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Mixtures of *Pseudomonas putida* CECT 5279 cells of different ages: Optimization as biodesulfurization catalyst

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ABSTRACT

Sulfur content in fossil fuels is known to be the most important anthropogenic cause of sulfur oxide emissions to the atmosphere. In order to avoid health, environmental and technical problems caused by this compound, legislation imposes restrictive limitations to fuel sulfur content. Biodesulfurization (BDS) can become a complementary technology to hydrodesulfurization (HDS) to face this situation. *Pseudomonas putida* CECT 5279 is a genetically modified microorganism which can act as a desulfurizing biocatalyst. This microorganism has the ability of performing the metabolic 4S pathway from *Rhodococcus erythropolis* IGTS8, in order to desulfurize DBT, as a model compound. Maximum *in vivo* activities of monooxygenase enzymes (DszA and DszC) are shown when late exponential growth phase is reached (23 h), while desulfinase enzyme DszB presents a maximum activity during the early exponential growth phase (5 h), as previously reported [1]. Also, it has been proved that the combined utilization of these two cell ages yields excellent results when used as biocatalyst for desulfurization [2]. The aim of this work is to optimize the ratio and total biomass concentration of both 5 h and 23 h growth time cells in a complex biocatalyst for desulfurization by performing resting cells biodesulfurization assays using dibenzothiophene (DBT) as sulfur model compound. The best combination of cells was determined aiming for the highest desulfurization in the shortest time of operation while investing the minimum concentration of biomass. A particular cell mixture, containing 66.7% of 23 h growth time cells, was found to work as the most effective desulfurization biocatalyst.

1. Introduction

Current living standards require a high demand of energy coming from fossil fuels [3,4]. Combustion of oil causes the emission of sulfur oxides, which are responsible for many well known environmental, health and material stability problems [5–7]. Quite restrictive legal limitations have been imposed in the European Union [8], United States [9] and other countries such as Japan and Canada [5] in order to avoid these noxious effects. Such low limits (around 10 ppm of sulfur [5,8,9]) can be achieved by employing different proposed technologies [10]. Among them, hydrodesulfurization (HDS) is the most widely employed. However, there is a group of aromatic sulfur-containing compounds, such as 4- and 4,6-alkyl DBT, and polyaromatic sulfur heterocycles, which show resistance to be completely removed by conventional HDS processes [11]. Improving sulfur compound removal involves not only the use of more severe conditions of pressure and temperature which can alter fuel final characteristics, but also the increase of investment in order to develop better and poisoning resistant catalysts [10,11]. Therefore, techniques involving milder operation conditions and reducing CO_2 production need to be studied and developed [5].

Biodesulfurization (BDS) is one of the emerging technologies which can satisfy many of the requirements to confront the disadvantages of conventional HDS. Such biological treatment involves the use of microorganisms, their enzymes or cellular extracts as catalysts in order to remove sulfur present in fuels [12–16]. BDS is based on the degradation of sulfur aromatic molecules through a process where mild conditions of temperature and pressure are employed. Under these premises, BDS technology can succeed in reducing energy waste [5], greenhouse gases exhausts and capital investment [16–19]. This technique is not proposed as an alternative process to HDS, but instead, as a technology that, when added to a previous HDS process could complement it and succeed in obtaining high quality fuels, while respecting existing regulations. BDS offers a high selectivity technique due to the use of biocatalytic enzymatic systems with the ability of reducing the generation of undesirable byproducts [15,16,20]. One of the strengths of biological desulfurization is the possibility of hardly alter the treated fuel because of the employ of microorganisms following

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sulfur selective pathways. Such biocatalysts avoid C–C bond cleavage helping to maintain the final properties of the fuel [5,15,16,21]. *Rhodococcus erythropolis* IGTS8 belongs to this group of microorganisms and it has become the most widely employed desulfurizing biocatalyst. This strain is able to complete an oxidative and non-destructive metabolic route [5,10,16,22–24] which is called 4S route, due to the transformation of dibenzothiophene (DBT) into a sulfur-free molecule, 2,2′-hydroxybiphenil (HBP), through four in series reactions [25,26]. DBT and its alkylated forms are employed as model compounds in desulfurization studies because of their abundance in fossil fuels [15,27] and particularly in heavier oil distillates [28,29]. These compounds present a special resistance to be removed by conventional HDS processes [12,13,29], thus, the oil after HDS usually contains DBT and its alkylated derivatives.

Current knowledge about 4S route includes information about both genes and enzymes needed for this transformation [25,30]. Two monoxygenases, DszC and DszA, and a desulfinase, DszB, participate in the conversion of DBT into HBP. Flavin-dependent monooxygenases DszC catalyses the first two steps of DBT oxidation into dibenzothiophene-5-oxide (DBTO) and consecutively into dibenzothiophene-5,5-dioxide (DBTO2). Monooxygenase DszA catalyses the transformation of $DBTO_2$ into 2-(2-hydroxybiphenyl)-benzenesulfinate (HBPS). The third enzyme involved in 4S route, desulfinase DszB, catalyses the last step of 4S route, which involves the conversion of HBPS into the final product, HBP [25,30,31]. Development of BDS in order to become an industrial scale process requires improving not only the process itself (through reactor design, optimization of operation conditions and down stream operation) [13], but the characteristics of biocatalysts as well [5,13,15,16,32,33]. Great effort has been made aiming for higher biodesulfurization activity or designing a recombinant biocatalyst with a stable activity [5,22,29,30,34,35]. The interest of obtaining recombinant bacteria deals with over-expressing the genes of the 4S route [30,36,37]. Therefore, many mutants have been constructed in *Pseudomonas* sp. [38–40] and other bacteria [41–45].

The microorganism employed in this work as biocatalyst is *Pseudomonas putida* CECT 5279, which is a genetically modified bacterium with the ability of expressing the 4S pathway owing to the fact that this strain carries the genes *dszABC* from *Rhodococcus erythtropolis* IGTS8, and a flavin-oxydo-reductase from *E. coli* (hpaC) [46,47]. Previous works allow compiling information about the behavior of this bacterial strain when it is employed as a desulfurizing biocatalyst. On one hand, a maximum desulfurizing capability is observed when cells collected at around 9 h of growth time are employed for DBT desulfurization in resting cell conditions [48,49]. A deep study on BDS of DBT using resting cells could prove that the transport of all 4S route intermediates across the cell membrane is not a mass transport controlling resistance [50], and that neither the intracellular concentrations of reducing cofactors nor the NADH dependent reductase HpaC have influence on the desulfurization rate [50]. Based on these data, *in vivo* enzymatic activities of DszA, DszB and DszC could be studied along the growth curve of *Pseudomonas putida* CECT 5279 [1]. This study proved the presence of maximum activities of both flavin-dependent monooxygenases, DszC and DszA, at around 23 h of growth time, and a maximum activity for the desulfinase DszB, reached at around 5 h of growth time of *Pseudomonas putida* CECT 5279. These different patterns of expression of monooxygenases DszA and DszC and desulfinase DszB throughout growth time, were found to explain the behavior of cells collected at 9 h of growth time [1].

The aim of this work is testing different complex biocatalysts composed by cells of *Pseudomonas putida* CECT 5279, collected at growth times of 5 and 23 h, varying the total biomass concentration and the proportion of the aforementioned cell ages. The results obtained in resting cell assays are compared to the response given by a simple biocatalyst previously optimized [48,49], formulated only using 9 h cells, employing analogous total biomass concentrations. Specific conversion rate is the parameter used to select one of the proposed cell mixtures as an adequate complex biocatalyst pursuing the highest DBT conversion into HBP, the minimum time of operation and the lowest biomass investment.

2. Materials and methods

2.1. Chemicals

Dibenzothiophene was purchased from Aldrich, HEPES buffer and IPTG (isopropyl-β-D-galactopyranoside) were purchased from Sigma. Deionized water (resistance = 18.2 Ω) was used to prepare all media and stock solutions.

2.2. Microorganism

Pseudomonas putida CECT 5279 was the bacterium used as biocatalyst. It was supplied by the Biological Research Center (CIB-CSIC-Madrid, Spain) [38]. Cultures were concentrated, resuspended with a glycerol in saline serum (50%) solution and conserved at −80 °C.

2.3. Biocatalyst production

In order to obtain comparative experimental results, a previously described standardized procedure was employed [48,49]. Frozen stocks were used as inocula and grown in Luria–Bertani (LB) rich medium, containing 1% tryptone, 0.5% yeast extract and 1% NaCl, in Erlenmeyer flasks, and maintained at 210 rpm and 30 °C in an orbital shaker for 12 h. A second growth was accomplished in LB medium under the same conditions for 4 h, after inoculating bacteria from the previous culture. These cells were employed as inoculum in a 2 L commercial bioreactor (BIOSTAT B, Braun Biotech). Biocatalyst production was carried out using basal salt medium (BSM) containing $NaH_2PO_4{\cdot}H_2O$, 4 g/L; K_2HPO_4, 4 g/L; $MgCl_2{\cdot}6H_2O$, 0.0245 g/L; $CaCl_2{\cdot}2H_2O$, 0.001 g/L; $MgSO_4$, 2 mM; glycerine, 2%; $FeCl_3{\cdot}6H_2O$, 0.001 g/L; NH_4Cl, 2 g/L. IPTG (0.2 mM) and tetracycline (25 μg/mL), as explained in previous works [48]. The carbon source employed in these experiments was glutamic acid 20 g/L [48,49]. Operational conditions employed were: 1 L/L/min of aeration, 200 rpm of stirrer speed and 30 °C of temperature [48,49]. Since it has been shown in a previous work that *Pseudomonas putida* CECT 5279 yields higher DBT conversion when glutamic acid is used as carbon source in a non-buffered medium [49], initial pH was adjusted but not controlled during the process. Cells were collected at selected growth times (5, 9 and 23 h) in order to execute biodesulfurization assays using different biocatalyst formulations as previously exposed. Under these conditions, average values of biomass concentration reached after 5 and 23 h of growth time were, respectively, 0.75 and 2.1 gDCW/L.

2.4. Resting cell assays

Resting cell desulfurization assays were carried out by using cells previously collected at selected growth times and conserved at −18 °C, after centrifugation and resuspension in a glycerol–saline (1:1) solution [1]. These cells were suspended in a 100 mL Erlenmeyer flask, containing 16 mL of 50 mM HEPES buffer (pH 8.0) and 25 μM DBT, varying total biomass concentrations from 0.7 to 4.2 gDCW/L. Desulfurization was performed at 30 °C, 210 rpm in orbital shaker for 180 min. Samples of 0.5 mL were collected periodically; samples were afterwards mixed with 0.5 mL of acetonitrile in Eppendorf tubes in order to stop the reactions and then centrifuged at 14,000 × *g* for 9 min. Finally, 0.75 mL of the supernatant was withdrawn for analysis.

2.5. Analytical methods

Biomass concentration was determined as optical density at 600 nm (OD600) measured in a Shimadzu UV–visible spectra-photometer (model UV-1603). HPLC was employed to analyze DBT and HBP. Samples were acidified by using HCl and then analyzed using a C-18 column (Kromasil 150 mm × 4.6 mm, 5 μm particles). The mobile phase was an initial mixture acetonitrile/water (50/50) at 1 mL/min initial flow rate. Peaks were monitored at different wavelengths (206 nm HBP and 234 nm for DBT). Calibrations were performed using commercial and highly purified DBT and HBP chemicals.

2.6. Evaluation of tested biocatalysts

Performance and effectiveness of proposed biocatalysts used in desulfurization assays were evaluated using the following parameters:

- *Maximum percentage of desulfurization* (X_{BDS}^{MAX}), this parameter indicates the maximum desulfurizing capability of cells obtained during the resting cell assay, according to the following equation:

$$X_{BDS}^{MAX} = \frac{C_{HBP}^{MAX}}{C_{DBT}^{0}} \times 100 \quad (1)$$

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where C^0_{DBT} is the initial concentration of DBT used to perform the resting cell assay (25 μM) and C^{MAX}_{HBP} is the maximum HBP concentration obtained during the time employed in the resting cell assay.

- *Time for maximum biodesulfurization* (t^{max}_{BDS}), indicates the time at which the maximum percentage of biodesulfurization has been reached in the resting cells assay.
- *Initial DBT removal rate*, R^0_{DBT}, which shows the initial rate of DBT transformation. It is calculated by applying differential method over the evolution of DBT concentration throughout time in order to estimate the value of DBT removal rate at time zero, according to the following expression:

$$R^0_{DBT} = \left.\frac{dC_{DBT}}{dt}\right|_{t=0} \quad (2)$$

- *Maximum productivity*, P^{MAX}_{BDS}, which relates maximum percentage of desulfurization to the time needed for this conversion as follows:

$$P^{max}_{BDS} = \frac{X^{MAX}_{BDS}}{t_{BDS_{MAX}}} \quad (3)$$

- *Specific conversion rate*, *E*, which regards the performance of each tested biocatalyst. It relates X^{MAX}_{BDS}, concentration of biomass, C_X and the time needed for this maximum achieved conversion, according to:

$$E = \frac{X^{MAX}_{BDS}}{t_{BDS_{max}} C_X} \quad (4)$$

According to the parameters discussed above, the formulation for this complex biocatalyst has been selected. This selection takes into account the highest conversion of DBT into HBP in the shortest operation time with the lowest possible amount of biomass for each tested combination of 5 and 23 h cells.

3. Results and discussion

Although the employed cells of *Pseudomonas putida* CECT 5279 collected at 9 h of growth time in resting cell assays showed the highest capability for desulfurization [1], the formulation of complex biocatalysts by combining cells of 5 and 23 h of growth time is suggested to offer better features due to the higher activities offered for desulfinase and monooxygenase enzymes, respectively. In order to determine an optimized formulation of complex biocatalyst, cells collected at 5 and 23 h were combined in different proportions as well as varying total biomass concentration. The results obtained using the aforementioned proposed biocatalysts were compared to the responses shown by 9 h cells when analogous total biomass concentrations are employed.

According to this, the experimental planning for this study of the complex biocatalyst and its comparison to the simple catalyst is shown in Table 1. This first table classifies each run according to the total biomass concentration used, C_X, the 23 h cell biomass fraction, Y^{23} (as shown in Eq. (5)) and the growth time, t_G, for cells employed in each experiment. In all cases, 25 μM DBT was utilized as initial sulfur model compound concentration. The other operational variables were maintained at a constant value, as mentioned in the BDS assay description.

In Figs. 1–4, values of X^{MAX}_{BDS}, $t_{BDS_{MAX}}$, R^0_{DBT} and P^{MAX}_{BDS}, respectively, for each tested total biomass concentration from 0.7 to 4.2 gDCW/L, are shown for both 9 h simple biocatalysts and a 5 and 23 h complex biocatalysts. When using complex biocatalysts, in order to highlight the relative composition of 5 and 23 h, variable Y^{23}, 23 h cell biomass fraction, is employed. Y^{23} shows the proportion of 23 h cell in the complex biocatalyst as shown by the following equation:

$$Y^{23} = \frac{C^{23}_X}{C^{5+23}_X} \quad (5)$$

In Fig. 5, values of specific conversion rate are represented for both 9 h simple catalysts and each 5 and 23 h biocatalyst proposed. Total biomass concentration and 23 h cell biomass fraction are the variables considered in this study.

Fig. 1 depicts a progressive increase in maximum percentage of biodesulfurization when higher biomass concentrations of 9 h cells are employed. Complete transformation of DBT into HBP is achieved when biomass concentrations higher than 2.1 gDCW/L are used. In

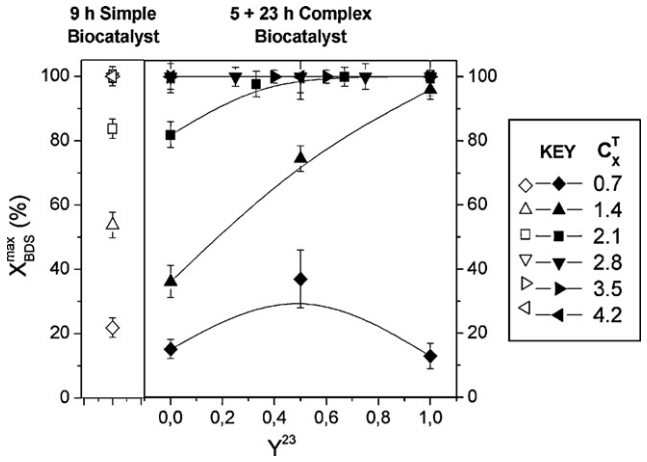

Fig. 1. Comparison of the values of X^{MAX}_{BDS} between a 9 h simple biocatalyst and different 5 and 23 h complex biocatalysts, employing total biomass concentrations between 0.7 and 4.2 gDCW/L.

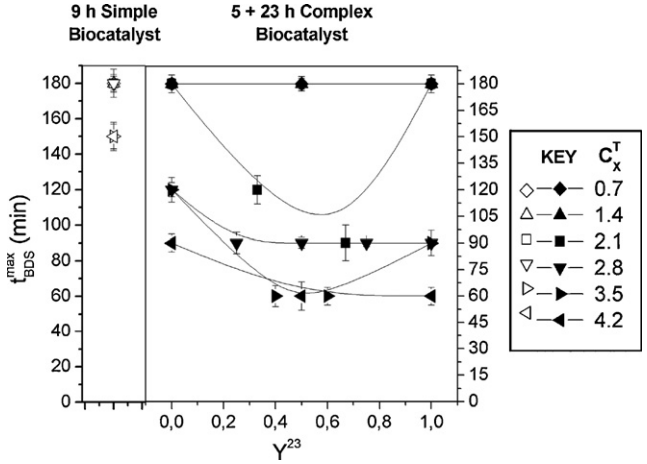

Fig. 2. Comparison of the values of $t_{BDS_{MAX}}$ between a 9 h simple biocatalyst and different 5 and 23 h complex biocatalysts, employing total biomass concentrations from 0.7 to 4.2 gDCW/L.

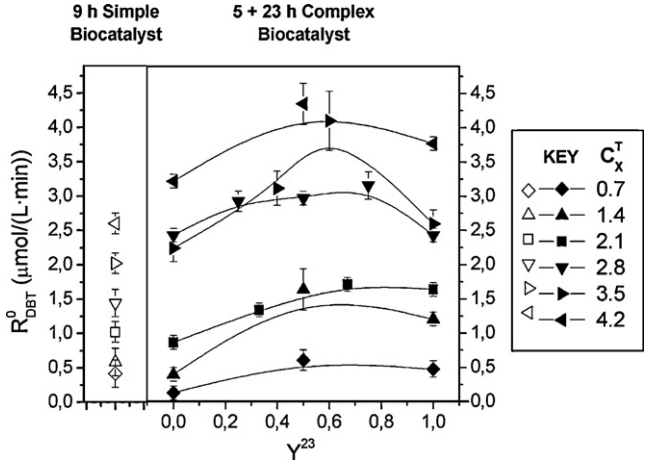

Fig. 3. Comparison of the values of R^0_{DBT} between a 9 h simple biocatalyst and different 5 and 23 h complex biocatalysts, employing total biomass concentrations between 0.7 and 4.2 gDCW/L.

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Table 1
Experimental schedule involving as variables the total biomass concentration (C_X^T), mass proportion of 23 h cells in the complex biocatalyst (Y^{23}) and growth time (t_G).

Run	Kind of biocatalyst	C_X^T (gDCW/L)	Y^{23}	t_G (h)
1	Simple	0.7	–	9
2	Complex	0.7	0.00	5+23
3	Complex	0.7	0.50	5+23
4	Complex	0.7	1.00	5+23
5	Simple	1.4	–	9
6	Complex	1.4	0.00	5+23
7	Complex	1.4	0.50	5+23
8	Complex	1.4	1.00	5+23
9	Simple	2.1	–	9
10	Complex	2.1	0.00	5+23
11	Complex	2.1	0.33	5+23
12	Complex	2.1	0.67	5+23
13	Complex	2.1	1.00	5+23
14	Simple	2.8	–	9
15	Complex	2.8	0.00	5+23
16	Complex	2.8	0.25	5+23
17	Complex	2.8	0.50	5+23
18	Complex	2.8	0.75	5+23
19	Complex	2.8	1.00	5+23
20	Simple	3.5	–	9
21	Complex	3.5	0.00	5+23
22	Complex	3.5	0.40	5+23
23	Complex	3.5	0.60	5+23
24	Complex	3.5	1.00	5+23
25	Simple	4.2	–	9
26	Complex	4.2	0.00	5+23
27	Complex	4.2	0.50	5+23
28	Complex	4.2	1.00	5+23

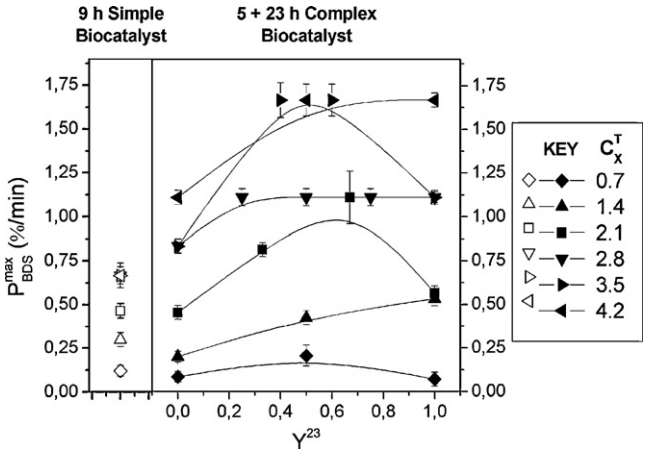

Fig. 4. Comparison of the values of P_{BDS}^{MAX} between a 9 h simple biocatalyst and different 5 and 23 h complex biocatalysts, employing total biomass concentrations from 0.7 to 4.2 gDCW/L.

the case of employing cells collected at 5 and 23 h of growth time, an analogous behavior is observed when total biomass concentration is raised. However, a higher percentage of biodesulfurization is achieved when the two cell qualities are combined ($0 < Y^{23} < 1$) than when using a simple biocatalyst consisting of cells of 9 h of growth time. According to this, neither a biocatalyst formed only by 5 h cells ($Y^{23} = 0$) nor a biocatalyst composed only by 23 h cells ($Y^{23} = 1$) offer better properties than a 9 h biocatalyst, when a total biomass concentration of 0.7 gDCW/L is employed. However, due to the combination of high desulfinase activity in 5 h cells and high monooxygenase activity in 23 h cells [1,2], the complex biocatalyst that combines these two kind of cells ($Y^{23} = 0.5$) achieve better behavior for BDS than the 9 h simple biocatalyst. That leads to a higher DBT conversion into HBP in the same time (180 min), and higher initial DBT removal rate and maximum productivity, as well. The employ of higher biomass concentrations shows the important contribution of 23 h cells to DBT removal. Despite the fact that this growth time presents a low desulfinase activity [1], increasing biomass concentration allows compensating this situation so that total DBT conversion into HBP is reached.

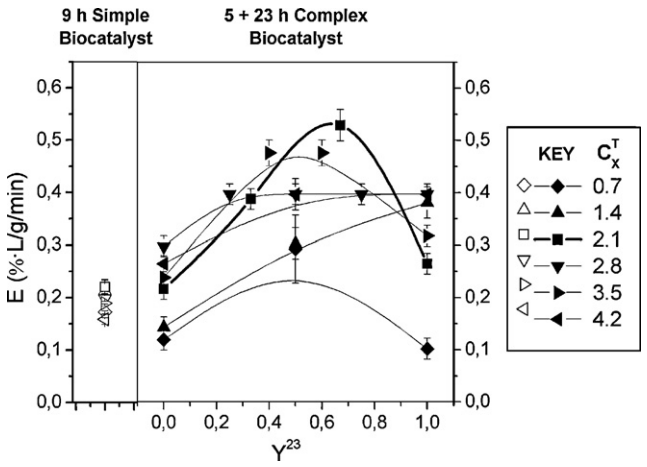

Fig. 5. Values of the specific conversion rate, *E*, for the different proposed 5 and 23 h complex biocatalysts varying total biomass concentration and the proportion of cells from each age.

In Fig. 2 needed times for maximum desulfurization, $t_{BDS_{MAX}}$, are presented for both simple and complex biocatalysts. The employ of simple biocatalysts only allows a light reduction in time when biomass concentrations higher than 2.8 gDCW/L are employed. In contrast, most combinations of 5 and 23 h cells constituting complex biocatalysts allow reducing needed time below 120 min. The combination of desulfinate activity in cells collected at 5 h of growth time and monooxygenase activities in 23 h cells, leads to the smallest needed times up to 60 min, as can be seen in the mentioned figure (at biomass total concentration higher than 2.8 g/L).

Similarly, Fig. 3 shows values obtained for initial DBT removal rate. There is an important improvement in DBT elimination when 5 and 23 h cells are combined in comparison with the employ of 9 h cells. Although biocatalysts formulated either only using 5 h cells or only 23 h cells cannot improve results from a simple biocatalyst when the lowest biomass concentration is employed (0.7 gDCW/L), the increase of cell concentration in a complex biocatalyst offers much better results than the ones obtained with a 9 h biocatalyst. Furthermore, Fig. 3 shows the advantages of combining two cell ages from the point of view of DBT removal rate, due to the mentioned desulfinase and monooxygenase activities working together. Therefore, the best R_{DBT}^0 values are obtained when Y^{23} ratios between 0 and 1 are employed.

Maximum productivity values are presented in Fig. 4 for both simple and complex biocatalysts. It is important to emphasize that combination of 5 and 23 h cells achieve much better results in all cases than the employ of a 9 h simple biocatalyst. In the latter case, increasing biomass concentration over 2.8 gDCW/L does not yield better results. However, combining high desulfinase activity of 5 h cells and high monooxygenase activities of 23 h cells offer the highest productivity values for each tested total biomass concentration. When only cells collected at 23 h of growth time are employed with the higher biomass concentration, high maximum productivity is maintained according to their low but even important desulfinase activity, which is compensated with the use of a high cell concentration. These different behaviors observed demonstrate the advantage of suitably exploiting the skills of 5 and 23 h cells in order to follow the different steps of the 4S pathway. The combination of cells with different predominant enzyme activities allows higher productivities than those where cells of a single growth time are used.

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Table 2
Comparison between a 9 h biocatalyst and the selected complex biocatalyst.

	9 h simple biocatalyst $C_X = 2.1$ g/L	5 + 23 h complex biocatalyst $Y^{23} = 0.67$; $C_X^T = 2.1$ g/L
X_{BDS}^{MAX} (%)	83.6	100.0
$t_{BDS_{max}}$ (min)	180	90
R_{DBT}^0 (μmol/(L min))	1.022	1.714
P_{BDS}^{MAX} (%/min)	0.465	1.111
E (% L/(g min))	0.221	0.530

As can be seen in Fig. 5, where values of specific conversion rate are represented for both 9 h simple biocatalysts and each 5 and 23 h biocatalyst proposed, this parameter is much larger when a complex biocatalyst is employed. From all the tested formulations, using both 5 and 23 h cells, one cell combination stands out among the rest. A complex biocatalyst formulated with a 1:2 ratio of 5 and 23 h cells ($Y^{23} = 0.67$) and a total biomass concentration of 2.1 gDCW/L, offers a complete DBT conversion in a reduced time with an acceptable concentration of invested biomass. Table 2 compares the parameter values obtained when the most active cell age for desulfurization [1] is employed in a simple biocatalyst to those when the selected formulation of a 5 and 23 h complex biocatalyst is used. Although 9 h simple biocatalyst achieves a high DBT conversion (ca. 84%), the selected complex biocatalyst allows complete transformation of DBT into HBP. Moreover, the time needed by 5 and 23 h cell biocatalyst in order to reach this value is doubled by the time needed by the 9 h cell for a lower percentage of desulfurization. Reached R_{DBT}^0 and P_{BDS}^{MAX} values are particularly higher in the complex biocatalyst than in the simple one.

4. Conclusions

The desulfurizating properties of complex biocatalysts composed by 5 and 23 h cells has been shown according to the respective desulfinase and monooxygenase activities contributed to the mixture [1]. The highest percentage of desulfurization, initial DBT removal rate and the lower time of desulfurization needed support this fact. The mixture composed by a 1:2 ratio of cells collected at 5 and 23 h of growth time of *Pseudomonas putida* CECT 5279, using a total biomass concentration of 2.1 gDCW/L showed the best capabilities for desulfurization in resting cells, among all the proposed biocatalyst formulations, according to the specific conversion rate, previously defined. Improving complex biocatalysts can offer better biodesulfurization processes, achieving higher DBT conversions and reducing time needed for biodesulfurization.

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Appendix A. Nomenclature

BDS biodesulfurization
C_j concentration of compound j (μM)
C_X^i concentration of biomass (gDCW/L), total or at i growth time
DBT dibezothiophene
DBTO dibenzothiophene-5-oxide
$DBTO_2$ dibenzothiophene-5,5-dioxide
E specific conversion rate ((% L)/(min gDCW))
HBPS 2-(2-hydroxybiphenyl)-benzenesulfinate
HBP 2-hydroxybiphenyl
HDS hydrodesulfurization
IPTG isopropyl-β-D-galactopyranoside
Mtoe millions of tons of oil equivalents
P_{BDS}^{MAX} maximum biodesulfurization productivity
R_{DBT}^0 initial DBT removal rate
$t_{BDS_{max}}$ time for maximum biodesulfurization (min)
t_G growth time (h)
X_{BDS} percentage of biodesulfurization (% BDS)
Y^{23} mass proportion of 23 h cells in the complex biocatalyst

Subindexes
DBT refers to dibezothiophene
HBP refers to 2-hydroxybiphenyl
X refers to biomass

Superindexes
0 refers to initial time
5 refers to 5 h growth cell time
5 + 23 refers to both 5 and 23 h growth cell time
9 refers to 9 h growth cell time
23 refers to 23 h growth cell time
MAX refers to maximum value
T refers to the total amount of cells

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