Production of a Biocatalyst of Pseudomonas p utida CECT5279 for DBT Biodesulfurization: Influence of the Operational Conditions

ARTICLE *in* ENERGY & FUELS · MAY 2005

Impact Factor: 2.79 · DOI: 10.1021/ef0400417

4 AUTHORS, INCLUDING:

A. Alcon
Complutense University of Madrid
34 PUBLICATIONS **412** CITATIONS
SEE PROFILE

Victoria E. Santos Mazorra
Complutense University of Madrid
55 PUBLICATIONS **1,142** CITATIONS
SEE PROFILE

Production of a Biocatalyst of *Pseudomonas putida* CECT5279 for DBT Biodesulfurization: Influence of the Operational Conditions

Ana B. Martin, Almudena Alcon, Victoria E. Santos, and Felix Garcia-Ochoa*

Dpto. Ingenieria Quimica, Facultad CC. Quimicas, Universidad Complutense, 28040 Madrid, Spain

Received April 7, 2004

The influence of working conditions on the growth batch of *Pseudomonas putida* CECT5279 has been studied, in regard to both the growth rate and the desulfurization capability accumulated in the cells. These operational conditions include pH conditions (buffered and nonbuffered media, using different carbon sources (glucose, citrate, and glutamic acid)), operating temperatures (26−32 °C), and different dissolved oxygen concentrations, due to different aeration conditions (different air flows, using enriched air, etc.). *Pseudomonas putida* CECT5979, which is a genetically modified microorganism (GMO), has the ability to convert dibenzothiophene (DBT) to 2-hydroxybiphenyl (HBP), desulfurizing the organic molecule. To get the best conditions to obtain desulfurizing cells, a parameter (D_{BDS}) that incorporates both biomass concentration and time to reach a particular percentage of desulfurizing capability (X_{BDS}) has been used. The optimum value of D_{BDS} has been obtained under the following working conditions: temperature, 30 °C; nonbuffered medium with glutamic acid as the carbon source; and, in relation to the dissolved oxygen concentration, the best conditions for growth are not the same as those required to get the highest desulfurizing activity. A kinetic model based on a logistic equation has been applied to describe biomass concentration during *P. putida* CECT5979 growth. Kinetic model parameters (μ and C_X^{max}) were obtained under several operating conditions. A model proposed in a previous work [Martin et al., *Energy Fuels* **2004**, *18*, 851−857] was applied to describe biodesulfurization capability evolution during growth. Predicted values of biomass concentration and biodesulfurizing capability percentage achieved by the cells can be obtained during bacteria growth, with values very similar to those found experimentally, in a wide interval of operating conditions.

Introduction

Recent regulations will drive sulfur levels in crude oil to <350 ppm and refiners are expected to get <10−15 ppm sulfur soon (2005−2007).[2]

Currently, petroleum refining is mainly based on the use of physicochemical processes and chemical catalysis operating under drastic conditions (high temperature and pressure). These processes are costly in energy and highly contaminating;[3] therefore, the application of a biodesulfurization (BDS) process after hydrodesulfurization (HDS), mainly for diesel oils, has attracted attention as a new ecotechnology to achieve more-efficient desulfurization.[4] This type of BDS is usually studied with model compounds, with dibenzothiophene (DBT) being the model compound used most often.

A wider range of literature is available on the biotransformation of DBT by *Rhodococcus erythropolis* IGTS8, which is the bacteria more frequently used in this type of study. The genes involved in DBT desulfurization constitute the Dsz pathway, which is also called the 4S pathway. The enzymes and genes of metabolic pathways for desulfurization have been elucidated.[5,6]

Genetic engineering has been used to improve the IGTS8 strain, in an effort to increase the DBT desulfurizing activity, allowing shorter residence times that are compatible with a commercial application.[7]

There are some studies that are developing genetically modified microorganisms (GMOs) capable of DBT desulfurization from bacteria unable to develop the 4S pathway. The first patent on the incorporation of the desulfurization genes into a *Pseudomonas* bacterium was issued in 1999 in the United States.[8] Another patent was awarded on the incorporation of a flavin reductase into an artificial operon to collect all the genes required for BDS into a single transcript.[9]

(1) Martin, A. B.; Alcon, A.; Santos, V. E.; Garcia-Ochoa, F. *Energy Fuels* **2004**, *18*, 851−857.
(2) Monticello, D. *Curr. Opin. Biotechnol.* **2000**, *11*, 540−546.
(3) Le Borgne, S.; Quintero, R. *Fuel Process. Technol.* **2003**, *81*, 155−169.
(4) Kirimura, K.; Furuya, T.; Nishii, Y.; Ishii, Y.; Kino, K.; Usami, S. *J. Biosci. Bioeng.* **2001**, *91* (3), 262−266.
(5) Denome, S. A.; lson, E. S.; Young, K. D. *Appl. Environ. Microbiol.* **1993**, *59*, 2837−2843.
(6) Oldfield, C.; Pogrebinski, O.; Simmonds, J.; Kulpa, C. F. *Microbiology* **1997**, *143*, 2961−2973.
(7) Pacheco, M. A.; Lange, E. A.; Pienkos, P. T.; Yu, L. Q.; Rouse, M. P.; Lin, Q.; Linguist, L. K. Presented at the Annual Meeting of the National Petrochemical and Refiners Association, San Antonio, TX, 1999, Paper AM-99-27.
(8) Darzins, A.; Xi, L.; Childs, J. D.; Monticello, D. J.; Squires, C. H. Dsz Gene Expression in *Pseudomonas* Hosts, U.S. Patent No. 5,952,208, September 14, 1999.

10.1021/ef0400417 CCC: $30.25
Published on Web 03/19/2005

In this work, *Pseudomonas putida* CECT5279, a GMO has been used, according with the procedure described elsewhere.[10] This bacteria carries the genes dszABC from *R. erythropolis* IGTS8, and the gene hpaC from *Escherichia coli* W.[11]

There are some works in the literature where operational conditions are taken into consideration;[12−16] however, all of them have desulfurization occurring while biomass growth is taking place; that is, a resting cells procedure is not used. These studies found in the literature are focused on optimizing the growth conditions using DBT or other sulfurated organic molecules as a sulfur source.[12−16] These works use several microorganisms (*Corynebacterium* sp.,[12,14] *R. erythropolis* IGTS8,[13] *Corynebacterium* P-32-C1,[15] *R. erythropolis* N1-36, N1-43[16]); all of them are different from that used in this work.

Most of the microorganisms that have been reported to degrade DBT in a C−S bond can do it at mesophilic temperatures, usually near 30 °C.[17−20] There are few reports of BDS under higher-temperature conditions.[21−27]

With relation to pH, most reports use values between 6.5 and 7.5 for *R. erythropolis* IGTS8.[18,20,27,28] The alternatives are buffering the medium or letting the pH change with growth time.

The purpose of this work is to perform an operating condition study during the growth of *P. putida* CECT5279 to obtain cells with the the highest desulfurizing capability. For this study, different temperatures, pH, and dissolved oxygen concentrations have been used.

In a previous work,[1] the medium composition influence on growth rate and desulfurizing capability of *P. putida* CECT5279 was established. In that work, the study of carbon and nitrogen sources was made to obtain the cells that have biocatalyst with the highest BDS capability. Glutamic acid (20 g/L) and ammonium chloride (670 ppm) were the sources yielding the best results. Nevertheless, in this work, the study of pH influence has been performed with three carbon sources (glucose, citrate, and glutamic acid), taking into account the possible influence of pH in the catabolism of the different carbon sources. Thus, these experiments have been performed both with a buffered medium (Tris 0.1 M) and with a nonbuffered medium.

Biomass growth was measured in all the runs, together with the desulfurizing capability developed by the cells. To measure desulfurizing capability, a standard resting cells desulfurization assay has been used. To emphasize these aspects, first, the different substrate into biomass yields (Y_{jX}) have been calculated, as well as a parameter, the biodesulfurizing degree (D_{BDS}) that has been previously proposed[1] to express the desulfurizing capability of the cells and the time spent in achieving it. Afterward, a growth kinetics model and a previously proposed[1] BDS model were applied. This growth kinetics model is applied to growth experimental data to obtain kinetic parameters μ (specific growth rate) and C_X^{max} (maximum biomass concentration) for the different runs conducted under different operational conditions. A BDS model was also applied to predict the biodesulfurizing percentage of DBT that can be achieved using *P. putida* CECT5279.

Materials and Experimental Procedure

Chemicals. The following chemicals were used (suppliers are listed in parentheses): d(+)-glucose, $CaCl_2 \cdot 2H_2O$, and $FeCl_3 \cdot 6H_2O$ (from Riedel-de Haën); tris buffer, glycerine, citrate, glutamic acid, and NH_4Cl (from Panreac); Hepes buffer, isopropyl β-D-thiogalactopyranoside (IPTG) and tetracycline (from Sigma); dibenzothiophene (DBT) (from Aldrich); tryptone and yeast extract (from Fluka); $NaH_2PO_4 \cdot H_2O$ and K_2HPO_4 (from Merck); and $MgCl \cdot 6H_2O$ and $MgSO_4$ (from Probus). Deionized water (with a resistance of 18.5 Ω) was used to prepare all media and stock solutions, except as otherwise indicated.

Microorganism. The microorganism used was *Pseudomonas putida* CECT5279, which was supplied by the Biological Research Center (CIB−CSIC-Madrid, Spain). Cultures were maintained on concentrated stock with a glycerol in saline serum (10%) solution.

Media. For inoculum buildup, a complex medium was used: LB (Luria-Bertani), containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. *P. putida* IGTS8 was cultured in a basalt salt medium (BSM) with the following composition:[6] $NaH_2PO_4 \cdot H_2O$, 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) were also added to the culture medium. The carbon source (20 g/L) was added afterward (glucose, glutamic acid, or citrate), depending on the experiment that was being performed. For the desulfurizing capability studies during microorganism growth, Hepes buffer 50 mM (pH 8) was used, and the model compound DBT (25 μM) was added.

Setup and Procedure. The following experimental procedure was used, as described in the previous work.[1] Inoculum was always prepared by means of a standardized method, to obtain comparative experimental results. Experiments regarding pH and temperature were performed in an orbital shaker (Gallenkamp, model INR-200); these experiments were con-

(9) Squires, C. H.; Ji, W.; Xi, L.; Ortego, B. C.; Pogrebinsky, O. S.; Gray, K. A.; Child, J. D. Method of Desulfurization of Fossil Fuel with Flavoprotein, U.S. Patent No. 5,985,650, November 16, 1999.
(10) Garcia-Ochoa, F.; Garcia-Calvo, E.; Garcia, J. L. Spanish Patent Office, No. P200301677, 2003.
(11) Galan, B.; Diaz, E.; Garcia, J. L. *Environ. Microbiol.* **2000**, *2* (6), 687−694.
(12) Omori, T.; Monna, L.; Saiki, Y.; Kodama, T. *Appl. Environ. Microbiol.* **1992**, *58* (3), 911−915.
(13) Olson, E. S.; Stanley, D. C.; Gallagher, J. R. *Energy Fuels* **1993**, *7*, 159−164.
(14) Gallagher, J. R.; Olson, E. S.; Stanley, D. C. *FEMS Microbiol. Lett.* **1993**, *107*, 31−36.
(15) Maghsoudi, S.; Vossoughi, M.; Kheirolomoom, A.; Tanaka, E.; Katoh, S. *Biochem. Eng. J.* **2001**, *8*, 151−156.
(16) Wang, P.; Krawiec, S. *Appl. Environ. Microbiol.* **1996**, *62* (5), 1670−1675.
(17) Abbad-Andaloussi, S.; Lagnel, C.; Warzywoda, A.; Monot, F. *Enzyme Microbiol. Technol.* **2002**, *6*, 1−9.
(18) Matsui, T.; Hirasawa, K.; Konishi, J.; Tanaka, Y.; Maruhashi, K.; Kurane, K. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 196−200.
(19) Omori, T.; Saiki, Y.; Kasuga, K.; Kodama, T. *Biosci. Biotechnol. Biochem.* **2001**, *59* (7), 1195−1198.
(20) Setti, L.; Farinelli, P.; Di Martino, S.; Frassinetti, S.; Lanzarini, G.; Pifferi, P. G. *Appl. Microbiol. Biotechnol*. **1999**, *52*, 111−117.
(21) Ishii, Y.; Konishi, J.; Okada, H.; Hirasawa, K.; Onaka, T.; Suzuki, M. *Biochem. Biophys. Commun*. **2000**, *270*, 81−88.
(22) Konishi, J.; Onaka, T.; Ishii, Y.; Suzuki, M. *FEMS Microbiol. Lett*. **2000**, *187*, 151−154.
(23) Kargi, F.; Robinson, J. M. *Biotechnol. Bioeng*. **1984**, *26*, 687−690.
(24) Furuya, T.; Kirimura, K.; Kino, K.; Usami, S. *FEMS Microbiol. Lett.* **2001**, *204*, 129−133.
(25) Kayser, K. L.; Cleveland, L.; Park, H. S.; Kwak, J. H.; Kolhatkar, A.; Kilbane, J. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 737−745.
(26) Finnerty, W. R. Microbial Desulfurization and Denitrogenation of Fossil Fuels. In *Proceedings of the 9th Energy Technology Conference, Washington, DC*; Hill, R. F., Ed.; Government Institute, Inc.: Rockville, MD, 1982; pp 883−890.
(27) Folsom, B. R.; Schieche, D. R.; Di Grazia, P. M.; Werner, J.; Palmer, S. *Appl. Environ. Microbiol*. **1999**, *65*, 4967−4972.
(28) Kaufman, E. N.; Borole, A. P.; Shong, R.; Sides, J. L.; Juengst, C. *J. Chem. Technol. Biotechnol*. **1999**, *74*, 1000−1004.

Table 1. Experiments Performed in This Work To Realize the Study of Working Conditions for *P. Putida* CECT5279

run	carbon source (20 g/L)	setup	T^a (°C)	buffer	Y_{CX} (g/g)	Y_{NX} (g/g)	D_{BDS}^{max} (%D (g of X) h^{-1} L^{-1})
			Orbital Shaker				
1	glutamic acid		30	no	1.57	0.16	21.65
2	glutamic acid		30	yes	2.45	0.63	13.57
3	glucose		30	no	2.03	0.12	1.14
4	glucose		30	yes	2.84	0.13	3.89
5	citrate		30	no	2.89	0.15	9.81
6	citrate		30	yes	3.26	0.20	5.88
7	glutamic acid		26	no	3.52	0.39	8.51
8	glutamic acid		28	no	1.76	0.46	14.04
9	glutamic acid		32	no	1.77	0.36	8.89
			Bioreactor				
10	glutamic acid	N_o =100 rpm, Q = 1 L/L/min, no enriched air	30	no	2.16	0.54	8.85
11	glutamic acid	N_o =100 rpm, Q = 3 L/L/min, no enriched air	30	no	2.0.38	0.38	10.07
12	glutamic acid	N_o =100 rpm, Q = 3 L/L/min, enriched air (75% pure O_2/25% air)	30	no	2.54	0.29	7.5

ducted in 50-mL Erlenmeyer flasks. In the experiments regarding dissolved oxygen, cells were cultured in a commercial fermentor (BIOSTAT B, Braun Biotech) with a working volume of 2 L. Sterilizable Ingold pH and dissolved oxygen probes, and a Braun sterilizable temperature probe, were used to monitor on-line and control the pH, temperature, and dissolved oxygen concentration in cultures during fermentations. Samples were withdrawn during growth and conserved at −18 °C after centrifugation and resuspension in 5 mL of a glycerol/NaCl (50/50) solution. These cells then can be used to perform the standard desulfurization assay (resting cells).

Standard Desulfurizing Assay. To estimate the desulfurizing capability of the cell, a resting cell assay was performed as follows. Cells were suspended in a 250-mL Erlenmeyer flask, containing 40 mL of 50 mM Hepes buffer (pH 8.0) and 25 μM DBT, with an initial concentration of 0.7 (g of X)/L. The desulfurization reaction proceeded at 30 °C and 210 rpm in an orbital shaker for 3 h. Before introducing cells and after 3 h of reaction, 0.5 mL samples were mixed with 0.5 mL of acetonitrile in Eppendorf tubes to stop the reaction and, afterward, were centrifuged at 14 000 *g* for 2 min; 0.9 mL of the supernatant then was taken and analyzed.

Analytical Methods. *Biomass Concentration*. A Shimadzu UV−visible spectrophotometer (model UV-1603) was used to measure cell concentration, which was determined from the optical density at 600 nm (OD_{600}).

Glucose Concentration. The concentration of glucose was analyzed by high-performance liquid chromatography (HPLC) (Kontron, model Sedex-45) using a light scattering detector. A Nucleosil NH_2 column (250 mm × 4.6 mm, with 5 μm particles) was used. The mobile phase was 75% acetonitrile (in deionized water), using a flow rate of 1 mL/min. The retention time was 6.9 min.

Glutamic Acid and Citrate Concentrations. The concentrations of both glutamic acid and citrate were measured with an enzymatic kit from Boehringer−Mannheim (Ref. 139092 and Ref. 130084, respectively).

Nitrogen Source Concentration. The ammonium concentration was monitored, using a selective ammonia electrode (Orion model 95-12).

DBT and HBP Concentrations. The concentrations of DBT and HBP in the resting cell assay were analyzed by HPLC (Hewlett−Packard, model 1100 HPLC), using a UV detector at 240 nm. A Kromasil C-8 column (150 mm × 4.6 mm, with 3 μm particles) was used. The mobile phase was 50% acetonitrile (in deionized water), with a flow rate of 1 mL/min and a temperature of 25 °C. The retention times of HBP and DBT were 5.9 and 14.0 min, respectively.

Experimental Results and Discussion

Twelve experiments were performed to study operating condition influences during *Pseudomonas putida* CECT5279 growth. Temperature (26−32 °C), pH (buffered and nonbuffered mediums), and different dissolved oxygen conditions (air-gas flows of 1 and 3 L/min and the use of enriched air) were changed in the different runs; the experimental values applied are given in Table 1.

To discriminate between the different operating conditions, the yields of different substrates into biomass (Y_{jX}), the percentage of desulfurizing capability of the cells (X_{BDS}), and the desulfurization development degree during growth (D_{BDS}) were calculated. These parameters—X_{BDS}, Y_{jX}, and D_{BDS}—which were proposed and used in a previous work,[1] are defined by the following equations.

Different yields were calculated according to

$$Y_{jX} = \frac{C_{j0} - C_j^{X_{max}}}{C_X^{max} - C_{X_0}} \qquad (j = C, N) \tag{1}$$

The percentage of desulfurization, measured as the HBP conversion (X_{BDS}), was determined according to

$$X_{BDS} = \frac{C_{HBP,3}}{C_{DBT,0}} \times 100 \tag{2}$$

where $C_{DBT,0}$ is the initial DBT concentration used to perform the standard BDS resting cell assay and $C_{HBP,3}$ is the HBP concentration obtained after 3 h of resting cell assay.

The degree of desulfurizing development during growth (D_{BDS}), to quantify the desulfurizing capability, taking into account all the variables that influence it (desulfurization percentage obtained at each time by resting cells, together with the biomass concentration reached), was calculated as

$$D_{BDS} = \frac{X_{BDS} C_X}{t_G} \tag{3}$$

where t_G is the time of growth needed to reach a biomass concentration C_X and X_{BDS} is the percentage of BDS

attained by the cells in the standard test of resting cells assay (according to eq 2).

pH Influence. The influence of the carbon source on *P. putida* growth and its desulfurization ability with several substrates (glutamate, pyruvate, citrate, glucose, glycerol, and an LB-rich medium) have been examined in previous works.[1,12,13,27] With the three first substrates, the microorganism exhibited a reasonable DBT desulfurization rate, but cells grown in the other three carbon sources were inefficient, in regard to the removal of sulfur from DBT.[11] Similar results were obtained in a previous work,[1] where the percentage of desulfurization was only 5% when the microorganism grew in BSM with glucose. Nevertheless, the pH value reached in that case was 4.9; for this reason, it seemed interesting to perform the same experiment in a buffered medium, to avoid this decrease in pH. On the other hand, the same study has been conducted with the other two carbon sources (citrate and glutamic acid), to check if pH influence on growth and desulfurizing ability is dependent on the carbon source that is used.

Other works have used different carbon sources, such as glucose,[12,15,16,27] ethanol,[12] and glycerol;[13−15] these works do not pay attention to pH influence, because they use a carbon substrate initial concentration of 2−5 g/L, and in those cases, the pH does not change to values that affect growth or the production of desulfurizing capability.[13−15] The work of Wang and Krawiec[16] involves the influence of pH using glucose (3 g/L); however, in that study, desulfurization is performed during growth and does not use resting cells. There is only one work[29] in which pH is controlled (using glucose, 2 g/L), but it was conducted in a continuous culture and then the pH control was due to the operation in a steady state and not by the addition of any substance. A previous report[30] is the only work that has involved the same problem; however, it used *Rhodococcus erythropolis* (the microorganism most employed in the literature). In this case, the pH influence is similar to that observed for *P. putida*.

The influence of pH has been studied using three different carbon sources (glutamic acid, glucose, and citrate) in buffered (with tris-HCl pH 7.5, 0.1 M) and nonbuffered medium (see Table 1, runs 1−6). All the experiments have been performed using an initial concentration of 20 g/L as the different carbon source and ammonium chloride as the nitrogen source.

Experimental results (biomass concentration, percentage of desulfurization, and degree of BDS during growth) are shown in Figure 1. As can be seen in Figure 1a and 1e, generally, the growth is better when the microorganism grows in a nonbuffered medium, attaining a value of 4.5 g/L (see Figure 1a). The maximum biomass concentration reached at the stationary phase was higher when the growth was conducted with glutamic acid as a carbon source in a nonbuffered medium. On the other hand, the lowest final concentration of biomass was obtained when citrate was used as carbon source in buffered medium, reaching a value of 2.5 g/L (Figure 1e).

The pH increases with time when citrate and glutamic acid are used as carbon sources but decreases to 4.90 and 5.92 with glucose in nonbuffered and buffered BSM, respectively (see Figure 1b and 1f).

The values obtained for the yields of the different substrates into biomass (Y_{CX} and Y_{NX}) are shown for each of the experiments in Table 1. The highest value of the parameter Y_{CX} is obtained in the run performed with citrate as the carbon source in a buffered medium (3.26). The parameter Y_{NX}, obtained with glutamic acid in a buffered medium, is higher than those obtained for the remainder of the experiments.

Figure 1c and 1g show the evolution of the percentage of desulfurization (X_{BDS}) reached in the desulfurization assay when different carbon sources are used in buffered and nonbuffered mediums. This parameter attains its highest value (64%) when glutamic acid is used as the carbon source in a nonbuffered medium. When growth is performed with glucose in a nonbuffered medium, the value of X_{BDS} is the lowest (5%); however, it increases to 20% when the same carbon source is used in a buffered medium.

The D_{BDS} values are given in Table 1 and in Figure 1d and 1h for the different runs. The maximum value of the D_{BDS} parameter is 21.65, which is obtained when glutamic acid is used as the carbon source in a nonbuffered medium. For the remainder of the experiments, the D_{BDS} value is always lower than that obtained in run 1. These results agree with those obtained when different carbon sources were checked in a previous work.[1]

Temperature Influence. Runs 1, 7, 8, and 9 have been performed to study the influence of temperature. The experimental results are given in Figure 2, where it can be observed that the bacterial growth is very similar in all cases, except when the temperature used is 32 °C; under this condition, the maximum cell concentration obtained is much lower than those obtained in the rest of the runs.

The highest value of the parameter Y_{CX} is obtained in the experiment that has been performed at 26 °C. The values of Y_{NX} obtained in the experiments performed at 26, 28, and 32 °C are similar and lower than those obtained in the run conducted at 30 °C.

The influence of the temperature on the desulfurizing capability can be observed in Figure 2b. The highest value of the parameter X_{BDS} is obtained when the microorganism grows at 30 °C in the exponential phase of growth (t_G = 9 h) and is conserved up to 15 h of growth; after that point, the desulfurizing capability decreases to 40%. Under the other temperatures, the percentage of desulfurization is always smaller than the value obtained working at 30 °C.

The highest values of the parameter D_{BDS}^{max} (21.65 and 14.04) correspond to the experiments that have been performed at 30 and 28 °C, respectively (see Figure 2c and Table 1).

Other works[12−15,27,29] that have utilized other microorganisms use the central temperature used in this work (30 °C); however, the influence of this variable on growth and desulfurizing capability production is not considered.

Influence of the Dissolved Oxygen Concentration. To study the influence of dissolved oxygen in both

(29) Honda, H.; Sugiyama, H.; Saito, I.; Kobayashi, T. *J. Ferment. Bioeng.* **1998**, *85* (3), 334−338.
(30) Del Olmo, C.; Santos, V. E.; Alcon, A.; Garcia-Ochoa, F. *Biochem. Eng. J.* **2004**, *22*, 229−237.

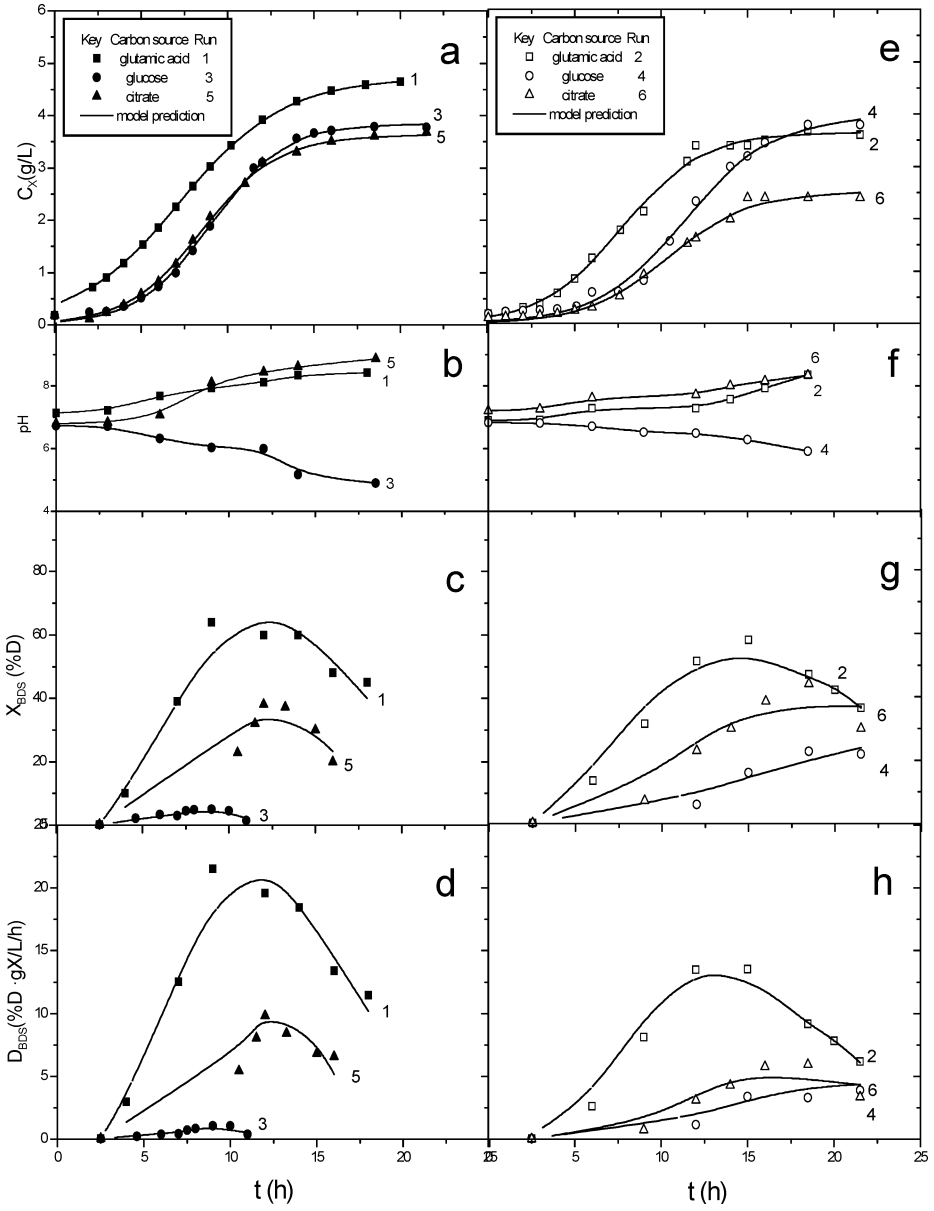

Figure 1. Evolution and model prediction of (a, e) biomass concentration, (b, f) pH (c, g) percentage of desulfurization, and (d, h) degree of desulfurizing development obtained in experiments performed with glutamic acid, glucose, and citrate. Data given in panels a−d are for a nonbuffered medium, and data given in panels e−h are for a buffered medium.

bacterial growth and desulfurizing capability, a bioreactor must be used, because the evolution of dissolved oxygen cannot be studied in an orbital shaker. For this study, three experiments were performed (denoted as runs 10, 11, and 12). In the first experiment, an air flow of 1 L/Lmin was used, whereas the air flow was 3 L/Lmin in the second experiment, and the same flow rate but with enriched air (75% in oxygen) was used in the third experiment.

Figure 3 shows the experimental results, where it can be observed that both growth rate and maximum cell concentration (Figure 3a) increase as the concentration of dissolved oxygen in the growth medium increases. The highest biomass concentration was obtained when enriched air was used.

Yield values calculated (see Table 1) show that the highest value of Y_{CX} was obtained when the microorganism grew with enriched air; however, the opposite occurs with Y_{NX}, which reaches the smallest value under those conditions.

The maximum amount of desulfurization (as a percentage) was obtained in the experiment that was conducted with an air flow of 1 L/Lmin, reaching a value of ∼80%. In the other two experiments, the parameter X_{BDS} is always smaller, with values of ∼50% in the experiment performed with an air flow of 3 L/Lmin and only 20% in the third run, with enriched air.

The highest value of the parameter D_{BDS} corresponds to the experiment that was performed with an air flow of 3 L/Lmin (D_{BDS} = 10.07). Figure 3d shows that the evolution of this parameter with growth time is similar in the experiments that have been performed without enriched air and slightly different when enriched air is used.

The only previous work where the concentration of dissolved oxygen is controlled is that by Honda et al.,[29] which is conducted in a continuous culture in the range of 2−5 mg/L of oxygen; however, as noted previously, they used *R. erythropolis.*

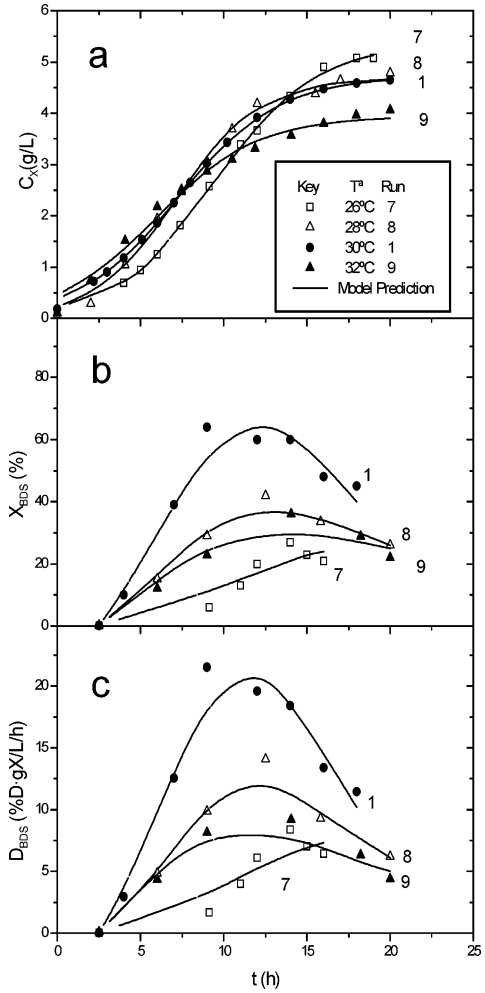

Figure 2. Evolution and model prediction of (a) biomass concentration, (b) percentage of desulfurization, and (c) degree of desulfurizing development obtained in experiments conducted at different temperatures.

Kinetic Modeling

Growth Kinetic Model. A kinetic model for microorganism growth was proposed in a previous work.[1] Experimental data of biomass concentration, relative to time, are fitted with the kinetic model:

$$\frac{dC_X}{dt} = \mu C_X\left(1 - \frac{C_X}{C_X^{max}}\right) \quad (4)$$

Equation 4 has been integrated, with the initial condition: $t = 0\ \therefore C_X = C_{X_0}$, yielding

$$C_X = \frac{C_{X_0}\exp(\mu t)}{1 - (C_{X_0}/C_X^{max})[1 - \exp(\mu t)]} \quad (5)$$

The fitting has been performed by nonlinear regression, using the Fischer and Student tests to evaluate the quality of the fitting. Moreover, the sum of square residuals, calculated according to eq 6, has been used as a representative of the fitting:

$$SSR = \frac{\sum_{i=1}^{N}(C_{X_i}^{exp} - C_{X_i}^{the})^2}{N} \quad (6)$$

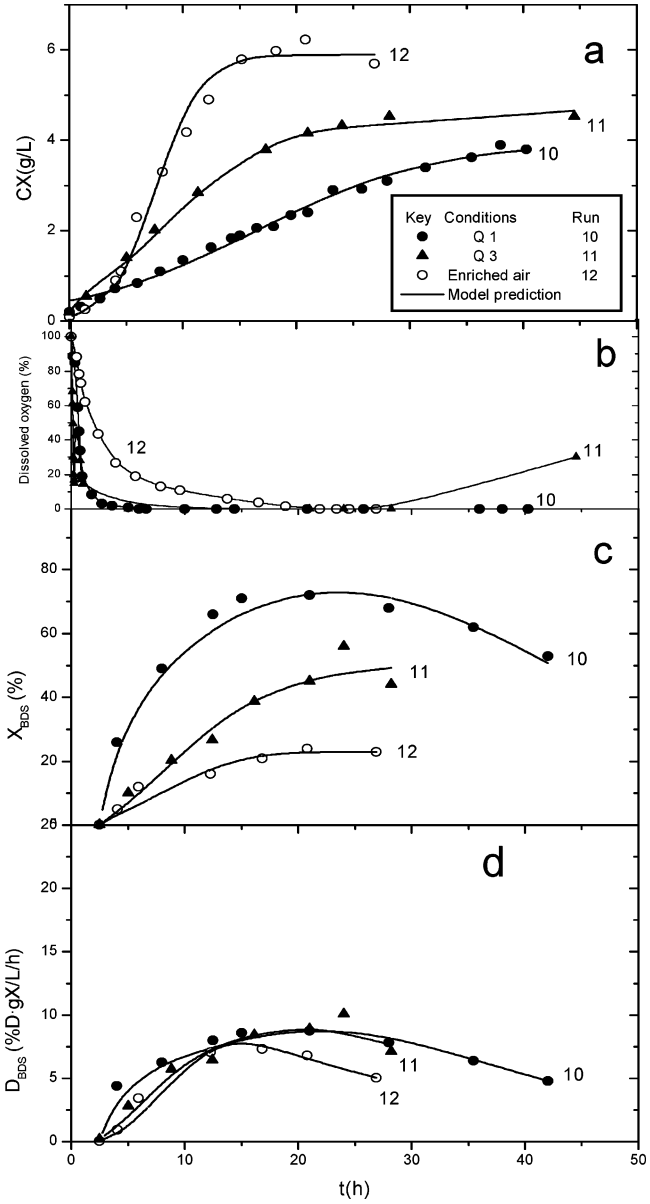

Figure 3. Evolution and model prediction of (a) biomass concentration, (b) percentage of desulfurization, and (c) degree of desulfurizing development obtained in the experiment conducted in the bioreactor with air flows of 1 L/Lmin.

The fitting results for all experiments performed in this work are summarized in Table 2, showing, in all cases, very good statistical parameter values.

For runs 1−6, which were conducted with different carbon sources in a buffered medium and a nonbuffered medium, the values of the specific growth rate μ are very similar (in the range of 0.34−0.36 h^{-1}), except when the carbon source used is citrate in a buffered medium; for this substrate, $\mu = 0.23\ h^{-1}$. The highest value of the parameter C_X^{max}, 4.70 g/L, is obtained when glutamic acid is used as the carbon source in a nonbuffered medium.

The values of the parameter μ are very similar in the experiments that have been conducted at 26 and 28 °C and higher than those obtained in the experiments that have been conducted at 30 and 32 °C. The maximum concentration of biomass attained at the stationary phase of growth is higher in the experiment performed

Table 2. Parameters Obtained by Fitting of Experimental Data to eq 4

	μ (h^{-1})		C_X^{max} ((g of X)/L)				95% confidence level	
run	value	Student's *t*	value	Student's *t*	Fischer's *F*	SSR	Student's *t*	Fischer's *F*
1	0.34 ± 0.05	13.88	4.70 ± 0.25	41.57	1814	7.6×10^{-3}	2.18	3.49
2	0.36 ± 0.05	18.38	3.65 ± 0.06	65.68	3225	8.2×10^{-3}	2.16	3.80
3	0.36 ± 0.03	30.15	3.84 ± 0.08	102.92	8251	8.4×10^{-3}	2.14	3.49
4	0.35 ± 0.06	13.21	3.97 ± 0.26	32.29	1245	16.1×10^{-3}	2.16	3.80
5	0.35 ± 0.03	31.42	3.63 ± 0.07	105.38	7989	7.2×10^{-3}	2.18	3.49
6	0.23 ± 0.05	14.45	2.51 ± 0.13	40.72	1620	6.2×10^{-3}	2.14	3.74
7	0.42 ± 0.08	28.29	5.32 ± 0.17	74.93	7775	0.5×10^{-3}	2.17	3.88
8	0.40 ± 0.06	14.28	4.68 ± 0.21	51.35	2042	0.2×10^{-3}	2.16	3.90
9	0.27 ± 0.04	9.13	3.93 ± 0.27	33.18	1040	0.3×10^{-3}	2.22	4.10
10	0.23 ± 0.05	13.25	3.97 ± 0.15	36.18	2601	0.6×10^{-3}	2.20	3.52
11	0.35 ± 0.07	18.52	4.40 ± 0.25	63.11	3521	1.6×10^{-3}	2.22	4.10
12	0.73 ± 0.09	21.05	6.23 ± 0.31	83.71	5512	1.2×10^{-3}	2.20	3.52

Table 3. Parameters Obtained by Fitting of Experimental Data to eq 7

	α (%D L/(g of X))		β (%D L/(g of X) h)				95% confidence level	
run	value	Student's *t*	value	Student's *t*	Fischer's *F*	SSR	Student's *t*	Fischer's *F*
1	33.72 ± 5.76	9.96	1.77 ± 0.66	4.18	184	5.17	2.44	5.14
2	18.77 ± 3.12	11.04	0.56 ± 0.09	5.61	222	3.26	2.57	5.79
3	4.03 ± 0.42	6.32	3.98 ± 0.04	5.82	464	0.61	2.57	5.79
4	4.86 ± 3.12	6.81	0.56 ± 0.09	5.14	308	0.42	2.77	9.55
5	13.34 ± 1.57	15.22	1.68 ± 0.35	9.69	543	3.72	2.57	5.79
6	12.86 ± 1.81	6.78	0		133	3.11	2.57	5.79
7	10.52 ± 1.97	8.22	0		191	0.82	2.44	5.14
8	14.96 ± 2.84	15.32	0.33 ± 0.07	6.22	393	0.97	2.77	6.94
9	13.58 ± 2.51	7.31	0.06 ± 0.02	2.74	130	2.06	2.77	6.94
10	35.09 ± 2.54	55.12	0.54 ± 0.05	6.82	1009	1.72	2.44	5.14
11	19.15 ± 1.64	25.36	0		356	1.25	2.44	5.14
12	15.20 ± 1.87	35.63	0		110	1.24	2.57	5.79

at 26 °C (5.32 g/L) and smaller in the experiment performed at 32 °C (3.93 g/L).

Significant influences can be observed on the parameters μ and C_X^{max} when different dissolved oxygen conditions were used (see Table 2). The value of both parameters increases when the concentration of dissolved oxygen increases: the highest values of μ (0.73 h^{-1}) and C_X^{max} (6.23 g/L) correspond to the experiment that was performed with enriched air and a flow rate of 3 L/Lmin.

Biodesulfurization Model. A previously proposed kinetic model[1] describing the development during growth of the desulfurizing capability has been applied to the experimental data obtained in this work. The equation proposed is a modification of the Luedeking−Piret[31] equation, as follows:

$$\frac{dX_{BDS}}{dt} = \alpha\frac{dC_X}{dt} - \beta C_X \qquad (7)$$

with the boundary conditions $t = 2.5$ h and $X_{BDS} = 0$, which means that a delay time in the development of desulfurization capability of the cells must be taken into consideration.

Experimental data are fitted to eq 7 using a fourth-order Runge−Kutta algorithm to integrate it, coupled to a simple-response nonlinear algorithm.[32] The model can be fit to all the runs that have been performed, showing good statistical parameters (Student's t-tests and Fischer's F-tests), as shown in Table 3. The prediction of D_{BDS} values can be performed using eq 3 but introducing theoretical values for both percentage of biodesulfurization capability (X_{BDS}) and biomass concentration (C_X). Figures 1−3 show the model predictions for both variables (X_{BDS} and D_{BDS}) as solid lines, yielding very good reproductions of the experimental data.

The value of the parameter α, considering the production of desulfurizing capability to be growth-associated, is similar when glucose and citrate are used as carbon sources in buffered and nonbuffered media; however, when the carbon source used is glutamic acid, the value of this parameter decreases from 33.72 (in a nonbuffered medium) to 18.77 (in a buffered medium). The tendency to decrease the desulfurizing capability during stationary growth phase (indicated by the parameter β) is higher when glucose is used as the carbon source in a nonbuffered medium.

Temperature has great influence on the development of the desulfurizing capability of *P. putida* CECT5279. When a temperature of 30 °C is used, the parameter α presents a value of 33.72 and the smallest value for this parameter, α = 10.52, is obtained in the experiment that has been conducted at 26 °C. On the other hand, the highest value of the parameter β is obtained when the microorganism grows at 30 °C, which means that the tendency to decrease the desulfurizing capability is higher when the cells grow at this temperature.

Studies about the influence of dissolved oxygen on desulfurizing ability show enormous differences in the parameter α; it is almost twice as high in the experiment that has been performed with an air flow of 1 L/Lmin, with respect to the values obtained in the other two experiments. The parameter β is almost zero in the three experiments, although the value of this parameter is slightly higher when an air flow of 1 L/Lmin is used.

Conclusions

The growth of *Pseudomonas putida* CECT5279 can be conducted with the medium and the procedure

(31) Luedeking, R.; Piret, E. L. *J. Biochem. Microbiol. Technol. Eng.* **1959**, *1*, 393−412.
(32) Marquardt, D. W. *J. Soc. Ind. Appl.* **1963**, *11*, 433−441.

previously described,[1] with the simultaneous development of a desulfurization capability in the cells.

Inside the experimental interval studied, the highest growth rate and maximum biomass concentration were obtained when glutamic acid was used as the carbon source in a nonbuffered medium; however, the value of the growth rate is very similar for the three carbon sources checked in buffered and nonbuffered media, except with citrate in a buffered medium, where the lowest biomass concentration is obtained. The highest percentage of desulfurization is obtained in a nonbuffered medium with glutamic acid, but it remains constant for more time in buffered media, although, in this case, the value of the desulfurizing capability is smaller. The results are different with glucose: in this case, when the microorganism grows in a nonbuffered basalt salt medium (BSM), it does not develop its desulfurizing ability; however, in a buffered medium, the microorganism reaches up to 20% desulfurization.

Temperature influences the growth of the microorganism. The highest values of the parameters μ and C_X^{max} are obtained when the cells are grown at 26 °C, and these parameters reach their lowest values at 32 °C. Nevertheless, the best desulfurization capability is obtained when the bacterium growth occurs at 30 °C.

Changing the oxygen transport rate in a bioreactor, the highest values of both specific growth rate μ and the maximum biomass concentration C_X^{max} are reached in the experiment that was performed with enriched air and the maximum flow rate, that is to say, with the maximum oxygen transfer rate. Nevertheless, these conditions, although positive for growth, are negative for desulfurization capability development. When the concentration of dissolved oxygen increases, the desulfurizing capability decreases. That is to say, the greater the microorganism growth, the smaller its desulfurizing capability.

The kinetic growth model can predict all the results of the experiments conducted in this work with very good fitting of the experimental data. The values of the kinetic parameters μ and C_X^{max}, which are given in Table 2, are in agreement with the experimental observations.

The biodesulfurization (BDS) model was also able to predict the BDS percentage prediction and desulfurization capability developed by the cells, yielding α and β values, according to experimental observations.

Growth and BDS models allow prediction of the D_{BDS} values, along with growth of *P. putida* CECT5279, when the cells were cultured under different operational conditions, according to eqs 4 and 7 and the parameter values given in Tables 2 and 3.

Acknowledgment. This work has been supported by MCyT-Plan Nacional de I+D-Programa de Procesos y Productos Quimicos, under Contract No. PPQ2001-1361-C02-01. The grant support for two of the authors by Comunidad Autonoma de Madrid (for A. A.) and by Ministerio de Ciencia y Tecnologia (for A. B. M.) is gratefully recognized.

Nomenclature

C_j = concentration of compound j ((g of j)/L)
DBT = dibenzothiophene
D_{BDS} = biodesulfurization development degree in growth (%D (g of X) h^{-1} L^{-1})
HBP = 2-hydroxybiphenyl
N = number of experimental data of each experiment
SSR = sum of square residuals referred to data number
X_{BDS} = percentage of biodesulfurization (% HBP), given by eq 2
Y_{jX} = yield of source j into biomass ((g of i)/(g of X)), defined by eq 1
t = time (h)

Greek Symbols

α = parameter of eq 7 (%D L/(g of X)), used to define the kinetics of the production of desulfurization capability, as associated to growth
β = parameter of eq 7 (%D L/g of X), used to define the kinetics of losing desulfurization capability
μ = specific growth rate (h^{-1})

Subindexes

C = refers to the carbon source
DBT = refers to dibenzothiophene
G = refers to the growth time
HBP = refers to 2-hydroxybiphenyl
N = refers to the nitrogen source
X = refers to the biomass
0 = refers to the initial value
3 = refers to the value after 3 h

Superindexes

exp = refers to the experimental values
max = refers to the maximum value of the parameter
the = refers to the theoretical values

EF0400417