 4.0–6.8), Na_2HPO_4–NaH_2PO_4 buffer (pH 5.8–8.0), Na_2HPO_4–KH_2PO_4 buffer (pH 7.73–8.67), Gly–NaOH buffer (8.6–10.4). The effects of organic solvents on the surface-displayed DSM were examined using methanol, diethylether, and DMSO at a concentration of 20 % (v/v). Relative activity was calculated by defining the respective original activity as 100 %. Residual activities were measured by the standard assay as described above. The residual activity of the surface-displayed enzyme at different concentrations of DMSO (20, 30, and 40 %) was also determined. The surface-displayed enzyme was incubated with DMSO for different durations, centrifuged at 4000×*g* for 90 s, and the pellet was suspended in preheated pNPB-C4; enzyme activity was assayed immediately. To eliminate the influence of background hydrolysis of the substrate under different conditions, the following controls were applied: (1) the compounds of the corresponding reaction mixture and the reaction conditions were the same (2) the DB403 spore without the surface-displayed DSM was used instead of the recombinant DB403 spore.

The spores are easily purified by centrifugation; therefore, the reusability of the surface-displayed protein was measured by determining the activity of the centrifuged recombinant spores. All reactions were conducted under optimal catalytic conditions (60 °C, pH 7.4) with 2 mM pNP-C4 as the substrate. After 1 reaction cycle, the spores were isolated by centrifugation at 4000×*g* for 90 min, and were reused for five cycles. Relative activity was calculated by defining the activity of first reaction as 100 %.

To further confirm that esterase-DSM was expressed on the *B. subtilis* spore surface, the activity of spore surface-displayed DSM under different treatments was determined using pNP-C4 as the substrate. Untreated surface-displayed DSM and DB403 (pHS-CotB) spores were used as controls. Purified recombinant spores were suspended in Tris–HCl buffer (pH 7.4) containing 0.25 % blomelain, trypsin, 50 U proteinase K, and 5 mM PMSF for 1 h at 37 °C. The reaction was started after *p*-nitrophenyl butyrate (pNP-C4) was added to the final concentration of 2 mM. After incubation at 65 °C for 2 min, the enzyme reaction was stopped by quickly freezing on ice. The mixture was centrifuged at 10,000×*g* for 30 s at room temperature and then quickly subjected to further analysis. To eliminate the influence of background hydrolysis of the substrate under different conditions, the following controls were applied: (1) the compounds of the corresponding reaction mixture and the reaction conditions were the same and (2) isopyknic Tris–HCl buffer (pH 7.4) was used without the spores. The activity of untreated surface-displayed DSM was defined as 100 %. All tests were performed in triplicate and three independent experiments were conducted. The data are expressed as mean ± standard deviation.

Result

Construction of pET28a-DSM and shuttle vector pHS-cotB-DSM

The esterase-*dsm* gene from *C. thermocellum* genomic DNA was PCR amplified, cloned into the PET-28a (+) cloning vector, and named pET28a-DSM. The expression vector, pHS-cotB-DSM, was constructed with the plasmid pLJ-(*E. coli–B. subtilis* shuttle vector) (Fig. 1b). We used the method of overlap PCR to amplify *cotB-dsm* which has a flexible linker (Gly–Gly–Gly–Gly–Ser) between the C terminus of the anchoring motif of *cotB* with the knocked out termination codon and the N terminus of the displayed enzyme DSM for the correct conformation (Fig. 1a). The relative flexibility of the short peptide allows significant movement of the structural domains, which is important to prevent the disturbance of the domain functions [43]. Each target gene was PCR amplified with specifically designed primers to ensure precise construction of recombinant

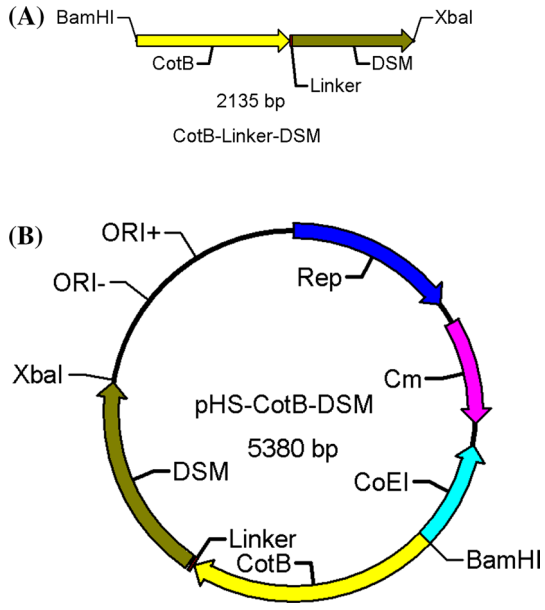

Fig. 1 Construction of the pHS-cotB-DSM **b** expression plasmid for cell surface display CotB-Linker-DSM was construct by overlap PCR **a** with the restriction enzymes of *Bam*HI and *Xba*I. The ORI+, ORI− and Rep represent the single-strand replication origin, the double strand origin and replication protein in *B. subtilis*, respectively. CoEI and Cm represent *E. coli* CoEI replicon and chloramphenicol-resistance marker, respectively

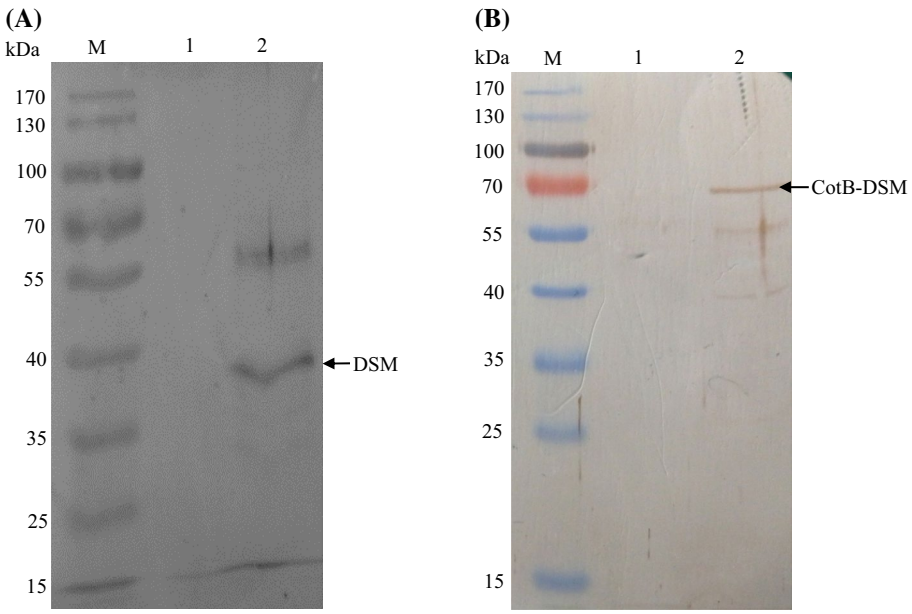

Fig. 2 Western blot analysis: **a** the specificity of the rat antiserum against DSM protein. M, protein molecular weight marker; *lane 1*, protein expressed with the empty pET-28a plasmid vector; *lane 2*, crude cell extract *E. coli* expressing recombinant DSM protein; Western blot analysis **b**: the recombinant spores expressing CotB-Tm1350DSM fusion proteins. *M*, protein molecular weight marker; *Lane 1*, proteins extracted from spores of *B. subtilis* DB403 (pHS-cotB); *Lane 2*, proteins extracted from purified recombinanted spores (pHS-CotB-DSM)

plasmids that were sequenced to ensure that no unintended mutation had occurred.

Identification of CotB-DSM displays on the spore surface of *Bacillus subtilis*

Western blot analysis was used to determine the specificity of the rat anti-DSM protein out of the total expressed protein from *E. coli* BL21 (pET28a-DSM) (Fig. 2a). The molecular weight of DSM was 35.2 kDa as calculated from the predicted amino acid sequence.

Previously prepared rat antiserum, anti-DSM, was used to verify the localization of CotB-DSM on the spore coat using western blot analysis (Fig. 2b). A band around 70 kDa was detected in the extracts from recombinant spores, while no similar band in the control lane was detected, which was consistent with the expected molecular weight of the fusion protein CotB-DSM (68 kDa). These data confirm the presence of CotB-DSM on the spore coat. To further confirm the surface-displayed DSM on the spore, the activity of the recombinant spores was determined by different treatments and using pNP-C4 as the substrate (Fig. 3). The activity was reduced to 24.7, 19.8, 13.4, 11.6 % after being treated with 0.25 % trypsin, blomelain, and 50U proteinase K, 5 mM PMSF, respectively. The DB403 (pHS-CotB) spores scarcely demonstrated any activity (14.5 %) than that of the untreated recombinant spores (100 %). The result suggested that DSM was displayed on the surface of spores.

Effect of temperatures on enzyme activity and stability

The optimum temperature of the spore surface-displayed DSM and the esterase-DSM was determined by subjecting

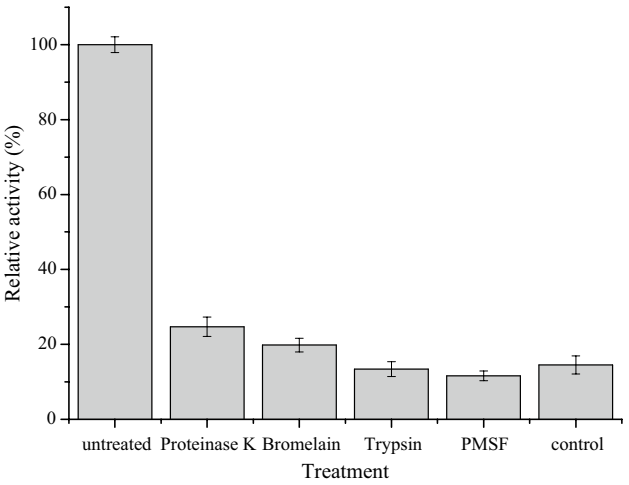

Fig. 3 The relative activity of spores with surface-displayed DSM under different treatment Untreated: the recombinant spores without treated. Purified recombinant spores were treated with 0.25 % trypsin, blomelain and 50U proteinase K, 5 mM PMSF; control: the spores of DB403 (pHS-CotB)-without treated. Residual activities were measured by the standard assay. The activity of untreated was defined as 100 %. All tests were implemented in triplicates from 3 independent experiments, and the data were expressed as mean ± deviations

to various temperatures from 30 to 80 °C in 50 mM Tris–HCl buffer (pH 7.4) (Fig. 4). The optimum activity of DSM was at 65 °C, while that of surface-displayed DSM was at 60 °C, which was consistent with the optimal growth temperature for *C. thermocellum* (60–65 °C). The thermostability of the spore surface-displayed DSM and esterase-DSM was detected at three different temperatures by measuring the residual activity with increasing incubation time of up to 300 min (Fig. 5). At pH 7.4, the

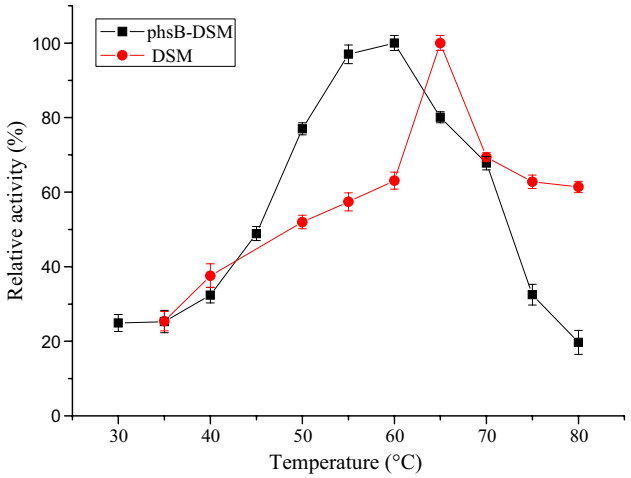

Fig. 4 Temperature optima of the spore-surfaced DSM (*boxes*) and the esterase-DSM (*circles*). Optima temperature was determined with pNP-C4 as substrates in 50 mM Tris–HCl (pH 7.4) at temperatures ranging from 30 to 80 °C. Spore-surfaced DSM, has optimal activity at 60 °C; DSM, has optimal activity at 65 °C, so activity at 60 and 65 °C was taken as 100 %, respectively. The values are means of three independent experiments

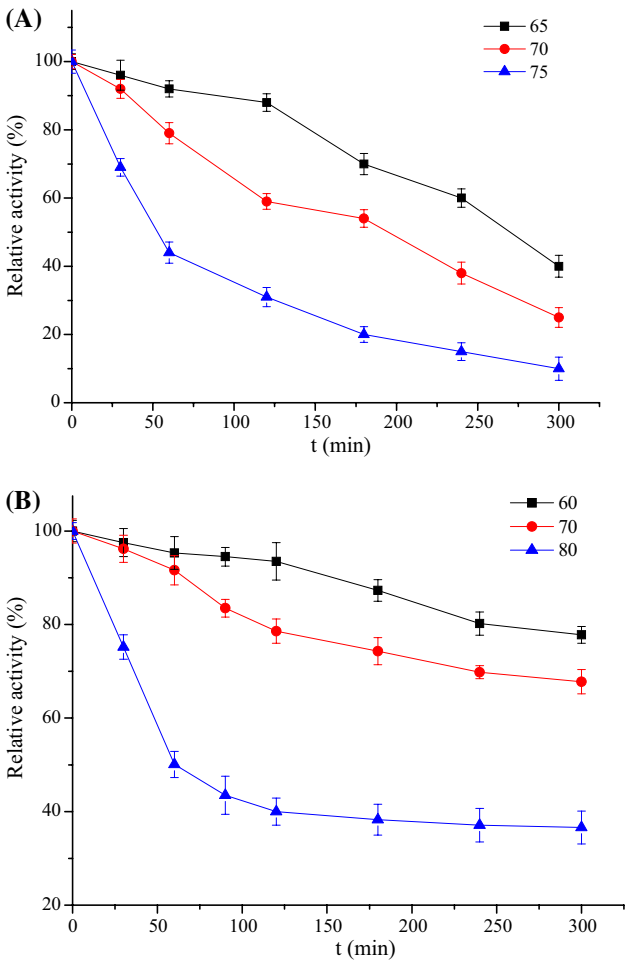

Fig. 5 Thermostability of esterase-DSM (**a**) and spore-surfaced DSM (**b**). The spore-surfaced DSM and the esterase-DSM was incubated at 60 °C (*boxes*), 70 °C (*circles*), 80 °C (*triangles*) and 65 °C (*boxes*), 70 °C (*circles*), 75 °C (*triangles*), respectively. And with pNP-C4 as substrates in 50 mM Tris–HCl (pH 7.4). Residual activity was measured using the standard assay. Activity before incubation was defined as 100 %

activity of the spore surface-displayed DSM was retained 78, 68, 37 % of its original activity after 300 min of incubation at 60, 70, 80 °C, respectively (Fig. 5b), while the esterase-DSM retained 41, 26, 10 % of its original activity after 300 min of incubation at 65, 70, 75 °C, respectively (Fig. 5a). To further characterize the properties of spore-surface enzymes and esterase-DSM, 60 and 65 °C, respectively, was chosen as the experimental temperature for subsequent analyses.

Effect of pH on enzyme activity

The surface-displayed DSM had maximal activity at pH 7.5 in sodium phosphate (50 mM), while the esterase-DSM had maximal activity at pH 6.8 (Fig. 6). The activity of surface displayed DSM at different pH changed slightly than that of the esterase-DSM.

Effect of organic solvents on enzyme activity

The effect of organic solvents on the surface-displayed enzyme and esterase-DSM was determined using pNPB-C4 as the substrate. After incubation in methanol, DMSO, and diethylether, the surface-displayed enzyme could retain about 72.2, 99, and 68.1 % of its activity, respectively, while esterase-DSM retained about 49.2, 50.3, and 32.6 % of its activity, respectively (Fig. 7). The effect and tolerance of different concentrations of DMSO on the surface-displayed enzyme activity were also determined. The result shows that the surface-displayed enzyme could retain high activity, about 99 and 80 % of its activity at the concentration of 20 and 40 % DMSO, respectively (Fig. 8a), and has a high stability in 20 % DMSO by retaining 65.2 % of its activity after 7 h of incubation (Fig. 8b). The surface-displayed enzyme also retained 46 and 35.6 % of its activity after 7 h of incubation in a concentration of 30 and 40 % DMSO, respectively.

Reusability and specific activity of spore surface-displayed DSM

The reusability of the surface-displayed DSM was measured by determining the activity of centrifuged recombinant spores with pNP-C4 as the substrate (Fig. 9). The recombinant spores could be used for up to 4 reaction

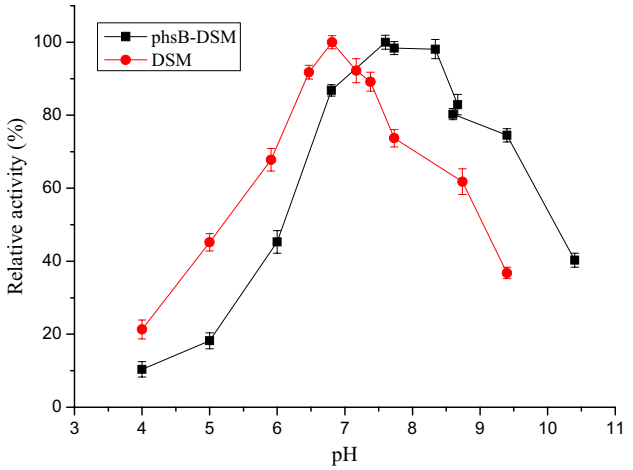

Fig. 6 PH optima of the spore-surfaced DSM (*boxes*) and the esterase-DSM (*circles*). pH optima at pHs from 4 to 10.4 was measured by standard assay. The buffers were Na_2HPO_4-Citric Acid buffer (pH 4.0–6.8), Na_2HPO_4–NaH_2PO_4 buffer (pH 5.8–8.0), Na_2HPO_4–KH_2PO_4 buffer (pH 7.73 to 8.67), Gly-NaOH buffer (8.6 to 10.4) with pNP-4C as substrate. Activity at pH 7.5 and 6.8 was taken as 100 %, respectively. The values are means of three independent experiments

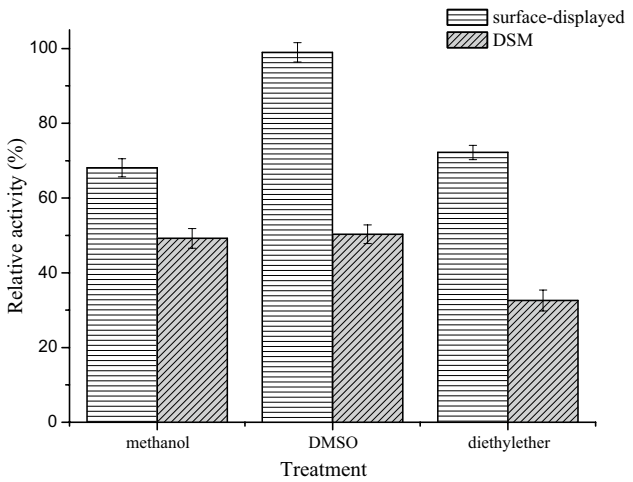

Fig. 7 The effects of organic solvents on the surface-displayed DSM and esterase-DSM. The concentration of methanol, diethylethern and DMSO is 20 % (v/v). The recombination spores (1.43×10^9 spores/ml) and esterase-DSM (15.25 μg/ml) was treatment at 37 °C with shaking of 220 rpm, 5 min. Residual activities were measured by the standard assay. The activity of untreated was defined as 100 %. All tests were implemented in triplicates from 3 independent experiments, and the data were expressed as mean ± deviations

cycles without any significant decrease in its activity. After 5 reaction cycles, the centrifuged recombinant spores retained 57 % of their original activity.

The activity of the spore surface-displayed DSM was also determined using pNP-C4 as the substrate under

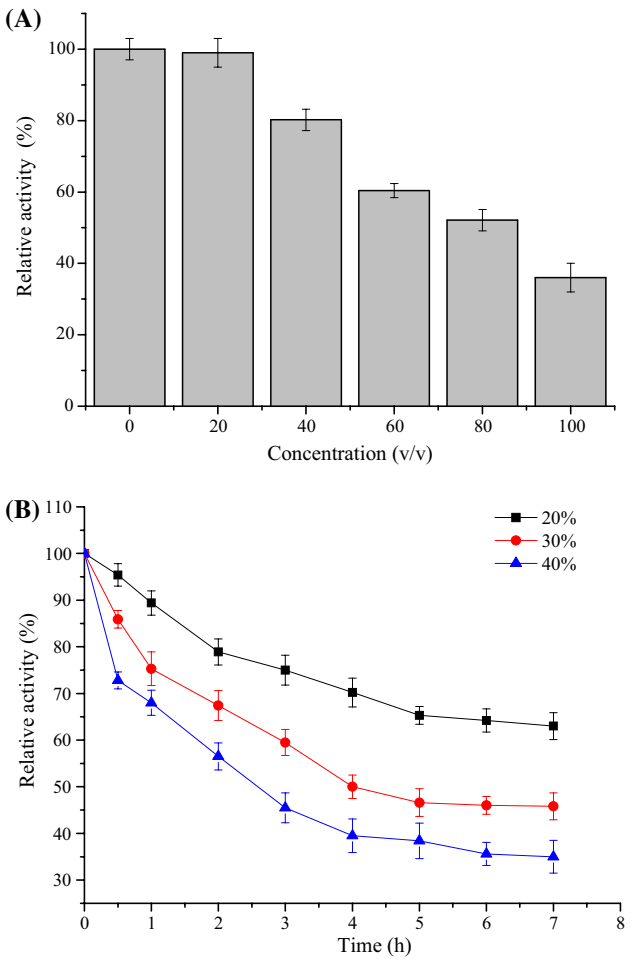

Fig. 8 The effect of different concentration of DMSO on the surface-displayed DSM. The spores was treatment at 37 °C with shaking of 220 rpm 5 min (**a**); and the stability of the spores was determined after incubation at different concentration of DMSO with shaking of 220 rpm at 37 °C. The activity without treatment was defined as 100 %. All tests were implemented in triplicates from 3 independent experiments, and the data were expressed as mean ± deviations

optimal temperature and pH conditions (60 °C, pH 7.5). The concentration of the recombinant spores was 5.72×10^{10} spore/ml while the specific activity was 3.0×10^{-9} U/spore.

Discussion

Some features of spore-forming bacilli, such as fast growth, relatively simple nutritional requirements, tolerance to harsh environment and efficient secretion of large amounts of proteins and other metabolites, make these organisms particularly suitable for industrial fermentations. Some species, such as *B. subtilis*, have also been used as hosts for the production of heterologous proteins [12, 26, 28]. On the other hand, due to the lack of enzyme stability mesophilic enzymes are often not well suited for harsh reaction

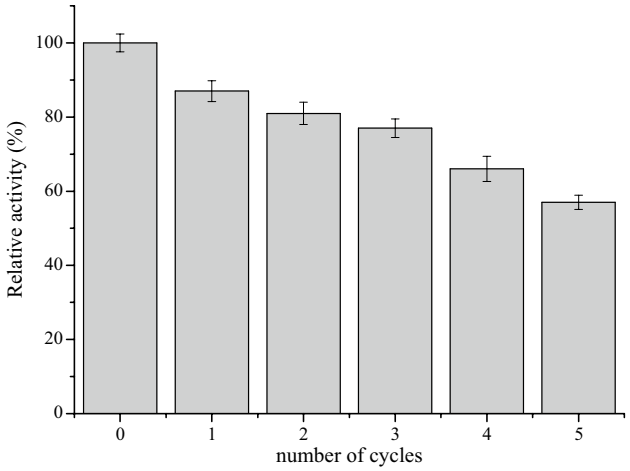

Fig. 9 Reusability of the surface-displayed DSM. The standard reaction mixture was pre-incubated at 60 °C for 2 min, then the first reaction was started after pNP-C4 was added. After incubated at 60 °C for 2 min, the enzyme reaction mixture was centrifuged, then quickly determined. The spores were cleaned using Tris–HCl buffer (50 mM, pH 7.4), then recovered to conduct the second reaction by centrifugation at 4000×*g* for 2 min at 4 °C. The relative activity was calculated by defining the activity of first reaction as 100 %. All tests were implemented in triplicates from 3 independent experiments, and the data were expressed as mean ± deviations

conditions used in industrial processes [22] such as high temperatures or the presence of organic solvents [9, 14]. The enzymes from thermophile bacteria have greatly influenced the field of biocatalysis because of their thermostability and chemical stability [39]. *Clostridium thermocellum* is a thermophilic bacterium that grows optimally at 60–65 °C, which makes it a potential source for thermostable enzymes. Some esterases from *C. thermocellum* have been identified and classified [7]. For suitable application of esterase in industrial production, we have displayed DSM on the *B. subtilis* spore surface using CotB as the anchoring motif and investigated the catalytic capability of the spore surface-displayed DSM using pNPB-C4 as the substrate.

Detailed genetic and morphological studies have shown that *B. subtilis* spore is surrounded by a multilayered coat [10], which encases the spore and helps to protect it from noxious environmental agents. It has previously been reported that at least 20 polypeptides are organized to form the double layers of the *B. subtilis* spore coat [23]. CotB was not only the first spore coat protein used to anchor heterologous proteins on the spore surface as anchoring motifs [20], but also considered as a more appropriate carrier to display heterologous proteins because the amount of recombinant protein expressed depends mainly on CotB and is not influenced by the nature of the heterologous part [2, 18]. Evidence from literature suggest that this would be the first report on displaying esterase on the surface of *B. subtilis* spores using CotB as the anchoring motif. The spore surface display system was usually constructed by double cross-over using recombinant integrative plasmid and the production of an amylase inactivated mutant to insert the target gene into the chromosomal DNA of *B. subtilis* [41]. However, this method has some shortcomings, such as complex operations, low success rate of conversion, and low-level expression. Shuttle vectors have been used to overcome such defects, but little attention has been devoted to the spore surface display system. The high copy number of *E. coli–B. subtilis* shuttle vector pEB03 was constructed based on the high-copy number vector pGJ103, which was used to display the enzyme on the spore surface demonstrating that the activity of the displayed enzyme was much high using pHP13 (low-copy number) as the expression vector [44].

In this study, we constructed an *E. coli–B. subtilis* shuttle vector pHS-cotB-DSM, based on the high-copy-number plasmid pLJ (based on the vector pGJ103). A 68-kDa fusion protein, CotB-DSM, was successfully displayed on the spore surface of *B. subtilis* DB403 and was confirmed by western blot analysis using a previously prepared rat antiserum and determination of enzyme activities.

We have purified and characterized the new esterase-DSM from *C. thermocellum*, and it has high thermostability and neutral pH, whereas the surface-displayed enzyme is more stable at high temperatures and high pH than the esterase-DSM. Relative to the esterase-DSM, the activity of surface-displayed DSM had a rather broad temperature and pH optimum range of 50–70 °C and 7–9.5, respectively, which was on an average 30–40 % higher than the glucuronyl esterase obtained from *Sporotrichum thermophile* [40]. The surface-displayed esterase could retain about 40 % of its activity after incubation at 80 °C for 5 h, which was twofold higher than the activity of esterase-XG2 from a metagenomic library [36] under the same conditions. The esterase-DSM showed some activity in organic solvent such as methanol, DMSO, and diethylether, but it has little activity without DTT (data not shown), while the retained activity of surface-displayed DSM without DTT was about 18.9, 49 and 39.6 % higher than that of the esterase-DSM with DTT in methanol, DMSO, and diethylether, respectively. After 7 h of incubation in DMSO, the residual activity was 63 % at the concentration of 20 % (v/v) and 45.8 at 30 % (v/v). The relative activity at 20 % (v/v) of DMSO is 30 % higher than the activity of thermostable esterase from the *Sulfolobus tokodaii* strain 7 [38]. In fact, the tolerance to DMSO and other organic solvents is important in organic synthesis [27, 37]. Therefore, the surface displayed esterase-DSM could be a potential candidate for industrial applications, such as synthetic organic chemistry. We predict that the displayed DSM on the surface of the spore coat protein CotB with the flexibility

linker (Gly–Gly–Gly–Gly–Ser) was beneficial to be used for effectively reducing the stereospecific blockade blocking of esterase on the spore surface and facilitating correct esterase conformation folding, increasing the stability and efficiency of display on the spore surface, thus, enhancing its activity.

The reusability of a biocatalyst is one of the most important factors to be considered for industrial application [5], and spores have the characteristic of easy purification by centrifugation. In our study, the activity of displayed esterase-DSM retained 77 % of its original activity after three rounds of reuse, which was higher than the immobilized lipase SMG1 on macroporous resin [42] and the spore surface-displayed Neu5Ac aldolase [44] similar to the CALB immobilized on PPS [1] after five rounds of reuse. Therefore, display of enzyme on the surface of *B. subtilis* spore as an effective approach of enzyme immobilization has a great potential application to sustain activity under harsh conditions used in biochemical industry.

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