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Extended kinetic model for DBT desulfurization using *Pseudomonas Putida* CECT5279 in resting cells

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ABSTRACT

Dibenzothiophene desulfurization by *Pseudomonas putida* CECT5279, genetically modified microorganism, in resting cells is studied. In previous works, operational conditions were established and a kinetic model describing the four serial reactions was proposed. Later studies showed the existence of two characteristic growth times of this bacterium, 5 and 23 h, offering maximum activities in the desulfinase and the monooxygenase enzymes of this route. The combination of cells collected at 5 and 23 h of growth time was proved to be a very effective biocatalyst for desulfurization in resting cells. In this work, the previously proposed kinetic model is extended and applied to these cells with different ages. Moreover, other extension is considered, taking into account the activity loss of the enzymes involved in 4S route, and the influence of biomass concentration employed. These extensions are of considerable importance in order to scale-up the process. The kinetic model developed is able to fit the experimental results for resting cell operation with cells of different ages, in different concentration taking into account the enzyme deactivation.

1. Introduction

Sulfur oxide emissions are responsible for many well-known problems on human health, environment and materials. More restrictive legal limitations about sulfur content in fossil fuels have been imposed in European Union [1], United States [2] and other countries such as Japan and Canada [3]. Many efforts are focused on reaching such low limits by developing different proposed technologies [4]. Hydrodesulfurization (HDS) is the most extensively employed method. However, some aromatic sulfur-compounds, such as 4- and 4,6-alkyldibenzothiophene DBT, and polyaromatic sulfur compounds, show resistance to be completely removed.

Biodesulfurization (BDS) is one of the emerging technologies proposed to solve these problems. BDS consists of the employ of microorganisms, their enzymes or cellular extracts as catalysts in order to remove sulfur present in fuels [5–9]. BDS has been presented as a complementary technique which, added to a previous HDS process. Among its advantages, BDS offers a high selectivity through the employ of microbial enzymatic systems with the ability of reducing the generation of undesirable byproducts [5,6,10], and selective routes to avoid C—C bond breakdown helping to maintain the final properties of the fuel [3,5,6,11,12].

Due to abundance of some aromatic sulfur in fossil fuels [5,13] (particularly in heavier oil distillates [14]) and their special resistance to be removed by conventional HDS processes [5,6,14], dibenzothiophene (DBT) and its alkylated forms are usually chosen as model compounds in desulfurization studies. 4S route is an oxidative and non-destructive metabolic pathway, carried out by *Rhodococcus erythropolis* IGTS8, which belongs to this kind of selective routes [2,3,9,15–17]. This route is formed by four serial reactions make up this route through the transformation of DBT into a free sulfur molecule, 2,2′-hydroxybiphenil (HBP) [18,19]. 4S route is catalyzed by two monoxygenases, DszC and DszA, and one desulfinase, DszB [17,20]. 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 [18,20,21]. This route has been found in other wild type microoganisms such as *Pantoea agglomerans* [22] or *Lysinibacillus sphaericus* [23].

One of the aspects to be improved in BDS in order to be developed as an industrial scale process involves obtaining better biocatalysts for desulfurization [3,6,8,9,24–26]. *Pseudomonas putida* CECT 5279 is the biocatalyst employed in this work. It is a genetically modified bacterium with the ability of expressing the 4S pathway due to carrying the genes *dszABC* from *Rhodococcus erythtropolis* IGTS8, and a flavin-oxydo-reductase from *Escherichia coli* (*hpaC*) [27,28]. Previous works focused on desulfurization of DBT using *P. putida* CECT5279 studied the influence of the medium composition and the conditional operations on the desulfurization capabilities of this bacterium. A maximum in DBT conversion is

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Nomenclature

BDS	biodesulfurization
C_j	concentration of compound (μM)
C_X^i	concentration of biomass (g L^{-1}), at i growth time
DBT	dibenzothiophene
DBTO	dibenzothiophene-5-oxide
$DBTO_2$	dibenzothiophene-5,5-dioxide
F_F	Fischer's F, statistical parameter
FMN	flavin mononucleotide (oxidized form) concentration of compound j (oxidized form)
$FMNH_2$	flavin mononucleotide (reduced form)
HBPS	2-(2-hydroxybiphenyl)-benzenesulfinate
HBP	2-hydroxybiphenyl
HDS	hydrodesulfurization
IPTG	isopropyl-β-D-galactopyranoside
k_i	kinetic parameter of reaction i (μM $min^{-1)}$
k_i'	modified kinetic parameter of reaction i (min^{-1})
K_i	affinity parameter of substrate of reaction i (μM)
K_i'	modified affinity parameter of substrate of reaction i (μM^{-1})
K_I	inhibition constant of Eq. (13) (μM)
K_I'	inhibition constant of Eq. (13) (μM^{-1})
Mtoe	millions of oil equivalent tons
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
r_i	rate of reaction i (μM/min)
R_j	rate of production of compound j (μM/min)
SSR	square sum of residuals. $SSR = \sum_{i=1}^{N} (C_j^{EXP} - C_j^{TEO})^2$
t_G	growth time (h)
t_S	Student t, statistica. parameter

Subindexes

DBT	refers to dibezothiophene
HBP	refers to 2-hydroxybiphenyl

Superindexes

0	refers to initial rate
5	refers to 5 h growth cell time
23	refers to 23 h growth cell time
EXP	refers to experimental value
TEO	refers to predicted value

achieved when cells collected at 9 h of growth time are employed for DBT desulfurization in resting cell conditions [29,30]. A later work carried out a deep study on BDS of DBT using resting cells and it could prove that the transport of all 4S route intermediates across the cell membrane is not a mass transport controlling resistance [31], and that neither the intracellular concentrations of reducing cofactors or the NADH dependent reductase HpaC have influence in the desulfurization rate [31]. As a result of the former, in that previous work [32] the evolution of *in vivo* enzymatic activities of DszA, DszB and DszC were measured along the growth curve of *P. putida* CECT5279 [32]. Maximum activities of both flavin-dependent monooxygenases, DszC and DszA, were found in cells of 23 h of growth time, and a maximum activity for the desulfinase DszB was observed in cells of 5 h of growth time of *P. putida* CECT5279. These different patterns of expression of monooxygenases DszA and DszC and desulfinase DszB along growth curve can explain the behavior of cells collected at 9 h of growth time, observed previously [32]. In a recent work [33], the possibility of combining cells collected at 5 and 23 h of growth time to formulate complex biocatalyst was studied. Biocatalysts containing 5 and 23 h cells in a 1:1 ratio were compared to simple biocatalysts, composed exclusively by 9 h cells, maintaining total biomass concentration as a constant. These combinations of cells collected at these cell ages, offered better desulfurizing capabilities for desulfurization than a 9 h simple biocatalyst [33].

Kinetic studies on BDS in literature are not frequently focused on the 4S route as a whole four serial reaction network. The most commonly studied step of this pathway is the first reaction, consisting on the disappearance of DBT, whose rate has been correctly described through a Michaelis–Menten kinetic equation [34–37]. Further analysis on kinetic knowledge about intermediate reactions of this route comes down to *in vitro* works studying the activities of both monooxygenases DszC [38] and DszA [11,39]. Particularly, the last step of this route presents high interest because it has been proved to act as the controlling step of the overall process [40,41]. It is also known that the final product of the 4S route, HBP, causes a competitive inhibition on the desulfinase enzyme, DszB, the enzyme of the last reaction [42,43].

Works focused on the study of whole 4S route are scarce in literature. In previous works, a kinetic model, able to describe both biomass growth of *P. putida* CECT5279 and BDS capability accumulation during cell growth, was proposed [29,30]. Later on, a model describing the evolution of every compound involved in 4S route (DBT, DBTO, $DBTO_2$, HBPS and HBP) for BDS in resting cells was proposed [44]. As neither HpaC activity nor reduced cofactor concentrations have been proved to limit desulfurization rate in resting cells conditions [27]; lateral reactions contributing to $FMNH_2$, needed for monooxygenases DszC and DszA, are not considered in this kinetic model. This model was able to describe the evolution of the mentioned compounds during resting cell assays employing cells of *P. putida* CECT5279 collected at different cellular ages [44].

The aim of this work is to extend and apply the previous kinetic model proposed [44] to the employ of two characteristic cell ages, 5 and 23 h, according to their proved high capabilities to carry out, respectively, the last step of the 4S route, and its first three reactions [32]. This kinetic model will be extended considering, on one hand, the possible loss of activity of the enzymes involved in each of the reactions of the 4S route during the time of desulfurization, and, on the other hand, the influence of biomass concentration employed of each kind of cells. As a result, the final obtained model must be able to describe BDS experiments carried out in resting cells conditions considering the deactivation of enzymes involved in 4S route during the operation time. In addition to this improvement, cell concentration contributed by these two characteristic cell ages will be taken into account. These refinements will allow the kinetic model to better describe the BDS process under the previously mentioned conditions.

2. Materials and methods

2.1. Chemicals

DBT and $DBTO_2$ were supplied by Aldrich, and HBP by Fluka, HEPES buffer and IPTG (isopropyl-β-D-galactopyranoside) were purchased from Sigma, and deionized water (resistance = 18.2 Ω) was used to prepare all media and stock solutions. DBTO and HBPS were not commercial compounds, it was necessary to perform the synthesis of both in our laboratory using previously described methods [18,45].

2.2. Microorganism

P. putida CECT5279 was the microorganism employed as biocatalyst for this study. It was supplied by the Biological Research