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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

Table 1
Kinetic equations for 4S reaction scheme.

i	r_i Previously proposed kinetic model [1]		r_i Extended kinetic model	
1	$\frac{k_1 \cdot C_{DBT}}{K_1 + C_{DBT}}$	(T1)	$\frac{k'_1 \cdot \exp(-S_1 \cdot t) \cdot C_{DBT}}{1 + K'_1 \cdot C_{DBT}} \cdot C_X$	(T5)
2	$\frac{k_2 \cdot C_{DBTO}}{K_2 + C_{DBTO}}$	(T2)	$\frac{k'_2 \cdot \exp(-S_2 \cdot t) \cdot C_{DBTO}}{1 + K'_2 \cdot C_{DBTO}} \cdot C_X$	(T6)
3	$\frac{k_3 \cdot C_{DBTO_2}}{K_3 + C_{DBTO_2}}$	(T3)	$\frac{k'_3 \cdot \exp(-S_3 \cdot t) \cdot C_{DBTO_2}}{1 + K'_3 \cdot C_{DBTO_2}} \cdot C_X$	(T7)
4	$\frac{k_4 \cdot C_{HBPS}}{K_4 \left(1 + \frac{C_{HBP}}{K_I}\right) + C_{HBPS}}$	(T4)	$\frac{k'_4 \cdot \exp(-S_4 \cdot t) \cdot C_{HBPS}}{1 + K'_5 \cdot C_{HBP} + K'_4 \cdot C_{HBPS}} \cdot C_X$	(T8)

Table 2
Experimental planning carried out.

Run	t_G (h)	C_X (g L^{-1})
1	5	0.7
2	5	1.4
3	5	2.1
4	5	2.8
5	5	3.5
6	5	4.2
7	23	0.7
8	23	1.4
9	23	2.1
10	23	2.8
11	23	3.5
12	23	4.2

Center (CIB-CSIC-Madrid, Spain). Cultures were concentrated, resuspended with a glycerol in saline serum (50%) solution and conserved at −80 °C.

2.3. Biocatalyst production

An standardized procedure [29,30] was followed in order to obtain comparative experimental results. Frozen stocks were employed as inoculum 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 step was accomplished in LB medium under the same conditions for 4 h, after inoculating bacteria from the previous culture. These cells were employed as inocula 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^{-1}; K_2HPO_4, 4 g L^{-1}; $MgCl_2{\cdot}6H_2O$, 0.0245 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 0.001 g L^{-1}; $MgSO_4$, 2 mM; glycerine, 2% (w/w); $FeCl_3{\cdot}6H_2O$, 0.001 g L^{-1}; NH_4Cl, 2 g L^{-1}. IPTG (0.2 mM) and tetracycline (25 μg/mL), as explained in previous work [29]. The carbon source employed in these experiments was glutamic acid 20 g L^{-1} [29,30]. Operational conditions employed were: 1 L/L/min of aeration, 200 rpm of stirrer speed and 30 °C of temperature. Since it has been shown in a previous work that *P. putida* CECT5279 yields higher DBT conversion when glutamic acid is used as carbon source in a non-buffered medium [30], pH was not either adjusted, or controlled during the process.

2.4. Resting cell assays

Resting cell desulfurization assays were carried out by using cells previously collected at 5 and 23 h of growth time and conserved at −18 °C, after centrifugation and resuspension in a glycerol–saline (50:50) solution [32,33]; 1 mL of this solution was inoculated into a 100 mL Erlenmeyer flask, containing 16 mL of 50 mM HEPES buffer (pH 8.0) and 25 μM DBT. Biomass concentrations of both 5 and 23 h cells were varied from 0.7 to 4.2 g L^{-1}. Desulfurization was carried out at 30 °C, 210 rpm in orbital shaker for 180 min. Samples of 0.5 mL were collected periodically. These 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 taken for analysis [32,33].

2.5. Analytical methods

Determination of biomass concentration was carried out measuring optical density at 600 nm (OD_{600}) using a Shimadzu UV-visible spectra-photometer (model UV-1603). HPLC was employed to analyze DBT, DBTO, $DBTO_2$, HBPS and HBP. Samples were acidified by using HCl and then analyzed employing 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. However, a concentration gradient in the mobile phase was employed in order to separate every compound in 4S route as described in a previous work [32]. Peaks were monitored at different wave lengths (234 nm for DBT and $DBTO_2$, 220 nm for DBTO 202 nm for HBPS and 206 nm for HBP) employing a diode array detector. Calibrations were performed using commercial and high purified standards of every compound.

2.6. Parameter calculation methods

Estimation of parameters has been carried out employing a non-linear regression algorithm, by means of NL2SOL method (Non Linear Least Square Solver), a variation of Newton method [46]. Single-response regression has been used in case of studying each reaction separately and multiple-response regression when applying it over the whole reaction network. The integral method was employed in all cases in order to integrate de kinetic equations from their differentiated forms [47]. The implicit Euler method [48] has been used as numerical method for integration. Statistical meaning of the obtained fits was evaluated through Fischer's *F* and Student's *t*-tests. The confidential interval of the parameters and the sum of square of residuals (SSR) has been calculated in order to evaluate the goodness of fits.

3. Kinetic model

4S route expressed by *P. putida* CECT5279 involves five reactions, including the four serial reactions and a lateral reaction producing $FMNH_2$ [17]. In a previous work was proved that the basal concentration of reduced equivalents does not limit the BDS rate [31]. As a result, the reaction scheme can be simplified into the following one [44]:

$$DBT + ½\,O_2 \xrightarrow{r1} DBTO \quad (1)$$

Table 3
Preliminary kinetic parameter values obtained in the application of reaction rate method for cellular ages of 5 and 23 h.

Parameter	t_G = 5 h			t_G = 23 h			Units
	Optimun value		Confidence limit	Optimun value		Confidence limit	
k'_1	7.59×10^{-2}	±	0.70×10^{-2}	9.94×10^{-2}	±	0.69×10^{-2}	($L g^{-1} min^{-1}$)
S_1	1.84×10^{-2}	±	0.33×10^{-2}	0.53×10^{-2}	±	0.33×10^{-2}	(min^{-1})
k'_2	0.146	±	0.009	0.311	±	0.049	($L g^{-1} min^{-1}$)
S_2	1.61×10^{-2}	±	0.18×10^{-2}	1.26×10^{-2}	±	0.71×10^{-2}	(min^{-1})
k'_3	2.14×10^{-2}	±	0.39×10^{-2}	0.195	±	0.051	($L g^{-1} min^{-1}$)
S_3	0.32×10^{-2}	±	0.24×10^{-2}	2.28×10^{-2}	±	0.77×10^{-2}	(min^{-1})
k'_4	0.469	±	0.200	0.826	±	0.327	($L g^{-1} min^{-1}$)
S_4	1.52×10^{-2}	±	0.40×10^{-2}	3.60×10^{-2}	±	0.69×10^{-2}	(min^{-1})

Table 4
Kinetic parameter values obtained in the application of production rate method for cellular age of 5 h.

Kinetic parameter					t_s		F_F		SSR
					Tab.	Tab.	Calc.	Tab.	
k'_1	7.59×10^{-2}	±	0.55×10^{-2}	($L g^{-1} min^{-1}$)	26.91	1.97			
S_1	1.62×10^{-2}	±	0.23×10^{-2}	(min^{-1})	13.81	1.97			
k'_2	0.153	±	0.023	($L g^{-1} min^{-1}$)	13.15	1.97			
S_2	1.75×10^{-2}	±	0.32×10^{-2}	(min^{-1})	10.90	1.97	1593.28	1.25	1.52
k'_3	4.07×10^{-2}	±	0.31×10^{-2}	($L g^{-1} min^{-1}$)	25.36	1.97			
S_3	1.44×10^{-2}	±	0.22×10^{-2}	(min^{-1})	13.00	1.97			
k'_4	0.859	±	0.046	($L g^{-1} min^{-1}$)	3.71	1.97			
S_4	2.36×10^{-2}	±	0.51×10^{-2}	(min^{-1})	9.16	1.97			

Table 5
Kinetic parameter values obtained in the application of production rate method for cellular age of 23 h.

Kinetic parameter					t_s		F_F		SSR
					Tab.	Tab.	Calc.	Tab.	
k'_1	9.72×10^{-2}	±	0.95×10^{-2}	($L g^{-1} min^{-1}$)	11.33	1.97			
S_1	0.45×10^{-2}	±	0.24×10^{-2}	(min^{-1})	3.68	1.97			
k'_2	0.290	±	0.072	($L g^{-1} min^{-1}$)	7.90	1.97			
S_2	1.28×10^{-2}	±	0.44×10^{-2}	(min^{-1})	5.76	1.97	333.58	1.24	2.36
k'_3	0.162	±	0.035	($L g^{-1} min^{-1}$)	9.15	1.97			
S_3	2.12×10^{-2}	±	0.33×10^{-2}	(min^{-1})	12.48	1.97			
k'_4	0.226	±	0.039	($L g^{-1} min^{-1}$)	11.33	1.97			
S_4	1.44×10^{-2}	±	0.32×10^{-2}	(min^{-1})	8.92	1.97			

$$DBTO + 1/2\,O_2 \xrightarrow{r2} DBTO_2 \quad (2)$$

$$DBTO_2 + 1/2\,O_2 \xrightarrow{r3} HBPS- + H^+ \quad (3)$$

$$HBPS^- + H_2O \xrightarrow{r4} HBP + SO_3^= + H^+ \quad (4)$$

These reactions were assigned kinetic equations according to the literature [34–37,42,43]. Therefore, Michaelis–Menten kinetics was assumed for reactions (1)–(3) and product competitive inhibition for reaction (4), as shown in the expressions (T1)–(T4) [44] gathered in Table 1.

In the present work, a generalization of the kinetic model exposed above is proposed in order to be able to describe the evolution of the compounds involved in 4S route (DBT, DBTO, $DBTO_2$, HBPS and HBP) for long periods of time. In order to achieve this, the loss of activity of the enzymes involved in this pathway during BDS process, need to be taken into account. The extension of the kinetic model will also consider the concentration of biomass associated to each different cell age employed during the assay. The hypothesis of deactivation of 4S-route enzymes comes up from the observation of a saturating evolution of both intermediate compounds and HBP when low biomass concentrations are employed. This suggests the possibility of a drop of enzymatic activity as process prolongs. According to all this, first order kinetics of deactivation is coupled in equations (T1)–(T4) while a term considering biomass concentration is also included, as it can be seen in expression (T5)–(T8), also gathered in Table 1.

As can be seen, previous hyperbolic expressions (T1)–(T4) were transformed by dividing both numerator and denominator by the affinity of each reaction (K_i) in order to avoid possible convergence problems during fitting.

According to this, the new extended kinetic model can be expressed in terms of rates of production for each key compound (DBT, DBTO, $DBTO_2$, and HBP) through expressions (5)–(8) (as explained in Appendix A).

$$R_{DBT} = \frac{dC_{DBT}}{dt} = -r_1 \quad (5)$$

$$R_{DBTO} = \frac{dC_{DBTO}}{dt} = r_1 - r_2 \quad (6)$$

$$R_{DBTO_2} = \frac{dC_{DBTO_2}}{dt} = r_2 - r_3 \quad (7)$$

$$R_{HBP} = \frac{dC_{HBP}}{dt} = r_4 \quad (8)$$

Being the change on the other compound of the 4S route, HBPS, calculated by stoichiometry or according to the following equation:

$$R_{HBPS} = \frac{dC_{HBPS}}{dt} = r_3 - r_4 \quad (9)$$

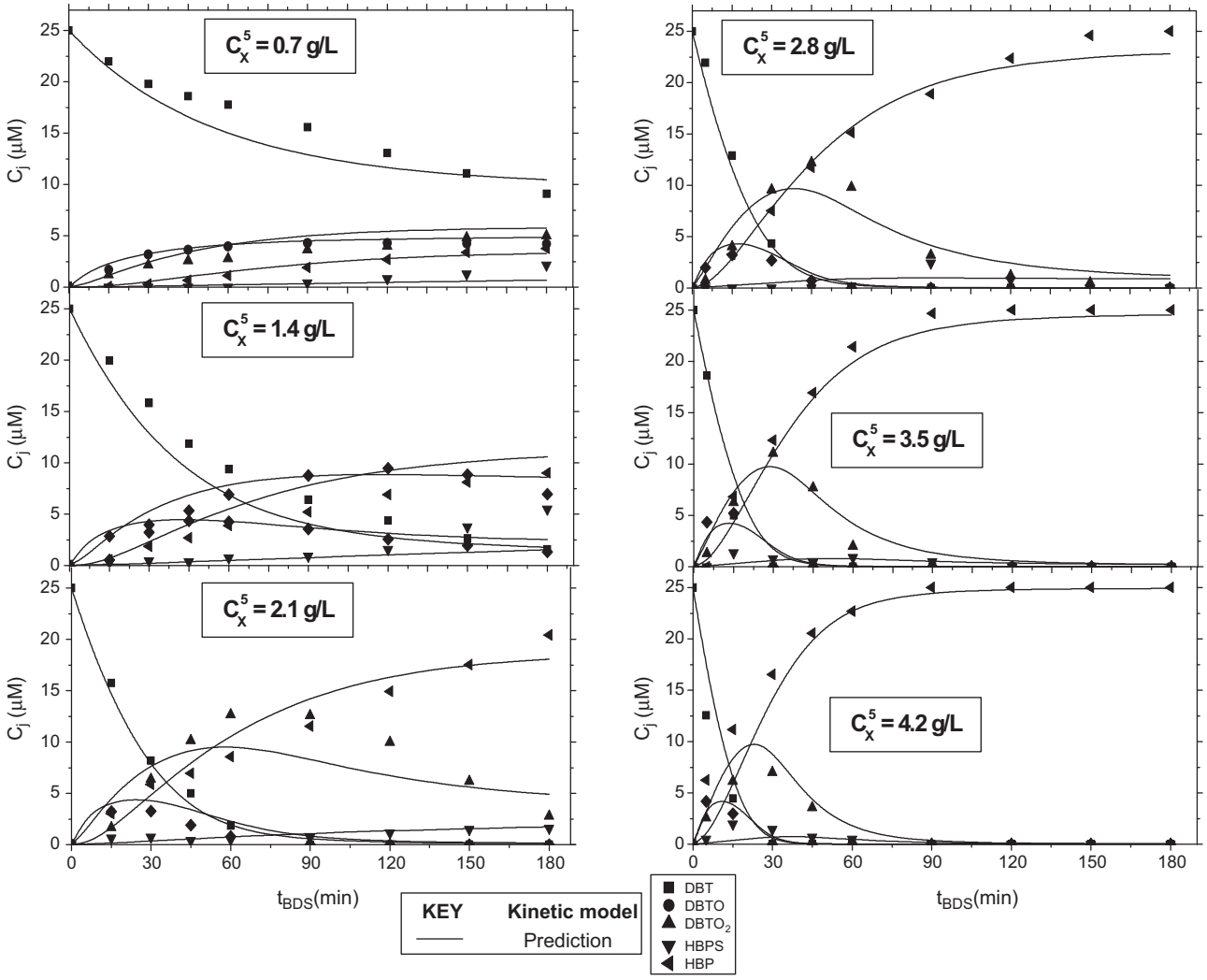

Fig. 1. Evolution of experimental data of DBT, DBTO, $DBTO_2$, HBPS and HBP for biomass concentrations between 0.7 and 4.2 g L^{-1}, and the prediction of kinetic model applying production rate method, when cells collected at 5 h of growth time are employed.

4. Results and discussion

The development of this kinetic model has been carried out in two steps. In a first stage, the reaction rate method (as explained in Appendix A), through a nonlinear simple-response regression [48], has been applied in order to compare the fit goodness when enzymatic deactivation is taken into account or left out, on each reaction studied separately. The second aim of this first stage consists of obtaining preliminary values for kinetic parameters. In a second stage, production rate method has been applied by using a non linear multi-response regression in order to obtain definitive parameters values. This methodology has been applied on two sets of experimental data, corresponding with cells of *P. putida* CECT5279 collected at 5 and 23 h of growth time, respectively. In Table 2, the experimental design is summarized. In all cases, 25 μM DBT was employed as initial sulfur model compound concentration. The other operational variables were maintained at a constant value, as mentioned before in the BDS assay description.

As a first approximation to the values of kinetic parameters, the reaction rate method has been applied over the four reactions involved in the 4S route. As explained before, a single response and non linear regression was employed in an integrated way. In order to achieve this DBT, DBTO, $DBTO_2$ and HBP have been chosen as key compounds for each of the four reactions of this route. In order to simplify parameter estimation, the mentioned reaction rate method can be applied [48]. The coincidence between the number of reactions to be studied and the number of key compounds allows relating reaction rates and production rates as shown in Appendix A.

Proposed extended kinetic equations have been checked following this methodology. Experimental results using both 5 and 23 h growth cells were employed to carry out non linear regressions studying each of the reactions of the 4S route separately. The goodness of fits allowed to discriminate whether considering deactivation of enzymes involved in each reaction or not, leads to improve the kinetic model. Substrate affinity parameters, K_i' and inhibitions constants, K_I' have been fixed for every reaction using previously obtained values of $K_1' = 0.153\,M^{-1}$, $K_2' = 0.266\,M^{-1}$, $K_3' = 0.109\,M^{-1}$, $K_4' = 9.18 \times 10^{-2}\,M^{-1}$ and $K_I' = 0.127\,M^{-1}$ [1].

4.1. Application of the reaction rates method

4.1.1. Cells collected at 5 h of growth time

The four reactions involved in 4S route have been studied separately. Experimental data of concentration of DBT, DBTO, $DBTO_2$ and HBP from desulfurization assays were employed, respectively, for each reaction. Simultaneous fitting on data using six

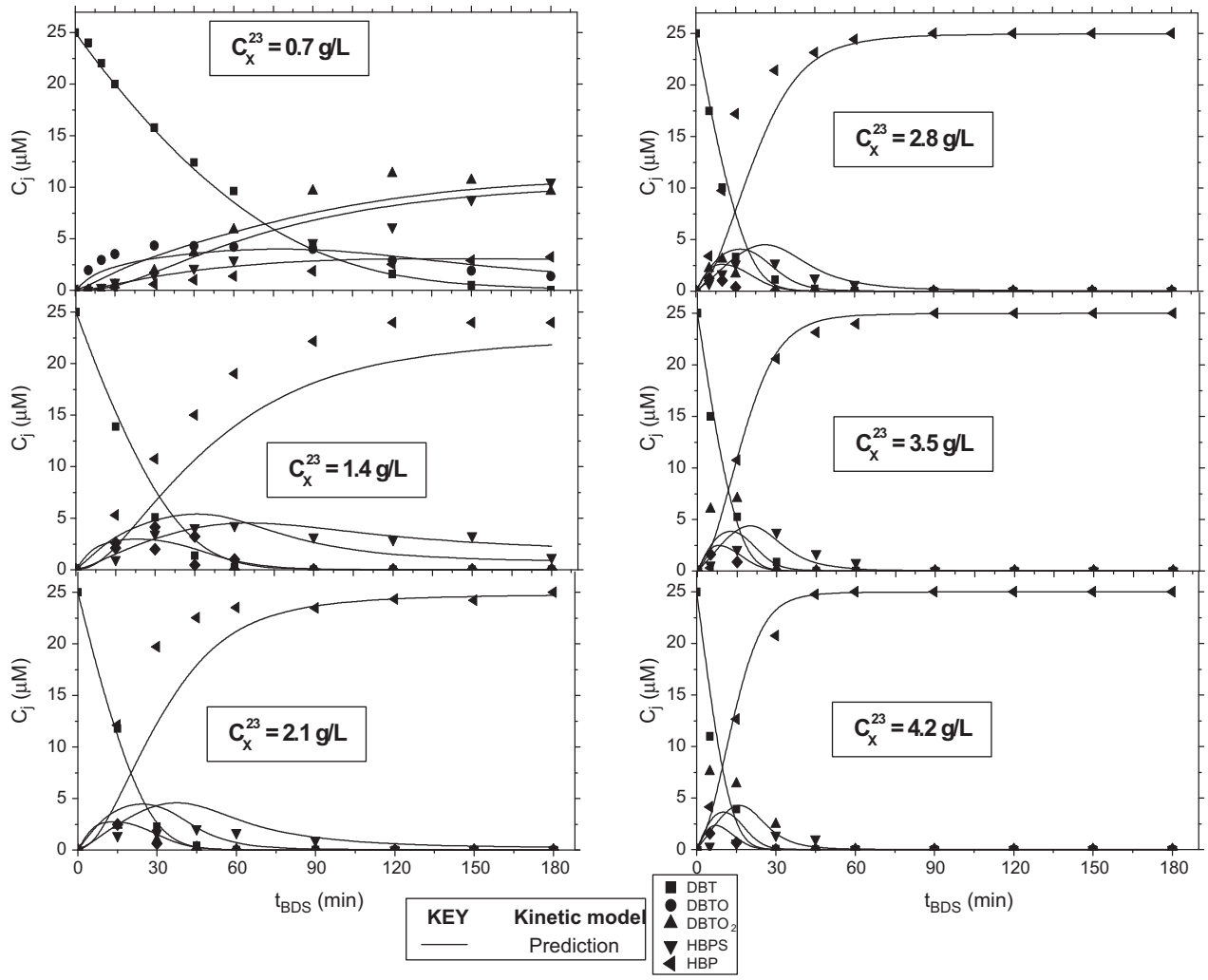

Fig. 2. Evolution of experimental data of DBT, DBTO, $DBTO_2$, HBPS and HBP for biomass concentrations between 0.7 and 4.2 g L^{-1}, and the prediction of kinetic model applying production rate method, when cells collected at 23 h of growth time are employed.

biomass concentrations between 0.7 and 4.2 g L^{-1} was carried out for each compound. In all cases, statistically significant results were obtained according to higher values in Fisher's *F* and Student's *t*-values in comparison to the ones tabulated for the same number of freedom degrees at a 95% confidence level. As it was expected, the employ of an additional parameter in order to take into account the enzyme deactivation allowed a better fitting of experimental data.

In all the cases, for the four reactions of the 4S route, fittings of the model to experimental data taking into account the enzyme deactivation were much better than if such deactivation is not considered. Mainly for the intermediate compounds (DBTO and HBPS), when enzyme deactivation is not considered, the fittings of the model to experimental data show a clear trend in the error. Moreover, model prediction for HBP concentration is good for experimental data at low biodesulfurization time, but data at long times, greater than 1 h operating in resting cells, are overestimated if the enzyme deactivation is not considered. Studying each reaction separately allowed for proving the advantage of taking into account the loss of activities of DszC, DszA and DszC for desulfurization of DBT in resting cell conditions employing cells of *P. putida* CECT5279 collected at 5 h of growth time.

4.1.2. Cells collected at 23 h of growth time

Hereafter, experiments of biodesulfurization employing 23 h cells were used to carry out an analogous study. As reported previously, statistically significant results were obtained in all cases according to higher values in Fisher's *F* and Student's *t*-values in comparison to the ones tabulated for the same number of freedom degrees at a 95% confidence level.

The evolutions of the 4S route compounds when 23 h cells were employed, with biomass concentrations between 0.7 and 4.2 g L^{-1}, are well fitted by the model. Again, if the possible loss of activity of DszC enzyme is taken into account, fittings results are closer to experimental values, but the difference is smaller than with 5 h cells. Again the differences are some bigger for the intermediates compounds of the 4S route.

From these results, it could be concluded that the employ of the reaction rate method on every reaction of the 4S route, both for 5 and 23 h cells, allowed the checking of this extended kinetic model, obtaining preliminary values for the kinetic parameters (Table 3).

4.2. Application of the production rates method

In this second step of calculus, definitive values for kinetic parameters have been obtained. As explained before, a multiple-response and non-linear regression was employed, through the production rate method, using the preliminary values obtained through reaction rate method shown above, as starting point. A four serial reaction scheme such as 4S route, whose steps are coupled, constitutes a multiple response system. Therefore, a

multiple-response method is expected to be the best methodology for kinetic parameters estimation.

Differential equations (5)–(9), presented above, express the production rate of DBT, DBTO, $DBTO_2$, HBPS and HBP, and can be integrated yielding Eq. (10). Thus, the concentrations of the main compounds involved in 4S route can be expressed in an integrated way, resulting in an integrated kinetic model (as shown in Appendix A).

$$C_j - C_j^0 = \sum_{i=1}^{NC} \nu_{ij} \cdot \int_{t_0}^{t} r_i \cdot dt \tag{10}$$

As it has been carried out in the previous stage of calculus, substrate affinity parameters, K_i' and inhibitions constants, K_I' have been fixed for every reaction using previously obtained values of $K_1' = 0.153\,M^{-1}$, $K_2' = 0.266\,M^{-1}$, $K_3' = 0.109\,M^{-1}$, $K_4' = 9.18 \times 10^{-2}\,\mu M^{-1}$ and $K_I' = 0.127\,\mu M^{-1}$ [44].

4.2.1. Cells collected at 5 h of growth time

Simultaneous fittings of the experimental data of the key compounds (DBT, DBTO, $DBTO_2$ and HBP) together with other 4S route compound (HBPS) were carried out using 5 h cells with a biomass concentration between 0.7 and 4.2 g L^{-1}. Table 4 shows the values of kinetic parameters obtained by this fitting. In Fig. 1 the experimental data and the model predictions are plot along time. Errors are set below 20% in almost all cases. Therefore, good predictions for every compound involved in 4S route have been obtained using a kinetic model that takes into account biomass concentration and deactivation of 4S-route enzymes, DszC, DszA and DszB, in the biomass concentration range studied.

4.2.2. Cells collected at 23 h of growth time

Kinetic parameters obtained by application of the production rate method with 23 h cells are compiled in Table 5. As can be seen in Fig. 2, appropriate predictions of DBT, DBTO, $DBTO_2$, HBPS and HBP have been obtained using the proposed kinetic model. In all cases, predictive errors below 20% reflect the goodness of fitting. The best results are obtained when using experimental data with the higher biomass concentrations (2.8, 3.5 and 4.2 g L^{-1}).

5. Conclusions

The good results obtained in the predictions of BDS data in resting cell conditions by a kinetic model which takes into account not only biomass concentration, but enzyme deactivation as well, are consistent with previous works [29–33,44]. Obtained parameter values are not only statistically significant, but coherent with previous knowledge about 5 and 23 h cell behavior [32] as well. Therefore, the advantage of using a multiple-response regression on a multiple-response system is shown. After comparing the values of the kinetic parameter values in Tables 4 and 5, higher monooxygenase activity is found in 23 h cells, as previously showed [33], it is also supported by higher values of k'^{23}_1, k'^{23}_2 (for enzyme DszC) and k'^{23}_3 (for enzyme DszA) than k'^{5}_1, k'^{5}_2 and k'^{5}_3, in cells of 5 h of growth time. Analogously, value of k'^{5}_4 is higher than k'^{23}_4 according to a previously reported higher enzymatic activity of desulfinase DszB in 5 h cells than in 23 h cells.

The lower parameter values of S_1^{23} and S_2^{23}, in comparison to those S_1^5 and S_2^5, can be due to a slower deactivation of monooxygenase DszC in 23 h cells than in cells collected at 5 h of growth time. This situation seems to be supported by the small differences between fitting ignoring and taking into account the loss of activity of this enzyme for the 23 h cells.

This extended kinetic model is useful for the description of DBT desulfurization by *P. putida* CECT5279 in resting cell conditions, when biomass concentrations between 0.7 and 4.2 g L^{-1} are employed. This model can be used in the study of BDS by characteristic cellular ages of 5 and 23 h of growth time. The interest of this model is in the behavior prediction of these cells in resting cell conditions, including the possible mixtures of cells collected at different ages.

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Appendix A.

A.1. Application of the reaction rate method to the 4S pathway

As previously explained, the following simplified reaction scheme can be considered for the desulfurizating 4S pathway [44]:

$$DBT + \tfrac{1}{2}\,O_2 \xrightarrow{r1} DBTO \tag{A.1}$$

$$DBTO + 1/2\,O_2 \xrightarrow{r2} DBTO_2 \tag{A.2}$$

$$DBTO_2 + 1/2\,O_2 \xrightarrow{r3} HBPS- + H^+ \tag{A.3}$$

$$HBPS^- + H_2O \xrightarrow{r4} HBP + SO_3^= + H^+ \tag{A.4}$$

DBT, DBTO, $DBTO_2$ and HBP have been chosen as key compounds for each of the four reactions of this route.

Usually production rate method is employed in order to determine reaction rate in complex reaction networks [47]. This method is based on the following equation:

$$\dot{R} = \dot{\nu} \cdot \dot{r} \tag{A.5}$$

where $\dot{R}$ is a vector containing the production rates of all the key compounds of the system; $\dot{\nu}$ is the matrix containing the stoichiometic coefficients involved in the reaction network, and $\dot{r}$ is the vector containing the reaction rates of all the reactions present in the network. According to the previous reaction scheme composed by expression (A.1)–(A.4), Eq. (A.5) can also be expressed as follows for each key compound involved in 4S pathway:

$$\begin{pmatrix} R_{DBT} \\ R_{DBTO} \\ R_{DBTO_2} \\ R_{HBP} \end{pmatrix} = \begin{pmatrix} -1 & 0 & 0 & 0 \\ 1 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \cdot \begin{pmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \end{pmatrix} \tag{A.6}$$

Thus, this equation system yields the following set of equations:

$$R_{DBT} = -r_1 \tag{A.7}$$

$$R_{DBTO} = r_1 - r_2 \tag{A.8}$$

$$R_{DBTO} = r_2 - r_3 \tag{A.9}$$

$$R_{HBP} = r_4 \tag{A.10}$$

As a first approximation to the values of kinetic parameters, the reaction rate method has been applied over the four reactions involved in the 4S route. A single response and non linear regression was employed in an integrated way. In order to simplify parameter estimation, the mentioned reaction rate method can be applied [47]. The coincidence between the number of reactions to

be studied and the number of key compounds allows to relate reaction rates and production rates as shown in the following matrix expression [47]:

$$\dot{r} = \dot{\nu}^{-1} \cdot \dot{R} \tag{A.11}$$

According to the reaction scheme made up by the reactions previously listed (A.1) and (A.4), the inverted matrix of stoichiometric coefficients, ν^{-1}, can be calculated as shown in expression (A.12).

$$\dot{\nu}^{-1} = \begin{pmatrix} -1 & 0 & 0 & 0 \\ -1 & -1 & 0 & 0 \\ -1 & -1 & -1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \tag{A.12}$$

In this way, Eq. (A.11) can also be expressed as follows:

$$\begin{pmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \end{pmatrix} = \begin{pmatrix} -1 & 0 & 0 & 0 \\ -1 & -1 & 0 & 0 \\ -1 & -1 & -1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \cdot \begin{pmatrix} R_{DBT} \\ R_{DBTO} \\ R_{DBTO_2} \\ R_{HBP} \end{pmatrix} \tag{A.13}$$

As a result of this matrix expression, the following system of equations can be inferred:

$$r_1 = -R_{DBT} = -\frac{dC_{DBT}}{dt} \tag{A.14}$$

$$r_2 = -R_{DBT} - R_{DBTO} = -\frac{dC_{DBT}}{dt} - \frac{dC_{DBTO}}{dt} \tag{A.15}$$

$$r_3 = -R_{DBT} - R_{DBTO} - R_{DBTO_2} = -\frac{dC_{DBT}}{dt} - \frac{dC_{DBTO}}{dt} - \frac{dC_{DBTO_2}}{dt} \tag{A.16}$$

$$r_4 = R_{HBP} = \frac{dC_{HBP}}{dt} \tag{A.17}$$

Every of these identities can be expressed in an integrated way, taking into account the following imposed condition: $t = 0 \therefore C^0_{DBTO} = C^0_{DBTO_2} = C^0_{HBP} = 0$ as seen below:

$$\int_0^t r_1 dt = -\int_{C^0_{DBT}}^{C_{DBT}} dC_{DBT} = C^0_{DBT} - C_{DBT}(t) \tag{A.18}$$

$$\int_0^t r_2 dt = -\int_{C^0_{DBT}}^{C_{DBT}} dC_{DBT} - \int_{C^0_{DBTO}}^{C_{DBTO}} dC_{DBTO} = C^0_{DBT} - C_{DBT}(t) - C_{DBTO}(t) \tag{A.19}$$

$$\int_0^t r_3 dt = \int_{C^0_{DBT}}^{C_{DBT}} dC_{DBT} - \int_{C^0_{DBTO}}^{C_{DBTO}} dC_{DBTO} - \int_{C^0_{DBTO_2}}^{C_{DBTO_2}} dC_{DBTO_2} = C^0_{DBT} - C_{DBT}(t) - C_{DBTO}(t) - C_{DBTO_2}(t) \tag{A.20}$$

$$\int_0^t r_4 dt = \int_{C^0_{HBP}}^{C_{HBP}} dC_{HBP} = C_{HBP}(t) \tag{A.21}$$

These expressions allow obtain values of $\int_0^t r_i dt$, which means calculating values of r_i at every time directly from experimental data, simplifying a multiple response problem into a single-response one. According to this, each reaction can be studied separately from integral data, concentration *vs* time. Concentration of HBPS was calculated from stoichiometry from results of DBT, DBTO, $DBTO_2$ and HBP.

$$C^0_{DBT} = C_{DBT} + C_{DBTO} + C_{DBTO_2} + C_{HBPS} + C_{HBP} \tag{A.22}$$

In the present work, the reaction rate method has been employed in its integrated way. Therefore, integrated values of reaction rates could be easily obtained from experimental data of the change in concentration of 4S pathway compounds with time. Determination of the values of initial reaction rates was carried out using the following extrapolation [41]:

$$\left.\frac{d\left(\int_0^t r_i dt\right)}{dt}\right|_{t\to 0} = r_i^0 \tag{A.23}$$

A.2. Application of the reaction rate method to the 4S pathway

Differential equations (14)–(17) in Table 1, express the production rate of DBT, DBTO, $DBTO_2$, HBPS and HBP, and can be integrated according to expression (A.24). Thus, the concentrations of compounds involved in 4S route can be expressed according to Eqs. (A.25)–(A.29). The proposed kinetic model can be made up in an integrated way as follows:

$$C_j - C_j^0 = \sum_{i=1}^{NC} \nu_{ij} \cdot \int_{t_0}^{t} r_i \cdot dt \tag{A.24}$$

$$C_{DBT} - C^0_{DBT} = -\int_0^t \frac{k'_1 \cdot \exp(-S_1 \cdot t) \cdot C_{DBT} \cdot C_X}{1 + K'_1 \cdot C_{DBT}} dt \tag{A.25}$$

$$C_{DBTO_2} = \int_0^t \frac{k'_2 \cdot \exp(-S_2 \cdot t) \cdot C_{DBTO} \cdot C_X}{1 + K'_2 \cdot C_{DBTO}} dt - \int_0^t \frac{k'_3 \cdot \exp(S_3 \cdot t) \cdot C_{DBTO_2} \cdot C_X}{1 + K'_2 \cdot C_{DBTO_2}} dt \tag{A.26}$$

$$C_{DBTO} = \int_0^t \frac{k'_1 \cdot \exp(-S_1 \cdot t) \cdot C_{DBT} \cdot C_X}{1 + K'_1 \cdot C_{DBT}} dt - \int_0^t \frac{k'_2 \cdot \exp(S_2 \cdot t) \cdot C_{DBTO} \cdot C_X}{1 + K'_2 \cdot C_{DBTO}} dt \tag{A.27}$$

$$C_{HBPS} = \int_0^t \frac{k'_3 \cdot \exp(-S_3 \cdot t) \cdot C_{DBTO_2} \cdot C_X}{1 + K'_3 \cdot C_{DBTO_2}} dt - \int_0^t \frac{k'_4 \cdot \exp(S_4 \cdot t) \cdot C_{HBPS} \cdot C_X}{1 + K'_4 \cdot C_{HBPS} + K'_5 \cdot C_{HBP}} dt \tag{A.28}$$

$$C_{HBP} = \int_0^t \frac{k'_4 \cdot \exp(S_4 \cdot t) \cdot C_{HBPS} \cdot C_X}{1 + K'_4 \cdot C_{HBPS} + K'_5 \cdot C_{HBP}} dt \tag{A.29}$$

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