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Production of Cyanocarboxylic Acid by *Acidovorax facilis* 72W Nitrilase Displayed on the Spore Surface of *Bacillus subtilis*

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Nitrilase is a valuable hydrolase that catalyzes nitriles into carboxylic acid and ammonia. Its applications, however, are severely restricted by the harsh conditions of industrial reaction processes. To solve this problem, a nitrilase from *Acidovorax facilis* 72W was inserted into an *Escherichia coli–Bacillus subtilis* shuttle vector for spore surface display. Western blot, enzyme activity measurements and flow cytometric analysis results all indicated a successful spore surface display of the CotB-nit fusion protein. In addition, the optimal catalytic pH value and temperature of the displayed nitrilase were determined to be 7.0 and 50°C, respectively. Moreover, results of reusability tests revealed that 64% of the initial activity of the displayed nitrilase was still retained at the 10^{th} cycle. Furthermore, hydrolysis efficiency of upscale production of cyanocarboxylic acid was significantly higher in the displayed nitrilase-treated group than in the free group expressed by *E. coli* (pET-28a-nit). Generally, the display of *A. facilis* 72W nitrilase on the spore surface of *Bacillus subtilis* may be a useful method for immobilization of enzyme and consequent biocatalytic stabilization.

Keywords: *Acidovorax facilis* 72W, nitrilase, *Bacillus subtilis*, immobilization, spore surface display

Introduction

Recently, there has been a growing interest in the use of enzymes as biocatalysts in industrial biocatalysis for the production of high-value products, such as pharmaceutical intermediates [1–3]. Many features, such as mild conditions without organic solvents, make them an interesting alternative for numerous conventional chemical processes. Numerous biocatalytic reactions can use: (a) free or immobilized enzymes; (b) whole-cell catalysts with preserved partial enzyme activities; or (c) direct fermentation for the production of products such as intermediates or final versions [4–6].

Nitrilase (E.C. 3.5.5.1), as an important hydrolase, could mediate biocatalytic reactions with high regioselectivity, stability and other benefits. Therefore, nitrilase is also attractive as a kind of mild "green" catalyst. In addition, various host-based systems, such as yeasts and bacteria, can be used for the expression of nitrilase [7]. However, the free nitrilase that has been used for decades in many biocatalytic preparation processes has various limitations, including the fact that it can be used only once for the required reaction as well as its instability in harsh environments [8–10]. Thus, many effective methods of enzyme immobilization have been developed in order to overcome these sorts of constraints. For example, the nitrilase-catalyzed hydrolysis of 2-methylglutaronitrile to 4-cyanopentanoic acid, an intermediate in the preparation of 1,5-dimethyl-2-piperidone, was conducted using microbial cell immobilization with greater than 98% regioselectivity at 100% conversion [11–13]. Nevertheless, process complexity, high cost and other constraints still exist in the preparation of immobilized nitrilase [14, 15].

Recently, the surface display system, as an effective immobilization tool, has already been widely applied to generate biocatalysts or biosensors, treat microbial infections and screen peptide libraries [16−20]. Several approaches have been developed and widely used, such as the construction of chimera-encoding gene fusions, which consist of a carrier protein and anchor a heterologous passenger protein, to display certain proteins in phages and bacteria [21−28].

Among all of the *Bacillus* species, *B. subtilis*, as a non-pathogenic bacterial species that can form an extremely resistant spore in an extremely nutrient-deprived environment, is currently being broadly and intensively studied for its application in displaying the enzymes or heterologous antigens on the surface of endospores [20, 29]. To date, more than 50 proteins have been discovered to be distributed on the spore surface, organized in an inner and outer layer. The outer layer contains 5 main proteins: CotA, CotB, CotC, CotF and CotG. This coat significantly enhances the spore stability under harsh conditions, such as high temperature and extreme pH [30]. Using coat proteins, such as CotB or CotG as fusion partners in spore display has become an effective immobilization tool [31, 32]. However, the application of *B. subtilis* spore surface display technology in the immobilization of high-value-added nitrilase has not yet been fully investigated and remains a topic of research.

Here, we report a method to immobilize *Acidovorax facilis* 72W nitrilase on the spore surface of *B. Subtilis* by using CotB as a fusion partner. The thermal stability, pH stability and reusability of the displayed nitrilase were also evaluated in order to assess the applicability of this process. In addition, we systematically compared the *A. facilis* 72W nitrilase, expressed in *E. coli* and displayed on the *B. subtilis* spore surface, in terms of upscale bioconversion efficiency.

Materials and Methods

Materials

The gene (GenBank: DQ444267.1) of *A. facilis* 72W nitrilase was synthesized by GENEWIZ. Inc (Suzhou, China). This gene was used for the expression of *A. facilis* 72W nitrilase. The strains, plasmids and primers used in this study are listed in Table 1. The other related reagents, such as DNA ligase and restriction enzymes, were provided by TaKaRa (China). Proteinase K, trypsin, bromelain, succinonitrile and related standards were purchased from Sigma (Germany). The horseradish peroxidase (HRP)-goat anti-rabbit IgG was bought from Jackson ImmunoResearch Laboratories, Inc. (USA).

Construction of Recombinant Plasmid

Plasmid pHS-CotB was constructed by ligating the *cotB* gene from *B. subtilis* 168, amplified by the primers P1-2 (Table 1) and digested with *Xma* I and *Spe* I, into the *E. coli–B. subtilis* shuttle vector. Using the gene of *A. facilis* 72W as a template, the nitrilase gene was amplified by primers P3-4 (Table 1). The desired recombinant plasmid pHS-CotB-nit was then successfully constructed by cloning the nit gene into plasmid pHS-CotB which had been pre digested with *Spe* I and *Xba* I.

Table 1. Strains, plasmids, primers used in this study.

Strain, plasmid, primer	Description	Source
Strains		
E. coli DH5α	Type strain	Gene copoeia
Bacillus subtilis DB403	His npr R2 npr E18 aprA	Bacillus Genetic Stock Center (BGSC)
Bacillus subtilis 168	Type strain	Bacillus Genetic Stock Center (BGSC)
Plasmids		
pHS	High-copy-number *E. coli-B. subtilis* shuttle vector with chloramphenicol resistance gene	This study
pHS-CotB	pHS with gene CotB	This study
pHS-CotB-nit	pHS-CotB with gene nit	This study
Primers		
P1	5′-TAGCCCGGGACGGATTAGGCCGTTTGTCC-3′	This study
P2	5′-CGGACTAGT*TGAACCCCCACC*TCCGTAGGGATGATTGAT-3′	This study
P3	5′- CGGACTAGTGTGGTTTCGTATAACAGCAAGT-3′	This study
P4	5′- TGCTCTAGACTACTTTGCTGGGACCGGT-3′	This study

[a]The italicized letters indicate the introduction of a flexible linker at the C terminus of the CotB structural gene product.

[b]The underlined letters indicate the introduction of restriction sites.

Preparation of Recombinant Spores

The recombinant *B.subtilis* DB403, cultivated in Difco-sporulation medium at 37°C for 24 h, was harvested and resuspended in a sodium phosphate buffer (100 mM, pH 7.4). The purified spores were then resuspended in phosphate buffer after sequential washing with 1 M NaCl and 1 M KCl. The free nitrilase was prepared using BL21 with vector pET28a.

Western Blotting

The pHS-CotB-nit expression was verified by western blot using a polyclonal antibody obtained from immunized rabbit using recombinant nitrilase as the antigen according to standard procedures. HRP-conjugated goat anti-rabbit IgG (Biodragon, Bejing) was used for immune detection of the fusion protein.

Verification of Nitrilase Display on the Spore Surface of *B. subtilis* DB403

The spores of *B.subtilis* DB403 with or without the recombinant plasmid (pHS-CotB-nit) were resuspended in three phosphate sodium buffer (PSB) which contains 0.1% proteinase K, trypsin, and bromelain, respectively, at 37°C for 1 h in order to evaluate its activity.

Flow cytometric analysis was further conuducted to verify the successful surface display of nitrilase on the spores of *B. subtilis* DB403. Purified spores were incubated overnight at 4°C in phosphate-buffered saline (PBS) (pH 7.4) containing nitrilase-specific antibody, and then incubated with FITC-conjugated goat anti-rabbit IgG for 1 h at 37°C after washing ten times. These spores were further examined under a FACStarPLUS flow cytometer (Becton Dickinson, USA) compared with *B. subtilis* DB403 (without pHS-CotB-nit).

Enzyme Activity Assays

The standard nitrilase activity was evaluated by mixing malononitrile (10 mM), PSB (pH 7.0), and the purified recombinant spore suspension in a total volume of 1 ml at 50°C. The mixed products, filtered by a 0.22-μm filter, were detected on a 7820A gas chromatograph equipped with a HP-5 column (Agilent, USA). The unit activity of nitrilase was defined as the amount of displayed nitrilase required to biocatalyze 1 μmol malononitrile in 1 min. Through serial dilution, the amount of spores could be directly counted from the plating medium.

Stability Evaluation of the Spore Surface-Displayed Nitrilase

In order to comprehensively understand the property stability and reusability of the nitrilase displayed on the spore surface, we further evaluated the effects of different chemicals, temperature or pH on the surface-displayed nitrilase. The effects of temperature ranging from 35°C to 65°C were evaluated by using malononitrile as the substrate. The stability of the displayed nitrilase was evaluated by incubating at 50°C, 55°C, and 60°C for 6 h. In addition, the effect of buffers at pH ranging from 3–9 on the displayed nitrilase was investigated at 50°C. Then the pH stability of the displayed nitrilase was tested in different buffers (pH 6.0, 7.0, 8.0) for 6 h at 50°C. With the purified native nitrilase as a control, we calculated the residual activity of the displayed nitrilase relative to the activity of the untreated ones (defined as 100%). In addition, the effects of several chemical reagents on the displayed nitrilase were studied by incubating in EDTA (1 mM), DTT (1 mM), PMSF (1 mM), methanol (20%, v/v), ethanol (20%, v/v), DMSO (20%, v/v) and SDS (1%,v/v), respectively. Under the standard activity assay, the reusability of the spore surface-displayed nitrilase was further tested after 10 cycles of use at 50°C and pH 7.0.

Upscale Bioconversion of Spore Surface-Displayed Nitrilase to Tomalononitrile, Succinonitrile and Glutaronitrile

In a typical procedure, a volume of 10 ml containing either 0.50 g of cell lyophilized powder of free nitrilase or 0.50 g of spore surface-displayed nitrilase lyophilized powder and 1 M substrate in PSB (pH 8.0) were mixed and reacted for 24 h. The products 2-cyanoacetic acid, 3-cyanopropionic acid, and 4-cyanobutyric acid were analyzed according to standard enzyme activity assays.

Data Analysis

The data were analyzed using GraphPad Prism 5. All measured variables were presented as means ± standard deviation (SD). Differences in all parameters were tested via one-way analysis of variance (ANOVA). $P < 0.05$ indicates statistical significance when testing all treatment groups versus the vehicle-treated control group.

Results

Construction of Recombinant Plasmid

The *A. facilis* 72W nit gene was synthesized as described in Materials and Methods. The cotB gene was amplified by PCR from *B. subtilis* 168 genome successfully. The obtained DNA fragments, which encodes the genes of nitrilase and CotB, was then ligated into the predigested shuttle vector in order to construct the pHS-CotB-nit plasmid (Fig. 1). The resulting expression constructs with the pHS-CotB-nit gene were further confirmed by DNA sequencing.

Determination of CotB-nit Expression on the *B. subtilis* Spore Surface

Western blot analysis was applied to determine whether the nitrilase was displayed on the *B. subtilis* DB403 spore surface. The molecular weights of CotB and nit were 59 and 36.9 kDa, respectively. An evident band with relative molecular mass around 96 kDa was found in lane 2, while there was no band in lane 3 (Fig. 2), indicating a successful surface display of nitrilase on the spore of *B. subtilis* DB403.

With malononitrile as a substrate, the activity of the

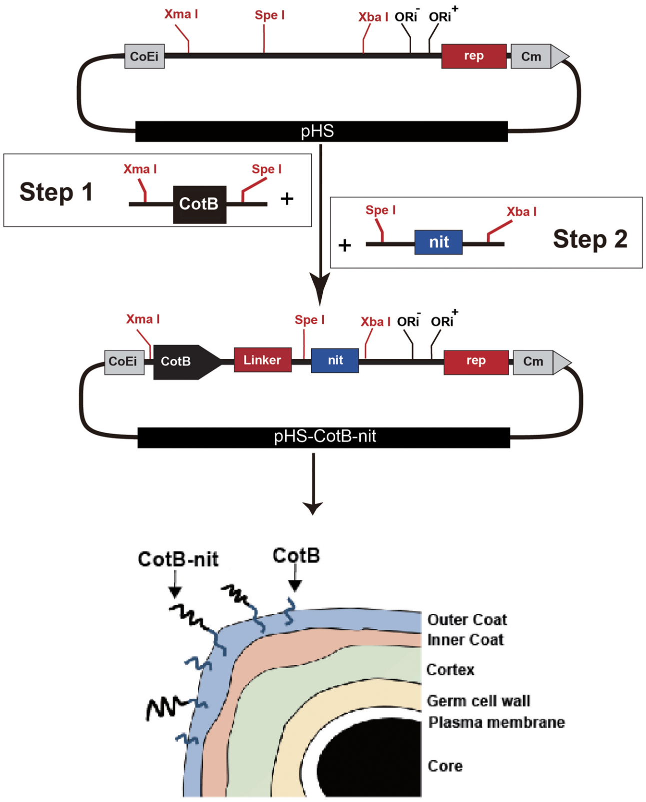

Fig. 1. Schematic diagram of the construction of recombinant vector pHS-CotB-nit.

CotB and nit represents the encoding gene of spore coat protein from *B. subtilis and* nitrilase from *A. facilis* 72W, respectively.

displayed nitrilase was determined after treatment with proteinase or phosphate buffer. Proteinase can digest the protein in the external spore environment, but cannot

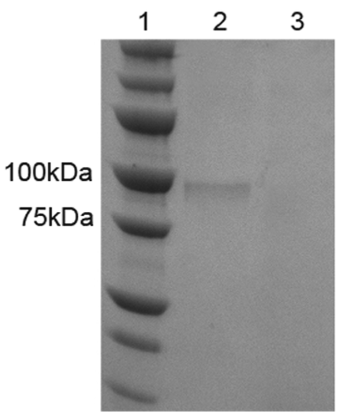

Fig. 2. Western blot analysis.

Line 1, marker; Lane 2, proteins extracted from recombinant spores of *B. subtilis* DB403 (pHS-CotB-nit); Lane 3, proteins extracted from spores of *B. subtilis* DB403.

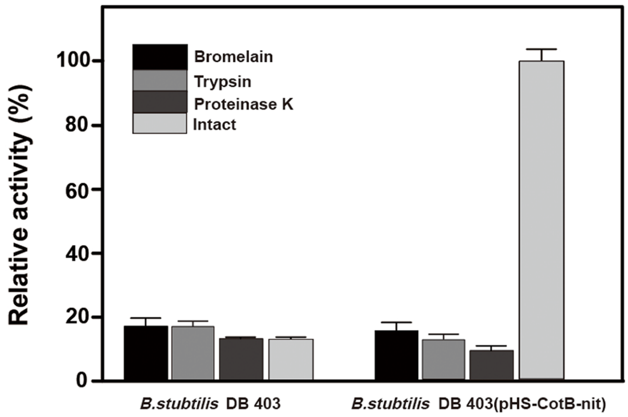

Fig. 3. Enzyme activity in proteolytic test.

Results are presented as means ± SD (n = 3 each group).

penetrate through the spore wall. The intact spores were noticeably more active than the spores treated with different proteinases (Fig. 3), verifying the nitrilase was indeed successfully displayed on the spore.

Flow cytometric analysis further confirmed that the nitrilase was on the spore surfaces. As shown in Fig. 4, fluorescence intensity of the spores (CotB-nit) was significantly increased compared with the control. This indicates that nitrilase was successfully displayed on the spore surface of *B. subtilis* DB403.

Optimum Reaction Temperature and Thermostability

The optimal temperature of the displayed nitrilase was carefully investigated in the range of 35–65°C. As illustrated in Fig. 5A, the maximum activity of the displayed

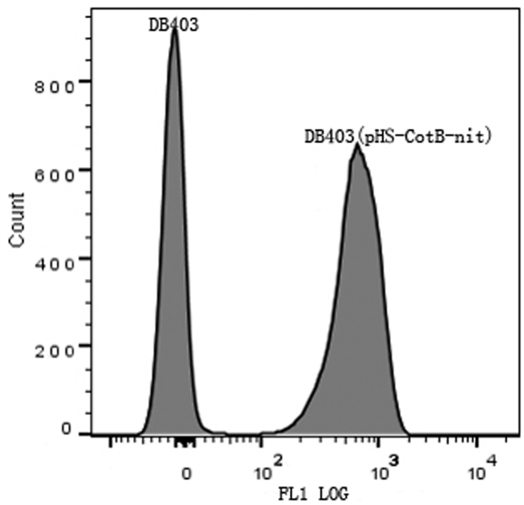

Fig. 4. Flow cytometric analysis of *B. subtilis* DB403 (control) and *B. subtilis* DB403 (pHS-CotB-nit).

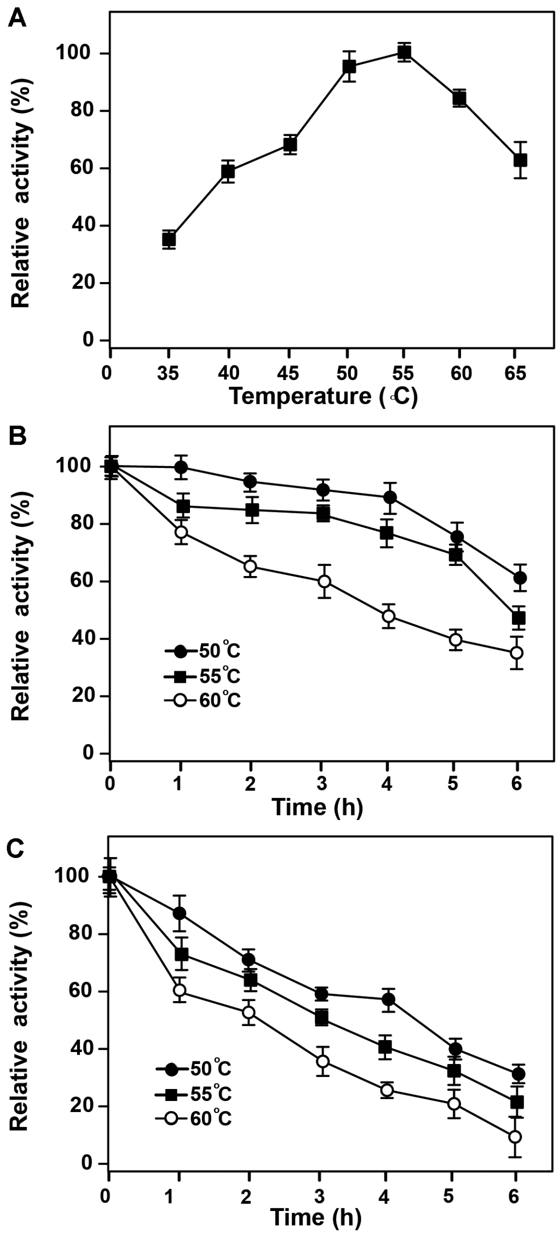

Fig. 5. Optimal temperature (**A**), thermal stability of the spore surface-displayed nitrilase (**B**) and the free nitrilase (**C**).
Results are presented as means ± SD (*n* = 3 each group).

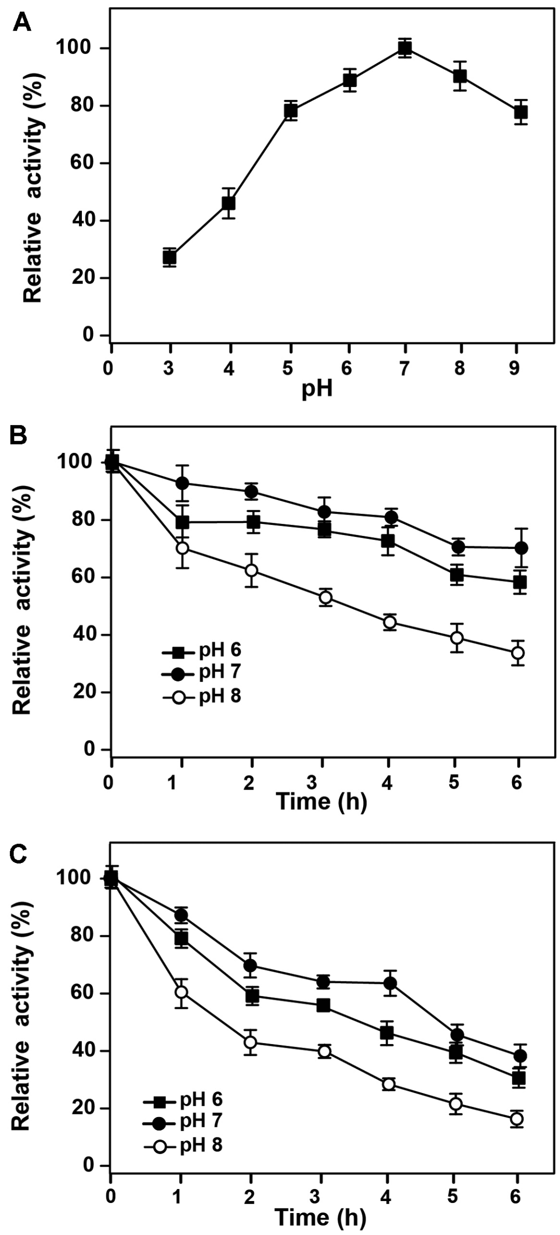

Fig. 6. Optimal pH (**A**), pH stability of the spore surface-displayed nitrilase (**B**) and the free nitrilase (**C**).
Results are presented as means ± SD (*n* = 3 each group).

nitrilase occurred at 50°C. For temperatures ranging from 50–60℃, the enzyme activity was not clearly affected and remained at an elevated level (more than 80%). When the temperature fell to 35℃, however, nitrilase activity decreased to less than 40% of its maximum value. Next, the thermostability of the displayed nitrilase was evaluated in buffers at 50°C, 55°C, and 60°C. After 6 h of incubation, the displayed nitrilase activity was still greater than 60% of its initial value at 50°C, but retained less than 60% and 40% of its original activity at 55°C and 60°C, respectively (Fig. 5B). Moreover, the non-immobilized nit retained only 32%, 22%, and 8%, respectively, of its original activity under the same conditions, which might be due to enzyme denaturation under high temperatures (Fig. 5C).

Table 2. Stability of spore surface-displayed nitrilase in various chemicals. (Results are presented as means ± SD, n = 3 each group).

Solution	Residual acitivity (%)	
	Spore surface-display nitrilase	Free nitrilase
EDTA	79 ± 5.6	70.4 ± 3.3
PMSF	75 ± 7.8	58.4 ± 4.1
SDS	55.5 ± 5.6	43.4 ± 3.9
DMSO	45 ± 5.1	18.4 ± 1.9
Methanol	70.7 ± 5.9	39.2 ± 2.6
Ethanol	82.9 ± 5.2	38.3 ± 3.2

Results are presented as means ± SD (n = 3 each group).

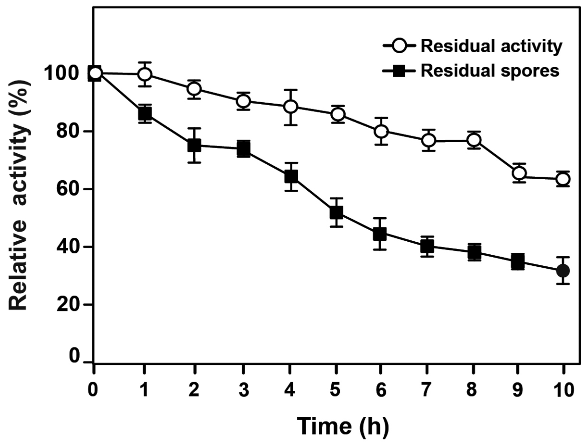

Fig. 7. Reusability tests of spore surface-dispalyed nitrilase. Results are presented as means ± SD (n = 3 each group).

Optimum Reaction pH and pH Stability

The activity of the displayed nitrilase maximized at pH 7.0 (Fig. 6A). Interestingly, for pH values ranging from 5–9, the enzyme activity was not significantly affected and remained at an elevated level, but dropped to less than 50% of its maximum value when the pH decreased to 3 or 4.

The pH stability tests in buffers at pH 6.0, 7.0, and 8.0 revealed that the displayed nitrilase still retained over 70% of its initial activity for 6 h at the optimal pH value of 7.0, but retained only about 60% and 40% at pH 6.0 and 8.0, respectively (Fig. 6B). After the 6 h incubation, the non-immobilized nitrilase retained only 46%, 58%, and 16% of its original activity at pH 6.0, 7.0, and 8.0, respectively (Fig. 6C).

Chemical Resistance of the Displayed Nitrilase

The durability of the displayed nitrilase in various chemicals is an important evaluation parameter. Not surprisingly, the displayed nitrilase was more stable than the native version in all of the tested chemical reagents, with the most significant changes occurring in the ethanol and methanol groups, which both rose more than 30% compared to the native nitrilase (Table 2). Notably, the stability of the free nitrilase was lower than that of the displayed nitrilase when EDTA was used as the solution. With the exception of SDS and DMSO, the displayed nitrilase maintained high activity (>70%) in all of the other chemicals, indicating an enhanced chemical resistance compared with the free version.

Reusability of Spore Surface-Displayed Nitrilase

Since reusability of surface-displayed enzymes is one of the most important characteristics during industrial application, we further evaluated the reusability of the displayed nitrilase in optimal temperature and pH conditions via repetitive enzymatic hydrolysis reactions. The displayed nitrilase maintained over 80% of its corresponding initial activity after at least 7 cycles (Fig. 7). However, the amount of recombinant spores decreased noticeably and was less than 40% of its initial level following 7 reaction cycles. After 10 complete reaction cycles, the residual activity and centrifuged recombinant spores retained were 64.8 ± 3.2% and 37.2 ± 5.2% of their original levels, respectively.

Upscale Bioconversion of Spore Surface-Displayed Nitrilase to Malononitrile, Succinonitrile and Glutaronitrile

As the substrate concentration increased to 1 μM in a reaction volume of 10 ml, spore surface-displayed nitrilase exhibited higher hydrolytic activity per unit mass than the whole-cell expressed by *E. coli* (pET-28a-nit) (Table 3). In addition, the yields of cyanocarboxylic acid by both the

Table 3. Upscale bioproduction of 2-cyanoacetic acid, 3-cyanopropionic acid and 4-cyanobutyric acid.

Biocatalyst	2-cyanoacetic acid (M)	3-cyanopropionic acid (M)	4-cyanobutyric acid (M)
BL21 (pET-28a-nit)	0.91 ± 0.05	0.82 ± 0.01	0.75 ± 0.1
Spore surface-displayed nitrilase	1 ± 0.02	0.94 ± 0.02	0.91 ± 0.02

Results are presented as means ± SD, n = 3 each group.

pET-28a-nit and spore surface-displayed nitrilase groups decreased with the increasing number of carbons. Moreover, the biotransformation efficiency was equal or close to 100% in all of the displayed nitrilase-treated groups. Not surprisingly, this efficiency was 91%, 82%, and 75% in the BL21 (pET-28a-nit)-treated groups corresponding to the hydrolysis of malononitrile, succinonitrile and glutaronitrile, respectively.

Discussion

Over the past several decades, nitrilase-based biocatalysis has shown some promising application possibilities that have drawn considerable attention from both scientists and entrepreneurs. Microbial catalysts with nitrile nitrilase enzyme activity can convert nitriles to specialty or commodity chemicals, as well as agrochemical or pharmaceutical intermediates. The application of nitrilase in chemical production has been extensively studied, revealing various nitrilase-producing organisms, such as bacteria, yeasts, and plants [4]. From the viewpoint of biotechnological application, nitrilase, as a green catalyst for the production of high-value-added compounds and other catalytic applications in mild conditions, is more controllable, with relatively low cost and high yield [7, 33, 34]. Immobilization of whole cells and purified enzymes can make the biocatalytic process more economical. The availability of immobilized biocatalytic recycling can greatly reduce the operational costs of production [35]. To date, most nitrile industrial biocatalysis processes have been performed using immobilized biocatalysts, which could significantly reduce production costs.

Recently, the spore surface-display system has been widely used in many industrial fields, including the immobilization of different catalytic enzymes. As an alternative to other existing methods for immobilizing enzymes, using *B. substilis* spores as the enzyme carriers in biocatalysis appears to be an effective choice [20, 24−26]-[30, 36−39]. Of these proteins, composed of a spore cot, CotB, a 59 kDa outer coat protein, is mainly utilized in biocatalysis since it is an effective anchor protein for spore surface display [25, 40].

In this study, we displayed nitrilase from *A. facilis* 72W on the spore surface of *B. subtilis* by using an immobilization method in which CotB was utilized as the fusion partner. The pHS, an *E. coli*–*B.subtilis* shuttle vector with high copy number, was chosen in order to improve the expression stability of nitrilase during the spore-forming process. As a result, CotB-nit, a 96 kDa fusion protein, was successfully displayed on the spore surface of *B. subtilis* DB403, as confirmed by western blot (Fig. 2), a proteinase treatment test (Fig. 3) and flow cytometric analysis (Fig. 4). Moreover, what deserves more investigation is that the expression of CotB-nit is not very high which may affect the overall application on an industrial scale. Therefore, we need to improve it in the follow-up study, by changing the expression vector or through optimization of expression conditions.

Reaction stability, including thermo- and pH stability, has been proven to be one of the key requirements for biocatalysts. Goldlust et al. demonstrated that inactivation of nitrilase only requires 10 min of heating at 40°C or higher and the activity of displayed nitrilase maximized at 50°C (Fig. 5A), whereas the previous study found that it was 45ºC for free ones [41]. After 6 h of incubation, the displayed nit still retained no less than 60% of its initial activity, while less than 60% and 30% were retained after incubation at 55°C and 60°C, respectively (Fig. 5B). In addition, the free nitrilase retained only 32%, 22%, and 8% of its corresponding original activity at 50°C, 55°C, and 60°C, respectively, indicating that the enzyme may have been denatured after the prolonged incubation at high temperatures (Fig. 5C). To further characterize the spore surface-displayed enzyme, we conducted subsequent experiments at 50°C. The highest activity of the displayed nitrilase was found at pH 7.0 (Fig. 6A). After incubation at pH 7.0 for 6 h, the displayed nitrilase still retained more than 70% of its initial activity, compared with less than 40% of the initial activity in the free nitrilase (Figs. 6B and 6C).

Enzymatic reactions in organic solvents, used as co-solvents to increase the solubility of substrates (*e.g.*, lipase-mediated reactions), have been extensively reported [42, 43]. High chemical resistance is essential for the application of the displayed nitrilase due to the poor solubility of most nitriles. Previous studies have proved that almost half of its initial activity is lost in the presence of either 5% n-hexane or 5−15% ethanol [42]. Therefore, the stability of nitrilase in aqueous environments as well as in organic solvents was carefully studied. Not surprisingly, the spore surface-displayed nitrilase exhibited both higher stability and 10–30% greater activity than free nitrilase (Table 2).

In terms of reusability, the displayed nitrilases still retained 83% and 64% of their initial activity after 5 and 10 cycles of use, respectively (Fig. 7). Moreover, 40% of the initial recombinant spores were still retained. Finally, we compared the *A. facilis* 72W nitrilase expressed in *E. coli* and the surface-displayed ones in terms of hydrolysis efficiency for the upscale bioconversion of dinitriles in the production of cyanocarboxylic acid (Table 3). The above test results of our surface-displayed nitrilase, especially for reusability and enlarged-scale hydrolysis efficiency, exhibited prominent advantages over several published immobilized choices and also proved that it has superb application potential [20, 24−26].

As a result, we demonstrated a successful enzyme immobilization using CotB as the fusion carrier for the expression of the nitrilase fusion protein on the *B. subtilis* spore surface, as verified by western blot analysis and activity measurement. Stability tests revealed that the thermo-, pH, and chemical stability of the displayed nitrilase were all significantly better than those of the free nitrilase. In addition, the displayed nitrilase could be reused up to 10 times with more than 60% residual activity. Furthermore, we conducted upscale experiments in order demonstrate that *A. facilis* 72W nit, using CotB as fusion partner and displaying on the spore surface, was more efficient and durable in transforming dinitriles of varying chain lengths to corresponding cyanocarboxylic acid than free nitrilase expressed by *E. coli*. In summary, this strategy of enzyme immobilization may be applicable to other plentiful enzymes in the organic synthesis industry in addition to *A. facilis* 72W nitrilase.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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