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ORIGINAL PAPER

Desulfurization of dibenzothiophene by *Bacillus subtilis* recombinants carrying *dsz*ABC and *dsz*D genes

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Abstract The desulfurization (*dsz*) genes from *Rhodococcus erythropolis* DS-3 were successfully integrated into the chromosomes of *Bacillus subtilis* ATCC 21332 and UV1 using an integration vector pDGSDN, yielding two recombinant strains, *B. subtilis* M29 and M28 in which the integrated *dsz* genes were expressed efficiently under the promoter, *Pspac*. The dibenzothiophene (DBT) desulfurization efficiency of M29 was 16.2 mg DBT l^{-1} h^{-1} at 36 h, significantly higher than that of *R. erythropolis* DS–3 and *B. subtilis* M28 and also showed no product inhibition. The interfacial tension of the supernatant fermented by M29 varied from 48 mN m^{-1} to 4.2 mN m^{-1}, lower than that of the recombinant strain, M28, reveals that the biosurfactant secreted from M29 may have an important function in the DBT desulfurization process.

Keywords *Bacillus subtilis* · Desulfurization · Dibenzothiophene · *dsz* genes · *Rhodococcus erythropolis*

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Introduction

The burning of fossil fuels contained sulfur compounds leads to environmental pollution such as acid rain. Biological desulfurization (BDS) using microorganisms to remove or lower the sulfur content in fuels, has been developed to decrease this pollution (Ohshiro and Izumi 1999). As a model compound, dibenzothiophene (DBT) is desulfurized to 2-hydroxybiphenyl (2-HBP) by the enzymes encoded by *dsz*ABC and *dsz*D genes from *Rhodococcus erythropolis* IGTS8. However, several factors, such as enzyme specificity, the desulfurization rate, environmental factors, product inhibition and the oil–water separation, may limit its commercial applications. BDS can be performed more efficiently by a number of techniques such as changing the promoter, co-expressing the flavin oxido-reductase gene (Ohshiro et al. 2002) and reconstructing the *dsz* promoter for reducing product inhibition (Matsui et al. 2002). Other possibilities also exist including engineering DszC for widening the substrate range (Arensdorf et al. 2002), expressing two sets of *dsz*B for enhancing the amount of rate-limiting enzyme (Nakayama et al. 2002), altering the fluidity of the cell membrane by mutation to improve the pre-digesting mechanism of DBT (Watanabe et al. 2003a) but, the method most widely used is expressing the *dsz* genes in a heterogenous host

such as a *Pseudomonas* sp. (Noda et al. 2003) or a *Mycobacterium* sp. (Watanabe et al. 2003b). However, there have not been any reports on the expression of the *dsz* genes in *B. subtilis* that is widely used in the industry as an optimal host, or on the effect of the biosurfactant on BDS process. In this study, we have established a better desulfurization system with higher desulfurization efficiency by cloning the *dsz* genes and integrating them into *B. subtilis* ATCC21332 and UV1. The recombinant strain, *B. subtilis* M29 was shown to secrete the biosurfactant into the culture media for making cells to touch organic phase more easily.

Materials and methods

Strains, plasmids and media

Rhodococcus erythropolis DS-3, identified and conserved in China General Microbiological Culture Collection Center (CGMCC No. 1465), was previously screened from the soil and the sewage in Gudao Oil Field, China. Expression vector, pHT315, integration vectors, pMUTIN4 and pDG1728, were kindly provided by *Bacillus* Genetic Stock Center (BGSC). *B. subtilis* UV1 was obtained as a mutant from *B. subtilis* ATCC21332 (BGSC), which lacks the capability of producing biosurfactant. *R. erythropolis* DS-3 was cultivated in Basal Salt Medium (BSM) as described in a previous method (Ma et al. 2002). *B. subtilis* strains were cultivated in a minimal medium (NK medium) containing 0.2 g $MgSO_4 \cdot 2H_2O$, 4.1 g KH_2PO_4, 14.3 g Na_2HPO_4, 4 g NH_4NO_3, 0.013 g $FeCl_3 \cdot 4H_2O$, 0.02 g $CaCl_2$, 0.005 g $MnCl_2 \cdot 2H_2O$ and 10 g glucose in 1 l distilled water (pH 7.2). *E. coli* was cultivated in Luria-Bertani (LB) medium. Antibiotics were used when needed at: 100 mg ampicillin l^{-1}, 25 mg erythromycin l^{-1}, 100 mg spectinomycin l^{-1} or 12.5 mg lincomycin l^{-1} (Sigma). *B. subtilis* were cultured at 30°C in a rotary shaker at 180 rpm. The *dsz*ABC and *dsz*D gene sequences of *R. erythropolis* DS-3 have been deposited in GenBank sequence database with the Accession Nos. DQ444325 and DQ444326, respectively.

Electroporation

Electroporated *B. subtilis* cells were pre-cultivated in a 500 µl recovery medium (LB medium containing 0.5 M sorbitol and 0.38 M mannitol) at 37°C for 3 h on a shaker with 100 rpm, and then streaked on a LB agar plate containing antibiotics.

DBT desulfurization assay and analysis of DBT concentration

The recombinant strains were cultured in 100 ml NK medium and *R. erythropolis* DS-3 in BSM medium at 30°C for 60 h on a shaker. 0.5 mM DBT in 10 ml *n*-hexadecane was added into the medium as a sulfur source. At intervals, 10 µl was extracted from the organic phase and analyzed by HPLC (Waters 600E) using a C18 column (5 μ, 3.9 × 150 nm) with a diode array detector at 244 nm. The mobile phase was a methanol/water (0.8:0.2, v/v) at 1 ml min^{-1}. The injection volume was 10 µl.

Results

Construction of the integration vector pDGSDN

As shown in Fig. 1, the integration vector, pDGSDN, was constructed by ligating a DNA fragment containing the promoter *Pspac* and *dsz* genes from pHTSDN and a linear plasmid pDG1728 generated by *Hin*dIII and *Eco*RI, which carries two partial fragments used for integration and is derived from an amylase gene (*amyE*) homologous to the chromosome of *B. subtilis*. All of the plasmids were amplified in *E. coli* DH5α grown with 100 mg ampicillin l^{-1}.

Screening for the recombinants containing the dsz genes-integrated

In *B. subtilis*, homologous recombination usually occurs between two homologous DNA fragments around 150 bp or longer. In this experiment the homologous recombination happened at the location of the amylase gene (*amy*E) on the

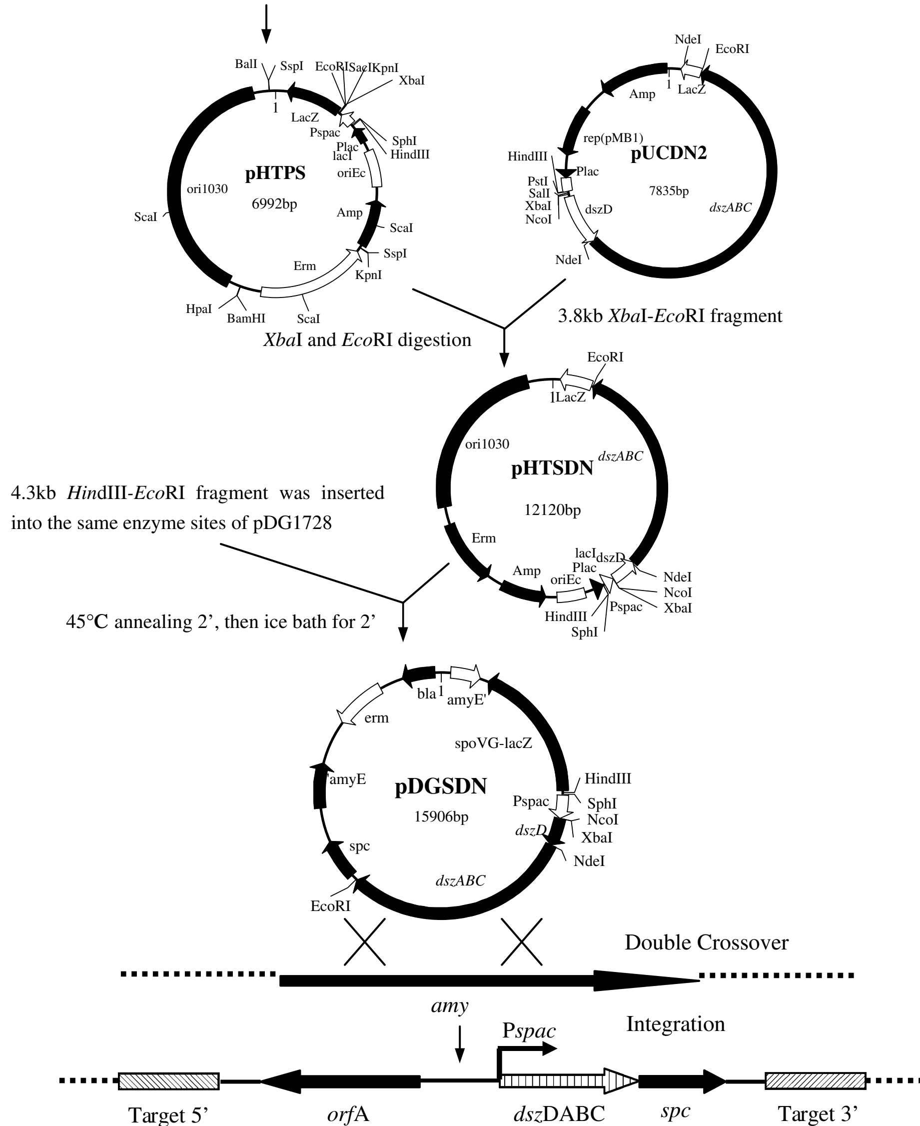

Fig. 1 Construction of integration vector pDGSDN. A 360 bp putative promoter element *Pspac* from a gram positive bacterium was amplified from pMUTIN4 using the sense primer with *Sph*I: 5′-TTCGGCATGCAAATTATGGGTGAA-3′, and the antisense primer with *Xba*I: 5′-GCGATCTAGATGGGTAACGCCAGG-3′. The PCR product was digested with *Sph*I and *Xba*I, and inserted into the pHT315 at the same restriction cleavage sites to construct the plasmid pHTPS. The pHTPS was digested with *Xba*I and *Eco*RI, and then ligated with a DNA fragment containing *dsz*D and *dsz*ABC from pUCDN2 cut with *Xba*I and *Eco*RI, to make pHTSDN. The integration vector pDGSDN was constructed by ligating a DNA fragment containing the promoter *Pspac* and *dsz* genes from pHTSDN digested with *Hin*dIII and *Eco*RI, and a linear plasmid pDG1728 generated by *Hin*dIII and *Eco*RI. This vector also contains ampicillin resistance gene (amp), erythromycin resistance gene (erm), spectinomycin resistance gene (spc), *E. coli* origin of DNA replication (*ori*) and *B. subtilis* origin of DNA replication (*ori*1030). The pDGSDN was transformed to *B. subtilis* using Micropulser electrophoresis (BioRad, USA)

chromosome of *B. subtilis* and resulted in the loss of its amylase activity while the *dsz* genes remained intact. After pDGSDN was electroporated, and integrated in vivo into the *amy*E gene on the chromosome of *B. subtilis* ATCC21332 and UV1, approximately 100 recombinants were

obtained, which were resistant to 100 mg spectinomycin l^{-1}. About 20 of them were further determined sensitive to 25 mg erythromycin l^{-1} and 12.5 mg lincomycin l^{-1}. They were then grown in NK medium supplemented with 0.5 mM DBT in *n*-hexadecane (10%, v/v), and two of the 20 recombinants were selected from *B. subtilis* UV1 and ATCC21332, respectively, designated M28 and M29. Both M28 and M29 successfully expressed the desulfurization genes *dsz*ABC, and flavin reductase gene, *dsz*D.

Southern blot

The genomic DNA from the recombinant *B. subtilis* M29 was completely digested with *Eco*RI/*Hin*dIII, *Eco*RI/*Nde*I, *Pst*I, *Xba*I, *Xho*I and *Hin*dIII, respectively (Fig. 2a). The digested

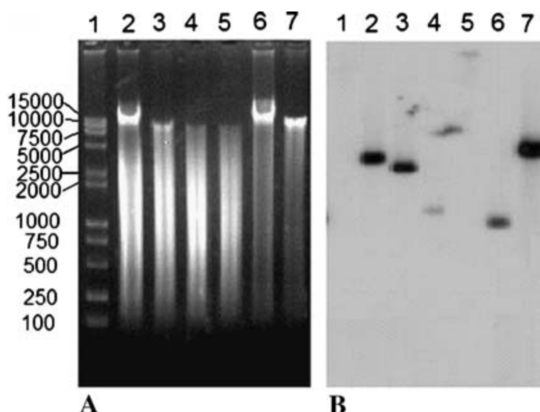

Fig. 2 Southern blot analysis of the recombinant M29 genomic DNA. A DNA fragment used as the radioactive probe, which located at 683–1276 bp of *dsz*C and was amplified with two primers as following: 5′-GAC-TTCCACAACGTCAAGGT-3′ (sense) and 5′-AGGAG GTGAAGCCGGGAAT-3′ (antisense), and radio-labeled with DIG DNA Labeling Kit (Roche). Hybridization was carried out under stringent conditions in 6× SSC at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The nylon membrane was washed in 0.1 ml 3× SSC–0.1% SDS at 65°C. (**A**) The total *B. subtilis* M29 genomic DNA digested with restriction enzymes and then separated by electrophoresis on a 0.8% agarose gel. Lane 2–7 shows the M29 genomic DNA samples digested by *Eco*RI/*Hin*dIII, *Eco*RI/*Nde*I, *Pst*I, *Xba*I, *Xho*I and *Hin*dIII, respectively. Hybridization signals in the middle of the gel or in the genomic DNA region are shown (**BZ**). The hybridization signal bands, in lane 2 (4.2 kb), lane 3 (3.6 kb), lane 6 (1.2 kb) and lane 7 (4.8 kb) correspond to the relevant fragments all containing the *dsz*C gene, respectively, and lane 1, 4 and 5 gave a clean background

genomic DNA samples were then subjected to Southern blot analysis. *Eco*RI, *Hin*dIII and *Xba*I were used for constructing the integration vector pDGSDN as described in Fig. 1. Each of the *dsz* genes has a single *Nde*I, *Pst*I and *Xho*I restriction cleavage site. Southern blot results are shown in Fig. 2b. There was the clear band in lane 2, 3, 6 and 7. The size of the band in lane 2 was equal to the total size of *Pspac*, *dsz*D and *dsz*ABC, and the 3.6 kb band in the lane 3 was identical to the size of *dsz*ABC. These results indicated that the fragment containing *dsz* genes was successfully integrated into the chromosome of *B. subtilis* M29. The same results were also validated in the *B. subtilis* M28.

DBT desulfurization by recombinant strains

Figure 3 shows the time course of the desulfurization of DBT in *n*-hexadecane by ATCC21332, M28, M29 and *R. erythropolis* DS-3. The strain DS-3 could desulfurize 0.42 mM DBT in 60 h. However, both M28 and M29 strains desulfurized 0.34 mM and 0.19 mM DBT, respectively, in 36 h. Cell populations dropped after 24 h, while the activity of the enzymes in the medium continually increased indicating a continuous desulfurization process in the cell culture. The selective desulfurization efficiencies of the strains M28 and M29 at 36 h were 8.4 mg and 16.2 mg DBT l^{-1} h^{-1},

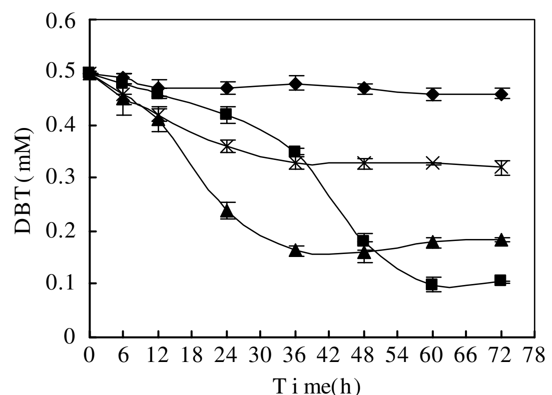

Fig. 3 Time course of DBT consumption by *B. subtilis* ATCC 21332, M28, M29 and *R. erythropolis* DS-3. The *B. subtilis* strains were cultured with a modified medium (NK2), the NK medium supplemented with 0.5 mM DBT dissolved in 10 ml *n*-hexadecane as the sulfur source for desulfurization assay. (♦) denotes *B. subtilis* ATCC21332, (×) M28, (▲) M29 and (■) *R. erythropolis* DS-3

while DS−3 was 13.1 mg DBT l^{-1} h^{-1} at 36 h and 16.7 mg DBT l^{-1} h^{-1} at 60 h. These results suggest that the desulfurization efficiency of M29 is significantly higher than that of M28 and DS-3 in 36 h. DS-3 could desulfurize more DBT in 60 h (84%) than M29 (62%).

Change of the medium's interfacial tension during desulfurization

As shown in Fig. 4, the interfacial tension between the aqueous and the oil phase in the media decreased from 48 mN m^{-1} to 4.2 mN m^{-1} by the recombinant M29 during the desulfurization process, lower than that of M28. [M28 was derived from a mutant strain *B. subtilis* UV1 that lacked the ability of producing surfactant in each desulfurization step.] The desulfurization efficiency of M29, denoted by sulfur removal from DBT in 36 h (0.26 mM), was higher than that of M28 (0.14 mM). The fermented broth with DBT in *n*-hexadecane was more completely emulsified by M29 than M28. Therefore these experimental results suggest that the biosurfactant might be necessary to allow the cells to make butter contact with the oil phase containing DBT so that the desulfurization process could be easily carried out, though the cells have the aqueous surface.

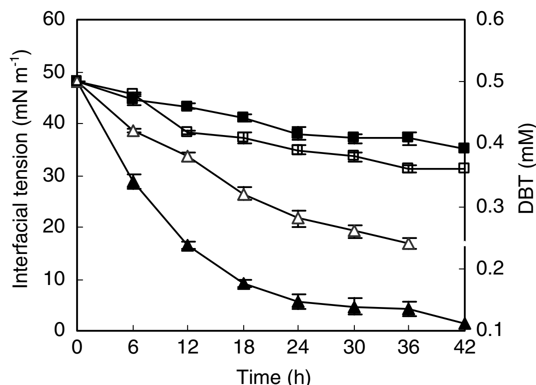

Fig. 4 Time course of interfacial tension between *n*-hexadecane and the medium during desulfurization by M28 and M29. The *B. subtilis* strains were cultured with NK2. The ferment medium was centrifuged in 8000*g* for 5 min to obtain a supernatant. The interfacial tension of the supernatant was detected by Processor Tensiometer K100 (Kruss, Germany). The solid patterns indicate the interfacial tension curve, and the hollow patterns represent the DBT consumption curve. (■) and (□) denote M29; (▲) and (Δ) indicate M28

Discussion

Previous studies have shown that the expression of *dsz* genes under the *dsz* promoter was completely repressed by 0.3 mM 2-HBP or 1.4 mM sulfate (Ma et al. 2002). In this work, the desulfurization process by the strain M29 could not be inhibited by sulfate or the metabolic product of DBT, 2-HBP, suggesting that the Dsz enzymes might be efficiently produced in the strain M29 under the *spac* promoter. It seems that the desulfurization efficiency of the recombinant *B. subtilis* strain was only limited by its growth period and its ability of contacting the oil phase for pre-digesting the substrate during the desulfurization process. Recombinant *B. subtilis* strains are widely used as one of the best expression hosts for producing foreign proteins, which suggests an advantage of using *B. subtilis* M29 for DBT desulfurization. It is likely that the desulfurization efficiency of the recombinant strains rests on the ability of cells to uptake the DBT or alkyl DBT from the media. Furthermore, a type of biosurfactant secreted by the host cell itself could also increase the ability of closing DBT from aqueous phase. By contrasting the DBT desulfurization rate of the two recombinant *B. subtilis* strains, M29 and M28, with their abilities of secreting the biosurfactant, we concluded that the biosurfactant produced by the host cell itself could increase the DBT desulfurization rate. A surfactant, like Tween 80, can indeed, increase the desulfurization rates of bacteria (Jiang et al. 2002), however, adding additional surfactants might cost more in an industrial process. A recombinant strain like M29, which can secrete biosurfactant itself, will benefit the DBT desulfurization process as a potential industrial strain. However, the mechanism of the DBT-uptake from the external environment into the cells is not yet clear. Further research is required to demonstrate why and how the biosurfactant in a growth medium can enhance cellular uptake of the water-insoluble compounds, and investigate the differences of the uptake mechanisms of *R. erythropolis* and *B. subtilis*.

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