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Biodesulfurization of dibenzothiophene by growing cells of *Pseudomonas putida* CECT 5279 in biphasic media

Ainhoa Caro, Karina Boltes, Pedro Letón *, Eloy García-Calvo

Dpto. Química Analítica e Ingeniería Química, Facultad de Ciencias, Universidad de Alcalá, 28871 Madrid, Spain

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ABSTRACT

Several studies have proven that natural or genetically modified bacteria, such as *Pseudomonas putida* strain, degrade recalcitrant organic sulfur compounds. However, from a practical point of view, the biodesulfurization (BDS) process has to be performed with really high proportions of organic solvents. In this work, the dibenzothiophene (DBT) was selected as recalcitrant model compound, and hexadecane as model organic solvent. It has been observed that *P. putida* CECT 5279 was able to desulfurize DBT even in the presence of 50% (v/v) of hexadecane. A concentration of 400 ppm of DBT was converted at a specific rate of generation of desulfurized final product, 2-hydroxybiphenyl (HBP), of 2.3 and 1.5 mg HBP L^{-1} (g DC L^{-1} h)$^{-1}$ for 27% and 50% (v/v) of hexadecane, respectively. Finally, the Haldane kinetic model was used to describe the process evolution. The study is relevant as it has been proven that the strain CECT 5279 is a potential biocatalyst for developing an efficient BDS process.

1. Introduction

The emissions of sulfur oxides provide serious environmental and health problems. Therefore, current environmental regulations are more and more strict, ordering very low sulfur contents in diesel and gasoline (McFarland, 1999; Monticello, 2000). The desulfurization technologies are responsible of the sulfur removal from crude oils and the hydrodesulfurization (HDS), reductive conventional physical–chemical technology, is nowadays the more effective and economic (Speight, 1981; del Olmo et al., 2005a). It has been reported that more than 20 millions of oil barrels are diary treated in the world by HDS (Ohshiro et al., 1999). Unfortunately, in the process remain recalcitrant molecules, mainly from the dibenzothiophene (DBT) chemical group (Monticello, 1998), which adds up to 60% of total sulfur content in crude oils (Watkins et al., 2003).

Biodesulfurization (BDS) is the generic term which defines all processes where microorganisms catalyze the desulfurization reaction (Monticello and Finnerty, 1985), removing the recalcitrant molecules under mild pressures and temperatures. It has been described that aerobic, anaerobic and facultative anaerobic microorganisms effectively degrade DBT and its analogous (McFarland, 1999), but also that anaerobic bacteria reach yields too low (Ohshiro and Izumi, 1999).

Rhodococcus erythropolis IGTS8 is a natural and aerobic Gram(+) bacterium which develops the most promising desulfurization pathway described. This biocatalyst removes selectively the sulfur from DBT, and the carbon skeleton remains intact (Kilbane and Jackowski, 1992; Omori et al., 1992; Olson et al., 1993). The detailed description of the pathway, defined as 4S-pathway, has been published previously (Gallagher et al., 1993; Oldfield et al., 1997). The 4S-pathway is energetically expensive due to a great consume of reducing equivalents and oxygen before the production of the free sulfur product (Tao et al., 2006). On the other hand, this bacterium shows a high tolerance to organic solvents.

It is currently assumed that the success of BDS as an alternative industrial approach to produce ultra low sulfur fuels depends on the design of microbial strains which remove sulfur in the presence of great proportions of organic solvents, with higher rate or longer stability of desulfurization activity even at high temperatures (Soleimani et al., 2007). Therefore, current research efforts are focused to isolate new extremophilic microorganisms or develop genetic modifications on natural strains (Xu et al., 2006), improving the design of solvent tolerant strains which metabolize a broad range of organic sulfur compounds (Gunam et al., 2006; Kilbane, 2006; Yu et al., 2006a,b), under high temperatures (Li et al., 2007) and with two-layer and continuous bioreactors (Yang et al., 2007).

Numerous studies about the BDS process are looking for solvent tolerant bacteria with great desulfurizing activities and several works have analysed *Pseudomonas* species (Setti et al., 1994, 1997; Luo et al., 2002; Martin et al., 2004; Alcon et al., 2005; Tao et al., 2006). In this sense, it has been previously proven that

* Corresponding author. Tel.: +34 91 8854974; fax: +34 91 8855088.
E-mail address: pedro.leton@uah.es (P. Letón).

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Pseudomonas putida CECT 5279 is a promising biocatalyst for the BDS process (Gallardo et al., 1997; Martin et al., 2004; Caro et al., 2007b, 2008). This Gram(−) aerobic type strain performs the 4S-pathway increasing the process efficacy because the amount of reducing equivalents consumed is lower (Gallardo et al., 1997). On the other hand, it has been frequently suggested in the literature that BDS process is also limited by substrate diffusion problems, the presence of inhibition effects and the necessity of cofactors re-generation. Therefore, it is necessary to identify the main limitations which reduce the BDS process yield with strain CECT 5279 and how these parameters affect the biocatalyst.

The main target of this work is the description of the influence of the DBT and the oil presence on the development of the BDS process by growing cells of the genetically modified *P. putida* CECT 5279 strain. Concerning biphasic media, when an acceptable yield is achieved it is preferable to work with the lowest water content and so, the influence of high oil proportions were evaluated. Moreover, the process was carried out with an important range of initial DBT concentrations, and the biocatalyst response was determined. The evolution of the desulfurization of DBT by growing cells in biphasic media had been previously described in the literature by a typical Haldane equation, as substrate inhibits both biocatalyst growth and 4S-pathway (Guchhait et al., 2005a,b). In this work it has been also proven that the BDS reaction by growing cells of CECT 5279 followed the Haldane model.

2. Materials and methods

2.1. Biocatalyst

The biocatalyst used was *P. putida* CECT 5279. This strain was developed by including the cluster of genes dszABC from *Rhodococcus erythropolis* IGTS8. The flavin oxidoreductase was obtained by cloning and expression the *hpaC* from *Escherichia coli*. The detailed description of the biocatalyst is reported by Gallardo et al. (1997). This aerobic Gram(−) strain was maintained on concentrated stocks with a solution of glycerol of 10% v/v in saline serum, at −80 °C.

Pseudomonas species were chosen as potential biocatalyst due to its high growth rate, metabolic diversity, its tolerance to biphasic media and the availability of genetic techniques (Tao et al., 2006).

2.2. Chemicals

Suppliers of the different chemicals used were as follows: L-glutamic acid, glycerol, NaCl, $NaH_2PO_4 \cdot H_2O$, $K_2HPO_4 \cdot 3H_2O$, NH_4Cl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, Tris hydroxymethyl aminomethane (TRIS), 2-propanol, ethanol and $MgSO_4 \cdot 7H_2O$, PANREAC; isopropyl β-D-thiogalacto-pyranoside (IPTG), tetracycline (TC), Agar, hexadecane, DBT, and HBP, SIGMA–ALDRICH; Yeast extract, FLUKA; Tryptone, PRONADISA. All other chemicals were of analytical grade. Deionizer water was used to prepare all media and solutions.

2.3. Experimental media

In this kind of processes, the knowledge about the optimum growth conditions is essential in order to obtain both high biocatalyst concentrations and high desulfurization capabilities (Martin et al., 2004; del Olmo et al., 2005a,b).

The Luria Bertani (LB) medium was the complex broth used for the first activation of the biocatalysts cells. This media (g L^{-1}) consisted in yeast extract 5, NaCl 10 and tryptone 10. The activation was done in 250 mL Erlenmeyer flasks, adding 250 μL of a concentrated frozen stock of the strain CECT 5279 into 50 mL of LB, with 150 μL of TC 10 g L^{-1}. For 12 h the flasks were agitated at 250 rpm and 30 °C on an orbital shaker. Afterward, 0.1 g dry cell weight L^{-1} were again inoculated in fresh LB, and agitated at the same operational conditions for additional 3 h. This media was next centrifuged at 7000 rpm, 5 min and 4 °C, removing the LB medium, and into NaCl 9 g L^{-1} re-suspended.

The BDS process by growing cells were always performance in a standard Basal Salt Medium (BSM), which had the following composition (g L^{-1}): $NaH_2PO_4 \cdot H_2O$ 4, $K_2HPO_4 \cdot 3H_2O$ 3, $MgCl_2 \cdot 6H_2O$ 0.0245, $CaCl_2 \cdot 2H_2O$ 0.001, $FeCl_3 \cdot 6H_2O$ 0.001 (Martin et al., 2004; Alcon et al., 2005). Carbon, nitrogen and sulfur sources were L-glutamic acid 20 g L^{-1}, NH_4Cl 2 g L^{-1} and DBT at different concentrations, respectively. TC 9.1 mg L^{-1} and IPTG 43.2 mg L^{-1} were also introduced in the growth medium as antibiotic and 4S-pathway inductor. BSM solution was always prepared with buffer TRIS, with the initial pH 8 (Martin et al., 2004). As organic phase it has been chosen hexadecane, because its significant presence in the diesel oil fraction. The operational conditions were 250 rpm and 30 °C.

2.4. Analytical methods

2.4.1. Cell concentration

Cell concentration was determined by converting the optical density value obtained in a Shimadzu (Model UV 1603) spectrophotometer at 600 nm in grams dry cell weight per litter (g L^{-1}). A conversion factor previously determined was used for both aqueous and biphasic broths. Under biphasic conditions, for the biomass concentration determination the aqueous phase was extracted free of oil.

2.4.2. BDS pathway compounds

All 4S compounds concentrations were measured by high-performance liquid chromatography (HPLC) VARIAN, equipped with a diode-array detector and an automatic injector.

For the analysis, 1.5 mL of sample was taken and centrifuged into Eppendorf tubes (aqueous samples with acetonitrile, in ½ dilutions) at 13 200 rpm, 5 min. When both phases were separated the compounds dissolved in the oil one were measured with a Kromasil C_{18} column (150 × 4.6 mm, 5 μm). In this case, isocratic elution was performed with a 55:45 (v/v) acetonitrile:water mobile phase at 1 mL min^{-1}, with 20 μL of injection volume. Both DBT and HBP were detected at 278 nm. Compounds concentrations in aqueous phase were quantified with a Luna C_8 column (150 × 4.6 mm, 3 μm) and isocratic elution with 50% acetonitrile and 50% water at 1 mL min^{-1}, and detection was realized with and UV detector at 239 and 210 nm for DBT and HBP, respectively. Other intermediates substrates of the pathway were also quantified, but the concentrations were negligible respect to DBT and HBP and so they are not presented in this communication.

3. Theoretical analysis

The values of biomass concentration were analyzed by applying a logistic equation (Eq. (1)), determining the kinetic parameters $C_{x_{max}}$ and μ (Martin et al., 2004; Alcon et al., 2005; del Olmo et al., 2005a,b) as follow:

$$C_X\ (\mathrm{g\,L^{-1}}) = \frac{C_{X0}e^{(\mu t)}}{1 - \left(\left(\frac{C_{X0}}{C_{X_{max}}}\right)(1 - e^{(\mu t)})\right)} \quad (1)$$

The evolution of the processes was analyzed by Haldane-type equations (Guchhait et al., 2005a,b; Caro et al., 2007a) (Eqs. (2)–(4)), taking into account that the pathway final product is stoichiometrically generated: