Phenol Degradation by *Ralstonia eutropha*: Colorimetric Determination of 2-Hydroxymuconate Semialdehyde Accumulation to Control Feed Strategy in Fed-Batch Fermentations

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Received 1 September 1998; accepted 17 May 1999

Abstract: Phenol biodegradation by *Ralstonia eutropha* was modeled in different culture modes to assess phenol feeding in biotechnological depollution processes. The substrate-inhibited growth of *R. eutropha* was described by the Haldane equation with a $K_s$ of 2 mg/L, a $K_i$ of 350 mg/L and a $\mu_{max}$ of 0.41 h$^{-1}$. Furthermore, growth in several culture modes was characterized by the appearance of a yellow color, due to production of a metabolic intermediate of the phenol catabolic pathway, 2-hydroxymuconic semialdehyde (2-hms) which was directly correlated to the growth rate and/or the phenol-degradation rate, because these two parameters are coupled (as seen by the constant growth yield of 0.68 g biomass/g phenol whatever the phenol concentration). This correlation between color appearance and metabolic activity was used to develop a control procedure for optimal phenol degradation. A mass-balance equation modeling approach combined with a filtering step using an extended Kalman filter enabled state variables of the biological system to be simulated. A PI controller, using the estimation of the phenol concentration provided by the modeling step, was then built to maintain the phenol concentration at a constant set-point of 0.1 g/L which corresponded to a constant specific growth rate of 0.3 h$^{-1}$, close to the maximal specific growth value of the strain. This monitoring strategy, validated for two fed-batch cultures, could lead, in self-cycling fermentation systems, to a productivity of more than 19 kg of phenol consumed/m$^3$/d which is the highest value reported to date in the literature. This system of monitoring metabolic activity also protected the bacterial culture against toxicity problems due to the transient accumulation of phenol. © 1999 John Wiley & Sons, Inc. CCC 0006-3592/99/040407-09

Keywords: *Ralstonia eutropha*; phenol inhibition; meta pathway; Kalman filter; Haldane equation

INTRODUCTION

Phenol is a hazardous pollutant, contained in industrial wastewater from many chemical plants, oil refineries, and agrochemistry plants. Biological treatment of industrial wastes is mostly based on aerobic-activated sludge systems, whenever toxic or recalcitrant molecules have to be dealt with. However, these biological waste-treatment systems are known to be sensitive to fluctuations in the phenol load. High-pollutant concentration is one of the principal causes of the recalcitrance of chemical products in the environment (Müller, 1992). However, these biological processes are generally operated on the basis of a few rudimentary physical parameters and on-line biomonitoring devices capable of quantifying the incoming load, detecting possible toxic pulses have yet to be developed (Verstraete and Top, 1992). This monitoring approach is particularly important in the case of inhibitory substrates such as phenol. Most microorganisms, able to use phenol as a sole source of carbon and energy, are substrate-inhibited at concentrations as low as 0.05 g/L (*Pseudomonas putida, Trichosporon cutaneum*) (Hill and Robinson, 1975), and this negative effect on microbial growth has been frequently modeled by substrate-inhibition kinetics (Sokol, 1987; Yang et al., 1975). The growth of *R. eutropha* in batch cultures is also characterized by a strong inhibitory effect of phenol with a threshold of 1.2 g/L above which growth is no longer possible (Léonard and Lindley, 1999). Additionally, the bacterial growth yield remains constant at 0.68 g biomass/g phenol whatever the phenol concentration, indicating that phenol degradation rate is directly coupled to growth, and that high-phenol degradation rates can only be attained if low-phenol concentrations are maintained. This constraint necessitates an on-line measurement of the phenol concentration in the bioreactor, coupled to a process-control strategy to adjust the phenol-feed inlet. The application of a fed-batch approach to treat such effluents provides a possible strategy comparable to the sequencing batch reactors proposed by Hughes and Cooper (1996), and avoids direct release into the environment of the treated effluent. Existing systems suffer from
a prolonged fermentation period directly attributable to the inhibitory effects caused by the high-initial phenol load. This could be avoided by an adequate feeding strategy but optimization of such a system requires a simple but reliable control loop. Phenol detection can be performed by chromatographic or spectrophotometric methods, or by analytical systems based on immobilized enzymes such as phenol hydroxylase or cells such as P. putida coupled with respirometric methods (Rainina et al., 1996). These analytical methods are sensitive, but limited by factors such as the concentration range, the inactivation of the support for biological techniques, time delays and their cost. They have been developed for off-line measurement, and actually automated phenol degradation processes are controlled only by the variations in dissolved oxygen concentration (Hughes and Cooper, 1996; Shishido and Toda, 1996).

In this work, a new approach has been investigated to control phenol degradation processes. This method is based on a physiological feature encountered in many microorganisms which catabolize aromatic compounds by the metacleavage pathway. This catabolic pathway, widely used for phenol degradation, is often characterized by the appearance of a yellow color in the culture medium, caused by the accumulation of a metabolic intermediate, the 2-hydroxymuconic semialdehyde, denoted 2-hms (Hughes and Bayly, 1983; Fujita and Ike, 1994). In a previous study on the physiology of phenol degradation by R. eutropha in continuous culture, it has been demonstrated that this accumulation of 2-hms can be attributed to the controlling influence of 2-hms dehydrogenase whose in vivo activity becomes substrate-saturated as the pathway flux increases in direct correlation with the growth rate (Léonard and Lindley, 1998).

In this study, the accumulation of 2-hms has been used to create an indirect sensor of phenol and biomass concentrations based on the direct on-line measurement of the yellow color of the culture supernatant and kinetic modeling of phenol degradation by R. eutropha. This original mode of estimating phenol concentration has been applied to the control of the phenol-feed rate for a fed-batch culture of R. eutropha, in which the residual phenol concentration in the reactor was maintained at a constant value by a proportional-integral (PI) controller.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

*Ralstonia eutropha* (ATCC 17697) was obtained from LMG (Brussels, Belgium) and used throughout the present study.

The mineral salts medium used for growth has been described previously (Ampe and Lindley, 1995). The basal salts medium was adjusted to 7 and sterilized by autoclaving at 121°C for 20 min. A stock solution of potassium phosphate (1M, pH 7) was autoclaved separately and added to the sterile mineral salts medium at a final concentration of 40 mM. Phenol was filter sterilized through membranes (pore size of 0.2 μm) and added to the medium prior to inoculation.

A 1.5-L bioreactor from Setric, Toulouse, France was used for batch and fed-batch cultures. The temperature was maintained constant at 30°C, the pH at 7 with automatic addition of KOH (3M) and the oxygen partial pressure was maintained above 60% of air saturation (i.e., > 0.132 mM oxygen dissolved in the medium) by modifying both the stirrer speed and the volumetric air-flow rate. The bioreactor was inoculated with a 10% (vol/vol) late-exponential-phase culture grown in shake-flask culture on phenol (5 mM).

A 3.5-L Chemap fermentor (working volume of 2 L) was used for chemostat cultures using protocols described previously (Léonard and Lindley, 1998).

For the on-line color measurements, the fermentation broth was separated from cells using a cross-flow microfiltration unit (A-Sep Module from Applikon, France): both cells and filtrate were returned to the reactor in a closed loop (Fig. 1). The permeate stream (flow rate: 18 mL/h) was extracted by a peristaltic pump and supplied to the continuous-flow cell for color measurement prior to return into the fermentor. The tangential recirculation of the medium (at a flow rate of 6 L/h) prevented the fouling at the surface of the 0.2-μm disc filter.

For fed-batch cultures, a concentrated phenol solution (50 g/L) was supplied by a peristaltic pump (minipulse 3, Gilson) under the control of the fed-batch procedure (Fig. 1).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Experimental set up: 1 = bioreactor; 2 = Phenol feed tank (50 g/L); 3 = peristaltic pump monitored by the microcomputer (8); 4 = peristaltic pump; 5 = microfiltration unit; 6 and 7 = colorimetric measurement system; 6 = reflectance spectrophotometer; 7 = dedicated microcomputer; 8 = monitoring microcomputer; 9 = controller (stirrer speed, pH, temperature, PO2).
Chemicals

All chemicals were analytical grade and obtained from Sigma Chimie (St. Quentin Fallavier, France) or C. F. Boehringer & Soehne (Mannheim, Germany) except for 2-hydroxymuconic semialdehyde which is not available commercially and was, therefore, synthesized by the procedure described by Feist et al (1969).

Off-line Analytical Methods

Biomass was measured by cell dry weight determination [dried to constant weight under partial vacuum (200 mbar at 60°C) for 24 h]. An average biomass formula of C_{1.74}H_{1.40}O_{0.46}N_{0.215} (with 3.6% ash content), determined by elemental analysis (Ecole de Chimie, Toulouse, France) was used for elemental recovery calculations.

The concentration of phenol was analyzed by HPLC using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and the following operating conditions: temperature, 65°C; mobile phase, 5 mM H_2SO_4 + 7% (vol/vol) CH_3CN; flow rate, 0.8 mL/min. Detection was made at 210 nm with a variable-wavelength UV detector, and quantification was made by peak integration. This analytical procedure had a detection threshold limit of 5 mg/L up to 0.75 g/L, and standard deviations of approximately 5% of the measured values when triplicate analyses were undertaken. For residual phenol concentrations in chemostat cultures, a colorimetric method (Folsom et al., 1990) was preferred because of a threshold limit of 0.1 mg/L and linearity until 10 mg/L.

On-Line Analytical Methods

The concentration of 2-hms was estimated by a color measurement with a Datacolor ACS ICS model CS3 reflectance spectrophotometer in the CIE Lab system. The Datacolor sensor was a dual-beam spectrophotometer with multi-lens capability designed to measure the percentage reflectance of samples in the visible wavelength region, from 380 nm to 720 nm. Calibration required measurement of the black trap and the white calibration standard. Three parameters could be determined in the CIE Lab color system (Santerre et al., 1994). The accumulation of 2-hms (ε_375 nm = 33000 cm^{-1}) during R. eutropha culture corresponded with an apparition of yellow color. To quantify the 2-hms concentration, the b parameter which defines the position of a sample on the blue/yellow axis in the CIE Lab color system (Hunter and Harold, 1987) was used. A linear correlation was established between the colorimetric parameter b and the 2-hms concentrations: [2-hms] = 0.022 b −0.11 (μg/L). A linear response was observed between color and 2-hms concentrations over the range of 0 to 0.75 μg/L (in this study concentrations of 2-hms never exceeded 55 μg/L).

The control of the spectrophotometer, parameter acquisition, storage, and printing using a prespecified format were managed by a dedicated microcomputer using the Chroma QC software of ACS Datacolor.

Process Monitoring

The bioreactor was linked to a PC/AT compatible microcomputer in charge of process monitoring and control (Queinnec et al., 1992). An RTI 815 board from Analog Devices was used for the communication between the computer and the process. The color measurement operation was initiated by this microcomputer with a sample interval of 5 min.

The “enter” key of the datacolor microcomputer keyboard which managed the different operations of a sample color measurement was activated by the monitoring computer through a digital output of the RTI 815 board. A two-way Converter/Buffer SXP-320 allowing parallel-to-serial-interface conversion was used to interface the printing parallel output of the Datacolor microcomputer with the RS232 line of the monitoring computer. The colorimetric b parameter was recovered and processed by the monitoring computer.

RESULTS AND DISCUSSION

Modeling of Phenol Degradation by Ralstonia eutropha

An automated phenol degradation process requires that an accurate and precise model of bacterial growth on this substrate exists. The general mass-balance equations for phenol degradation by R. eutropha can be written as:

\[ \frac{dX}{dt} = \mu X - \frac{Q_{in}}{V} X \]
\[ \frac{dS}{dt} = -q_s X + \frac{Q_{in}}{V} (S_{in} - S) \]
\[ \frac{dP}{dt} = v_p X - \frac{Q_{in}}{V} P \]
\[ \frac{dV}{dt} = Q_{in} - Q_{out} \]

where X is the concentration of bacteria, S is the phenol concentration, P is the 2-hms concentration, and \( \mu, q_s, \) and \( v_p \) are, respectively, the rates of biomass formation, phenol consumption, and 2-hms accumulation. The different modes of culture (batch, fed-batch, chemostat) can be directly coupled to these general mathematical expressions for which, however, both the specific growth models and kinetic parameters values must be determined. Evaluation of biokinetic constants and the relationship between kinetic parameters are not a function of the culture mode because they are derived from characteristic properties of a given microorganism. However, the growth of microorganisms proceeds by a complex series of biochemical reactions dependent upon many environmental reactions (temperature, pH, substrate concentration, product concentration, dissolved oxygen, growth inhibition, etc.) and a suitable modeling approach has to take into account the effects of vary-
ing conditions. Although the fermentation strategy (batch, fed-batch, chemostat, etc.) does not modify the intrinsic kinetic characteristics of a microorganism, different kinetics models of varying complexity are often used to draw attention to the biological phenomena directly resulting from the imposed constraints, notably in regard to the influence of inhibitory concentrations of either substrates or products associated with a particular fermentation strategy.

Chemostat culture, which provides a good approximation of the culture conditions encountered in continuous depolulation processes, allows substrate-limited growth to be studied. Indeed, if effluent treatment is to be efficient, growth capacity ought to be carbon-limited, thereby ensuring low-outlet concentrations of the effluent. Additionally, this mode of culture is a suitable method to evaluate the correlations that exist between kinetics parameters. The general model, Eq. (1) is rewritten for the chemostat by considering that a constant volume is maintained \( Q_{in} = Q_{out} \) and at steady state \( \frac{dX}{dt} = 0 \), the dilution rate \( D = \frac{Q_{in}}{V} \) is equal to the growth rate. Substrate-limited growth of R. eutropha has previously been described by a Monod-type model (Léonard and Lindley, 1998) because the consequences of phenol inhibition can be neglected under such conditions. However, this is not possible in batch cultures (\( Q_{in} = Q_{out} = 0 \)) or in fed-batch cultures (\( Q_{out} = 0 \)), and the growth behavior of R. eutropha on phenol in such systems obeys substrate-inhibition characteristics, frequently modeled by the Haldane expression (Folsom et al., 1990; Hughes and Cooper, 1996; Reuss and Goetz, 1992; Sokol, 1987; Villadsen, 1999).

\[
\mu = \mu_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}}
\]

(2)

It should be noted that this equation, though more complex than is strictly necessary, describes also the behavior of carbon-limited chemostat cultures. The parameters involved in the Haldane model have been identified using a simplex method, to obtain: \( \mu_{max} = 0.41 \) h\(^{-1} \), \( K_s = 350 \) mg/L and \( K_i = 2 \) mg/L. This model is plotted in Figure 2 and compared with growth kinetics evaluated both on chemostat and batch cultures. The low \( K_s \) value illustrates the high affinity of this strain for phenol and is in agreement with the values determined from carbon-limited continuous cultures of several other efficient phenol-degrading micro-organisms for which estimated \( K_s \) values range from 0.9 to 2.7 mg/L (Folsom et al., 1990; Müller and Babel, 1995; Yang and Humphrey, 1975).

Further, once polyhydroxybutyrate accumulation had been taken into consideration, a constant active-biomass yield was obtained (Léonard and Lindley, 1998) in which \( \mu \) and \( q_s \) were correlated by a linear relationship:

\[
Y_{xs} = \frac{q_s}{\mu}
\]

(3)

with \( Y_{xs} \) constant and equal to 0.68 g/g. Irrespective of the phenol concentration, biomass yield was constant (0.68 g/g) and, unlike P. putida (Hill and Robinson, 1975), no significant phenol-dependent maintenance phenomenon was found. The phenol-dependent decrease in both specific rates of growth and phenol consumption in R. eutropha has been attributed to hydrophobic perturbation of the membrane and a resulting decrease in phenol hydroxylase activity (Léonard and Lindley, 1999). Figures 3 and 4 illustrate the coherence of the modeling in batch and fed-batch cultures, respectively. Considering an initial state \( (X_0, S_0, P_0) \), the biomass \( (X) \) and the substrate \( (S) \) concentrations can be predicted reiteratively by using a Runge Kutta of order-4 integration of balance Eq. (1) with relationships, Eq. (2) and Eq. (3). Note that, in fed-batch cultures, the aim is to obtain high phenol degradation rates by controlling the
phenol concentration at acceptable (noninhibitory) values. As the specific phenol-consumption rate is correlated to the growth rate, this purpose was attained by ensuring a rapid and exponential growth rate of 0.3 h−1, close to the maximum specific growth value of the microorganism on phenol. This constant specific-growth rate was maintained by applying an exponential-feed rate of phenol with the help of the on-line colorimetric measurement. The growth of *R. eutropha* was exponential (μ = 0.3 h−1) and a quasi-constant residual phenol concentration of 0.1 g/L was maintained in the bioreactor (Fig. 4) in agreement with the predictions based upon Eq. (2).

In the three culture operations, 2-hms, visualized by a characteristic yellow color, was detected during phenol degradation. In chemostat culture, this intermediate accumulated as a nonlinear function of the dilution rate (Léonard and Lindley, 1998). In batch cultures, as specific rates of growth and substrate consumption increase, the specific color production rate (v_p) increases in parallel until maximal experimental rates of μ (0.36 ± 0.02 h−1), q_c (0.54 ± 0.02 g/g/h) were reached. The specific production rate of 2-hms (v_p) as measured by the appearance of yellow color can then be related to μ by a polynomial approximation. Best data fitting of v_p with respect to μ was obtained with a 5-order polynomial as:

\[ v_p = b_0 + b_1 \mu + b_2 \mu^2 + b_3 \mu^3 + b_4 \mu^4 + b_5 \mu^5 \]  

(4)

with \( b_0 = -0.085, b_1 = 3.03, b_2 = 5.43, b_3 = 0.046, b_4 = -6.21 \) and \( b_5 = 297 \). However, a linear relationship \( v_p = b_0 + b_1 \mu \), or even \( v_p = b_1 \mu \) (because physically, \( v_p = 0 \) when \( \mu = 0 \)), would be more appropriate to relay the specific growth rate and production rates, even if high degree terms allow some non-linearities in the spectrophotometer to be considered when used eventually in a larger range of product concentrations. Although not justified by physical considerations, a non zero value for \( b_0 \) corresponds better to data fitting. A second relation between \( v_p \) and \( \mu \) was then as:

\[ v_p = b_0 + b_1 \mu \]  

(5)

with \( b_0 = -0.085 \) and \( b_1 = 13 \). The linear relationship between \( \mu \) and \( v_p \) is not surprising in fed-batch culture conditions because the growth rate is quasi-constant throughout the entire culture. The difference between the \( v_p \) value in chemostat cultures (1.32 Ub/g cells/h) and in fed-batch cultures (3.81 Ub/g of cells/h) for identical growth rates can be explained by the nature of the physiological constraints specific to each system and more specifically, the degree of substrate limitation imposed.

All the culture strategies employed confirm that the colorimetric parameter \( b \) is a good manner to assess the growth rate. Further, because the rate of phenol consumption is a linear function of the growth rate, the rate of yellow-color production (\( v_p \)) (equivalent to 2-hms accumulation) can also be taken to assess the phenol-degradation rate. Therefore, on-line colorimetric measurements might be exploited to control the phenol-feed rate in a fed-batch strategy. Two fed-batch cultures performed under identical conditions show a good reproducibility of the biological reaction (Fig. 5) and validate the hypothesis that yellow color can be used to predict and thereby, control the phenol-degradation rate.

**Reconstruction the Non-Measured State Variable—Kalman Filtering**

The above experiments show that colorimetric measurement is correlated to both the growth rate and the phenol-consumption rate. This on-line measurement was used to design a software sensor to overcome the lack of reliable on-line sensors for either biomass or phenol concentrations. The algorithm designed for this purpose, the observer, is based on the general dynamical model described in the previous section. The observer design employed an extended Kalman filter as has been used widely in biotechnology process-control strategies (Marsili-Libelli, 1989; San and Stephanopoulos, 1984). The design of the extended Kalman observer was based upon the minimization of mean-square observation error, under the constraint of the linearized tangent approximation of the nonlinear dynamical model described above. It allows state estimation using a continuous-process model and discrete measurements.

Consider the general non-linear dynamical representation of the form:

\[ \dot{x}_i = F(x_i, u_i, t) + w_i \]  

(6)

where \( F \) is a nonlinear function of the state vector \( x \) of dimension \( n \) and of the control input \( u \) of dimension \( m \), \( w \) is Gaussian white noise zero mean and covariance matrix \( Q_n \), and \( t \) is the time variable.
The discrete observation equation is given by:

\[ y_k = H \hat{x}_{k|k-1} + v_k \]  

(7)

where \( H \) is the observation matrix of dimension \((pxn)\), \( y \) is the observation vector of dimension \( p \) (measurement vector), \( v \) is a Gaussian white noise with zero mean and covariance matrix \( R_k \) and \( k \) corresponds to the iteration at time \( t_k \). The algorithm is based on the steps of (Queinnec et al., 1999):

- Prediction of the state and measurement estimate \( \hat{x}_{k|k-1} \) and \( \hat{y}_{k|k-1} \) based on (6) and (7);
- Linearized approximation of \( F \) and \( H \) around the state estimated (Taylor approximation of order 1);
- Prediction of the positive semi-definite covariance matrix \( P_{k|k-1} \);
- Determination of the observation gain;
- Readjustment of the state estimate \( \hat{x}_{k|k} \) and of the covariance matrix \( P_{k|k} \).

The extended Kalman filter is an iterative procedure. The state estimate \( \hat{x}_{k|k} \) and the covariance matrix \( P_{k|k} \) are used as initial condition for the next iteration. The essential difficulty of such an approach for on-line optimal state estimation lies in the choice of noise covariance \( R_k \) and \( Q_t \) and in the initialization of the covariance matrix \( P_{t0} \) which are considered as synthesis parameters. The extended Kalman filter has been applied in this article to the four states fed-batch model, Eq. (1) with measurement vector \( y_k = [P_k V_k]^T \) and input variable \( u_t = Q_t \). The numerical values of the covariance matrices are given by:

\[ Q_t = \text{diag} \{ 0.01, 0.001, 0.5, 0.5 \} \]
\[ R_k = \text{diag} \{ 0.5^2, 0.06^2 \} \]
\[ P_{t0} = \text{diag} \{ 0.01, 0.01, 0.1, 0.1 \} \]

Figure 6 shows that despite the imperfect batch modeling (dotted line) caused essentially by the use of the chemostat relationship, Eq. (4) between \( \mu \) and \( v_p \), the optimal estimate given by the extended Kalman filter (solid line) enabled phenol concentration during fed-batch culture to be estimated. The observed phenol and biomass concentrations obtained from extended Kalman filtering (solid lines) are...
coherent with the experimental measurements (black squares and triangles, respectively). At 7 h, a disturbance occurred (operational problem with the influent phenol pump), and the Kalman filter was no longer able to observe the experimental data, because the amount of phenol really fed to the culture did not coincide with the value predicted from the pump speed. Without this step of observation, the batch model predicts a phenol limitation. Note that the filtering operation is particularly difficult because a decrease of the evolution of the colorimetric parameter b (equivalent to 2-hms accumulation) corresponds to one of two different interpretations, i.e., either to a limitation of phenol when \( S < (K_s K_i)^{0.5} \), or to an accumulation when \( S > (K_s K_i)^{0.5} \).

**Controlled Fed-Batch Cultures**

As has been seen previously, the phenol-degradation rate is correlated to both growth rate and phenol concentration. Improving the productivity of phenol degradation processes necessitates that a constant residual phenol concentration can be maintained in the reactor to ensure an adequate rate of feeding for rapid growth without exceeding the critical concentration which would provoke growth inhibition. In fed-batch fermentation, this can be done with an exponential rate of phenol inflow. The major difficulty is that the operating point is not steady state, but an unstable set point (a small error on the flow-rate profile leads to limitation or accumulation, provoking diminished productivity or collapse of the system). A closed-loop control strategy has, therefore, to be applied to adjust the flow rate \( Q_t \) from the error \( e_t \) between the phenol set point \( S^* \) and the phenol estimate \( \hat{S}_t \) given by the extended Kalman filter (see previous section). A proportionnal-integral PI controller has been chosen. Such an approach has been often used and is now known in the biochemical engineering community, because constant gain regulators are generally easy to implement, and there are few parameters to tune. Note, however, that the requirements must not be too restrictive and that further improvements may be expected from the use of advanced control approaches. The general expression of such a controller is given by:

\[
Q_t = K_p e_t + \frac{K_p}{T_i} \int_0^t e(\tau) d\tau
\]  

where \( K_p \) is the gain and \( T_i \) the integration time. Discrimination of Eq. (8) at times \( k \) and \( k-1 \) and combination of the expression allows the practical implementation of the PI controller with the following law:

\[
Q_k = Q_{k-1} + K_p (S_{k-1} - S_k) + \frac{K_p}{T_i} (S^* - S_k)
\]  

with the sampling period \( T \). The numerical parameters of the controller have been determined by simulation as \( T = 5 \) min, \( T_i = 0.3 \), \( K_p = 0.2 \).

To test this control system, a fed-batch culture was run so as to maintain a constant growth rate of 0.3 h\(^{-1} \) without manual intervention. According to the Haldane equation, this growth rate corresponds to a constant phenol concentration of 0.1 g/L (solution in the inhibition area). Experimental data (Fig. 7) validate this modeling approach to control the biotechnological process. Over a 7-h period, a constant growth rate of 0.31 ± 0.1 h\(^{-1} \) was maintained. The estimated phenol concentration was close to the experimental value (Fig. 7B) and the rates of both growth and of 2-hms production were exponential. This exponential profile of growth is unusual for phenol-consuming batch processes due to the inhibitory effect of this substrate, but illustrates that high productivity can be achieved for this fermentation by appropriate control of feed rate (we obtained a productivity of 9.3 kg of phenol consumed/m\(^3\)/d in the experiment plotted in Figure 7).

Self-cycling fermentations are often used in biotechnological depollution processes (Hughes and Cooper, 1996). This mode of culture management allows semi-continuous operating conditions of industrial interest. Conditions in the stirred-tank fermentor resulted in an overall oxygen-transfer coefficient \( (K_{La}) \) equal to 350 h\(^{-1} \). In these conditions, a
self-cycling fermentation for a process equipped with our control system operating with an initial biomass of 0.6 g/L and a set point of 0.1 g/L of phenol, would allow four cycles per day to be obtained if oxygen mass-transfer limitations are to be respected. This would lead to a productivity of 19.2 kg of phenol consumed/m³/d. Such biodegradation performance is significantly higher than those previously reported due to the minimization of the phenol inhibition effect on each cycle. The best productivity mentioned in the literature is about 14.5 kg of phenol consumed/m³/d obtained by Hughes and Cooper (1996) in a self-cycling fermentation process (with the same initial biomass concentration of 0.6 g/L).

CONCLUSION

The controlled fed-batch process presented in this work exhibits higher productivity than previously reported biodegradation systems. On-line monitoring of substrate concentration prevents phenol accumulations and avoids cell activity being affected by inhibition phenomena. Our monitoring approach is based both on the yellow color measurement of the culture supernatant (due to 2-hms accumulation) and on the biochemical modeling of phenol degradation. The mathematical model, based on mass-balance equations with an inhibitory substrate combination, provides a good fit with experimental. According to this model, the phenol concentration can be estimated on-line by an extended Kalman filter, and a PI controller was built to maintain the specific growth (and by extension the phenol concentration) at a constant set point. This monitoring strategy has been validated for fed-batch cultures and could be easily adapted to other processes, for which the accumulation of color can be correlated to the growth of the microorganism. However, within the specific framework of phenol degradation, the successful application of this strategy will depend upon the specific metabolic pathways employed. The yellow color (2-hms) is specific to the meta fission pathway, which although the most commonly encountered catabolic pathway involved in phenol degradation, is not the only such pathway.

This technique could also prove useful to avoid washout events in chemostat cultures that occur sometimes when the dilution rate is increased. Li and Humphrey (1989) showed that limit constraints exist which must be respected in regard to shift up of dilution rate. Too large a step provoked an inhibitory accumulation of phenol that, in turn, led to washout even when the imposed dilution rate is below the maximum specific growth rate. Our modeling approach could be useful to prevent this washout of the reactor by directly controlling the feed strategy in response to the rate-dependant accumulation of yellow color.

NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>( b )</td>
<td>colorimetric parameter (CIE Lab references)</td>
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<tr>
<td>( D )</td>
<td>dilution rate (h⁻¹)</td>
</tr>
<tr>
<td>( K_p )</td>
<td>gain of PI controller</td>
</tr>
<tr>
<td>( K_{\text{max}} )</td>
<td>inhibition constant (g/L⁻¹)</td>
</tr>
<tr>
<td>( P )</td>
<td>net-specific growth rate (h⁻¹)</td>
</tr>
<tr>
<td>( Q_m )</td>
<td>2-hms concentration (g/L⁻¹)</td>
</tr>
<tr>
<td>( Q_{\text{out}} )</td>
<td>outlet flow rate (L/h⁻¹)</td>
</tr>
<tr>
<td>( q_s )</td>
<td>specific substrate consumption rate (g of phenol/g of cells/h⁻¹)</td>
</tr>
<tr>
<td>( S )</td>
<td>substrate concentration (g/L⁻¹)</td>
</tr>
<tr>
<td>( S_m )</td>
<td>inflowing substrate concentration (g/L⁻¹)</td>
</tr>
<tr>
<td>( S_{\text{in}} )</td>
<td>inflowing substrate concentration (g/L⁻¹)</td>
</tr>
<tr>
<td>( T_i )</td>
<td>integration time of the PI controller</td>
</tr>
<tr>
<td>( V )</td>
<td>reactor volume (L)</td>
</tr>
<tr>
<td>( Y_{XXS} )</td>
<td>growth yield defined as the ratio of the mass cells formed to the mass of substrate consumed.</td>
</tr>
</tbody>
</table>

Greek symbols

- \( \mu \) : specific growth rate (h⁻¹)
- \( \mu_{\text{max}} \) : maximum specific growth rate
- \( \mu^* \) : net-specific growth rate
- \( \nu_p \) : specific yellow color production rate (U b/g of cells/h⁻¹)

References


