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Biodegradation dynamics of high catechol concentrations by *Aspergillus awamori*

Veselin Stanchev [a], Ivanka Stoilova [b], Albert Krastanov [b,*]

[a] *Department of Automatics, Information and Control Systems, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria*
[b] *Department of Biotechnology, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria*

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Abstract

The biodegradation process of high catechol concentrations by *Aspergillus awamori* was investigated. The values of the kinetic constants for a model of specific growth rate at different initial conditions were determined. At 1.0 g/L catechol concentration, the biodegradation process proceeded in the conditions of substrate limitation. At higher catechol concentrations (2.0 and 3.0 g/L) a presence of substrate inhibition was established. The dynamics of the specific catechol degradation rate was studied and the values of catechol and biomass concentrations, maximizing the specific catechol degradation rate, were estimated analytically. The specified ratio catechol/biomass could serve as a starting base for determination of the initial conditions for a batch process, for specifying the moment of feeding for a fed-batch process, and for monitoring and control of a continuous process by the aim of time-optimal control.

Keywords: *Aspergillus awamori*; Biodegradation; Catechol; Growth kinetics

1. Introduction

Enormous amounts of wastes are accumulated in the environment as a result of human life and industrial activity. These wastes are of great diversity, including solids, liquids and gases, organic or inorganic compounds, low or high molecular weight polymers, chemicals of different origin, radioactive compounds, etc. In most cases they are not distributed as isolated compounds but occur as composite parts of complex aggregates. On the background of the high industrial rate and the dynamics of natural resources exploitation, these toxic compounds are a real threat to plant, animal and human existence.

Some of the pollutants such as polyethylene, polypropylene, polystyrene, polyvinyl chloride, polyethylene tereftalate, caoutchouc, gums, glues, etc. are recyclable and this represents one of the possibilities for their removal from the environment. Vastly more complicated is the problem with the wastes deposited from the chemical industry, since they are strongly toxic and most of them are water-soluble compounds. On the other hand, water is a concomitant component in the chemical industry. The application of physical methods for wastewater treatment is limited and the use of chemical methods leads to a secondary contamination with chemicals or by-products.

A possibility for wastewater treatment is the application of biotechnology methods. The ability of some microbial strains to degrade toxic compounds is widely known. Most of the bioremediation technologies are based on the biodegradation ability of bacteria, whereas the application of yeast and fungi strains is not considered in detail. Fungi are widely spread in nature and are capable to degrade complex natural substances such as lignin, cellulose, and chitin. Fungal strains are more easily adaptable in comparison to bacteria and are capable to grow in extreme conditions: insufficiency of nutrient substances, low pH values, limited water content, etc. [1]. It should be noted that fungi are capable to survive in the presence of different xenobiotics toxic for other strains. Biosynthesis of extracellular oxidative and degrading enzymes enables their tolerance for high concentrations of various toxic compounds. A great number of articles focus on fungi ability to degrade different hydrocarbons, including xenobiotics. However, data about direct application of fungal strains in bioremediation processes and in environmental protection is still insufficient.

* Corresponding author. Tel.: +359 326 03607; fax: +359 326 44102.
E-mail address: abt-kr@rocketmail.com (A. Krastanov).

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Nomenclature

k	serial number of observations
K_i	inhibition constant (g/L)
K_s	saturation constant (g/L)
n	total number of observations
Q	specific substrate degradation rate (h^{-1})
S	substrate concentration (g/L)
t	process time (h)
X	biomass concentration (g/L)
Y	yield coefficient (–)

Greek letters

μ	specific growth rate (h^{-1})
μ_m	maximum specific growth rate (h^{-1})

Metabolism of aromatic hydrocarbons, including phenol and its derivatives, is studied in details in the case of prokaryotic cells [2], and especially about bacterial strains of *Pseudomonas* species [3,4]. The number of yeast strains capable of degrading monoaromatic compounds is comparatively small.

The ability of *Candida*, *Rodotorula* and *Trichosporon* species to metabolize aromatic compounds are described in literature [5–8]. Different *Trichosporon* strains are intensively studied and indicated as perspective for application in wastewater and soil treatment. An important part of these researches concerns the specific enzymes, responsible for biodegradation process.

Some filamentous fungi such as *Penicillium*, *Aspergillus*, *Fusarium*, *Graphium* and *Phanerochaete* are also known to degrade aromatic compounds [9–11]. Santos and Linardi [12] reported isolation and investigation of 30 different fungi strains, tolerant to phenol. The presence of phenol hydroxylase and catechol 1,2-dioxygenase activity was proved in the cells of 15 strains of the species *Fusarium*, *Aspergillus*, *Penicillium* and *Graphium*, cultivated in phenol containing medium.

The reported data demonstrate the perspective of filamentous fungi application for environment protection and prove the need of selection and investigation of such strains in the aim of their application as biocatalysts in environment detoxification.

Fungal whole cells are gaining importance for their use in the wastewater treatment systems and the potentiality of a fungal strain *Aspergillus awamori* to degrade high amount of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol is taken up for our previous publication [13]. Also, the microorganism growth kinetics using Haldane's growth model was investigated. The mathematical methods for experimental data processing can be used for specification of the model type for investigation of the biodegradation process. On this base, a controllable process can be worked out for the aim of maximum effectiveness of toxic compounds biodegradation.

Taking into consideration that catechol biodegradation is not very well investigated (in contrast to phenol biodegradation), the scope of the present research is as follows:

- Investigation on the opportunities for biodegradation of high catechol concentrations by *A. awamori* strain using mathematical modeling for description of growth kinetics.
- Determination of kinetic constants values in the model on the basis of experimental data, achieved for different initial concentrations of catechol, used as a sole carbon source in the nutrient medium.
- Dynamics investigation of the variation of the specific catechol biodegradation rate by *A. awamori* in the presence of substrate inhibition.
- Specifying of the parameters or their ratios, necessary for the aim of bioprocess monitoring and control in a continuous mode.

2. Materials and methods

2.1. Microorganism and growth medium

A strain of *A. awamori* NRRL 3112, obtained from US Department of Agriculture, University of Illinois, USA was used throughout this study. The organism was grown on slants on medium of the following composition (g/L): malt extract 3.0, yeast extract 3.0, peptone 5.0, glucose, and agar 20. The organism on the slants was allowed to grow for 72 h at temperature of 30 °C and then stored at 4 ± 1 °C for further use.

2.2. Medium for degradation studies

The studies on biodegradation were performed in Czapekdox medium, which had the composition (g/L) as sodium nitrate 2.0, potassium phosphate (dibasic) 1.0, potassium chloride 0.5, magnesium sulfate heptahydrate 0.5, and ferrous sulfate heptahydrate 0.01. The initial pH of the medium was adjusted to 5.5 using 1.0 M NaOH or 1.0 M HCl. All media contained catechol as a sole carbon source in concentrations of 1.0, 2.0, and 3.0 g/L.

2.3. Growth of the organism

The 14-day culture in spore form, from the slants was used as inoculum for the liquid medium (1×10^5 conidia/mL medium). To find out the growth phases of the organism, equal volume of inoculum (1×10^5 conidia/mL medium) was inoculated into 50 mL liquid medium in flasks and agitated on a shaker (240 rpm) at temperature of 30 °C. Samples were collected at every 12 h interval and centrifuged at 5000 rpm for 20 min to separate the cells. The wet weight of the cells was determined.

For each experiment, the ability of catechol for polymerization was tested by incubating it at the respective reaction conditions and analyzing its concentration by HPLC. No formation of polycatechols was detected for the investigated time period.

2.4. Analytical methods

The dry weight of the cells was determined by ULTRA X apparatus for drying.

The content of catechol was determined by using Folin–Ciocalteu reagents and confirmed by HPLC. One millilitre sample or standard solution was added to 10.0 mL distilled water and 1.0 mL of Folin–Ciocalteu reagents. The mixture was then allowed to stand for 5 min and 2.0 mL sodium carbonate was added to the mixture. After 1 h in darkness, the absorbance at 750 nm was measured [14]. The HPLC analyses were performed in C_{18} 10 μm Bondapac Column (3.9 mm × 300 mm) and Waters 484 UV detector (260 nm). The mobile phase was methanol:water (70:30), flow rate 0.2 mL/min and temperature 22 °C.

The computing procedures were performed by using a package of applied software programs of own development in the software medium of Matlab [15,16] and Eureka. Graphic performance was done by the means of Microsoft Excel 2000. Experimental data was preliminary smoothed by the method of the fourth differences and approximated with splines of third order [15,17].

3. Results and discussion

3.1. Description of the model

The mathematical model for the investigation of catechol biodegradation by *A. awamori* in a batch mode is represented with a system of four nonlinear differential Eq. (1):

$$\begin{aligned}
&\frac{dX(t)}{dt} = \mu(t)X(t) && (1.1)\\
&\frac{dS(t)}{dt} = -\frac{1}{Y}\frac{dX(t)}{dt} && (1.2)\\
&\left|-\frac{dS(t)}{dt}\right| = QX(t) && (1.3)\\
&\mu(t) = \mu_m \frac{S(t)}{K_s + S(t) + S^2(t)/K_i} && (1.4)
\end{aligned} \qquad (1)$$

The analytical dependence $\mu(t) = f[S(t)]$ was expressed by Andrews and Harris model [18–20], which described biomass growth in the presence of substrate inhibition (1.4). The choice of the model was done on the grounds of a preliminary analysis of experimental data. It was found that the function $\mu(t)$ has an extremum, especially pronounced at catechol concentrations of 2.0 and 3.0 g/L. The structure of (1) a priori presumed, that *A. awamori* used the substrate for maintenance of life activity and biomass growth, and not for accumulation of metabolites.

The dynamics of biodegradation was investigated at catechol concentrations of 1.0, 2.0, and 3.0 g/L [13]. The experimental points corresponding to the lag phase duration (about 24 h) were removed from the data set. For this reason the concentrations of catechol and biomass at $t = 24$ h are marked respectively with S_{0i} and X_{0i}. The values at the end of the process are noted S_{endi} and X_{endi}, where i is the number of experiment ($i = 1$–3).

The numeral values of the kinetic constants were determined by the means of a nonlinear optimization procedure, minimizing the following criterion:

$$J = \sum_{k=1}^{n}(S_{mod,k} - S_{exp,k})^2 + \sum_{k=1}^{n}(X_{mod,k} - X_{exp,k})^2 \rightarrow \min \qquad (2)$$

where the subscripts mod and exp are the values of the parameter, predicted by the model and achieved experimentally. The solution of (1) depends significantly on the initial conditions and the calculating step, since local extremums satisfying (2) could be found.

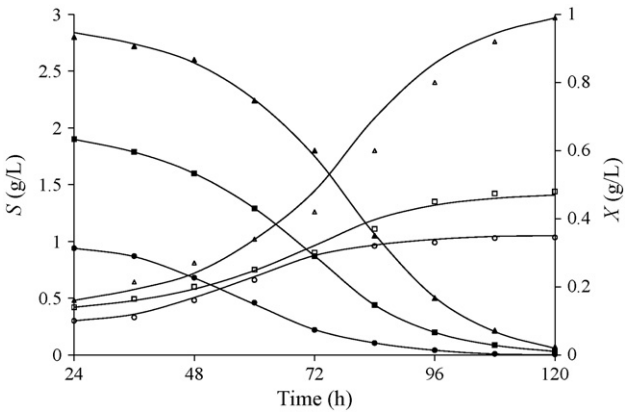

Fig. 1. Dynamics of catechol biodegradation and biomass growth at different initial conditions: (●) −1.0 g/L, (■) −2.0 g/L, and (▲) −3.0 g/L catechol. The hollow symbols present biomass concentration at the respective substrate concentration and the model values are marked with solid lines.

3.2. Catechol biodegradation at different initial conditions

The experimental and predicted values for catechol and biomass concentrations at different initial conditions [13] are presented graphically in Fig. 1. It is obvious that the predicted by the model values were close to the experimental data. Despite, it should be noted that a tendency for increase in the minimum of (2) was observed, when catechol concentration was increased. $J_{min,1} = 8 \times 10^{-4}$ (g/L)^2 and $J_{min,2} = 7 \times 10^{-4}$ (g/L)^2 were not only of the same order, but were also significantly similar in their values, whereas $J_{min,3} = 1 \times 10^{-2}$ (g/L)^2 was considerably higher. Besides, in the last case it was harder to find a simple solution from the computing procedure. On the basis of these observations, it was supposed that the mechanism of biodegradation was altered or metabolite by-products were accumulated, when high catechol concentrations were applied. This assumption was not expressed by the model used. It is difficult to prove this hypothesis analytically, since a lot of mechanisms for benzene ring opening are possible.

The numeral values of the kinetic parameters in (1) for the investigated catechol concentrations are presented in Table 1.

The values of μ_m in the model were found to decrease with the increase in catechol concentration, which corresponded to Eqs. (1.1) and (1.4). This tendency was easy to explain, when taking into consideration the biological aspects of the process. On one hand, the high values of S premised expression of substrate inhibition. On the other hand, catechol exhibited a toxic

Table 1
Numeral values of the kinetic constants in the model at different initial conditions

Catechol (g/L)	Initial conditions (g/L)	Parameter				
		μ_m (h^{-1})	K_s (g/L)	K_i (g/L)	Y (–)	Q (h^{-1})
1.0	$S_{01}=0.94$, $X_{01}=0.10$	0.06331	0.9223	1.0490	0.26554	0.02613
2.0	$S_{02}=1.90$, $X_{02}=0.14$	0.04787	0.9860	1.0145	0.17597	0.04004
3.0	$S_{03}=2.80$, $X_{03}=0.16$	0.04322	1.0207	0.9856	0.29626	0.02810

effect on the microbial cells, which could lead to partial inactivation of the enzyme systems or even decease of the population. Probably at least these two factors limited the life activity of *A. awamori* and respectively catechol biodegradation.

The enhancement in K_s values, noticed with the increase in substrate concentrations (Table 1) could be easily explained by the formulations of the classical Michaelis–Menten theory.

At all investigated catechol concentrations, the values of K_i were about 1 and they decreased in a significantly narrow range (Table 1). This fact showed that process inhibition was more likely due to the high catechol concentration than to its toxic effect on the biomass. In Eq. (1.4), S participated in the second order and in two of the cases $S_{0i}>1$. For these reasons, the values of the ratio $S^2(t)/K_i$ were significantly increased ($0.884 \rightarrow 3.610 \rightarrow 7.840$) and remained considerably high for a great period of process time.

The analysis of the function $\mu(t)$ (Fig. 2.) showed that in the case of 1.0 g/L catechol concentration the dynamics of the process coincided with the dynamics of Monod's model [20], though the estimating procedure was done on the basis of Andrews and Harris model (1.4). This was due to the values of S_{0i} and K_i. Only in this case $S_{0i}=0.94<1$ g/L, and $K_i=1.049$ g/L. Therefore, the ratio $S^2(t)/K_i<1$, which led to a reduction of the initial model to Monod's one. Practically at these initial conditions the starting point lay to the right of the extremum. Definitely it could be concluded that in the case of 1.0 g/L catechol, the biodegradation process proceeded in the conditions of substrate limitation. When 2.0 and 3.0 g/L catechol were applied, the biodegradation proceeded in the presence of substrate inhibition.

The lower value of Y_2 in comparison to Y_3 (Table 1) was another reason to consider that *A. awamori* strain consumed the carbon source after ring opening and transformation of catechol to other easier assimilating metabolites. The increase in S_{03} with 47.4% in relation to S_{02} led to a relative increase in biomass concentration ΔX_{03} with 152% towards ΔX_{02}. This fact is hard to explain in another manner. Y_1 is not a subject of discussion for reasons explained upwards.

Similar considerations could be made with regard to Q. In the case of catechol concentration of 2.0 g/L, the specific substrate degradation rate reached a maximum value (0.04004 h^{-1}).

3.3. Dynamics of the specific catechol biodegradation rate

The investigation of Q in the process time gave more detailed information about catechol biodegradation by *A. awamori*. A graphic interpretation of the results is presented in Fig. 3. The dynamics of Q versus the ratio S/X for running time instants at different initial conditions is presented in Fig. 4.

The values of some parameters observed in the maximum point for each one of the processes are presented in Table 2. The parameters were determined by working out an optimization problem of respective database and appropriate restrictions. The module of dS/dt cleared the difference between the signs of the left and right parts of (1.3) and did not change the quantitative relation of the parameters.

In the dynamics of the process, Q reached values above three times higher than the ones presented in Table 1, but this was noticed for a relatively shorter time intervals. The maximum value of the specific substrate degradation rate was achieved again in the case of catechol concentration of 2.0 g/L. The extremums of $Q(t)$ and $\mu(t)$ were shifted to the right of the

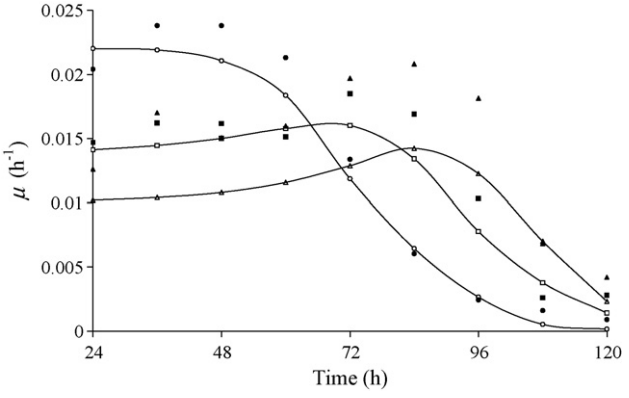

Fig. 2. Dynamics of the function $\mu(t)$ at different initial catechol concentrations: (●) 1.0 g/L, (■) 2.0 g/L, and (▲) 3.0 g/L. The model values are presented with solid lines and hollow symbols.

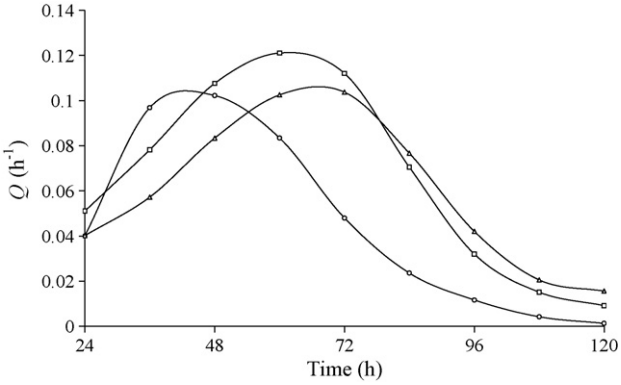

Fig. 3. Dynamics of specific substrate degradation rate at different initial catechol concentrations: (◯) 1.0 g/L, (□) 2.0 g/L, and (△) 3.0 g/L.

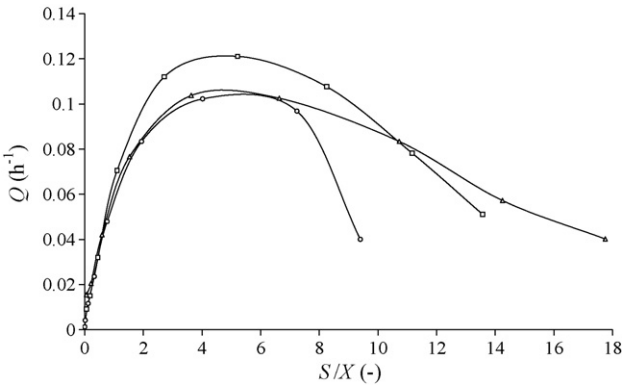

Fig. 4. Specific substrate degradation rate versus the ratio of *S*/*X* at different initial catechol concentrations: (◯) 1.0 g/L, (□) 2.0 g/L, and (△) 3.0 g/L.

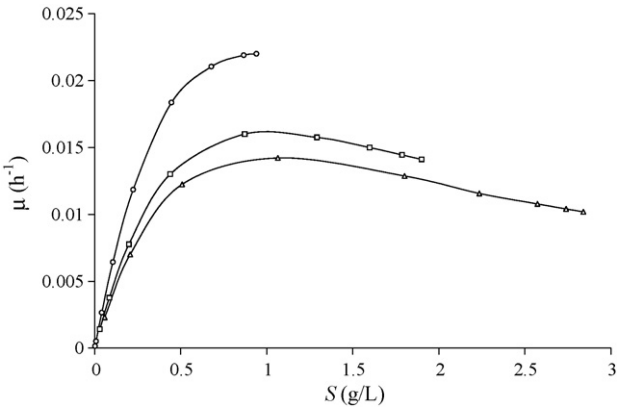

Fig. 5. Specific growth rate versus substrate concentration at different initial catechol concentrations: (◯) 1.0 g/L, (□) 2.0 g/L, and (△) 3.0 g/L according to the model.

process time, when catechol concentration was increased. The reason for this fact was considered to be the decrease in microbial growth rate at the expense of μ_m, K_i and S_0, which led to an increase in biodegradation time.

Significantly valuable information was obtained from the ratio $S_{Q\max}/X_{Q\max}$. In all cases, it was found to be about 5 (Table 2). Probably catechol biodegradation is performed by more than one enzyme. This hypothesis may be verified by applying a new model, instead of Eq. (1.4). This possibility is a subject of research in a future work. However, the more important fact is that the biodegradation process will reach a working point for $S(t)/X(t)$ earlier or later in the process time and this will maximize $Q(t)$.

The specified ratio may be used as a starting base for determination of the initial conditions for the batch process and for specifying the moment of feeding for a fed-batch process by the aim of time-optimal control. It could also serve as a parameter for monitoring and control of the process, when it is performed in a continuous mode.

The values of $Q_{\max}$ (Table 2) presumed that there was an absolute extremum $Q^{\max}_{\text{predicted}}$ in the area of S variation within the range of 0.744–1.987 g/L. For these reasons, the data was approximated with the following second ordered polynomials:

$$Q_{\max} = -0.0427 \times S^2 + 0.1171 \times S + 0 \times 0421 \quad (3)$$

$$Q_{\max} = -0.9544 \times X^2 + 0.5428 \times X + 0.0451 \quad (4)$$

The conditions maximizing the polynomials and the value of the ratio *S*/*X* were as follows: $Q^{\max}_{\text{predicted}} = 0.1224\,\text{h}^{-1}$, at $S = 1.3712$ g/L for (3); $Q^{\max}_{\text{predicted}} = 0.1223\,\text{h}^{-1}$, at $X = 0.2844$ g/L for (4); and $S/X = 4.8214$.

The above value of *S*/*X* was close to the results, presented in Table 2. In the next stage of the research, the statistical difference between $Q^{\max}_{\text{predicted}}$ and the value obtained from the observation points was studied. For that purpose, the Student criterion for the hypothesis of the mathematical expectation equality was used. It was expressed in the following way [21]:

$$t = \left| \frac{\bar{Q}_{\max} - Q^{\max}_{\text{predicted}}}{S_{Q_{\max}}} \sqrt{n_{Q_{\max}}} \right| \quad (5)$$

where $\bar{Q}_{\max} = (1/n)\sum_{i=1}^{n} Q_{\max,i}$, $Q^{\max}_{\text{predicted}} = 0.1224$, $S_{Q_{\max}} = \sqrt{1/(n-1)\sum_{i=1}^{n}(Q_{\max,i} - \bar{Q}_{\max})^2}$, $n_{Q_{\max}} = 3$.

The value of $t_{\text{crit}} = 2.92$ was determined from Student's *t*-test at the significance level of 95% and degree of freedom $\nu = n_{Q_{\max}} - 1 = 2$. As $t = 2.15 < t_{\text{crit}}$, it was concluded that the values of $Q^{\max}_{\text{predicted}}$, $Q_{\max,1}$, $Q_{\max,2}$, and $Q_{\max,3}$ were not statistically different. For these reasons, we considered that the maximum specific catechol degradation rate did not exceed $0.1224\,\text{h}^{-1}$.

3.4. Reliability of the model developed

The reliability of the kinetic constants and the model developed was evaluated by applying the function $\mu = f(S)$ (Fig. 5). Considering (1.4), it was easy to draw analytical expressions for the values of $S = S_{\text{opt}}$ and $\mu = \mu_{\text{opt}} = \mu_{\max}(S)$:

$$S_{\text{opt}} = \sqrt{K_i K_s} \quad (6)$$

$$\mu_{\text{opt}} = \mu_{\max}(S) = \mu_m \frac{\sqrt{K_i}}{2\sqrt{K_s} + \sqrt{K_i}} \quad (7)$$

Table 2
Values of some parameters in the maximum point at different initial conditions

Catechol (g/L)	Initial conditions (g/L)	Parameter				
		$Q_{\max}$ (h^{-1})	$S_{Q\max}$ (g/L)	$X_{Q\max}$ (g/L)	$t_{Q\max}$ (h)	$S_{Q\max}/X_{Q\max}$ (–)
1.0	$S_{01} = 0.94$, $X_{01} = 0.10$	0.1054	0.744	0.1514	44.22	4.914
2.0	$S_{02} = 1.90$, $X_{02} = 0.14$	0.1216	1.236	0.2577	61.78	4.796
3.0	$S_{03} = 2.80$, $X_{03} = 0.16$	0.1061	1.987	0.4146	66.89	4.793

Table 3
Reliability evaluation of kinetic constants

Catechol (g/L)	Initial conditions (g/L)	Parameter			
		Estimated analytically[a]		Estimated experimentally[b]	
		S_{opt} (g/L)	μ_{opt} (h^{-1})	S_{extr} (g/L)	μ_{extr} (h^{-1})
1.0	$S_{01} = 0.94$, $X_{01} = 0.10$	–	–	0.792	0.0239
2.0	$S_{02} = 1.90$, $X_{02} = 0.14$	1.000	0.0161	0.898	0.0186
3.0	$S_{03} = 2.80$, $X_{03} = 0.16$	1.003	0.0143	1.125	0.0208

[a] Estimated by Eqs. (6) and (7).
[b] Estimated experimentally, based on Figs. 1 and 2.

If the kinetic constants were the right solution of (1), and not only a local minimum of (2), the analytically estimated values of S_{opt} and μ_{opt} should match the experimental data about $\mu(t)$ (Fig. 2) and respectively $S(t)$ (Fig. 1). The calculation results are presented in Table 3.

In the case of 2.0 and 3.0 g/L catechol concentrations, the similarity of the results calculated by the two methods was comparatively high. The relative error for the parameters, derived from (1) was estimated towards the corresponding experimental values. The maximum relative error for S was found to be 11.3% (2.0 g/L catechol) and the one for μ was 31.2% (3.0 g/L catechol). Data about catechol concentration of 1.0 g/L was not included in Table 3, since the function did not have an extremum at these initial conditions and Eqs. (6) and (7) were not valid.

Almost in all observation points, the experimental value of μ exceeded the model value (Fig. 2). This fact confirmed once again the hypothesis that the biodegradation process was accompanied by transformation of catechol to secondary metabolites, which were not identified at this stage of the investigation. It was found that the dynamic error of μ increased with the increase in substrate concentration.

However, the mathematical problems connected with the estimation of Eq. (1) derivative in the presence of a discreet database should be taken into consideration. It should be noted that the final result included some error, accumulated as a result of the multiple mathematical processing of the experimental data.

Considering the above reasons, it can be concluded that the values of the kinetic constants reflected adequately the specificity of catechol biodegradation. They may be applied for investigation and control of the process.

4. Conclusions

The process of biodegradation of catechol (a sole carbon source in the nutrient medium) by *A. awamori* was investigated and the values of kinetic constants for a model of specific growth rate (Andrew and Harris model) were determined. It was established that at 1.0 g/L catechol concentration, the biodegradation process proceeded in the conditions of substrate limitation. On the contrary, when 2.0 and 3.0 g/L catechol were used, the process proceeded in the presence of substrate inhibition.

The investigation of the dynamics of the specific catechol degradation rate showed that its maximum value did not exceed 0.1224 h^{-1}. The values of catechol and biomass concentrations, ensuring a maximum of the specific catechol degradation rate, were estimated analytically.

The optimal value of the ratio catechol/biomass, maximizing the specific catechol degradation rate, was determined. This parameter could serve as a starting base for determination of the initial conditions for a batch process, for specifying the moment of feeding for a fed-batch process, and for monitoring and control of a continuous process by the aim of time-optimal control. In all cases, the process control on the basis of this parameter would minimize the time interval for reaching a steady-state, at a maximum value of the specific catechol degradation rate.

Acknowledgements

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