Optimization of a Desulfurizing Biocatalyst by Combining Cells of Different Cell Age of *Pseudomonas putida* CECT 5279

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Biodesulfurization is proposed as an alternative technology able to reduce sulfur content in fossil fuels, in order to aminish sulfur oxide emissions to the atmosphere. In this work, a genetically modified microorganism, *Pseudomonas putida* CECT5279, is employed as a desulfurizing biocatalyst. The combination of two cell age biomass can be used in order to optimize an effective biodesulfuring catalyst. Hereby, 5 hours and 23 hours were the selected growth times when cells were collected, combined in different biomass concentrations and tested by carrying out resting cells assays. In these experiments dibenzothiophene (DBT) was used as sulfur model compound. Biodesulfurization percentage, X_{BDS}, initial DBT elimination rate, R_{DBT}^0, employed biomass, C_x, and time for desulfurization, t_{BDS}, were the chosen parameters in order to search for the best biocatalyst. After this study, the mixture of 0,7 and 1,4 gDCW/L biomass concentration were selected for 5 hours and 23 hours growth time cells respectively, because of the best effectiveness achieved with formulation for a biodesulfurizing catalyst.

1. Introduction

World energy expense overcame 7900 Mtoe in 2005 and it is showing an annual increase of 2.2% (International Energy Agency, 2007). Population consumption of fossil fuels causes an important emission of sulfur oxides to the atmosphere. These compounds are recognized to be involved in many environmental, health and material stability problems. Therefore, more and more restrictive legal limitations about sulfur content in fossil fuels have been imposed in order to control emissions in combustion processes. For instance, European Union has fixed maximum sulfur content at 10 ppm for 2009 in diesel fuel (European Directive, 2003). Many technologies have been proposed to achieve these low limits (Babich and Moulijn, 2003). Among them, hydrodesulfurization (HDS) has been the most extensively employed. However, the severe conditions of pressure and temperature needed to be employed in deep HDS cause alterations in fuel final characteristics (Babich and Moulijn, 2003).

As a proposed technique, biodesulfurization (BDS), joined to a previous HDS process, could succeed in obtaining high quality fuels, and respecting in force regulations, as well. This technique, consisting on employing microorganisms (both wild and genetically modified) as catalysts, allows degrading sulfur aromatic molecules using mild conditions of temperature and pressure, avoiding C-C bond breakdown, and maintaining fuel properties. The main model compound employed in BDS studies is dibenzothiophene (DBT), which is transformed through 4S metabolic route (Oldfield et al., 1997) into 2-hydroxybiphenyl (HBP), removing the molecular sulfur while maintaining hydrocarbon skeleton.

In this work, the combination of cells from a GMO, *Pseudomonas putida* CECT 5279, is proposed for the formulation of a biocatalyst in a BDS process. The aim of this work is the optimization of an effective biocatalyst. The experimental variables considered in this study were biomass concentration and cell age.

2. Materials and Methods

2.1 Microorganism
The bacterial strain employed, *Pseudomonas putida* CECT5279 (Galán, 2001), have been supplied by Dr. José Luis García (Biological Research Center, CIB-CSIC, Spain). Cultures were maintained on frozen concentrated stock with glycerol in serum solution.

2.2 Biocatalyst production
Cells employed as biodesulfurizing catalyst were obtained in a 2L stirred tank bioreactor using a BSM medium (Martin et al., 2004), at 30°C, 1 L/L/min of air flow rate and 200 rpm of stirrer speed, following an standard method in order to obtain comparative and reproducible experimental results (Martin et al., 2005). Two growth time cells were collected in order to carry out BDS resting cells assays: 5 and 23 hours, because of their maximum abilities to produce HBP or to remove DBT, respectively, as previously observed (Calzada et al., 2007).

2.3 BDS assays
BDS resting cells experiments were conducted in an orbital shaker at 30°C and 210 rpm, using 100 mL Erlenmeyer flasks containing 16 mL HEPES medium with 25 μM DBT as sulfur substrate. 5 and 23 hours growth time cells were added in different proportions and biomass concentrations, as shown in Table 1.

2.4 Analytical methods
Biomass evolution was monitored by measuring absorbance at 600 nm (with a UV 1630 SHIMADZU spectrometer). Samples collected during BDS assays were centrifugated and acidified with HCl. HPLC-UV-diode Array (with a C-18 Komasil, 5μm, 150 x 4.6 mm column) was employed to analyze the evolution of DBT and HBP. Both mobile phase concentration (acetonitrile:water from 30 to 70%) and flow (from 1 to 2 ml/min) gradients were used in order to achieve 4S compounds separation.

Run	Y^{23}	C^5_x (gDCW/L)	C^{23}_x (gDCW/L)	C_x (gDCW/L)
U11	0,00	0,7	0,0	0,7
U12	1,00	0,0	0,4	0,7
U21	0,00	1,4	0,0	1,4
M22	0,50	0,7	0,7	1,4
U23	1,00	0,0	1,4	1,4
U31	0,00	2,1	0,0	2,1
M32	0,33	0,7	1,4	2,1
M33	0,67	1,4	0,7	2,1
U34	1,00	0,0	2,1	2,1

Table 1. Experimental design.

2.5 Mathematical methods

Differential method was applied in order to estimate DBT elimination rate at zero time, for each BDS assay. Experimental data were treated by using OriginPro ® 7.5 software so that fit calculations for initial rates were carried out.

3. Experimental results and discussion

3.1. Experimental results

An experimental design combining the two cell age biomass concentration was proposed in order to find out the best biocatalyst formulation, as shown in Table 1. That means maximizing DBT transformation while minimizing biomass waste and time of BDS. Total dry weight cell concentrations from 0,7 to 2,1 g/L were employed to carry out biodesulfurization assays.

As an example, evolution of DBT and HBP along BDS time for one of these assays is shown in Figure 1. In this case, 100% percentage of BDS is reached so that 25 mM initial DBT concentration is completely transformed into HBP.
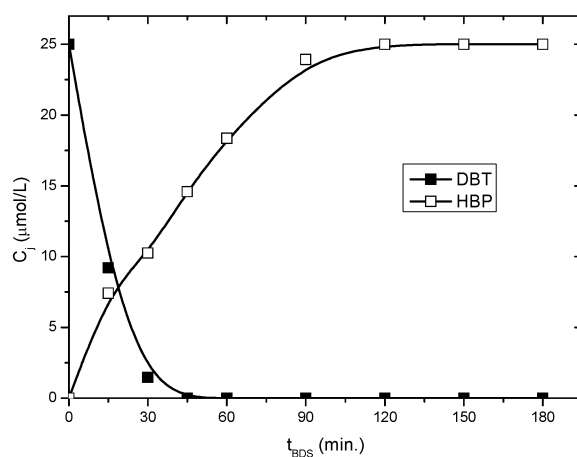

Figure 1. DBT and HBP evolution obtained from a BDS assay.

3.2. Discussion

Evolution of both DBT and HBP concentration were analyzed and studied in each assay by using the following parameters.

Maximum biodesulfurization percentage

$$X^{\max}{}_{BDS} = \frac{C^{\max}{}_{HBP}}{C^{0}{}_{DBT}} \cdot 100 \tag{1}$$

Initial DBT elimination rate

Differential method was employed so that initial DBT elimination ($R_{DBT}{}^{0}$) rate is estimated as follows:

$$R_{DBT}{}^{0} = -\frac{dC_{DBT}}{dt}\bigg|_{t=0} \tag{2}$$

Concentration of biomass

Total biomass concentration was expressed in dry cell weight as the contribution of both kind of cell concentration.

$$C_{x} = C_{x}{}^{5} + C_{x}{}^{23} \tag{3}$$

Specific desulfurization grade

E represents the goodness of each tested cell combination. It relates $X^{max}{}_{BDS}$, C_X and $t_{BDS}^{X^{max}_{BDS}}$.

$$E = \frac{\left[\dfrac{X^{\max}{}_{BDS}}{t_{BDS}^{x^{max}_{BDS}}} \right]}{C_{x}} \tag{4}$$

23 hours growth time mass fraction of cells, Y^{23}

Y^{23} shows the mass fraction that 23 hours growth time cells represent in the biocatalyst formulation.

$$Y^{23} = \frac{C_{X}{}^{23}}{C_{X}{}^{5} + C_{X}{}^{23}} \tag{5}$$

Values of parameters X^{max}_{BDS}, R_{DBT}^{0} and E were represented versus Y^{23}, as shown in Figures 2 to 4, in order to compare the different cell mixtures and their abilities to become a good BDS biocatalyst.

BDS percentage is increased when higher biomass concentrations is used for biocatalyst formulation, as it can be seen in Figure 2. However, 23 hours growth time cell contribution is always higher than 5 hours growth time ones, because of their bigger ability to remove DBT (Calzada et al., 2007).

As shown in Figure 3, total biomass concentration has a similar influence on initial DBT elimination rate. Using only one cell age used in biocatalyst formulation does not obtain the highest values for R_{DBT}^{0}. Instead, certain combinations of 5 and 23 hours growth times achieve higher initial DBT elimination rates.

Finally, specific desulfurization grade is represented in figure 4. For 0,7 and 1,4 gDCW total biomass, the higher the value of Y^{23} is, the biggest the E factor becomes. However, when 2,1 gDCW/L is employed, a maximum is observed by using a Y^{23} of 0,67.
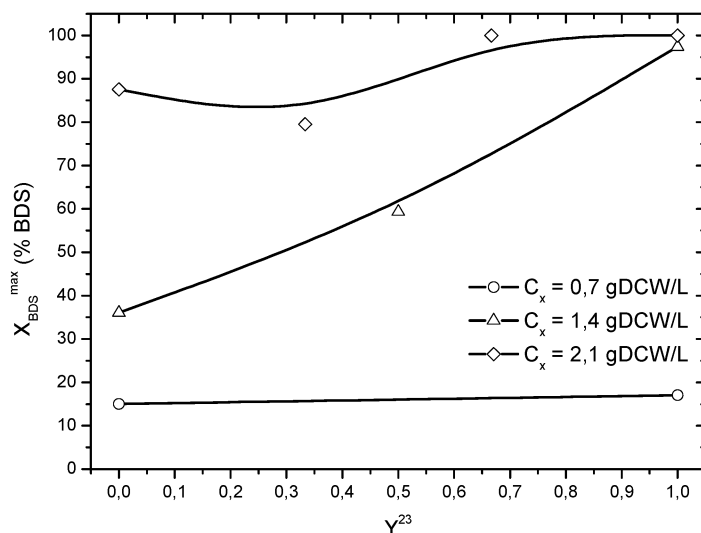

Figure 2. X^{max}_{BDS} versus 23 growth time mass factor of cells for different total biomass concentration.
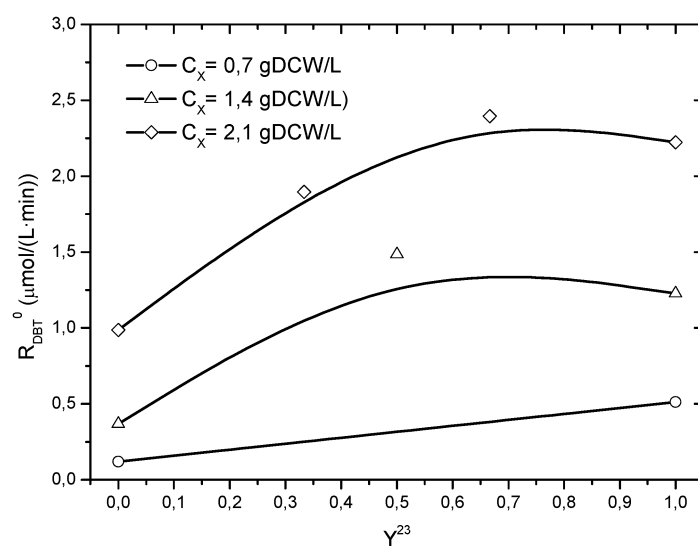
Figure 3. Initial DBT elimination rate versus 23 growth time mass factor of cells for different total biomass concentration.
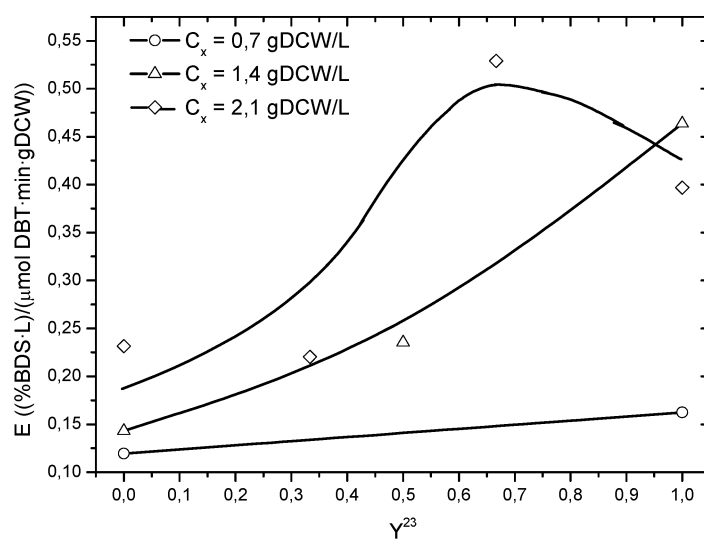

Figure 4. Biocatalyst effectiveness factor versus 23 growth time mass factor of cells for different total biomass concentration.

4. Conclusions

Experimental results show the possibility of optimizing a biocatalyst by mixing two age cells of *Pseudomonas putida* CECT5279. Complete transformation of DBT in a minimized reaction time is achieved by mixing different biomass concentration of both higher DBT removal activity cells and higher HBP production activity ones.

Combining 5 and 23 hours growth time cells, in 0,7 and 1,4 gDCW/L respectively, is found to be the best combination. This biocatalyt formulation gets 100% DBT conversion, while reducing BDS time. In addition, this cell combination achieves higher initial BDS elimination rate than 23 hours cells used alone. This combination have been selected because of the *E* maximun shown among the tested combinations.

5. Nomenclature

BDS = biodesulfurization
C^i_x = concentration of biomass (gDCW/L), total or at *i* growth time
C_j = concentration of compound *j* (μmol/L)
DBT = dibezothiophene
E = specific desulfurization grade (($\%$BDS·L)/(·min·gDCW))
HBP = 2-hydroxybiphenyl
HDS = hydrodesulfurization
R_{DBT}^0 = initial DBT removal rate (μmol/(L·min))
R_{HBP}^0 = initial HBP production rate (μmol/(L·min))
t^k_{BDS} = biodesulfurization time (min), at any or *k* condition
X^{max}_{BDS} = maximum percentage of biodesulfurization ($\%$BDS) given by equation (3)
Y^{23} = 23 hours growth time mass fraction of cells, which can be defined as:

Subindexes
BDS = biodesulfurization
DBT = refers to dibezothiophene
HBPS = refers to 2-(2-hydroxybiphenyl)-benzenesulfinate (sulfinate)
HBP = refers to 2-hydroxybiphenyl

Superindexes
0 = refers to initial time
5 = refers to 5 hours growth cell time
23 = refers to 23 hours growth cell time
X^{max}_{BDS} = refers to maximum percentage of biodesulfurization

6. Acknowledgements

7. References

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