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ORIGINAL PAPER

Description of by-product inhibiton effects on biodesulfurization of dibenzothiophene in biphasic media

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Abstract As several authors have reported previously, the Biodesulfurization of hydrodesulfurization recalcitrants, such as dibenzothiophene, is not yet commercially viable because mass transfer limitations and feedback inhibition effects are produced during the conversion. This work has been focused to investigate the inhibition process in aqueous and oil-water systems with two different aerobic biocatalysts types, *Rhodococcus erythropolis* IGTS8 and *Pseudomonas putida* CECT 5279. The results obtained have proven that global DBT desulfurization process using CECT 5279 was not clearly deactivated due to final product accumulation, under the experimental conditions assayed. Consistently, the desulfurization pattern has been described with the Michaelis-Menten equation, determining the kinetic parameters. On other hand, the assays have shown that important mass transfer limitations produced the decrease of the yields obtained with this $Gram^-$ strain in biphasic media. With strain IGTS8 it was observed lower mass transfer problems, but contrary the reaction was severely affected by the final product accumulation, in both aqueous and biphasic systems. Therefore it has been proposed an enzymatic kinetic model with competitive inhibition to describe the BDS evolution pattern when this $Gram^+$ strain was used.

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Nomenclature

BDS	Biodesulfurization
C	Concentration (μM)
DBT	Dibenzothiophene
DBTO	DBT-sulfoxide
$DBTO_2$	DBT-sulfone
DC	Dry cell
HBP	2-hydroxybiphenyl
HBPSi	2′-hydroxybephenyl-2-sulfinate
HDS	Hydrodesulfurization
I	Inhibitor concentration (μM of HBP)
K	Kinetic constant (μM)
P	Product (HBP) concentration (μM)
q_S	Specific substrate consume rate (mmol substrate·$KgDC^{-1}$ h^{-1})
S	Substrate concentration (μM)
X	Biomass concentration (g DC L^{-1})
X_{BDS}	Biodesulfurization percentage (%)
y	Fitting model parameter
Subindexes	
i	Intermediate
I	Inhibition over main substrate
I′	Inhibition over intermediate substrate
max	Maximum
r	Remove
S	Substrate, saturation

Introduction

Current regulations are ordering strict low sulfur contents in fuels so that sulfur oxides emissions drop significantly, as they are responsible of acid rain and air pollution (McFarland 1999; Monticello 2000). Nowadays, crude oils dependency becomes middle-distillate fractions more important and great efforts and investments in desulfurization technologies have been developed (Rashtchi et al. 2006).

Hydrodesulfurization (HDS) is the technology used to desulfurize fuels, removing the sulfur in the presence of metallic catalysts, hydrogen gas and very high pressures and temperatures (H. del Olmo et al. 2005a). However, recalcitrant molecules such as dibenzothiophene (DBT) and its derivatives, which add up to 60% of total sulfur found in petroleum products (Monticello 1998), remain intact after this conventional chemical treatment (Watkins et al. 2003). Biodesulfurization (BDS) is the generic term which defines all pathways where microorganisms catalyze reactions where sulfur is removed (Monticello and Finnerty 1985). In literature, several works have suggested that complementing the HDS and BDS technologies it is possible to achieve very low sulfur fuels.

BDS is carried out either under anaerobic or aerobic conditions, but it has been proven that the anaerobic reaction yield is very low (Ohshiro et al. 1999). On other hand, there are three different aerobic BDS pathways, and one of them results in the selective carbon–sulfur bond cleavage (Ohshiro et al. 2005). This non-destructive pathway has been called 4S pathway, and *Rhodococcus erythropolis* was the first strain isolated effectively capable to develop it (Kilbane and Jackowski 1992; Omori et al. 1992; Olson et al. 1993).

In Fig. 1 it is shown the 4S pathway. DBT molecule is transformed by two consecutive oxidation steps catalyzed by mono-oxygenase DszC enzyme to DBT-sulfoxide (DBTO) and DBT-sulfone ($DBTO_2$). After, it is produced a sulfone reductive hydroxylation reaction, catalyzing the DszA protein and, thanks to DszB enzyme, finally the free sulfur final product 2-hydroxybiphenyl (HBP) is stoichiometrically formed, and sulphate or sulphite are accumulated (Gallagher et al. 1993; Oldfield et al. 1997). The fourth enzyme implicated, DszD, guarantee the $FMNH_2$ contribution involved in mono-oxygenase reactions.

Several mesophilic bacteria develop the 4S pathway, such as *Corynebacterium* (Omori et al. 1992; Maghsoudi et al. 2001), *Gordonia* (Oldfield et al. 1998; Jia et al. 2006), *Nocardia* (Chang et al. 2000), *Escherichia* (Le Borgne and Quintero 2003) and *Pseudomonas* (Luo et al. 2002; Guobin et al. 2006). Nevertheless, *Rhodococcus* sp. is the mostly investigated up to now (Monticello 2000; H. del Olmo et al. 2005a; Ohshiro and Izumi 1999; Kilbane and Jackowski 1992; Honda et al. 1998; Guchhait et al. 2005). Concerning to the operation modes, aerobic BDS conversion can be carried out by growing cells (Gallagher et al. 1993; Honda et al. 1998; Guchhait et al. 2005), free cell extracts (Ohshiro et al. 1994) and also resting cells (Ohshiro et al. 1999; Gallagher et al. 1993; Oldfield et al. 1997; Maghsoudi et al. 2001; Jia et al. 2006; Luo et al. 2002). The highest DBT conversion levels reported in literature has been achieved with resting cells (Ohshiro et al. 1996; Konishi et al. 1997).

There are two relevant reviews recently published (Kilbane II 2006; Xu et al. 2006) focuses on progress of biodesulfurization and in understanding how microbes degrade S, N and O heterocycles. More important problems for biodesulfurization application at industrial scale are commented as well. Briefly, the presence of high proportions of organic solvents can kill most of microorganism. In this sense, an important research line goes to the development of more resistant and efficiency biocatalyst that can work in biphasic reaction media. (Takada et al. 2005; Tao et al. 2006). On the other hand, the problem of low solubility of the aromatic sulfur compounds in the aqueous fraction of media was studied as is reported by Feng et al. (2006).

In literature, there are few research works where the aerobic BDS of DBT has been compared under aqueous and biphasic conditions. In a previous publication (Caro et al. 2007) it has been described the influence of the oil fraction, the biocatalyst concentration and the initial DBT concentration on the desulfurization yield, with both *Rhodococcus erythropolis* IGTS8 and *Pseudomonas putida* CECT5279 strains, in aqueous and biphasic reaction media. Therefore, in this work it has been investigated this reaction kinetics in both media types. The biocatalysts used under resting cell conditions have been a $Gram^+$ *Rhodococcus erythropolis* IGTS8 strain, which presents high tolerance to organic

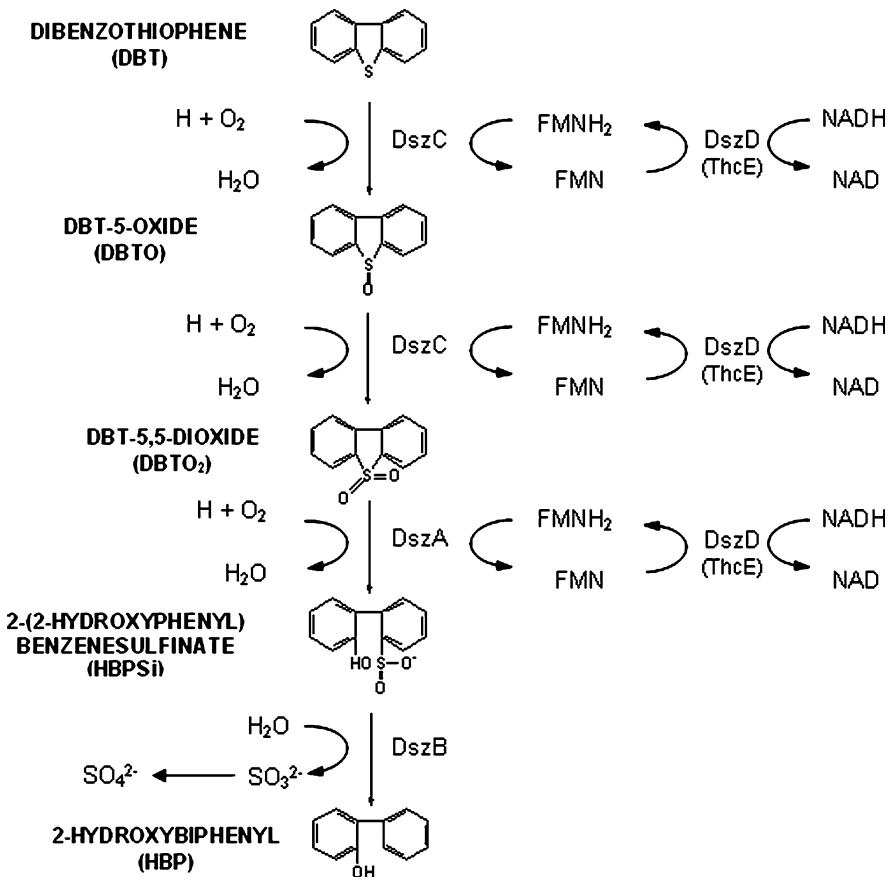

Fig. 1 Schematic 4S pathway evolution

media, and a genetically modified Gram$^-$ *Pseudomonas putida* CECT5279. This strain is more energetically effective than IGTS8 as it consumes less reducing equivalents to degrade the DBT molecule (Gallardo et al. 1997). The main aim of this work is the description of the end-product inhibition effects. HBP is a potent 4S pathway inhibitor (Monticello 2000) but, to our knowledge, no previous works have described in detail its influence.

Materials and methods

Materials

Suppliers of the different chemicals used were as follows: L-Glutamic acid, D(+)-glucose, glycerine, 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 Hydroximetil Aminomethane (TRIS), ethanol and $MgSO_4 \cdot 7H_2O$,PANREAC; *N*-[2-Hydroxyethil] piperazine-*N*′-[2-ethane-sulfonic acid (HEPES) buffer, isopropyl *β*-D-thiogalacto-pyranoside(IPTG), tetracycline (TC), Agar, Dimethylsulfoxide (DMSO), hexadecane, DBT, DBT-sulfone ($DBTO_2$) and HBP, SIGMA-ALDRICH; Yeast extract, FLUKA; Tryptone, PRONADISA. DBT-sulfoxide (DBTO) and 2′-hydroxybephenyl-2-sulfinate (HBPSi) were synthesized and supplied by Complutense University, (Madrid, Spain). Deionizer water was used to prepare all media. DBT and all other 4S compounds were dissolved in ethanol. All other chemicals were of analytical grade.

Media

Luria Bertani (LB) broth is a complex medium used for the inoculum built up, which consists in yeast extract 5 g l^{-1}, NaCl 10 g l^{-1} and tryptone 10 g l^{-1}. The basal salt medium (BSM) has been used for the growing steps. This medium has been described

previously in literature (H. del Olmo et al. 2005a; Martin et al. 2004), and it is composed by NaH_2PO_4 H_2O 4 g l^{-1}, $K_2HPO_4 \cdot 3H_2O$ 3 g l^{-1}, $MgCl_2 \cdot 6$ H_2O 0.0245 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.001 g l^{-1}, $FeCl_3$ $6H_2O$ 0.001 g l^{-1}, glycerol 2 g l^{-1}.

Microorganisms

The microorganisms used were *Rhodococcus erythropolis* IGTS8, and *Pseudomonas putida* CECT 5279, both supplied by the Biological Research Center, (CIB, Madrid, Spain). *Pseudomonas putida* CECT 5279 has the genes dszA, dszB and dszC cloned from *R. erythropolis* IGTS8 and the flavin-oxido reductase, *hpaC* from *Escherichia coli* (Alcon et al. 2005). This strain develops a similar *Rhodococcus*'s pathway (Gallardo et al. 1997). The IGTS8 strain was maintained at 4°C on LB-Agar plates, while CECT 5279 strain was kept on a concentrated stock with a solution of glycerol 10% v/v in saline serum, at −80°C.

Biocatalysts built up

The protocols followed for the biocatalysts growth have been previously reported (H. del Olmo et al. 2005a; Martin et al. 2004). The growth media used with both strains have different carbon and sulfur sources in order to optimize both the final cell concentration and the desulfurization activity. A detailed description of specific media and operative conditions for both strains are presented below.

Rhodococcus erythropolis *IGTS8*

First at all, it was obtained the inoculum by two following activation steps in Luria Bertani broth (LB), of 24 and 12 h. After this activation, the LB media was centrifuged at 7,000 r.p.m., 15 min and 20°C and into NaCl 9 g l^{-1} re-suspended. Next 0.25 g l^{-1} of the inoculum were incubated in a 2 l bioreactor that contained BSM media and glucose 20 g l^{-1}, NH_4Cl 2 g l^{-1} and DMSO 0.01 g l^{-1} as carbon, nitrogen and sulfur sources, respectively (H. del Olmo et al. 2005a). Operational conditions were 30°C, 250 r.p.m. and aeration 1 l l^{-1} min^{-1}. The pH was controlled at 8 adding TRIS and NaOH 0.1 M at the beginning of the fermentation. Growth is stopped after 19 h when the exponential growth phase was rinsed (H. del Olmo et al. 2005b; Wang et al. 1996), and then cells were pelleted at 7,000 r.p.m. and 20°C for 15 min, for the posterior re-suspension in glycerol-NaCl 50%. After cells were kept frozen at −18°C.

Pseudomonas putida *CECT 5279*

In this case, the inoculum was also activated in LB broth, but herein the steps were of 10 and 3 h, respectively. Then the media was centrifuged at 7,000 r.p.m., 5 min and 4°C and into NaCl 9 g l^{-1} re-suspended. A 2 l bioreactor is inoculated with 0.25 g l^{-1} of inoculum and BSM media, with L-glutamic acid 20 g l^{-1}, NH_4Cl 2 g l^{-1}, $MgSO_4$ 0.44 g l^{-1}, TC 9.1 mg l^{-1} and IPTG 43.2 mg l^{-1}, inductor of 4S pathway enzymes production (Martin et al. 2004). Operational conditions were the same as described above. Here, pH was controlled adjusting to 8 with NaOH 0.1 M before of inoculation. Growth was stopped after 5.5 h at the end of the exponential growth phase (Gallardo et al. 1997; Alcon et al. 2005), and pellet formation was carried out at 7000 r.p.m., 4°C and only 5 min, and then re-suspended and maintained at −18°C.

Biocatalyst concentration measure

Cell concentrations were determined from optical density values obtained in a Shimadzu (Model UV 1603) spectrophotometer at 600 nm. Those values were converted to grams dry cell weight per litter (gDC l^{-1}) using a factor previously determined.

Analysis of 4S-pathway compounds

All 4S compounds concentrations were measured in both aqueous and organic phases by high-performance liquid chromatography (HPLC) VARIAN with diode-array detector and an automatic injector. To analyze by HPLC 1.5 ml of sample was centrifuged at 13,200 r.p.m., 5 min, into eppendorf tubes with acetonitrile, in ½ dilution. When both phases were

separated the organic one was measured with a Kromasil C_{18} column (150 × 4.6 mm, 5 μm). Elution was performed with a 55:45 (v/v) acetonitrile:water mobile phase at 1 ml min^{-1}, in order to measure DBT, $DBTO_2$, HBPSi, and HBP at 278 nm, with 20 μl of injection volume. Aqueous phase of the samples was analyzed 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}, with 60 μl of injection volume, was used. DBT, DBTO, $DBTO_2$ were detected at 239 nm, and HBPSi and HBP at 210 nm.

Biocatalytic desulfurization by resting cell systems

All reactions were carried out in Erlenmeyer flasks with resting cells of both IGTS8 and CECT 5279 in aqueous and biphasic conditions. The flasks were agitated on a rotary shaker at 250 r.p.m. and 30°C. The reaction media have included HEPES buffer 12 g l^{-1} (at pH 8), glycerol from the inoculum as necessary energy source (Le Borgne et al. 2003) and either DBT or 4S pathway intermediate compounds, dissolved in ethanol, as substrates. Due to the extremely low solubility of these compounds, the BDS substrates are normally added dissolving in ethanol (Le Borgne et al. 2003) or in 2-propanol (H. del Olmo et al. 2005a; Martin et al. 2004) when the reaction media was an aqueous buffer. Alcohol proportions were always lower than 1% v/v, avoiding possible toxic effects reported previously (Kobayashi et al. 2001; Yan et al. 2000). Moreover, in biphasic reaction media the organic solvent used was hexadecane, because its high presence in diesel oil fraction (Maghsoudi et al. 2001).

In order to describe the HBP presence effects, it has been carried out a previous set of discrete experiments with both biocatalysts in 100 ml Erlenmeyer flasks. In these assays DBT, DBTO, $DBTO_2$ and HBPSi were used independently as substrates into an aqueous resting cell media. Different initial HBP concentrations were also added. Afterward, the 4S pathway evolution was followed both in aqueous and biphasic media, using always DBT as substrate and also different HBP initial concentrations.

The reactions results have been presented either as specific consume rates or BDS percentages (X_{BDS}), which has been defined as the ratio between the HBP concentration produced and the initial substrate concentration (H. del Olmo et al 2005a, b; Martin et al. 2004).

Finally, for both biocatalysts the BDS process has been described with equations based on the Michaelis Menten type kinetics. The kinetic parameters reported in literature have been adapted to generate the estimated concentration-time curves. Differential equations were solved numerically using a Felhberg fourth–fifth order Runge-Kutta method with Maple 9.0. Moreover, it has been also determined the volumetric mass transfer coefficients of DBT and HBP due to the important affection of mass transfer limitations under biphasic conditions.

Results and discussion

Influence of HBP accumulation over the initial specific consume rates

The analysis of the effects that the presence of HBP produce on each step of 4S biodesulfurization pathway was performed determining the specific consume rates of the four substrates at a very short reaction time. The reaction media were prepared as it has been described above, adding 2 gDC l^{-1} of biocatalyst concentration. Substrate concentrations were varied from 5 to 50 μM. Meanwhile, it was included HBP concentration from zero to 50 μM into the reaction media. All reactions were stopped after 15 min and the 4S compounds concentrations were determined in order to obtain initial disappearance rates.

Figure 2 shows the relation between the specific consume rates determined for 15 min and the initial substrate concentrations, with initial HBP concentrations of zero and 50 μM.

The results with *P. putida* CECT 5279 have proven that there were no inhibition effects over both mono-oxygenase DszC, when it converts DBT to DBTO, and DszA enzymes. Nonetheless, when DBTO and HBPSi were the substrates, the desulfurization activity was clearly inhibited over 50 μM of initial HBP concentration. Likewise, with *R. erythropolis* IGTS8 the inhibition effects were only observed again when the desulfurization substrates were either DBTO or HBPSi.

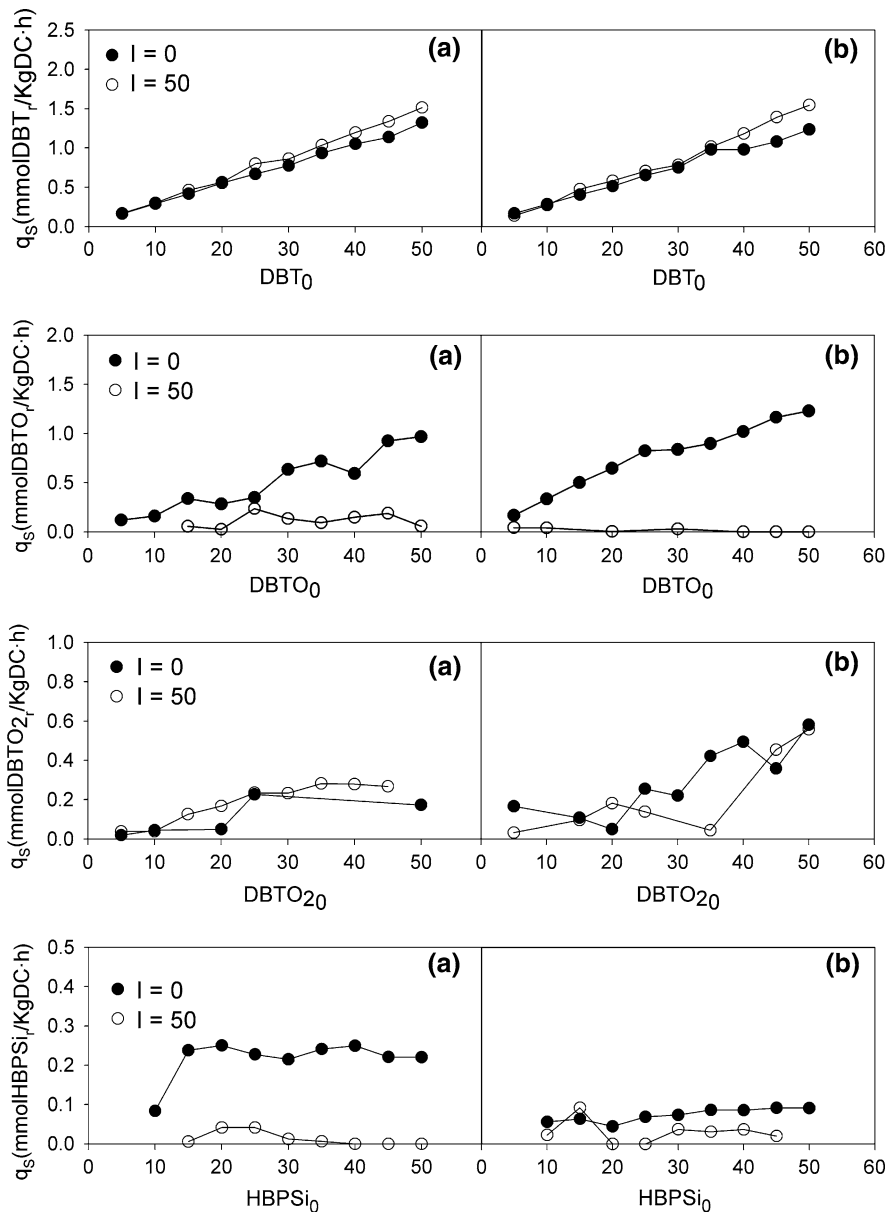

Fig. 2 Specific initial substrate consume rates with (**a**) *Pseudomonas putida* CECT 5279 and (**b**) *Rhodococcus erythropolis* IGTS8, in aqueous conditions. Substrate concentrations in μM

With both biocatalysts it has been observed that lower specific consume rates were achieved when 4S intermediates were used as substrates instead of DBT. The slowest and controlling step is the last one, as other authors have proposed previously (Gray et al. 1996). Furthermore, with CECT 5279 the specific consume rates of $DBTO_2$ and HBPSi achieved a stable value into the concentration range evaluated, while IGTS8 only rinsed this value with HBPSi.

This previous study has proven that HBP reduces the BDS activity in both biocatalyst types, by decreasing the activities of enzymes during the second and fourth 4S pathway steps. Monooxygenase DszC enzyme has DBT and DBTO as substrates, but probably, DszC present greater affinity by DBT as it can be concluded from the saturation constant determined by Lineweaver-Burk equation of a value twice higher for DBTO. In addition to these considerations it has been quantified an HBPSi specific consume rate three times higher with CECT 5279 than with IGTS8, while specific consume rates of the other 4S pathway compounds were very similar. Therefore, it has also

proven that flavin-oxido reductase, *hpaC*, was more effective than dszD (Alcon et al. 2005).

BDS inhibition study

This set of experiments was carried out into 250 and 500 ml Erlenmeyer flasks under aqueous and biphasic conditions, respectively. The biocatalysts concentration was 1 gDC l^{-1} and DBT was always the reaction substrate. The BDS process evolution was analyzed by quantifying the concentration of 4S pathway compounds during the desulfurization reactions and estimating the BDS percentages (X_{BDS}).

In aqueous BDS experiments the substrate concentrations were 25 or 10 μM, for CECT 5279 and IGTS8 respectively. In these cases, the HBP initial concentrations investigated were varied from 0 to 40 μM. Meanwhile, biphasic assays were prepared with oil to water ratio of 1, using the same media described above except for the substrate and inhibitor concentrations. The initial concentration of DBT added was 271 μM in these assays, and the initial HBP concentration range varied from 0 to 220 μM of HBP. These different concentrations answer to the extremely low solubility of DBT and HBP into aqueous media.

As it is observed in Fig. 3, with *Pseudomonas putida* CECT 5279 neither aqueous nor biphasic reactions were affected by the HBP presence, for the evaluated concentrations range. Under aqueous conditions the BDS percentage achieved was 60%, independently of the initial inhibitor concentration, and only near 20% in biphasic media. The lower yield achieved under biphasic condition has been related in literature with the incapability of *Pseudomonas* strains to be adhered to the oil-water interface (Abbad-Andaloussi et al. 2003). On other hand, with aqueous media the yield values became stable from 300 min. However, with biphasic systems the BDS conversion was slower and even after 420 min the BDS percentages did not become constant. Moreover, after 24 h the yields only reached 24% of conversion, (data not shown).

Likewise, in Fig. 4 it is possible to observe the desulfurization percentages obtained during the BDS process with *Rhodococcus erythropolis* IGTS8 in both aqueous and biphasic assays. As the initial HBP concentrations went up, the X_{BDS} values obtained decreased, showing the inhibition effect in both aqueous and biphasic media. In this figure it has been shown that, under aqueous conditions, from a concentration of 15 μM of initial HBP it was produced a clear inhibition effect, which resulted in a maximum conversion delay of 50 min approximately, achieving a yield 20% lower than assays with less initial HBP.

When biphasic media were investigated, again the first remarkable result was the dramatic decrease of the BDS percentages, in comparison with the aqueous assay, even although the DBT initial concentration were different. In this case the inhibition effect was also evident, but the inhibitor concentration which produced an important drop of the BDS yield was 220 μM of HBP. Moreover, the maximum conversion was achieved near 420 min with lower inhibitor concentration, but with 220 μM of HBP there was a delay of several hours, having a yield loss of 10%. Comparing both biocatalysts it has been obtained higher desulfurization percentages with IGTS8 under biphasic conditions than with CECT 5279. According to the results obtained it is possible to suggest that IGTS8 has shown a higher intolerance to HBP presence than CECT 5279, whereas this strain was more affected by mass transfer limitations.

4S pathway evolution under aqueous conditions

In literature it has been currently proven that the DBT biodesulfurization pattern is represented by a Michaelis-Menten equation (Rashtchi et al. 2006; Jia et al. 2006; Luo et al. 2002; Kobayashi et al. 2001), when resting cell systems are used. Therefore, in this study it has been proposed the following equation in order to describe the DBT conversion with *Pseudomonas putida* CECT 5279:

$$\frac{dS}{dt} = -X \cdot q_{S_{max}} \cdot \left(\frac{S}{S + K_S} \right) \quad (1)$$

In addition to DBT and HBP in all aqueous experiments appeared $DBTO_2$, which disappeared after near 200 min, and also DBTO and HBPSi. However, the evolutions of these last compounds have been not considered for the kinetic model, assuming that both intermediates achieved the pseudo-steady state during the reaction.

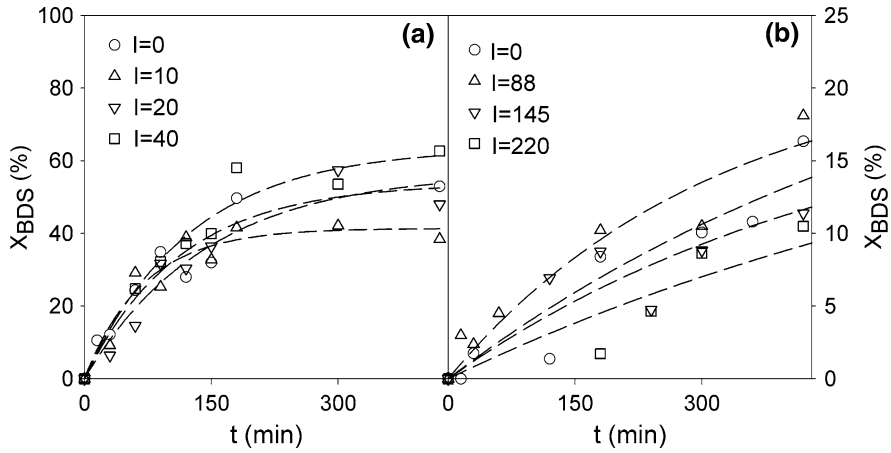

Fig. 3 X_{BDS} percentages achieved with *Pseudomonas putida* CECT 5279 in (**a**) aqueous and (**b**) biphasic media, and several inhibitor concentration in μM (I)

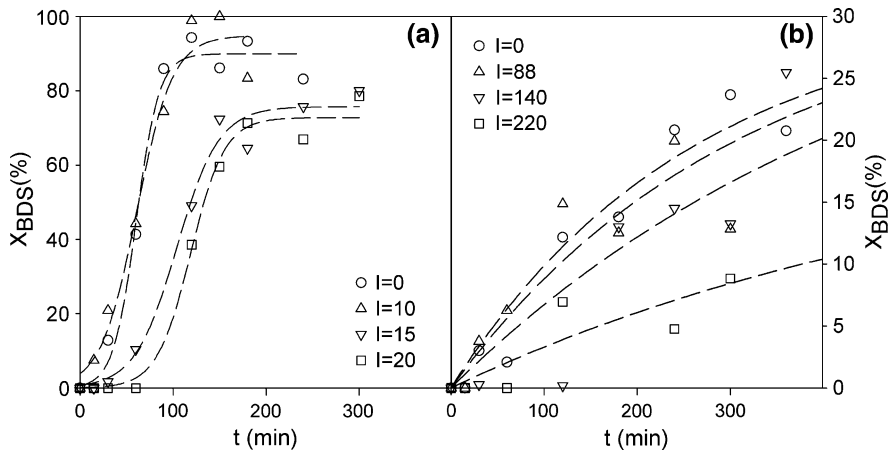

Fig. 4 X_{BDS} percentages achieved with *Rhodococcus erythropolis* IGTS8 in (**a**) aqueous and (**b**) biphasic media, and several inhibitor concentration in μM (I)

The $DBTO_2$ evolution has been expressed as:

$$\frac{dS_i}{dt} = \left(X \cdot q_{S_{max}} \cdot \left(\frac{S}{S + K_S}\right)\right) - \left(X \cdot q_{Si_{max}} \cdot \left(\frac{S_i}{S_i + K_{Si}}\right)\right) \quad (2)$$

and finally, the HBP production has been defined with the following equation:

$$\frac{dP}{dt} = \left[X \cdot q_{Si_{max}} \cdot \left(\frac{S_i}{S_i + K_{Si}}\right)\right] \cdot y \quad (3)$$

On other hand, with *R. erythropolis* IGTS8 it has been proposed the use of a typical kinetic model for enzymatic reactions with competitive inhibition, based on Michaelis-Menten equation. Several authors have reported previously that the desulfurization pattern of DBT by *Rhodococcus* sp. follows Haldane type kinetics with growing cells (Guchhait et al. 2005), and a Michaelis-Menten equation with resting cells (Kobayashi et al. 2001).

The DBT conversion rate with IGTS8 has been described here as:

$$\frac{dS}{dt} = -X \cdot q_{S_{max}} \cdot \left(\frac{S}{S + K_S \cdot \left(1 + \frac{P}{K_I}\right)}\right) \quad (4)$$

Only under aqueous conditions, in the media it was quantified DBTO during the first 150 min. Its evolution has been described by Eq. 5:

$$\frac{dS_i}{dt} = \left(X \cdot q_{S_{max}} \cdot \left(\frac{S}{S + K_S \cdot \left(1 + \frac{P}{K_I}\right)}\right)\right) - \left(X \cdot q_{Si_{max}} \cdot \left(\frac{S_i}{S_i + K_{S_i} \cdot \left(1 + \frac{P}{K'_I}\right)}\right)\right) \quad (5)$$

Moreover, it has been not detected significant concentrations of $DBTO_2$ and HBPSi, neither in aqueous nor in biphasic media. So, the pseudo-steady state was assumed again for these compounds.

Finally, HBP production has followed this equation:

$$\frac{dP}{dt} = \left[X \cdot q_{Si_{max}} \cdot \left(\frac{S_i}{S_i + K_{S_i} \cdot \left(1 + \frac{P}{K_I'}\right)} \right) \right] \cdot y \qquad (6)$$

The kinetic constants used with both CECT 5279 and IGTS8 strains have been obtained by modifying kinetic parameters reported in bibliography (Jia et al. 2006), which have fitted the experimental data observed. Table 1 shows these constants.

The parameter *y* was a necessary fitting parameter. It has been included with a value of 0.5 as the mass balances were closed in no aqueous assays, probably because some 4S pathway compounds were accumulated inside the cells. In order to prove this consideration the cells were broken down and the compounds found inside the cells were quantified. When both strains reacted into aqueous media, it has been found accumulated into the intracellular medium the entire 4S pathway compounds, especially HBP. The concentration of these compounds rinsed up more than 10% of the initial DBT concentration added.

Figures 5 and 6 show the good agreement obtained between experimental and calculated data.

4S pathway evolution under biphasic conditions

Figures 7 and 8 show the evolution of the 4S pathway with both CECT 5279 and IGTS8, under biphasic conditions.

Table 1 Kinetic BDS constants obtained with *Pseudomonas putida* CECT 5279 and *Rhodococcus erythropolis* IGTS8 in the DBT desulfurization under aqueous conditions

Parameter	CECT 5279	IGTS8
q_{Smax} (mmolDBT KgDC^{-1} min^{-1})	0.55	0.25
K_S (μM)	0.15	0.35
K_I (μM)	–	0.50
q_{Si} (mmolIC KgDC^{-1} min^{-1})*	0.15	0.10
K_{Si} (μM)	0.15	0.15
K_I' (μM)	–	0.80
X (g DC l^{-1})	1.00	1.00
y (−)	0.50	0.50

* IC: intermediate compound, $DBTO_2$ and DBTO for CECT 5279 and IGTS8, respectively

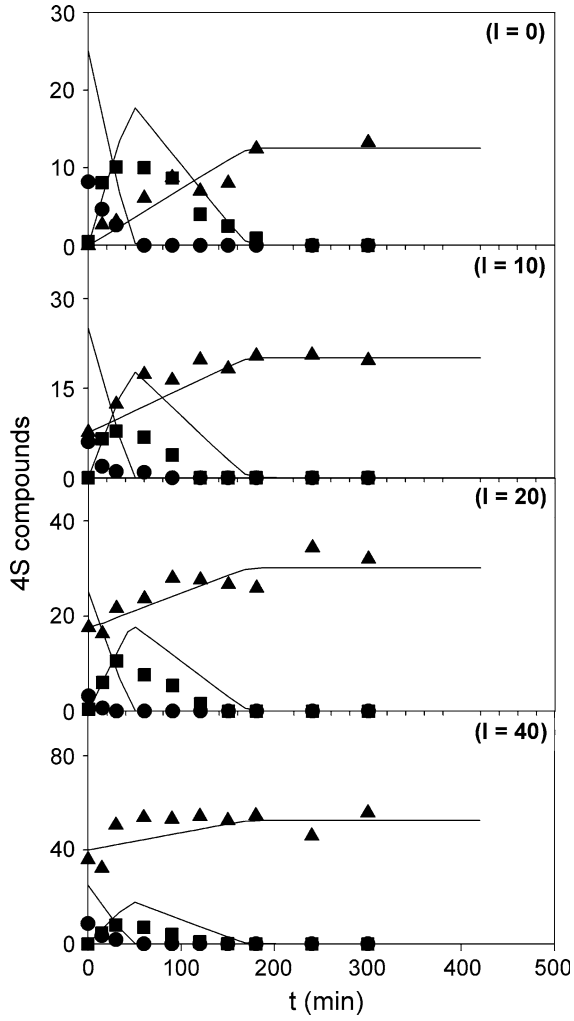

Fig. 5 BDS evolution simulation with *Pseudomonas putida* CECT 5279 in aqueous conditions and several inhibitor concentrations (I). (●) DBT, (■) $DBTO_2$, (▲) HBP, (—) estimated. All concentrations in μM

In both figures it is possible to observe a sharp deceleration of the DBT conversion rate and so, a significant decrease of the HBP production rate. The higher limitation to the DBT transference into the aqueous phase avoided the possibility to follow the actual reaction rates. On other hand, in the presence of organic solvent, none intermediate compound was quantified, even at very short reaction times. Only the strain CECT 5279 produced little amounts of sulfone, which were accumulated into the extra-cellular medium. Even though the mass balances were almost closed in the biphasic assays, cells were also disrupted and the intra-cellular media were analysed. Only little amounts

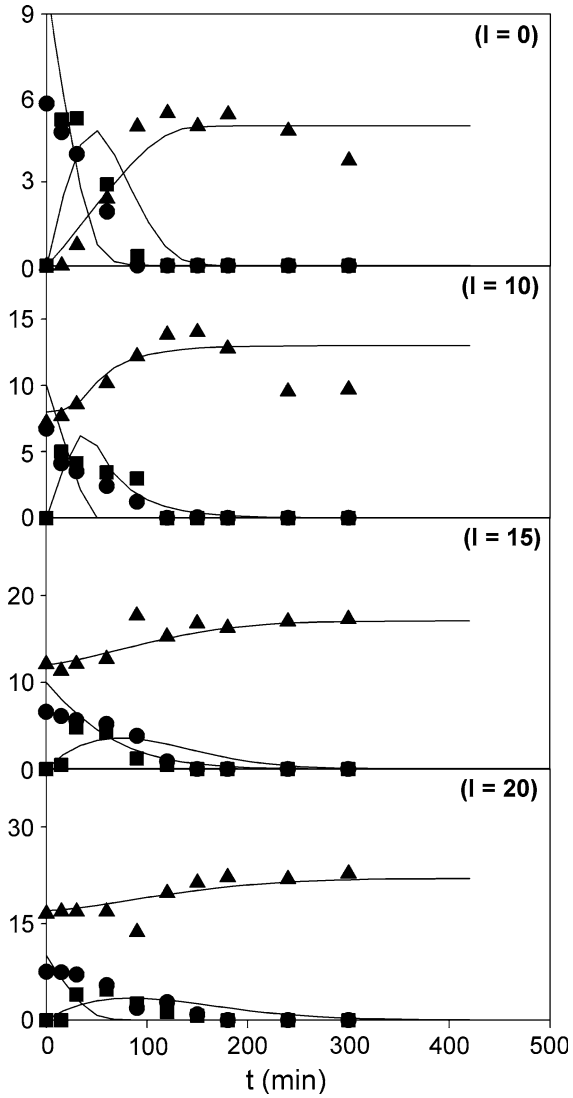

Fig. 6 BDS evolution simulation with *Rhodococcus erythropolis* IGTS8 in aqueous conditions and several inhibitor concentrations (I). (●) DBT, (■) DBTO, (▲) HBP, (—) estimated. All concentrations in μM

of DBT were found inside the cells, and $DBTO_2$ with CECT 5279 strain. Moreover, almost no HBP was quantified in both intra-cellular and aqueous medium as this compound is practically as soluble in oil as DBT.

Following the criterion described before, it was used the same kinetic models to simulate the evolution curves for the biphasic conversions. However, the kinetic parameters used had to be modified. For CECT 5279, 0.25 mmolDBT·KgDC^{-1} min^{-1}, 750 μM, 0.007 mmol$DBTO_2$·KgDC^{-1} min^{-1}, 0.15 μM and 1 g DC l^{-1} were the values for $q_{S_{max}}$, K_S, q_{S_i}, K_{S_i} and X, respectively. The fitting parameter y was 1 as the mass balance was closed in all cases. With IGTS8 the parameters had values of 0.25 mmolDBT·KgDC^{-1} min^{-1}, 150 μM, 100 μM, 1 g DC l^{-1} and 1 for q_{Smax}, K_S, K_I, X and y, respectively. The agreement between experimental and calculated data has been shown in Figs. 7 and 8. To fit the experimental evolutions it was necessary to increase the K_S constants, which is related to the lower mass transfer rates. On other hand, with IGTS8 the inhibition effect was lower in the presence of oil than under aqueous conditions, as HBP migrated to the oil phase Yang and

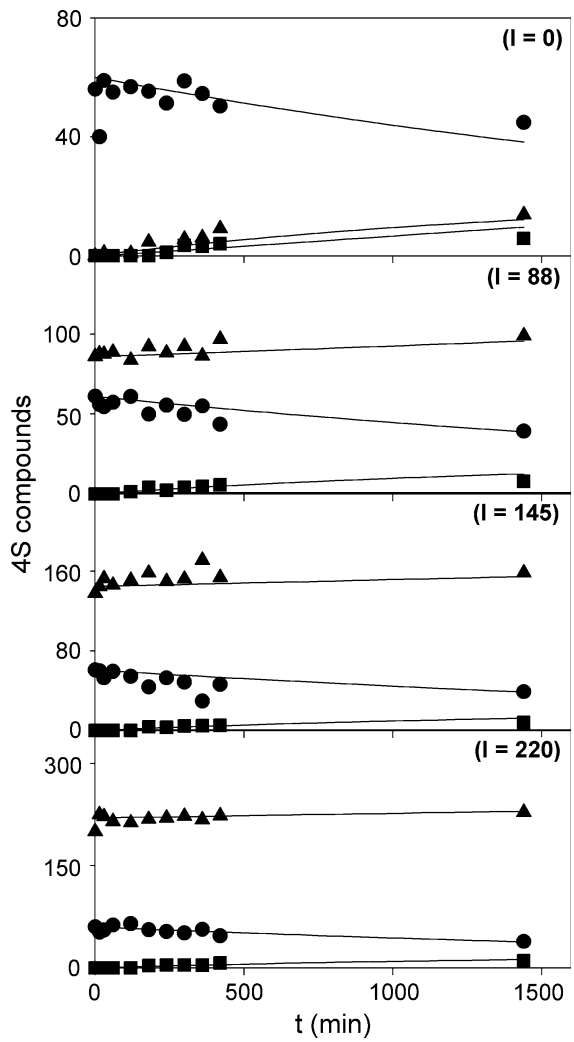

Fig. 7 BDS evolution simulation with *Pseudomonas putida* CECT 5279 in biphasic conditions and several inhibitor concentrations (I). (●) DBT, (■) $DBTO_2$, (▲) HBP, (—) estimated. All concentrations in μM

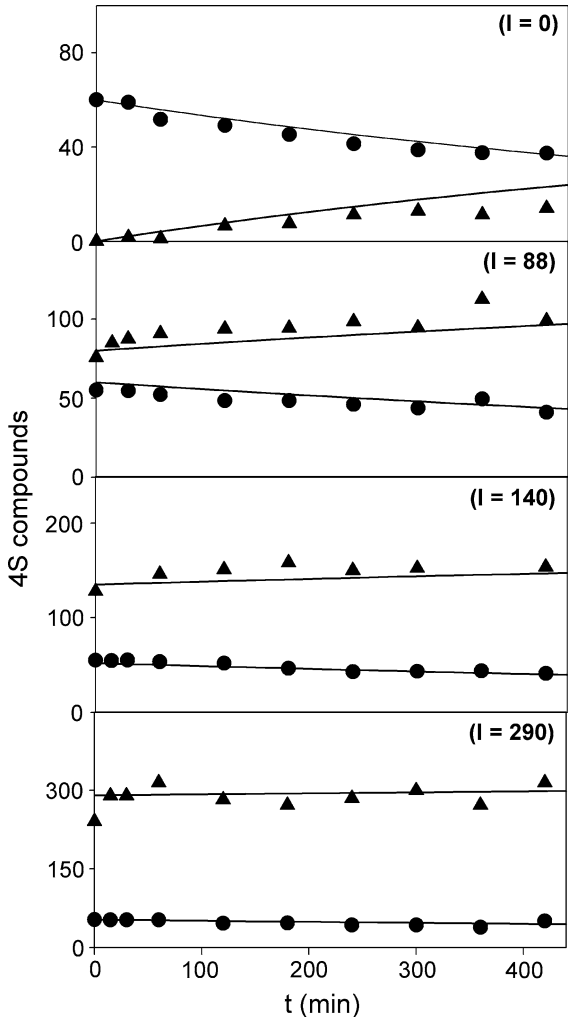

Fig. 8 BDS evolution simulation with *Rhodococcus erythropolis* IGTS8 in biphasic conditions and several inhibitor concentrations (I). (●) DBT, (▲) HBP, (—) estimated. All concentrations in μM

Marison (2005). This result agreed with the K_I determined.

However, as the kinetic parameters under these conditions are only apparent kinetics, it has been determined graphically the volumetric mass transfer coefficients (K) of DBT and HBP, taking into account the double-film model and supposing that the concentration in aqueous phase of these compounds was negligible. In this case the transfer rate only depends on the concentration of the compounds in the oil phase, and mass transfer controls the process. The rates of biodegradation of DBT and production of HBP were described by the equations presented below. The values of the volumetric mass transfer coefficients are shown in Table 2.

$$-\frac{dDBT}{dt} = K_{DBT} \times C_{DBT} \quad (7)$$

$$\frac{dHBP}{dt} = K_{HBP} \times C_{HBP} \quad (8)$$

In literature it has been previously described the DBT mass transfer from n-dodecane to water, according to the double-film model (Jia et al. 2006).

For CECT 5279, neither the values of K_{DBT} nor the values of K_{HBP} were affected by the presence of the inhibitor in oil media. This strain was not capable to be linked to the oil-water interface, and so it had no contact with the HBP molecules. Moreover, the volumetric mass transfer coefficients of HBP had the same magnitude order than the K_{DBT} values. For IGTS8 it has been obtained similar K_{DBT} values for the whole HBP concentrations range investigated. However, the volumetric mass transfer coefficients of HBP had shown a continuous decrease as the initial HBP concentration added went up, which has proven

Table 2 Diffusion coefficients of DBT and HBP during the BDS conversion under biphasic conditions with strains CECT 5279 and IGTS8

Inhibitor concentration (μM)	CECT 5279		IGTS8	
	$K_{DBT} \times 10^5$ (min^{-1})	$K_{HBP} \times 10^5$ (min^{-1})	$K_{DBT} \times 10^5$ (min^{-1})	$K_{HBP} \times 10^5$ (min^{-1})
0	48	85	120	91
80	59	70	97	72
140	92	91	87	13
220	77	84	99	7.1

the strong effect of the feedback inhibition when cells of IGTS8 were used. The lack of variation into the K_{DBT} values was in concordance with the results explained in Sect. "Influence of HBP accumulation over the initial specific consume rates", as DBT consume was not affected by the accumulation of HBP under aqueous conditions. On other hand, as the value of K_{HBP} dropped the mass balance could not be closed, and probably $HBPS_i$ was accumulated inside the cells. This compound was not quantified when the cells were disrupted as its concentrations would be near the analytical detection limit.

Conclusions

Under aqueous conditions, it has been proven that HBP was a potent inhibitor of the second and fourth steps of the 4S pathway, when CECT 5279 and IGTS8 were used as resting cells. However, the behaviour of both strains respect to the inhibition effects was different when the global DBT biodegradation was followed. With CECT 5279 it was observed no decrease of the BDS yield when HBP was added, under the experimental conditions assayed, both in aqueous and biphasic media. Contrary, IGTS8 was strongly affected from very low HBP concentrations, when the reaction medium was aqueous. HBP is soluble into the oil phase and therefore, in biphasic media the feedback inhibition effects were avoided below concentrations of HBP higher than 140 μM.

The biodegradation of DBT by resting cells of CECT 5279 was well described with the Monod equation. Likewise, with IGTS8 a typical kinetic model for enzymatic reactions with competitive inhibition was effective to fit the evolution of the 4S pathway.

On other hand, the study has shown that mass transfer controlled the biodegradation under biphasic conditions. A dramatic drop in the bioconversion rate was observed and so, apparent kinetic parameters could be determined. Moreover, as both microorganisms types contrast considerably in their capabilities to be adhered to the oil-water interface, it was possible to observe that mass transfer limitations were less important with IGTS8 than with CECT 5279, as IGTS8 takes up compounds dissolved in oil. The experimental volumetric mass transfer coefficients determined have proven that the accumulation of HBP had no effects over the degradation by CECT 5279, and however inhibits the production of HBP when IGTS8 was used instead.

With the results obtained in this study it is possible to suggest that the biodesulfurization process has to be improved by avoiding either mass transfer limitations or inhibition problems, depending on the biocatalysts employed, developing appropriate bioreactor designs in order to achieve high and stable desulfurization activities.

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