Available online at www.sciencedirect.com
ScienceDirect
Journal of Hazardous Materials 151 (2008) 780–788
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

# A kinetics study on the biodegradation of synthetic wastewater simulating effluent from an advanced oxidation process using *Pseudomonas putida* CECT 324

M.M. Ballesteros Martín [a,*], J.A. Sánchez Pérez [a], F.G. Acién Fernández [a], J.L. García Sánchez [a], J.L. Casas López [a], S. Malato Rodríguez [b]

[a] *Department of Chemical Engineering, University of Almería, 04120 Almería, Spain*
[b] *Plataforma Solar de Almería-CIEMAT, Carretera de Senés km 4, 04200 Tabernas, Almería, Spain*

Received 16 January 2007; received in revised form 11 June 2007; accepted 14 June 2007
Available online 19 June 2007

**Abstract**

Bacterial growth on mixed substrates is employed for wastewater treatment. Biodegradation kinetics of *Pseudomonas putida* CECT 324 growth on formic acid, vanillin, phenol and oxalic acid mixtures is described. The experiments were carried out in a stirred-tank fermentor in batch mode at different temperatures (25, 30 and 35 °C) and pH (5, 6 and 7). The four compounds selected are typical intermediates in pesticide-contaminated water treated by advanced oxidation processes (AOPs). The toxicity of intermediates was investigated for a combined AOP-biological treatment, and the minimum DOC inhibitory concentration of the intermediate mixture was 175 ppm. The resulting biodegradation and growth kinetics were best described by the sum kinetics with interaction parameters (SKIP) model. Phenol and oxalic acid inhibit *P. putida* growth, and formic acid consumption strongly affects the biodegradation of oxalic acid. At all the temperatures tested and at pH between 5 and 7, *P. putida* CECT 324 was able to degrade the four substrates after culture times of 30 h at 30 °C and pH 7, which were the best conditions, and after 70 h, under the worst, at 35 °C.


*Keywords:* *Pseudomonas putida* CECT 324; Biodegradation kinetics; Advanced oxidation process; Aromatic compounds; Carboxylic acids

## 1. Introduction

One of the most alarming phenomena affecting water sources is the growing accumulation of anthropogenic substances that are barely biodegradable due to a lack of water treatment systems capable of diminishing the concentration of toxic substances representing an acute or chronic chemical risk. The inability of conventional biological wastewater treatments to effectively remove many toxic pollutants shows that new treatment systems are needed. Rigorous pollution control and legislation in many countries has resulted in an intensive search for new and more efficient water treatment technologies. In the European Union, water policy is presently undergoing considerable change. The Framework Directive on Water [1] provides a policy tool that enables this essential resource to be sustainably protected. Among other measures, surface water deterioration must be prevented, and bodies of water protected and restored, good chemical and ecological condition of such water achieved and pollution from discharges and emissions of hazardous substances reduced by 2015. It has recently been shown that partial oxidation of toxic compounds by advanced oxidation processes (APOs) (that involve generation and subsequent reaction of hydroxyl radicals, $^{\bullet}OH$) may substantially increase wastewater biodegradability [2–7]. Even though wastewater treatments using AOPs have been shown to be highly efficient, their operation is still quite expensive (tens of € $m^{-3}$) [7–9]. Therefore, combination of an AOP as a preliminary treatment, followed by an inexpensive biotreatment, would seem to be an economically attractive option.

There are many publications dealing with integrated chemical and biological oxidation processes for wastewater for a variety of single compounds (e.g., aromatics, pesticides, antibiotics,

* Corresponding author. Tel.: +34 950014148; fax: +34 950015484.
*E-mail address:* mmenta@ual.es (M.M.B. Martín).

0304-3894/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2007.06.053

**Nomenclature**

| | |
|---|---|
| $I_{ij}$ | interaction constant ($L\,mg^{-1}$) |
| $J$ | objective function (dimensionless) |
| $k_d$ | Decay coefficient ($h^{-1}$) |
| $K_{S,i}$ | saturation constant for substrate $i$ ($mg\,L^{-1}$) |
| $K_{S\,ap,i}$ | apparent saturation constant for substrate $i$ ($mg\,L^{-1}$) |
| $K_{I,i}$ | inhibition constant for substrate $i$ ($mg\,L^{-1}$) |
| $K_{S\,Th,i}$ | Theoretic saturation constant for substrate $i$ ($mg\,L^{-1}$) |
| $S_i$ | $i$ substrate concentration ($mg\,L^{-1}$) |
| $S_j$ | $j$ substrate concentration ($mg\,L^{-1}$) |
| $X$ | biomass concentration ($mg\,L^{-1}$) |
| $X(i)_{mod}$ | simulated state space vector ($mg\,L^{-1}$) |
| $X(i)_{exp}$ | experimental state space vector ($mg\,L^{-1}$) |
| $Y_{X/S_i}$ | yield coefficient on substrate ($g\,g^{-1}$) |

*Greek letters*

| | |
|---|---|
| $\mu_i$ | specific growth rate for substrate $i$ ($h^{-1}$) |
| $\mu_{max,i}$ | maximum specific growth rate for substrate $i$ ($h^{-1}$) |
| $\mu_{max\,ap,i}$ | apparent maximum specific growth rate for substrate $i$ ($h^{-1}$) |
| $\mu_{max\,Th,i}$ | theoretic maximum specific growth rate for substrate $i$ ($h^{-1}$) |

synthetic dyes, etc.) [10,11] and multicomponent feed streams (effluents from textile mills, paper mills, tannery, vinasse and olive mills, etc.) [3,12]. However, it must be kept in mind that some intermediates of the chemical reaction could be as toxic as the parent substance or even more so [3,13–15]. Therefore, toxicity of the intermediates mixture formed as the chemical reaction proceeds must be evaluated. A recent review of the current technologies for integrated chemical and biological processes in the literature by Gogate and Pandit [16] reported that for quantification of performance with a mixed culture, mainly activated sludge, most papers consider global variables such as $BOD_5$, COD and TOC. For microbial activity, biomass production and respiration rates are commonly reported, and gas phase composition has also been analysed, especially in small scale bioreactors [15,17]. Little has been written about the kinetics of the integrated oxidation processes or the toxic or inhibitory properties of chemical oxidation intermediates [18]. In wastewater, the occurrence of organic chemical mixtures makes the degradation of one component strongly affected or inhibited by other compounds [19–21].

This work is aimed at studying the biodegradation of a mixture of compounds using the bacterial strain *Pseudomonas putida* CECT 324, a model microorganism for biological treatment. In this research, the growth medium contains two aromatic compounds, vanillin and phenol, and two carboxylic acids, formic acid and oxalic acid, as these substances are frequently found only partly oxidized in the mixture of pesticide residues during the photocatalytic process [22–24]. The aqueous solution of these compounds forms a synthetic wastewater simulating effluents from an AOP. Phenol degradation by *P. putida* is well documented in the literature [25–27]. However, as far as we know, the *P. putida* CECT 324 potential for degrading vanillin and carboxylic acids has not yet been explored. While this pure culture system lacks the complexity of activated sludge, it is a reasonable starting point for the development and validation of mathematical models for integrating AOP and biological treatment units.

In view of the acidic conditions at the pre-treatment (AOP) outlet and the strong effect that will have on the biological state of the culture, two controlled pH conditions were assayed (5 and 7). In a combined treatment, an intermediate neutralization step will be necessary, so these experiments are conceived as a first check of the extent of neutralization. A third experiment was carried out without pH control during culturing, starting out at pH 6. The temperature selected for the pH study was 30 °C, reported by Hill and Robinson [28] and Yang and Humphrey [29] as optimum for phenol biodegradation by *P. putida*. At the best pH, the temperature effect was tested at 25 and 35 °C, covering a 10-degree interval around the reported optimum of 30 °C.

Kinetics models taking several substrates into account were tested, mainly those that capture the strong interaction between chemically similar species and the substrate inhibition effect. The sum kinetics with interaction parameters (SKIP) model [30], was found to be a suitable starting point.

## 2. Materials and methods

### 2.1. Microorganism and culture media

*P. putida* CECT 324 was acquired from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, Valencia, Spain). Cultures were grown in $1\,g\,L^{-1}$ beef extract at pH 7.2, $2\,g\,L^{-1}$ yeast extract, $5\,g\,L^{-1}$ peptone, $5\,g\,L^{-1}$ NaCl, and $15\,g\,L^{-1}$ agar powder, and were kept in glycerol at −70 °C.

### 2.2. Flask cultures

The effect of DOC concentration on the growth of *P. putida* was studied in triplicate in shake flasks. Cultures were incubated for 72 h at 30 °C on a rotary platform shaker (150 rpm, 2.6 cm stroke) in 100-mL Erlenmeyer flasks filled with 20 mL of synthetic wastewater. Before addition of the carbon source, the composition of the medium was: $0.5\,g\,L^{-1}$ $NH_4Cl$, $0.5\,g\,L^{-1}$ $K_2HPO_4$, $0.5\,g\,L^{-1}$ $KH_2PO_4$, $0.5\,g\,L^{-1}$ $MgSO_4{\cdot}7H_2O$, and $10\,mL\,L^{-1}$ of trace mineral solution [21]. Completing the synthetic wastewater, the carbon source was a 1:1:1:1 molar mixture of vanillin, phenol, oxalic acid and formic acid. This ratio was maintained throughout the experiments. For instance, to start at 150 ppm DOC concentration, individual species concentrations were: $111.84\,mg\,L^{-1}$ vanillin, $69.18\,mg\,L^{-1}$ phenol, $66.18\,mg\,L^{-1}$ oxalic acid and $33.84\,mg\,L^{-1}$ formic acid, yielding 70.56 ppm DOC from vanillin, 52.92 ppm DOC from phenol, 17.64 ppm DOC from oxalic acid and 8.82 ppm DOC from formic acid. Initial DOC ranged from 25 to 500 ppm.

The flasks were inoculated with 30 μL of bacterial stock at −70 °C. Culture medium (except vanillin, phenol, formic acid and oxalic acid) and materials were autoclaved at 126 °C for 20 min.

### 2.3. Stirred-tank fermentor

Fermentations were done in a 5-L working-volume bioreactor (Bioflo III, New Brunswick Co., USA) with an internal vessel diameter of 0.17 m, four baffles, rounded bottom and a broth height to vessel diameter ratio of 1.4. Agitation was provided by two Ruston turbines (300 rpm). A pipe sparger aerated the culture at 1 vvm. Following the method reported by Casas López et al. [31], a maximum oxygen uptake rate of 60 mg $O_2$ $L^{-1}$ $h^{-1}$ was measured during culturing. The minimum dissolved oxygen concentration was 7.8 mg $L^{-1}$, and therefore, it can reasonably be assumed that oxygen was not a growth-rate-limiting substrate in our experiments. Automatic sampling was done by a Sigma 900 Portable Sampler. Five millilitres of bacteria stock at −70 °C was directly inoculated. Experiments were performed in duplicate with repeated runs at separated times for an accurate assessment of variability. Batches were cultured for 96 h.

### 2.4. Analytical methods

Biomass concentration was measured by optical density (OD) at 600 nm [32] using a spectrophotometer (Unicam, UV 2, Cambridge, UK). The relationship between dry weight and optical density was found to be ($X$ (g $L^{-1}$) = 0.4033$OD_{600}$ − 0.0057, $r^2$ = 0.988).

Vanillin, phenol, oxalic and formic acid concentrations were measured by high-pressure liquid chromatography (Shimadzu Lc10, Kyoto, Japan). Samples were filtered though 0.20-μm syringe filters (Millex®-GN, Millipore, Ireland) to remove bacteria before HPLC injection. Vanillin and phenol concentration were determined with an XTerra MS $C_{18}$ (150 mm × 4.6 mm, 5 μm) column at a mobile phase flow rate of 1 mL $min^{-1}$ with a 60% MeOH-40% $H_2SO_4$ 0.01 M isocratic elution. A Synnergi Hidro-RP $C_{18}$ (250 mm × 4.6 mm, 4 μm) column running with a mobile phase flow rate of 1 mL $min^{-1}$ with 20 mM $KH_2PO_4$ and pH 2.9 was used for carboxylic acid determination. The sample injection volume was 20 μL in all cases.

Mineralisation was monitored by measuring the total organic carbon (TOC) by direct injection of filtered samples (determining dissolved organic carbon, DOC) into a Shimadzu-5050A TOC analyser provided with an NDIR detector and calibrated with standard solutions of potassium phthalate.

## 3. Results and discussion

### 3.1. Inhibitory concentration of the compound mixture

To characterise a biodegradation process, the amount of organic compounds that can be treated must be known. The organic load can be quantified by means of the DOC concentration in the wastewater. When combining an AOP and biological oxidation, the inhibitory effect of degradation intermediates on the microorganism must be taken into account. The work reported in this paper studied the effect of the total DOC concentration on the growth of *P. putida*. Keeping the mixture ratio the same, the total substrate concentration was quantified as the equivalent DOC supplied to the culture. Shake-flask experiments were carried out at the following DOC concentrations: 25, 50, 75, 100, 125, 150, 175, 200, 300, 400 and 500 ppm with the results as plotted in Fig. 1. Up to 150 ppm, bacterial growth was observed, followed by a dramatic drop in biomass concentration at 175 ppm. In this sense, an average biomass yield of 0.93 ± 0.08 g $g^{-1}$ was obtained from 25 to 150 ppm DOC compared to 0.17 ± 0.06 g $g^{-1}$ at 175 ppm DOC and decreasing at higher carbon concentrations. This behaviour was observed at three culture times, 24, 48 and 72 h. Therefore, 175 ppm was considered the minimum inhibitory DOC concentration for the selected degradation intermediates for *P. putida* cultures. Further stirred-tank experiments were conducted at 150 ppm. By determining the minimum inhibitory concentration, work can be performed in a DOC range in which the lack of cell acclimatization will not interfere with the experiments. The goal in this study, is not DOC degradation, but the relative kinetics of substrate consumption. Lag-phases as long as 1 week have been reported to occur during degradation of phenol at an initial concentration of 700 mg $L^{-1}$ using well-acclimated *P. putida* [27]. In this study, only a few hours were enough to adapt the cells to the new environment.

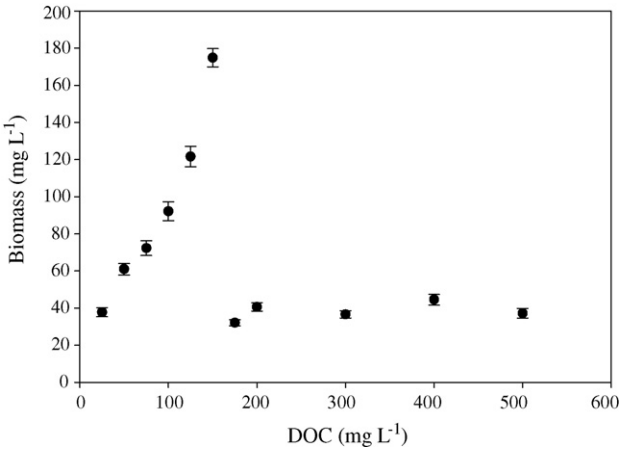


Fig. 1. Effect of vanillin, phenol, oxalic acid and formic acid substrate mixture concentration, on growth of *P. putida* CECT 324 after culturing for 24 h at 30 °C. Vertical bars represent the standard deviation on triplicates.

### 3.2. Effect of pH

The effect of pH on the biodegradation capability of *P. putida* is shown in Figs. 2–4. Different substrate uptake patterns were observed and sequential consumption was evidenced by depletion time for each chemical substance: formic acid and vanillin were consumed almost simultaneously during a short period between 13 and 18 h of culturing at pH 5 (Table 1). The mean of all the experimental formic acid and vanillin depletion times is 15.8 ± 1.3 h. This time is in the range observed for toluene and benzene biodegradation by *P. putida* F1 at 30 °C, and although

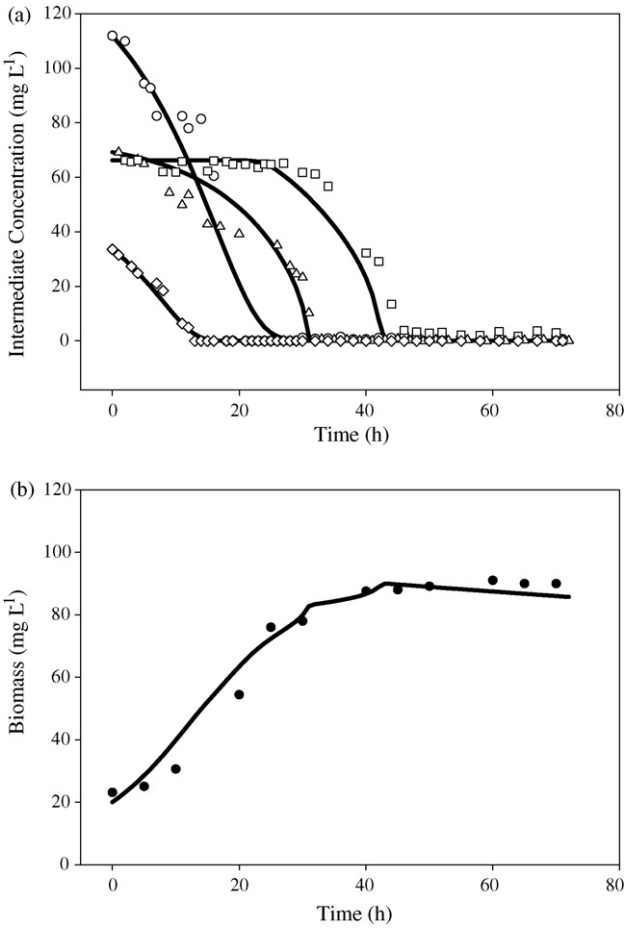


Fig. 2. Results from a batch culture of *P. putida* CECT 324 on a formic acid–vanillin–phenol–oxalic acid mixture at 30 °C and pH 5. Solid lines show model estimations. (a) (◊) Formic acid, (◯) vanillin, (△) phenol and (□) oxalic acid concentrations. (b) (●) Biomass concentration.

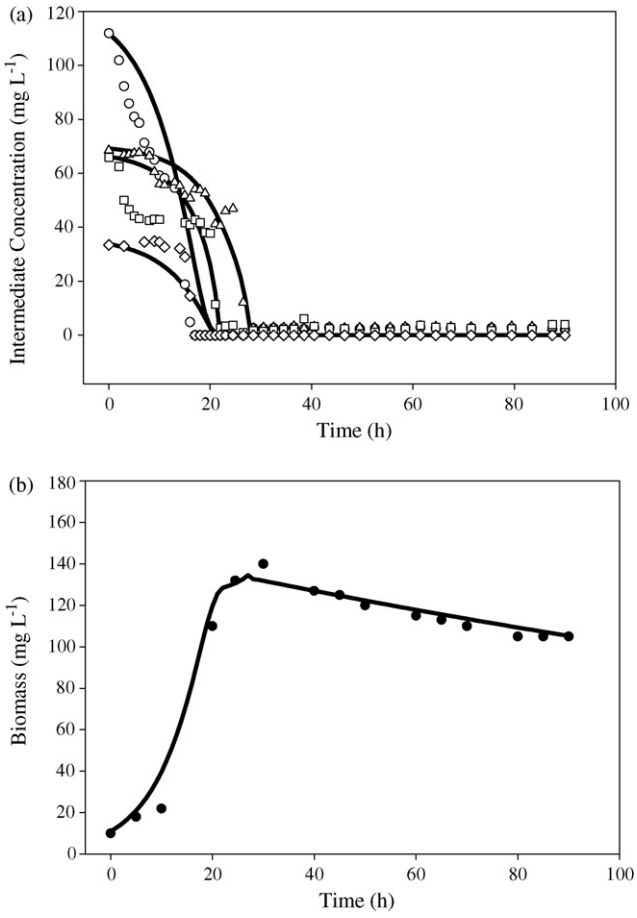


Fig. 3. Results from a batch culture of *P. putida* CECT 324 on a formic acid–vanillin–phenol–oxalic acid mixture at 30 °C and pH 7. Solid lines show model estimations. (a) (◊) Formic acid, (◯) vanillin, (△) phenol and (□) oxalic acid concentrations. (b) (●) Biomass concentration.

the pH was not controlled, it remained in the 6.7–6.9 range. Initial substrate concentrations were 40 mg $L^{-1}$ [33].

Variation in depletion time was wider for phenol and oxalic acid. At pH 7, 28 h were enough for total consumption of organic carbon, phenol being the last degraded carbon source. At pH 5, depletion time for phenol was close to that found at pH 7, although it took 24 h longer for oxalic acid. The experiment with uncontrolled pH resulted in a growth curve significantly different from controlled pH, regardless of the pH setpoint (Fig. 4). Vanillin and formic acid were consumed in 16 h. At this time, pH dropped to 4.7 from the initial 6.0. Total phenol consumption was reached after culturing for around 46 h, with pH down to 4.0. At the end of the culturing period, pH rose to 6 again when growth ceased due to lack of nutrients. The change in two pH units during culturing did not affect molecular vanillin ($pK_a = 7.38$ at 298 K), phenol ($pK_a = 9.98$ at 298 K) or formic acid ($pK_a = 3.77$ at 298 K) as both aromatic substances remained alkaline until consumption, and the formic acid was dissociated. Only oxalic acid was affected in its second dissociation when pH dropped to 4 because it was so close to $pK_{a_2}$ ($pK_{a_1} = 1.30$; $pK_{a_2} = 4.30$ at 298 K).

Qualitative analysis of the experimental results provides a framework for an approximate mathematical model. At all pH assayed, vanillin and formic acids are degraded in the early hours of culturing, giving rise to exponential biomass growth. Once both substrates are depleted in the medium, an adaptation phase is observed, and cells are finally able to degrade phenol and oxalic acid.

Purely competitive inhibition kinetics alone cannot explain the unusual interaction observed, and the nature of the substrate interaction in the mixture is not well understood. The use of sum kinetics with an empirical interaction seems to be a reasonable approach. The SKIP model was first proposed by Yoon et al. [30] and recently used by Reardon et al. [33] and Abuhamed et al. [26] to describe the biodegradation kinetics of benzene, toluene and phenol as mixed substrates with *P. putida*.

Table 1
Effect of pH on substrate degradation by *P. putida* CECT 324 at 30 °C

| pH | Experimental depletion time (h) | | | |
|---|---|---|---|---|
| | Formic acid | Vanillin | Phenol | Oxalic acid |
| 5 | 13 | 18 | 32 | 46 |
| 7 | 17 | 17 | 28 | 22 |
| 6 uncontrolled | 14 | 16 | 46 | 53 |

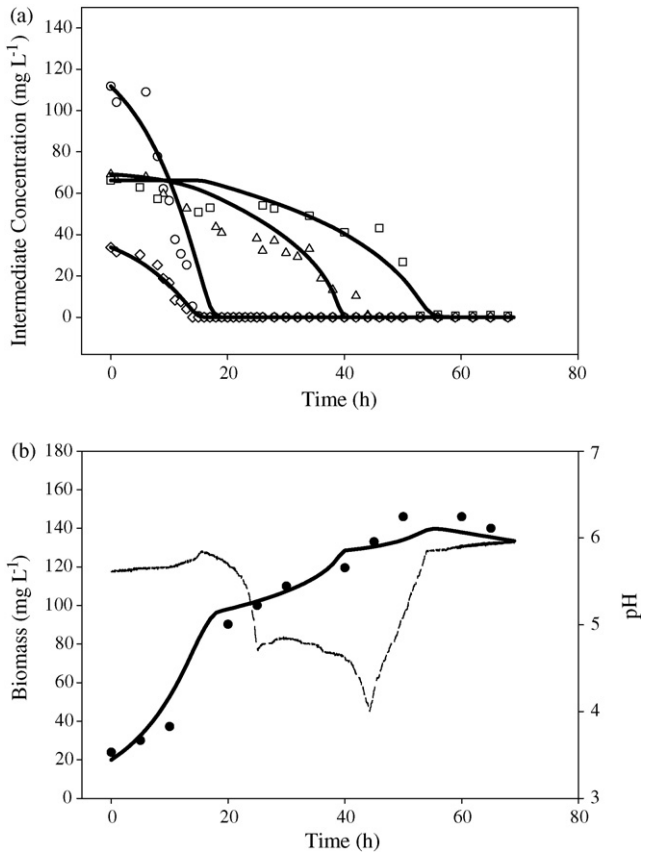


Fig. 4. Results from a batch culture of *P. putida* CECT 324 on a formic acid–vanillin–phenol–oxalic acid mixture at 30 °C. Initial pH was 6 and remained uncontrolled during culturing. Solid lines show model estimations. (a) (◊) Formic acid, (◯) vanillin, (△) phenol and (□) oxalic acid concentrations. (b) (●) Biomass concentration and pH (dashed line).

In this kinetics study, all models consisted of a specific growth rate equation, $\mu$, as a function of substrate concentration and a substrate removal rate equation, taking into account the substrate biomass yield. Vanillin and formic acid are considered non-inhibitory compounds and so are represented by Monod's non-inhibitory kinetics equation as shown below for substrate $i$:

$$\mu_i = \frac{\mu_{\max,i} S_i}{K_{S,i} + S_i} \tag{1}$$

where the constant $K_{S,i}$ indicates the ability of the microorganism to grow at low levels of substrate $i$.

Phenol inhibition had been observed in all previously reported studies and was also found in our experiments. As oxalic acid behaviour was similar, phenol and oxalic acid were considered growth-inhibitory compounds, and their kinetics were fitted to Andrews' growth model due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates [34]. In order to find out whether there is any kind of interaction between vanillin and phenol or formic acid and oxalic acid, an interaction parameter was introduced in the phenol and oxalic acid kinetic expressions. Therefore, the inhibitory-interaction growth kinetics equation is as follows:

$$\mu_i = \frac{\mu_{\max\ \mathrm{ap},i} S_i}{K_{S\ \mathrm{ap},i} + S_i} = \frac{\mu_{\max\ \mathrm{Th},i} S_i}{K_{S\ \mathrm{Th},i} + S_i(1 + (S_i/K_{\mathrm{I},i}) + I_{ij} S_j)} \tag{2}$$

where

$$\mu_{\max\ \mathrm{ap},i} = \frac{\mu_{\max\ \mathrm{Th},i}}{(1 + (S_i/K_{\mathrm{I},i}) + I_{ij} S_j)} \tag{3}$$

and

$$K_{S\ \mathrm{ap},i} = \frac{K_{S\ \mathrm{Th},i}}{(1 + (S_i/K_{\mathrm{I},i}) + I_{ij} S_j)} \tag{4}$$

$K_{\mathrm{I},i}$ is a substrate $i$ inhibition constant. The interaction parameter $I_{ij}$ indicates the degree to which substrate $j$ affects the biodegradation of substrate $i$ (the higher it is, the stronger the interaction).

The initial SKIP model for the overall specific growth rate, $\mu$, in the four-substrate mixture is:

$$\mu = \frac{\mu_{\max\ \mathrm{Th,F}} S_\mathrm{F}}{K_{S\ \mathrm{Th,F}} + S_\mathrm{F}} + \frac{\mu_{\max\ \mathrm{Th,V}} S_\mathrm{V}}{K_{S\ \mathrm{Th,V}} + S_\mathrm{V}} + \frac{\mu_{\max\ \mathrm{Th,P}} S_\mathrm{P}}{K_{S\ \mathrm{Th,P}} + S_\mathrm{P} + (S_\mathrm{P}^2/K_{\mathrm{I,P}}) + I_{\mathrm{PV}} S_\mathrm{P} S_\mathrm{V}} + \frac{\mu_{\max\ \mathrm{Th,O}} S_\mathrm{O}}{K_{S\ \mathrm{Th,O}} + S_\mathrm{O} + (S_\mathrm{O}^2/K_{\mathrm{I,O}}) + I_{\mathrm{OF}} S_\mathrm{O} S_\mathrm{F}} \tag{5}$$

for $i$ = F, V, P and O for formic acid, vanillin, phenol and oxalic acid, respectively.This model was used to write the equations for the change in biomass concentration

$$\frac{\mathrm{d}X}{\mathrm{d}t} = (\mu - k_\mathrm{d})\, X \tag{6}$$

and each substrate concentration over time:

$$\frac{\mathrm{d}S_i}{\mathrm{d}t} = -\frac{\mu\, X}{Y_{X/\mathrm{S}_i}} \tag{7}$$

All model parameters were determined by fitting the proposed differential equations to the experimental data using Matlab™ (Version 6.5). The Matlab Optimization Toolbox was used to find the model parameters. Constrained nonlinear programming was used for the search. The target function to be minimized was defined as a function of the vectorized state space, $X_{\mathrm{exp}}$, that is, all substrate data plus the biomass off-line readings. Note that each state could have a different number of points. Simulation points were picked at the same time as the data and arranged in the same order to conform the vectorized simulated state space vector, $X_{\mathrm{mod}}$. The objective function, $J$, is then defined as weighted squared differences, as follows:

$$J = \sum_{i=1}^{N} \left( \frac{X(i)_{\mathrm{exp}} - X(i)_{\mathrm{mod}}}{X(i)_{\mathrm{exp}}} \right)^2 \tag{8}$$

The state vector contains the biomass and substrate data collected following the experimental procedure. The model state vector was sampled at the same times by defining an appropriate time vector to numerically solve the differential equations mentioned above. To account for a reasonable number of parameters in the search, a sensitivity analysis had previously been performed, in which the death rate of biomass was found to be the

Table 2
SKIP model growth kinetics parameter values

| | Maximum specific growth rate ($h^{-1}$) | | Yield coefficient on substrate ($g\,g^{-1}$) | | Saturation constant ($mg\,L^{-1}$) | | Inhibition constant ($mg\,L^{-1}$) | | Interaction constant ($L\,mg^{-1}$) | | Decay coefficient ($h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| pH 5 | $\mu_{max\,Th,F}$ | 0.03 | $Y_{X/F}$ | 0.26 | $K_{S\,Th,F}$ | 6.43 | | | | | $k_d = 0.002$ |
| | $\mu_{max\,Th,V}$ | 0.05 | $Y_{X/V}$ | 0.34 | $K_{S\,Th,V}$ | 16.63 | | | | | |
| | $\mu_{max\,Th,P}$ | 0.09 | $Y_{X/P}$ | 0.24 | $K_{S\,Th,P}$ | 1.67 | $K_{I,P}$ | 4.96 | $I_{PV}$ | 0.03 | |
| | $\mu_{max\,Th,O}$ | 0.14 | $Y_{X/O}$ | 0.16 | $K_{S\,Th,O}$ | 2.52 | $K_{I,O}$ | 1.61 | $I_{OF}$ | $10^5$ | |
| pH 7 | $\mu_{max\,Th,F}$ | 0.03 | $Y_{X/F}$ | 0.98 | $K_{S\,Th,F}$ | 4.38 | | | | | $k_d = 0.003$ |
| | $\mu_{max\,Th,V}$ | 0.10 | $Y_{X/V}$ | 0.65 | $K_{S\,Th,V}$ | 13.70 | | | | | |
| | $\mu_{max\,Th,P}$ | 0.09 | $Y_{X/P}$ | 0.29 | $K_{S\,Th,P}$ | 4.44 | $K_{I,P}$ | 4.43 | $I_{PV}$ | 0.03 | |
| | $\mu_{max\,Th,O}$ | 0.21 | $Y_{X/O}$ | 0.11 | $K_{S\,Th,O}$ | 19.75 | $K_{I,O}$ | 1.00 | $I_{OF}$ | 0.01 | |
| pH 6 uncontrolled | $\mu_{max\,Th,F}$ | 0.03 | $Y_{X/F}$ | 0.53 | $K_{S\,Th,F}$ | 1.29 | | | | | $k_d = 0.004$ |
| | $\mu_{max\,Th,V}$ | 0.07 | $Y_{X/V}$ | 0.50 | $K_{S\,Th,V}$ | 4.10 | | | | | |
| | $\mu_{max\,Th,P}$ | 0.09 | $Y_{X/P}$ | 0.50 | $K_{S\,Th,P}$ | 2.05 | $K_{I,P}$ | 4.85 | $I_{PV}$ | 0.03 | |
| | $\mu_{max\,Th,O}$ | 0.15 | $Y_{X/O}$ | 0.48 | $K_{S\,Th,O}$ | 41.17 | $K_{,O}$ | 1.80 | $I_{OF}$ | 518 | |

Effect of pH.

most determinant parameter in the simulation. This was always included in the parameter sets that were tested with the optimization algorithm. Upper and lower levels were taken from literature, and were typically the yield coefficient and specific growth rates.

Table 2 gives the model parameter values for pH experiments. A general analysis shows that the maximum specific growth rates for each substrate are low, and that the best value is for oxalic acid. At controlled pH (pH 5 and 7) biomass yields on the substrate are close to those for formic acid and vanillin and decrease for phenol and oxalic acid and for uncontrolled pH experiments $Y_{X/S}$ was around 0.5. $K_S$ values are in the range found for substrates in the literature [26,28,33,35,36] and no general trend was observable for any growth medium ingredient. For the sake of comparison, $K_S$ at 30 °C for phenol ranged from <1.0 with *P. putida* ATCC 17484 [28], where phenol was the only carbon source, to 32 $mg\,L^{-1}$ with *P. putida* F1 [33] in a mixture of toluene, phenol and benzene. With the same mixture and bacterial strain, Abuhamed et al. reported a $K_S$ of 18 $mg\,L^{-1}$ for phenol [26]. Maximum specific growth rate ranged from 0.051 $h^{-1}$ [26] to 0.534 $h^{-1}$ [28]. The main model parameters seem to be the inhibition constant and the interaction parameter. At pH 7, the two interaction parameters are very low, pointing out that interaction between substrates is negligible (see Fig. 3). This was always the case for $I_{PV}$ (0.03 $L\,mg^{-1}$) which is not affected by operating parameters. Thus, phenol was consumed regardless of vanillin concentration. Nonetheless, at pH 5 and 6 (uncontrolled), $I_{OF}$ is high, giving rise to delayed consumption of oxalic acid, as the growth rate would remain low if there were a concentration of formic acid present in the medium. Confirming the initial model hypothesis, phenol and oxalic acid caused inhibition as $K_I$ is relatively low in comparison to substrate concentrations. A high $K_I$ indicates that inhibition can only be observed in a high concentration range. In this sense, oxalic acid inhibits *P. putida* growth at lower concentrations than phenol.

With regard to substrate removal rates given by Eq. (7), the maximum uptake rates of 12.3 and 15.7 $mg\,L^{-1}\,h^{-1}$, were found for phenol and oxalic acid, respectively, with 9.6 $mg\,L^{-1}\,h^{-1}$ for vanillin and 3.5 $mg\,L^{-1}\,h^{-1}$ for formic acid at pH 7. These values decreased at pH 5 to 11.0, 9.2, 5.8 and 2.8 $mg\,L^{-1}\,h^{-1}$ and at uncontrolled pH to 6.14, 6.03, 4.1 and 3.6 $mg\,L^{-1}\,h^{-1}$ for phenol, oxalic acid, vanillin and formic acid, respectively. Maximum removal rates are in the range reported in literature for phenol, but no data have been found for the other substrates. Phenol degradation rates by *P. putida* CCRC14365 at 30 °C were 28.1 and 19.4 $mg^{-1}\,h^{-1}$ for free and immobilized cell systems, respectively, in a batch reactor [25]. In shake-flask cultures, *Pseudomonas pseudomallei* degraded phenol at 13.85 $mg\,L^{-1}\,h^{-1}$, while 26.16 $mg\,L^{-1}\,h^{-1}$phenol degradation rate was obtained with *P. aeruginosa*. Moreover, the effect of various salts was studied and the phenol degradation rate was up to 1.53 times faster with *P. aeruginosa* [37].

### 3.3. Effect of temperature

Culturing of *P. putida* at 25 and 35 °C produced a marked delay in the uptake of phenol and oxalic acid, even though pH was kept optimum (Table 3; Figs. 5 and 6). This effect was stronger at 35 °C, for which phenol (66 h) and oxalic acid (70 h) biodegradation times were the longest.

Model parameters are shown in Table 4 as a function of temperature. Initial specific growth rates calculated by Eq. (5) show a limited effect of temperature on growth in the range tested (0.11 $h^{-1}$ at 25 °C, 0.12 $h^{-1}$ 30 °C and 0.10 $h^{-1}$ at 35 °C). In fact, specific growth rates, yield coefficients and saturation constants are consistent with those found at 30 °C. Nonetheless, at 25 and 35 °C, an increase in $I_{OF}$ is observed, being very high at 35 °C as discussed earlier at pH 5. This increase in the interac-

Table 3
Effect of temperature on substrate degradation by *P. putida* CECT 324

| Temperature (°C) | Experimental depletion time (h) | | | |
|---|---|---|---|---|
| | Formic acid | Vanillin | Phenol | Oxalic acid |
| 25 | 15 | 14 | 54 | 46 |
| 35 | 14 | 18 | 66 | 70 |

Table 4
SKIP model growth kinetics parameter values

| | Maximum specific growth rate ($h^{-1}$) | | Yield coefficient on substrate ($g\,g^{-1}$) | | Saturation constant ($mg\,L^{-1}$) | | Inhibition constant ($mg\,L^{-1}$) | | Interaction constant ($L\,mg^{-1}$) | | Decay coefficient ($h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 °C | $\mu_{max\,Th,F}$ | 0.05 | $Y_{X/F}$ | 0.99 | $K_{S\,Th,F}$ | 18.18 | | | | | $k_d = 0.003$ |
| | $\mu_{max\,Th,V}$ | 0.09 | $Y_{X/V}$ | 0.98 | $K_{S\,Th,V}$ | 15.29 | | | | | |
| | $\mu_{max\,Th,P}$ | 0.05 | $Y_{X/P}$ | 0.49 | $K_{S\,Th,P}$ | 3.90 | $K_{I,P}$ | 4.14 | $I_{PV}$ | 0.03 | |
| | $\mu_{max\,Th,O}$ | 0.15 | $Y_{X/O}$ | 0.24 | $K_{S\,Th,O}$ | 19.91 | $K_{I,O}$ | 0.88 | $I_{OF}$ | 357 | |
| 35 °C | $\mu_{max\,Th,F}$ | 0.05 | $Y_{X/F}$ | 0.78 | $K_{S\,Th,F}$ | 20.19 | | | | | $k_d = 0.015$ |
| | $\mu_{max\,Th,V}$ | 0.09 | $Y_{X/V}$ | 0.92 | $K_{S\,Th,V}$ | 10.24 | | | | | |
| | $\mu_{max\,Th,P}$ | 0.06 | $Y_{X/P}$ | 0.66 | $K_{S\,Th,P}$ | 3.66 | $K_{I,P}$ | 4.38 | $I_{PV}$ | 0.03 | |
| | $\mu_{max\,Th,O}$ | 0.17 | $Y_{X/O}$ | 0.43 | $K_{S\,Th,O}$ | 34.65 | $K_{I,O}$ | 4.96 | $I_{OF}$ | $10^5$ | |

Effect of temperature.

tion parameter justifies the delay in oxalic acid consumption. As discussed above, oxalic acid caused slightly stronger inhibition than, phenol except at 35 °C, when the two $K_I$ values are similar. A decay rate of around 0.003 $h^{-1}$ was found except at 35 °C. At this high temperature, $k_d$ was higher than at 25 and 30 °C due to the mesophilic behaviour of *P. putida* [38].

An analogous effect of temperature on degradation rate was observed. Decreasing culture temperature to 25 °C caused a decrease in maximum substrate removal rates to 6.3, 8.1, 8.4 and 2.3 $mg\,L^{-1}\,h^{-1}$ while 35 °C produced the lowest rates of 3.6, 7.2, 8.0, 7.2 and 2.1 $mg\,L^{-1}\,h^{-1}$ for phenol, oxalic acid, vanillin and formic acid, respectively.

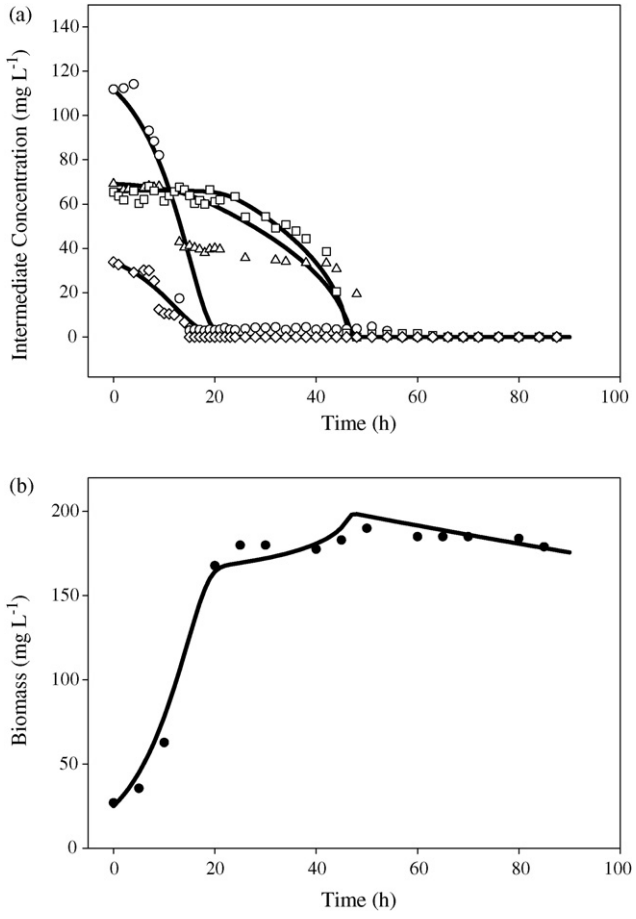


Fig. 5. Results from a batch culture of *P. putida* CECT 324 on a formic acid–vanillin–phenol–oxalic acid mixture at 25 °C and pH 7. Solid lines represent model estimations. (a) (◊) Formic acid, (◯) vanillin, (△) phenol and (□) oxalic acid concentrations. (b) (●) Biomass concentration.

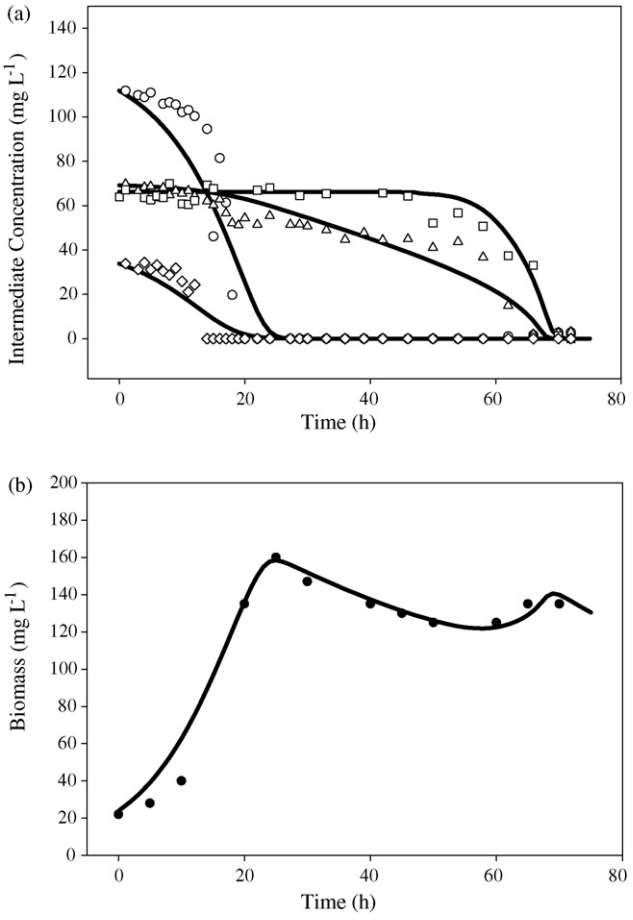


Fig. 6. Results from a batch culture of *P. putida* CECT 324 on a formic acid–vanillin–phenol–oxalic acid mixture at 35 °C and pH 7. Solid lines represent model estimations. (a) (◊) Formic acid, (◯) vanillin, (△) phenol and (□) oxalic acid concentrations. (b) (●) Biomass concentration.

## 4. Concluding remarks

As a general conclusion, it must be remarked that at all the temperatures tested and pH between 5 and 7, *P. putida* was able to degrade the four substrates in culture times of between 30 h

under the best conditions, 30 °C and pH 7, and 70 h under the worst, at 35 °C. The goal of this study was to explore the relative variation in kinetics of bacterial cultures in complex media. The kinetic approach described reveals the variety of consumption patterns that take place during biodegradation of complex substrate mixtures, such as the incompletely oxidized effluents of advanced oxidation processes. A change in temperature of a few degrees gives rise to quite different behaviour of individual substrate uptake patterns. Management of residence time in a biological oxidation plant must consider the major change in biodegradation kinetics with environmental conditions. The typical treatment time in an urban wastewater plant (≈10 h) may be insufficient for biooxidation of residues from an AOP plant. A complete specific system design may be required and close control of the chemical oxidation step would be necessary for proper chemical-biological plant operation.

## Acknowledgements

This research was supported by the Ministry of Education and Science, Spain (PPQ2003-07596-C03-02 and CTQ2006-14743-C03-03) and Junta de Andalucía (Andalusian Govt.) PAI (CVI 263).

## References

[1] European Commission, Directive 2000/60/EC of the European Parliament and of The Council of 23 October 2000 establishing a framework for Community action in the field of water policy, Off. J. Eur. Commun., L 327/1, 2000.

[2] M. Rodríguez, V. Sarriá, S. Espulgas, C. Pulgarín, Photo-Fenton treatment of a biorecalcitrant wastewater generated in textile activities: biodegradability of the photo-treatment solution, J. Photochem. Photobiol. A: Chem. 151 (2002) 129–135.

[3] V. Sarriá, S. Parra, N. Adler, P. Péringer, N. Benitez, C. Pulgarin, Recent developments in the coupling of photoassisted and aerobic biological processes for the treatment of biorecalcitrant compounds, Catal. Today 76 (2002) 301–315.

[4] A.M. Amat, A. Arqués, H. Beneyto, A. García, M.A. Miranda, S. Seguí, Ozonation coupled with biological degradation for treatment of phenolic pollutants: a mechanistically based study, Chemosphere 53 (2003) 79–86.

[5] S. Contreras, M. Rodríguez, F. Al Momani, C. Sans, S. Esplugas, Contribution of the ozonation pre-treatment to the biodegradation of aqueous solutions of 2.4-dichlorophenol, Water Res. 37 (2003) 3169–3171.

[6] P. Hörsch, A. Speck, F.H. Frimmel, Combined advanced oxidation and biodegradation of industrial effluents from the production of stilbenzene-based fluorescence whitening agents, Water Res. 37 (2003) 2748–2756.

[7] M. Bressan, L. Liberatorre, N. D'Alessandro, L. Tonucci, C. Belli, G. Ranalli, Improved combined chemical and biological treatments of olive oil mill wastewaters, J. Agric. Food Chem. 52 (2004) 1228–1233.

[8] C. Pulgarín, M. Invernizzi, S. Parra, V. Sarria, R. Polaina, P. Péringer, Strategy for the coupling of photochemical and biological flow reactions useful in mineralization of biocalcitrant industrial pollutants, Catal. Today 54 (1999) 341–352.

[9] A.E. Da Hora Machado, T.P. Xavier, D.R. de Souza, J.A. de Miranda, E.T.F. Mendonsa-Duarte, R. Ruggiero, L. de Oliveira, C. Sattler, Solar photo-Fenton treatment of chip board production waste water, Sol. Energy 77 (2005) 583–589.

[10] G. Bertanza, C. Collivignarelli, R. Pedrazzani, The role of chemical oxidation in combined chemical–physical and biological processes: experiences of industrial wastewater treatment, Water Sci. Technol. 44 (2001) 109–116.

[11] I.A. Alaton, S. Dogruel, E. Baykal, G. Gerone, Combined chemical and biological oxidation of penicillin formulation effluent, J. Environ. Manage. 73 (2004) 155–163.

[12] J. Beltrán-Heredia, J. Torregrosa, J. García, J.R. Domínguez, J.C. Tierno, Degradation of olive mill wastewater by the combination of Fenton's reagent and ozonation processes with an aerobic biological treatment, Water Sci. Technol. 44 (2001) 103–108.

[13] V.A. Sakkas, I.M. Arabatzis, I.K. Konstantinou, A.D. Dimou, T.A. Albanis, P. Falaras, Metolachlor photocatalytic degradation using $TiO_2$ photocatalysts, Appl. Catal. B: Environ. 49 (2004) 195–205.

[14] E. Evgenidou, I. Konstantinou, K. Fytianos, T. Albanis, Study of the removal of dichlorvos and dimethoate in a titanium dioxide mediated photocatalytic process through the examination of intermediates and the reaction mechanism, J. Hazard. Mater. B 137 (2006) 1056–1064.

[15] T. Essam, M.A. Amin, O. El Tayeb, B. Mattiasson, B. Guieysse, Sequential photochemical-biological degradation of chlorophenols, Chemosphere 66 (11) (2007) 2201–2209.

[16] P.R. Gogate, A.B. Pandit, A review of comparative technologies for wastewater treatment II: hybrid methods, Adv. Environ. Res. 8 (2004) 553–597.

[17] T. Essam, M.A. Amin, O. El Tayeb, B. Mattiasson, B. Guieysse, Solar-based detoxification of phenol and *p*-nitrophenol by sequential $TiO_2$ photocatalysis and photosyntetically aerated biological treatment, Water Res. 41 (8) (2007) 1697–1704.

[18] S. Ledakowicz, M. Solecka, R. Zylla, Biodegradation, decolourisation and detoxification of textile wastewater enhanced by advanced oxidation processes, J. Biotechnol. 89 (2001) 175–184.

[19] H. Shim, S.-T. Yang, Biodegradation of bencene, toluene, ethylbenzene, and *o*-xylene by a coculture of *Pseudomonas putida* of and *Pseudomonas fluorescens* immobilized in a fibrous-bed bioreactor, J. Biotechnol. 67 (1999) 99–112.

[20] M.-K. Chang, T.C. Voice, C.S. Criddle, Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and *p*-xylene by two *Pseudomonas* isolates, Biotechnol. Bioeng. 41 (1993) 1057–1065.

[21] H. Shim, E. Shin, S.T. Yang, A continuous fibrous-bed bioreactor for BTEX biodegradation by a co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens*, Adv. Environ. Res. 7 (2002) 203–216.

[22] S. Malato, J. Cáceres, A. Agüera, M. Mezcua, D. Hernando, J. Vial, A.R. Fernández-Alba, Degradation of imidacloprid in water by photo-Fenton and $TiO_2$ photocatalysis at a solar pilot plant: a comparative study, Environ. Sci. Technol. 35 (2001) 4359–4366.

[23] I.K. Konstantinou, T.A. Albanis, Photocatalytic transformation of pesticides in aqueous titanium dioxide suspensions using artificial and solar light: intermediates and degradation pathways, Appl. Catal. B: Environ. 42 (2003) 319–335.

[24] J.M. Herrmann, Heterogeneous photocatalysis: state of the art and present applications, Top. Catal. 34 (2005) 49–65.

[25] T.P. Chung, H.Y. Tseng, R.S. Juang, Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems, Process Biochem. 38 (2003) 1497–1507.

[26] T. Abuhamed, E. Bayrakta, T. Mehmetoglu, Ü. Mehmetoglu, Kinetics model for growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation, Process Biochem. 39 (2004) 983–988.

[27] A. Kumar, S. Kumar, S. Kumar, Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194, Biochem. Eng. J. 22 (2005) 151–159.

[28] G.A. Hill, C.W. Robinson, Substrate inhibition kinetics: phenol degradation by *Pseudomonas putida*, Biotechnol. Bioeng. 17 (1975) 1599–1615.

[29] R.D. Yang, A.E. Humphrey, Dynamic and steady state studies of phenol biodegradation in pure and mixed culture, Biotechnol. Bioeng. 17 (1975) 1211–1235.

[30] H. Yoon, G. Klinzing, H.W. Blanch, Competition for mixed substrates by microbial populations, Biotechnol. Bioeng. 19 (1977) 1193–1210.

[31] J.L. Casas López, E.M. Rodríguez Porcel, I. Oller Alberola, M.M. Ballesteros Martin, J.A. Sánchez Pérez, J.M. Fernández Sevilla, Y. Chisti, Simultaneous determination of oxygen consumption rate and volumetric oxygen transfer coefficient in pneumatically agitated bioreactors, Ind. Eng. Chem. Res. 45 (2006) 1167–1171.

[32] S.J. Wang, K.C. Loh, Modeling the role metabolic intermediates in kinetics of phenol biodegradation, Enzyme Microb. Technol. 25 (1999) 177–184.
[33] K.F. Reardon, D.C. Mosteller, J.D.B. Rogers, Biodegradation kinetics of benzene, toluene and phenol as single and mixed substrate for *Pseudomonas putida*, Biotechnol. Bioeng. 69 (2000) 386–400.
[34] J.F. Andrews, A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates, Biotechnol. Bioeng. 10 (1968) 707–723.
[35] S. Seker, H. Beyenal, B. Salih, A. Tanyolac, Multi-substrate growth kinetics of *Pseudomonas putida* for phenol removal, Appl. Microbiol. Biotechnol. 47 (1997) 610–614.
[36] S. Dikshitulu, B.C. Baltzis, G.A. Lewandowski, S. Pavlou, Competition between two microbial populations in a sequencing fed-batch reactor: theory, experimental verification, and implications for waste treatment applications, Biotechnol. Bioeng. 42 (1993) 643–656.
[37] M. Afzal, S. Iqbal, S. Rauf, Z.M. Khalid, Characteristics of phenol biodegradation in saline solutions by monocultures of Pseudomonas aeruginosa and Pseudomonas pseudomallei, J. Hazard. Mater. 149 (2007) 60–66.
[38] R. Margesin, P. Bergauer, S. Gander, Degradation of phenol and toxicity of phenolic compounds: a comparison of cold-tolerant *Arthrobacter* sp. and mesophilic *Pseudomonas putida*, Extremophiles 8 (2004) 201–207.