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Analysis of Dibenzothiophene Desulfurization in a Recombinant *Pseudomonas putida* Strain▽

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Biodesulfurization was monitored in a recombinant *Pseudomonas putida* CECT5279 strain. DszB desulfinase activity reached a sharp maximum at the early exponential phase, but it rapidly decreased at later growth phases. A model two-step resting-cell process combining sequentially *P. putida* cells from the late and early exponential growth phases was designed to significantly increase biodesulfurization.

Biodesulfurization (BDS), based on the application of microorganisms that selectively remove sulfur atoms from organosulfur compounds, appears to be a viable technology to complement the traditional hydrodesulfurization of fuels (9, 12, 18, 21, 22). Dibenzothiophene (DBT) has been widely employed as the model compound of polycyclic organic sulfur components in fuels. DBT desulfurization in *Rhodococcus erythropolis* IGTS8, a model bacterium used in BDS, is catalyzed through the 4S pathway composed of two-component, flavin-dependent monooxygenases, i.e., the DszA and DszC oxygenase components and the DszD flavin reductase, and a desulfinase, DszB. DszC catalyzes DBT transformation into dibenzothiophene sulfoxide (DBTO) and dibenzothiophene-sulfone ($DBTO_2$), whereas DszA further oxygenates $DBTO_2$ to 2-hydroxybiphenyl-2-sulfinic acid (HBPS). In the last step of this pathway, DszB hydrolyzes HBPS into 2-hydroxybiphenyl (HBP) and sulfite (4, 5, 10–13, 20, 21). The 4S pathway has been shown to exist in a wide variety of bacterial genera, such as *Paenibacillus*, *Pseudomonas*, *Corynebacterium*, and *Mycobacterium*, among others (9, 11, 12, 21, 22).

Several bottlenecks that limit the commercialization of BDS have been identified, and different strategies based on designing recombinant Dsz pathways and microorganisms have been accomplished to alleviate such limitations (3, 7, 9, 11, 12, 14, 15, 19, 22). In an attempt to expand our understanding of the BDS process at a molecular level, the activity of the three 4S route enzymes in the recombinant biocatalyst *Pseudomonas putida* CECT5279, which harbors the *R. erythropolis* IGTS8 *dszABC* genes in plasmid pESOX3 and the *hpaC* gene encoding the *Escherichia coli* flavin mononucleotide:NADH HpaC reductase (a DszD equivalent) inserted in its chromosome (6), has been determined.

Optimal desulfurization capacity of the *P. putida* recombinant cells. To determine the optimal BDS capacity of *P. putida* CECT5279, cells were cultured in a bioreactor (2 liter) using basal salt medium, containing 2 mM $MgSO_4$ as a sulfur source and 20 g liter^{-1} glutamic acid as a carbon source, as previously described (17). Samples collected at different growth times were used for resting-cell desulfurization assays using the substrates for all the 4S route enzymes (DBT, DBTO, $DBTO_2$, and HBPS). Cells (0.7 g dry cell weight [DCW]/liter) were suspended in 50 mM HEPES buffer (pH 8.0) and 10 μM substrate, and desulfurization was carried out at 30°C for 2 h. Samples (0.25 ml) were collected, and the concentrations of DBT, DBTO, $DBTO_2$, HBPS, and HBP were determined by high-performance liquid chromatography as previously described (16, 17). The BDS capability (X_{BDS}) of the cells was determined as the percentage of desulfurization according to the equation $X_{BDS} = (C_{HBP,2}/C_{DBT,0}) \times 100$, where $C_{DBT,0}$ is the initial concentration of DBT (μM) and $C_{HBP,2}$ is the HBP concentration (μM) after a 2-h resting-cell assay. Figure 1A shows that the maximum X_{BDS} (85% BDS) was reached with *P. putida* CECT5279 cultures at the mid-exponential phase (cells grown for 10 h), in clear contrast with the behavior observed for *R. erythropolis* IGTS8 cells that reached the maximum X_{BDS} at the stationary phase of growth (4). To gain some insights into this peculiar BDS behavior, the activities of the three enzymes DszA, DszB, and DszC were monitored by resting-cell assays (samples were taken every 5 min for half an hour) along the growth curve of *P. putida* CECT5279. It has been previously pointed out that the membrane transport of all the 4S route intermediates, the intracellular concentration of reducing cofactors, and the activity of the recombinant HpaC reductase (a DszD functional equivalent) did not influence the desulfurization process rate (2). The activity of each enzyme (a_j) (μmol/min/g DCW) was defined as the specific time-zero reaction rate according to the equation $a_j = dC_j/(C_x \times dt)_{t=0}$, where C_j is the concentration of the enzyme substrate j (μmol/liter) and C_x is the concentration of the biomass (g DCW/liter) employed in resting-cell assays (8). The experimental data (C_j versus time) were fitted to a first-order decay exponential equation. Figure 1B shows that the activities of the three enzymes followed clearly different patterns along the growth curve, which explains why X_{BDS} of the recombinant cells reaches the maximum level after 10 h of growth. Thus, al-

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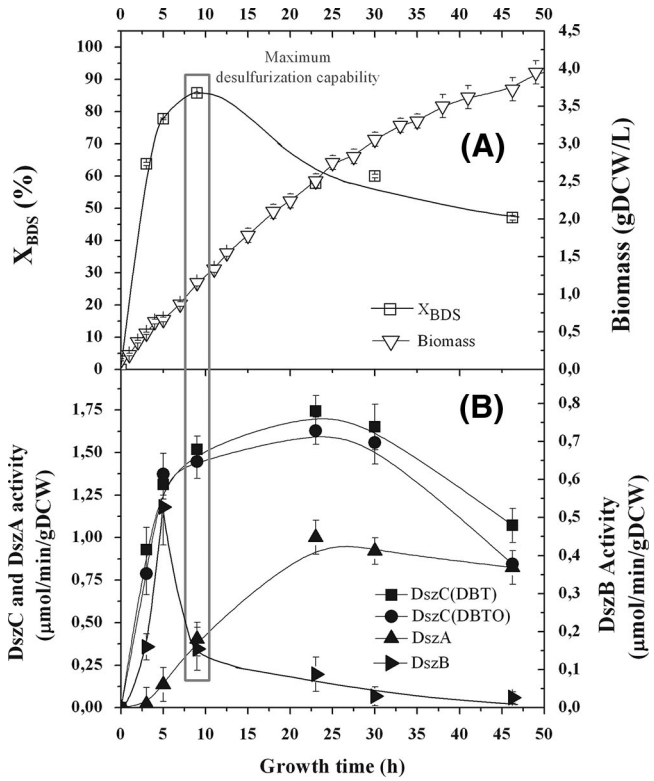

FIG. 1. Desulfurization ability (A) and DszA, DszB, and DszC enzyme activities (B) in *P. putida* CECT5279 cells along the growth curve. The standard deviations of three different experiments are shown.

though after 5 h of growth the activities of DszB and DszC are near their maximum, the activity of DszA is still very low to support an efficient desulfurization and, subsequently, is acting as the BDS limiting step. Five hours later, the cells reach the optimal combination between the decreasing activity of DszB and the increasing activity of DszA, and therefore they are able to desulfurize DBT at the maximum rate. After that, the activity of DszB decreases, acting as the new limiting BDS step (Fig. 1B). Since the genes *dszABC* are expressed as an operon under the control of the *Ptac* promoter in plasmid pESOX3 (7), the differential activity profiles shown by the DszA, DszB, and DszC enzymes along the growth curve in *P. putida* CECT5279 can be due to a still-unknown posttranscriptional regulation mechanism, e.g., mRNA processing, translation efficiency, etc., that deserves further studies.

To study further the drastic decrease in DszB activity after 5 h of growth (Fig. 1B), we checked whether it could be due to a rapid proteolysis of DszB along the growth curve. However, the amount of DszB protein increased along the growth curve (Fig. 2), strongly suggesting that the enzyme does not disappear from the host cells after 5 h of growth. Thus, the inactivation of the desulfinase activity rather than the degradation of the DszB protein appears to account for the observed drop and lack of HBPS desulfinase activity after the early exponential growth phase of the recombinant *P. putida* CECT5279 cells. Different mechanisms for DszB inactivation, e.g., oxidative stress (2), can be foreseen, and further work needs to be

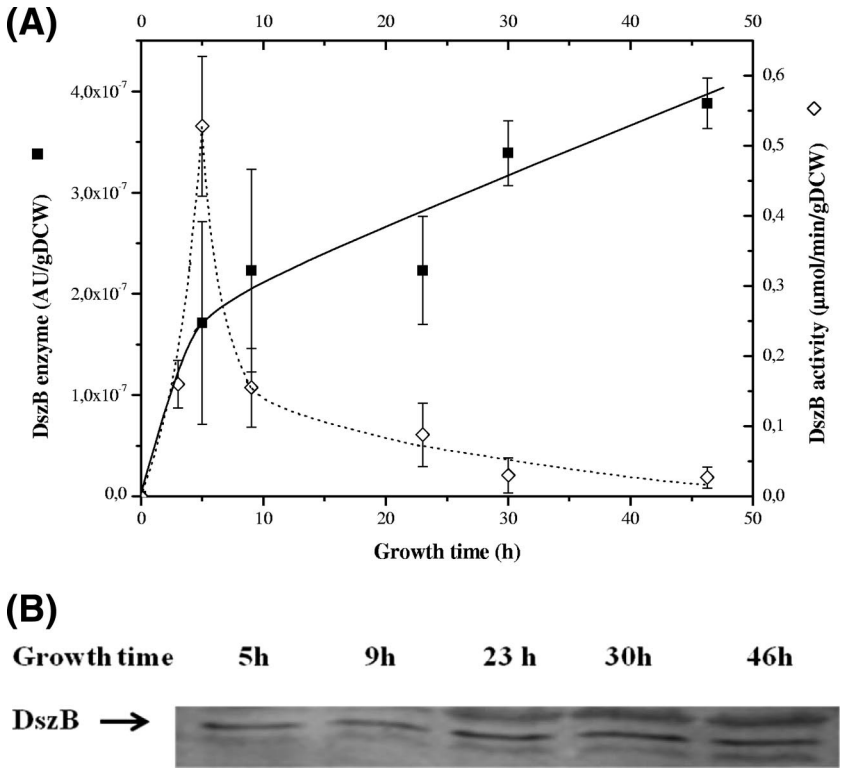

FIG. 2. DszB expression pattern in *P. putida* CECT5279. (A) DszB production and activity along the growth curve. The bars indicate the standard deviations of three different experiments. (B) Western blotting of crude extracts from cells collected at different growth times was performed by using an anti-DszB serum. The specific proteins were identified using anti-rabbit horseradish peroxidase-labeled secondary antibody with ECL Western blotting chemoluminescent reagent (Amersham). The location of the DszB protein band is indicated with an arrow.

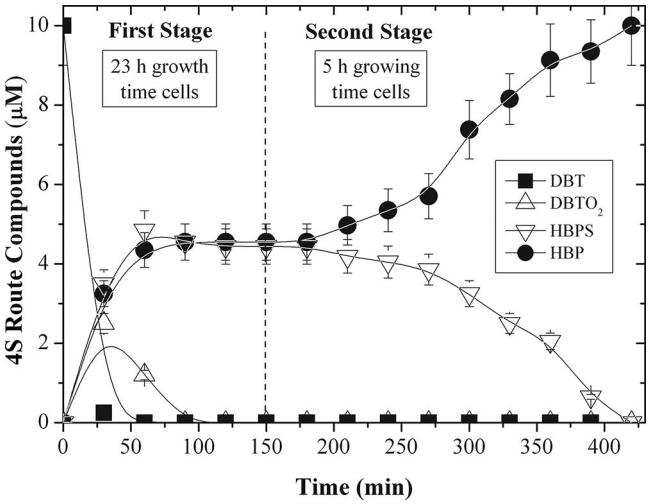

FIG. 3. A two-step BDS process employing *P. putida* CECT 5279 cells collected at two different growth stages. The concentration of the 4S route compounds was monitored throughout the resting-cell process. The bars indicate the standard deviations of three different experiments.

accomplished to identify the actual reason for such inactivation.

Designing a novel two-step desulfurization process. A two-step resting-cell process was designed to increase the BDS efficiency of the *P. putida* CECT5279 recombinant cells (Fig. 3). In the first step, *P. putida* CECT5279 cells (0.7 g DCW/liter) grown for 10 h, showing the maximum activities of DszA and DszC monooxygenases, were able to transform all DBT into a 50% mixture of HBPS and HBP after 75 min, and this transformation rate did not increase further even after 150 min of reaction. In the second step, the cells were removed from the reaction medium, and 0.7 g DCW/liter of cells from a *P. putida* CECT5279 culture grown for 5 h and, hence, presenting the maximum DszB activity were added. Immediately after the addition, the remaining HBPS was efficiently converted into HBP showing a complete transformation after 400 min of the whole resting-cell process (Fig. 3). It should be noted that the second step proceeds more slowly than the first step, likely because the activity of DszB is partially inhibited by the high concentration of HBP (1).

In summary, we have shown in this work that the availability of an active DszB enzyme is a key factor that finally controls the optimal BDS process in a recombinant *P. putida* strain. Although the DszB protein is produced along the growth curve at a constant rate, its activity reaches a sharp maximum at the early exponential phase and rapidly decreases at the middle exponential phase, hampering an efficient transformation of HBPS into HBP. To overcome this problem, we have designed a model two-step BDS resting-cell process using *P. putida* cells from the late and early exponential growth phases for the first and second steps, respectively. In this way, the efficiency of the BDS process could be increased so that the desulfurization time and biomass were minimized. This work contributes, therefore, new insights on recombinant biocatalyst behavior and provides a glimpse of new perspectives in BDS.

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REFERENCES

1. **Alcón, A., A. B. Martin, V. E. Santos, E. Gómez, and F. Garcia-Ochoa.** 2007. Kinetic model for DBT desulfurization by resting whole cells of *Pseudomonas putida* Cect5279. Biochem. Eng. J. **38:**486–495.
2. **Alcón, A., V. E. Santos, A. B. Martin, P. Yustos, and F. Garcia-Ochoa.** 2005. Biodesulfurization of DBT with *Pseudomonas putida* CECT5279 by resting cells: influence of cell growth time on reducing equivalent concentration and HpaC activity. Biochem. Eng. J. **26:**168–175.
3. **Darzins, A., L. Xi, J. D. Childs, D. J. Monticello, and C. H. Squires.** September 1999. Dsz gene expression in *Pseudomonas* hosts. U.S. patent 5,952,208.
4. **del Olmo, C., V. E. Santos, A. Alcón, and F. Garcia-Ochoa.** 2005. Production of a *Rhodococcus erythropolis* IGTS8 biocatalyst for DBT biodesulfurization: influence of operational conditions. Biochem. Eng. J. **22:**229–237.
5. **Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young.** 1994. Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. J. Bacteriol. **176:**6707–6716.
6. **Galán, B., E. Diaz, and J. L. García.** 2000. Enhancing desulfurization by engineering a flavin reductase-encoding gene cassette in recombinant biocatalysts. Environ. Microbiol. **2:**687–694.
7. **Gallardo, M. E., A. Ferrández, V. de Lorenzo, J. L. García, and E. Díaz.** 1997. Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. J. Bacteriol. **179:**7156–7160.
8. **García-Ochoa, F., A. Romero, V. E. Santos, and C. O. Rodríguez.** 1993. Studies of the use of the differential method in the determination of kinetic models for complex reactions. Int. Chem. Eng. **33:**634–648.
9. **Gray, K. A., G. T. Mrachko, and C. H. Squires.** 2003. Biodesulfurization of fossil fuels. Curr. Opin. Microbiol. **6:**229–235.
10. **Gray, K. A., O. S. Pogrebinsky, G. T. Mrachko, L. Xi, D. J. Monticello, and C. H. Squires.** 1996. Molecular mechanisms of biocatalytic desulfurization of fossil fuels. Nat. Biotechnol. **14:**1705–1709.
11. **Gupta, N., P. K. Roychoudhury, and J. K. Deb.** 2005. Biotechnology of desulfurization of diesel: prospects and challenges. Appl. Microbiol. Biotechnol. **66:**356–366.
12. **Kilbane, J. J.** 2006. Microbial biocatalyst developments to upgrade fossil fuels. Curr. Opin. Microbiol. **17:**1–10.
13. **Lee, W. C., T. Ohshiro, T. Matsubara, Y. Izumi, and M. Tanokura.** 2006. Crystal structure and desulfurization mechanism of 2-hydroxybiphenyl-2-sulfinic acid desulfinase. J. Biol. Chem. **281:**32534–32539.
14. **Li, G. Q., S. S. Li, M. L. Zhang, J. Wang, L. Zhu, F. L. Liang, R. L. Liu, and T. Ma.** 2008. Genetic rearrangement strategy for optimizing the dibenzothiophene biodesulfurization pathway in *Rhodococcus erythropolis*. Appl. Environ. Microbiol. **74:**971–976.
15. **Ma, T., G. Li, J. Li, F. Liang, and R. Liu.** 2006. Desulfurization of dibenzothiophene by *Bacillus subtilis* recombinants carrying *dszABC* and *dszD* genes. Biotechnol. Lett. **28:**1095–1100.
16. **Martín, A. B., A. Alcón, V. E. Santos, and F. Garcia-Ochoa.** 2004. Production of a biocatalyst of *Pseudomonas putida* CECT5279 for dibenzothiophene (DBT) biodesulfurization for different media compositions. Energy Fuels **18:**851–857.
17. **Martín, A. B., A. Alcón, V. E. Santos, and F. Garcia-Ochoa.** 2005. Production of a biocatalyst of *Pseudomonas putida* CECT5279 for DBT biodesulfurization: influence of the operational conditions. Energy Fuels **19:**775–782.
18. **Monticello, D. J.** 2000. Biodesulfurization and the upgrading of petroleum distillates. Curr. Opin. Biotechnol. **11:**540–546.
19. **Noda, K. I., K. Watanabe, and K. Maruhashi.** 2003. Recombinant *Pseudomonas putida* carrying both the *dsz* and *hcu* genes can desulfurize dibenzothiophene in *N*-tetradecane. Biotechnol. Lett. **25:**1147–1150.
20. **Piddington, C. S., B. R. Kovacevich, and J. Rambosek.** 1995. Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8. Appl. Environ. Microbiol. **61:**468–475.
21. **Soleimani, M., A. Bassi, and A. Margaritis.** 2007. Biodesulfurization of refractory organic sulfur compounds in fossil fuels. Biotechnol. Adv. **25:**570–596.
22. **Xu, P., B. Yu, F. L. Li, X. F. Cai, and C. Q. Ma.** 2006. Microbial degradation of sulfur, nitrogen and oxygen heterocycles. Trends Microbiol. **14:**398–405.