

Phenol Biodegradation Using a Repeated Batch Culture of *Candida tropicalis* in a Multistage Bubble Column

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ABSTRACT. As in many other microorganisms, the growth rate of *C. tropicalis* is affected by phenol. Besides, when the yeast is aerobically cultivated in a medium containing phenol, using a bubble column, the yeast cell flotation phenomenon occurs, which makes the continuous operation of this type of reactor difficult. Therefore, a system of phenol degradation, which recycles the biomass separated by flotation, was devised in this work. In order to reduce the substrate toxicity observed at high phenol concentrations, the bubble column used in the biodegradation studies was fed in a semibatch mode. So, a semicontinuous system was implemented to treat effluents with relatively high concentrations (> 9,000 ppm) of phenol, by replacing periodically about 22% of the bioreactor operational volume. The phenol removal efficiencies obtained with this system were higher than 98.7%.

Key words: *Candida tropicalis*, phenol biodegradation, bubble column, froth flotation.

RESUMEN Como en muchos otros microorganismos, la velocidad de crecimiento de *C. tropicalis* es afectada por fenol. Además, cuando la levadura se cultiva aeróbicamente en una columna de burbujeo, en un medio que contiene fenol, ocurre el fenómeno de flotación celular, lo que dificulta la operación continua de este tipo de reactor. Por consiguiente, en este trabajo se propone un sistema de degradación de fenol en el cual se recicla la masa celular separada por flotación. Además, con la finalidad de reducir la toxicidad del fenol, la columna de burbujeo que se utilizó en los estudios de biodegradación se alimentó en forma semilote. Por ello, se implementó un sistema semicontinuo (cultivo por lote repetido) para tratar efluentes con concentraciones relativamente altas (> 9,000 ppm) de este compuesto, mediante el reemplazo periódico de aproximadamente el 22% del volumen de operación del reactor. Las eficiencias de remoción de fenol que se obtuvieron con este sistema fueron superiores a 98.7%.

Palabras clave. *Candida tropicalis*, phenol biodegradation, bubble column, froth flotation.

INTRODUCTION

Wastewaters generated by the chemical, petrochemical and steel industries frequently contain high concentrations of phenolic compounds,⁸ which represent a serious ecological problem due to their widespread use, toxicity and occurrence throughout the environment.¹²

Aerobic processes of biological treatment are generally preferred to degrade these substances,¹³ due to the low costs associated with this option, as well as to the possibility of a complete mineralization of the xenobiotic.⁷

It has been demonstrated that various toxic organic compounds are not eliminated by the conventional biological effluent treatment systems, due to the presence of relatively high concentrations of easily biodegradable substances. Furthermore, the treatment of small volumes of concentrated toxic compounds at the site of emission, using specific microbial strains and better reactors, is preferable as this procedure allows a higher control over the

process and higher removal efficiencies than those obtained in conventional treatment plants.²⁹

Phenolic compounds degradation may be carried out by eukaryotic and prokaryotic organisms. The latter, under aerobic (oxygen as electron acceptor) or anaerobic (nitrate, sulfate, metal ions or carbon dioxide as electron acceptor) conditions. Aerobic biodegradation of many classes of aromatic compounds is common and proceeds through the key intermediate, catechol. Eukaryotic microorganisms produce catechol from phenol via an epoxide and a transdiol using a monooxygenase.³¹ Prokaryotes introduce the entire oxygen molecule by a dioxygenase reaction forming first a cis-diol. Anaerobically, the aromatic ring is not oxidized, but reduced. The key intermediate in this pathway is a cyclohexanone.⁵

Phenol degradation by microbial pure and mixed cultures have been actively studied.^{1,2,3,4,7,8,12,13,14,18,24,25,26,27,29} Most of the cultures tested are capable of degrading phenol at low concentrations.⁸



Most studies on phenol degradation have been carried out with bacteria, mainly from the *Pseudomonas* genus.^{1,11,12,14,17,31} A great number of yeast and filamentous fungi strains have a phenol degrading capacity. Among the yeast strains, *Candida tropicalis* has been the most studied. *C. tropicalis* is a hydrocarbonoclastic yeast able to degrade phenol, phenol derivatives and aliphatic compounds, at relatively high phenol concentrations (3,000 ppm).^{8,22,23,27,28} However, as in many other microorganisms, phenol inhibits the *C. tropicalis* growth and can also cause cellular lysis.²⁷

Current technology for the biodegradation of toxic compounds involves the use of microorganisms in batch and continuous processes, using either suspended or immobilized cultures. The main drawback associated with batch operation is that the initial substrate concentration must be very low, affecting the process productivity. In continuous cultures, low dilution rates are necessary to avoid instability or low conversion. In processes with immobilized cells, problems of nutrient and oxygen transfer are mainly observed.⁷

Gas-liquid contact towers are reactors which require lower energy inputs per unit weight of oxygen transferred to the culture medium, than the mechanically agitated reactors. For this reason, they have been used as a more attractive technological alternative for the biological treatment of wastewaters, than the conventional activated sludge systems and sparged stirred reactors.⁹

To improve the oxygen transfer efficiency of the gas-liquid contact systems, Kitai et al.²¹ and Brauer⁶ proposed the use of multistage contact towers with perforated plates as diffusers. Here, a high degree of turbulence is caused between the plates, increasing the gas holdup and the gas-liquid interfacial area.

When *C. tropicalis* is cultivated in mechanically agitated reactors, a film of yeast cells is deposited on the shaft and walls of the reactors, so the concentration of cells suspended in the culture medium is lower than the forecasted. The cultivation of this yeast in bubble columns or airlift reactors, even at high liquid circulation rates, is difficult since cell adsorption on the foam occurs, causing a higher cell concentration in the foam than in the bulk liquid broth.²⁸ Adsorption of substances to foam is known as froth flotation.²¹

This work examines a novel approach for the treatment of high strength phenolic wastes. As phenol degradation by *C. tropicalis* is carried out under aerobic conditions and thus a high oxygen transfer rate to the culture medium is required, a gas-liquid multistage contactor was used for the yeast cultivation. Also, in order to increase the cell concentration in the bulk liquid broth and thus, the phenol degradation efficiency, the reactor was operated completely full and the biomass carried on the foam was recycled to the lower part of the column, once the froth had been broken down by magnetic stirring. Furthermore, to reduce the toxic effects of phenol on the yeast cells, it is advisable to

use reaction systems (semicontinuous, continuous or fed-batch cultures) where low levels of phenol are kept; therefore, a semicontinuous culture (repeated batch culture) was used in this work, where approximately 22% of the culture volume was regularly exchanged for fresh medium. The above proposals allowed obtaining high phenol removal efficiencies.

MATERIAL AND METHODS

Microorganism. *C. tropicalis* was used throughout this work. It was obtained from the Colección de Cultivos del Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. The short-term storage of the yeast was on Sabouraud agar slants at 4°C.

Culture media. Growth liquid media contained phenol at different concentrations ranging from 1,000 to 9,200 ppm. The concentrations of the remaining media components were based on the amount of phenol added. Thus, the culture media were supplemented with: (NH₄)₂SO₄, 0.5 g; KH₂PO₄, 0.25 g; MgSO₄·7H₂O, 0.075 g; CaCl₂, 0.0075 g and yeast extract, 0.0375 g, per gram of phenol.

The culture medium for the inoculum preparation contained 1,000 ppm of phenol. The medium was sterilized by autoclaving (121°C for 20 min) and phenol was added after sterilization. The inoculum of *C. tropicalis* was grown in 500 ml Erlenmeyer flasks containing 100 ml of the aforementioned culture medium. Incubation took place in a shaker (54 rpm) at 28°C for 12 h. The cell suspension so obtained was used to inoculate the culture media.

Culture media for the biodegradation studies carried out in the bioreactor contained phenol at different concentrations in the range of 1,930-9,200 ppm and were not sterilized.

Bioreactor and Culture Conditions. The bioreactor used was a multistage bubble column, with a total volume of 3.6 liters. The schematic diagram of the reactor is shown in Fig. 1.

The column consisted of four stages made of Pyrex glass. Each one of the three lower stages had a height of 160 mm and an internal diameter of 82 mm. The top stage (fourth stage) was 158 mm high, with an internal diameter of 105 mm. At the base of each stage, there was a porous glass diffuser (with a pore size of 30-40 mm) of the same diameter than that of the stage. Each stage had ports through which culture samples could be taken. Air and liquid (culture medium) were supplied to the respective ports at the bottom part of the column. A constant air flow rate of 6 l/min, equivalent to a superficial gas velocity of 2.0 cm/s, was used.

The culture broth contained in each compartment (stage) of the column was well agitated by aeration; some air spaces were formed underneath each porous glass diffuser. These air spaces caused a non-continuous flow of the liquid broth and kept each compartment independent,

Table 1. Enrichment ratios of *C. tropicalis* cells in the repeated batch culture.

Time (h)	Biomass concentration in the foam (g/l)	Biomass concentration in the Reactor (g/l)	Enrichment ratio
44.5	0.68	0.228	2.98
47.0	0.58	0.228	2.54
62.5	0.50	0.28	1.78
66.5	0.63	0.233	2.71
77.5	0.56	0.313	1.79
81.5	0.57	0.420	1.36
87.5	0.83	0.500	1.66
97.5	1.01	0.535	1.88
105.5	1.41	0.693	2.04

Table 2. Phenol degradation efficiency in the semicontinuous culture of *C. tropicalis*

Number of medium replacements	$t_f - t_o$ (a) (h)	Φ_R (b) (ppm)	Φ_o (c) (ppm)	Φ_f (d) (ppm)	Phenol removal efficiency (%)
	61.72	6095	1390	1.8	99.8
1	31.83	4884	1114	3.7	99.6
2	49.83	5525	1260	5.2	99.6
3	32.75	5213	1189	5.4	99.5
4	63.75	6849	1480	15	98.9
5	43.50	6840	1560	7.4	99.5
6	58.00	7322	1670	1.7	99.8
7	66.17	7848	1790	4.0	99.7
8	42.58	7936	1810	6.4	99.6
9	59.00	7756	1769	9.4	99.4
10	36.34	8147	1858	23	98.7
11	48.25	9208	2100	16	99.2

(a) Lapse of time between one medium replacement and the next one. (b) Phenol concentration in the fresh medium added to the bioreactor in each medium replacement. (c) Phenol concentration in the bioreactor immediately after the medium replacements. (d) Phenol concentration at the end of each batch culture (immediately before the next medium replacement).

interfering with the mixing of the liquid between them. There was also some foam rising from the surface of the broth in the air spaces underneath the diffusers. Most part of the culture broth apparently flowed upward to the upper compartment in a frothy state rather than in a mass liquid form, and consequently, the cells were considered to be carried on the foam.

The liquid volume in the reactor (V_c) throughout the entire operation was approximately 2.85 liters and was determined by measuring the volume of the liquid drained from the reactor once the phenol degradation process was

over. The foam formed during the yeast culture aeration, which contained a significant biomass concentration, was removed through an overflow outlet located in the upper part of the fourth stage and sent to a collecting tank, where it was broken down through magnetic stirring. The liquid obtained via this process was reintroduced into the column through a port located in stage one (the lowest stage) by means of a peristaltic pump (Masterflex, Cole Parmer) operating with a flow rate of 6.2 l/h.

In the foam collecting tank, the volume of liquid obtained by breaking down the foam (V_f) remained practi-

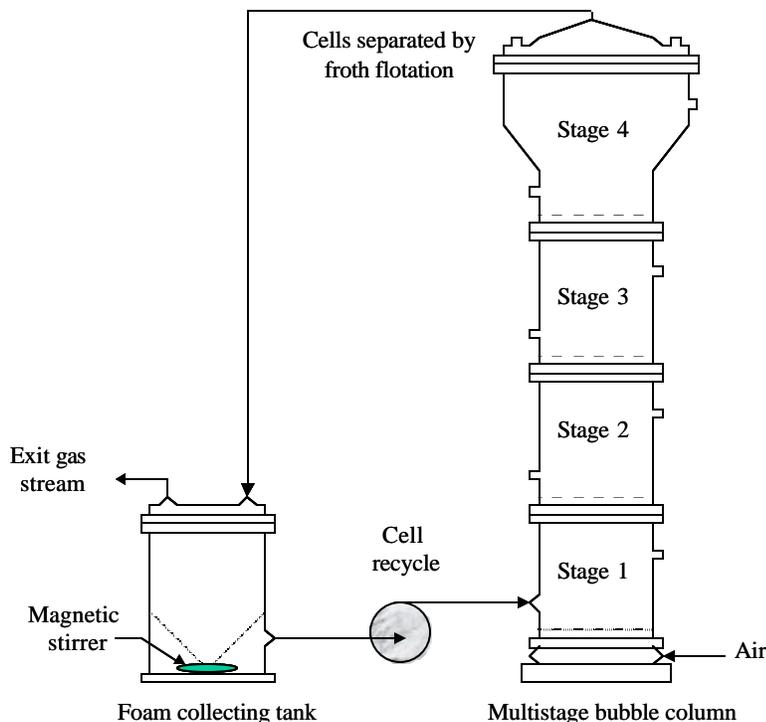


Fig. 1. Schematic diagram of the multistage bubble column.

cally constant throughout the operation, with a value of 0.25 l. Therefore, the operational volume of the system (V_s) was of 3.1 liters, as $V_s = V_c + V_f$.

During the phenol degradation studies, the column was fed in a semibatch mode. For that reason, the first culture was initiated in batch mode and once the phenol present in the culture medium had been almost completely consumed by the *C. tropicalis* cells, approximately 0.7 l of culture medium was regularly removed from the reactor and an equal volume of fresh medium was added, with phenol concentrations varying between 1,930 and 9,200 ppm.

These semicontinuous culture system studies were carried out at room temperature ($24 \pm 2^\circ\text{C}$); the pH was not controlled during the operation and reached levels as low as 3.0.

Fermentation took place under unsterile conditions. The yeast cultures were subject to periodical observations through a microscope to ensure that they were not contaminated with undesirable microbial species.

To estimate the phenol losses by stripping, the column was operated completely full with culture medium containing about 1,000 ppm of phenol in a batch mode, which was not inoculated with the *C. tropicalis* cell suspension. The off-gas from the column was passed through a NaOH solution (pH 10) and the absorbed phenol in the alkaline solution was measured by the method described below. In this control experiment, the column was operated under the same conditions as in the biodegradation studies.

Cell Concentration. Cell concentrations were determined by optical density and dry weight measurements. The optical density measurements were carried out at a wavelength of 600 nm, using a spectrophotometer (Bausch & Lomb). The dry cell weight was determined by filtering the culture samples through preweighed 1.2 μm filters (Whatman GF/C), which were subsequently dried at 95°C to constant weight. The filtrates were used to determine phenol concentrations.

Phenol Concentration. Phenol concentration was determined by the 4-aminoantipyrine method, according to the procedures described in the Standard Methods for the Examination of Water and Wastewater¹⁶.

RESULTS

In the first semicontinuous *C. tropicalis* culture, the culture medium was partially replaced five times (about 22% of the culture medium was drawn out after 47, 62.5, 77.5, 87.5 and 97.5 h of the experimental run) with fresh medium containing a phenol concentration of approximately 1,930 ppm, over a span of 105 h.

The biomass and residual phenol concentrations in the bulk liquid broth in each stage of the column and in the liquid obtained from the foam broken down through magnetic stirring, were initially determined.

Fig. 2 shows the cell concentration in each stage of the column as well as in the foam collecting tank. It can be

seen in this figure that the cell concentration varied in each stage and in the collecting tank, being the tank the place where the highest biomass concentration was observed.

By contrast, phenol concentration was practically uniform in each stage and in the foam collecting tank, as shown in Fig. 3, where it is also shown the phenol concentrations obtained in the control experiment carried out to estimate the phenol losses by stripping.

The amount of phenol added to the column at the beginning of the control experiment was of 3007 mg (970 mg/l), at the end of the run remained about 2542 mg (820 ppm) and so, the phenol removed by stripping was of about 465 mg, over a span of 105 h. Throughout the phenol biodegradation studies, approximately 9,700 mg of phenol were added to the bubble column and all of them was degraded by the *C. tropicalis* cells.

From these results, it is evident that the amount of phenol removed by stripping was very low in comparison with the total phenol biodegradation.

The correlation between the concentration of cells in the foam (x_f) and the average concentration of biomass (x) in the bioreactor is known as enrichment ratio³⁰ ($E = x_f/x$). In this study, the average biomass concentration in the column was ascertained averaging the cell concentration obtained in each stage of the reactor ($x = \sum x_i/4$, where x_i is the biomass concentration obtained in each stage). Enrichment ratio values were estimated at different stages during the culture process, from 44.5 h onwards. At this time, a significant increase of the yeast biomass concentration was registered in the bioreactor. The enrichment ratios of *C. tropicalis* cells, in the repeated batch culture, are shown in Table 1. It can be observed that the biomass concentration in the foam collecting tank was between 1.36 and 2.98 times higher than the average biomass concentration in the column.

To determine the system stability, bioreactor studies with a semicontinuous *C. tropicalis* culture were carried out for 25 days. Initially, the reactor was filled with 0.7 l of culture medium, which contained a phenol concentration of 6,095 ppm, and the remaining volume (2.4 liters) with water and inoculum, with a resultant phenol concentration of 1,390 ppm in the reactor. The bioreactor studies with batch culture of the yeast were carried out for 61.72 h. At this time, the first replacement of culture medium (0.7 l) for fresh medium took place, and the batch culture continued. During the 600 operating hours of the bioreactor, a total of 11 replacements of culture medium for fresh medium with phenol concentrations between 4,880 and 9,200 ppm were carried out. Table 2 shows the lapse of time from one replacement to the next one, the phenol concentration in the fresh medium added to the bioreactor in each replacement, the phenol concentration in the bioreactor immediately after replacements, the phenol concentration at the end of each batch culture (immediately before the next medium replacement), and the efficiencies of phenol biodegradation.

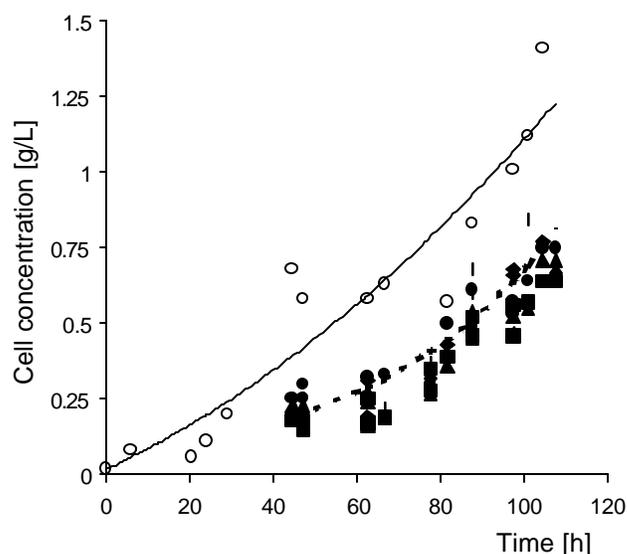


Fig. 2. Cell concentration in each stage of the column and in the foam collecting tank during the repeated batch culture of *C. tropicalis*.

○, foam collecting tank; ◆, Stage 1; ■, Stage 2; ▲, Stage 3; ●, Stage 4).

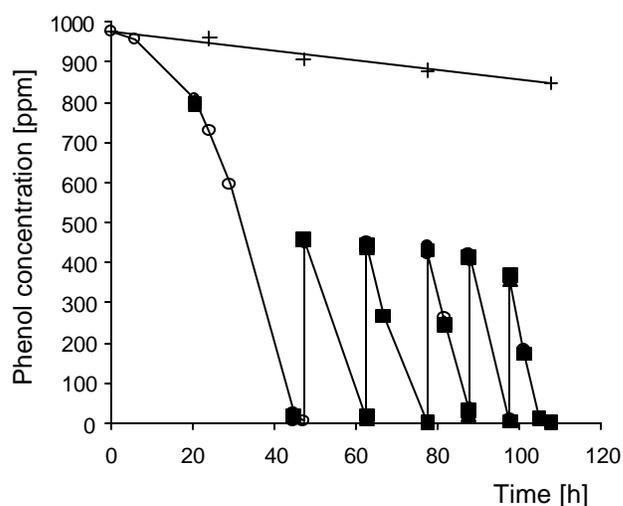


Fig. 3. Phenol concentration in each stage of the column and in the foam collecting tank during the semicontinuous culture of *C. tropicalis* and the phenol concentration to estimate the losses by stripping.

○, foam collecting tank; ◆, Stage 1; ■, Stage 2; ▲, Stage 3; ●, Stage 4; +, phenol concentration in the control experiment in order to determine phenol losses by stripping.



As the initial phenol concentration was increased, it was observed that the biodegradation time increased too. However, phenol removal efficiencies higher than 98.7% were obtained.

Besides, none of the yeast cultures became contaminated in the bioreactor; it may be due to the antiseptic nature of phenol.

DISCUSSION

Throughout the semicontinuous culture of *Candida tropicalis*, the difference between the cell concentration along the column and in the foam collecting tank became evident. The highest biomass concentration was found in the foam collecting tank which may be due to the *C. tropicalis* cells were carried on the foam formed in the aerated cultures of the yeast. This resulted in that high values of enrichment ratios were obtained. These values are higher than those reported for *Candida lipolytica* ($E = 1.29$), *Candida utilis* ($E = 0.72$) and *Escherichia coli* ($E = 0.86$) when they were cultivated in n-paraffin, ethanol and glucose, respectively.²¹ The foam separation of *C. tropicalis* cells would significantly reduce the biocatalyzer separation costs.

It is considered that the enrichment ratio is influenced by the physical properties of the culture broth and the microorganism.²¹ The *C. tropicalis* floating capacity has not been studied yet, but it may be due to the hydrophobicity of the cell surface or to the accumulation of some reserve carbohydrates, as in the *Anabaena flosaquae* cyanobacteria.²⁰

The phenol removed by stripping was found to be less than 4.8% of the total phenol degradation; therefore, it can be concluded that phenol removal in the multistage bubble column was due almost entirely to biodegradation.

Due to the inhibitory and lytic effects of phenol on the *C. tropicalis* yeast strain used in these studies,²⁷ the phenol degradation rate varied according to the phenol concentration of the fresh medium added to the reactor. Thus, as the initial phenol concentration increased the biodegradation time increased too and the degradation rate decreased. In spite of these facts, the phenol removal efficiencies were invariably high, and consistently estimated to be between 98.7 and 99.8 %.

A little information about phenol degradation efficiencies is found in the available literature. For equivalent initial phenol concentrations, the phenol removal efficiencies obtained in this work were higher than those reported for the free suspension systems of *Pseudomonas putida* ATCC 49451¹⁰ and *P. putida* ATCC 17484¹⁵ and similar to those reported for the suspension culture of *P. putida* ATCC 11172⁷ and for the immobilized mixed bacterial culture formed by *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia* sp. and *Yersinia* sp.¹⁹

Although the biological treatment processes of effluents usually use mixed cultures of microorganisms, it is

feasible to implement them with pure cultures. In the present study, a stable operation of the lab-scale system was attained for long periods of time (approximately 3.6 weeks) with no contamination by undesirable microorganisms, despite its functioning under non-aseptic conditions (unsterilized air, bioreactor and culture medium). Furthermore, the system allows the use of high phenol concentrations in the medium added to the reactor, without unduly affecting the degradation efficiency of the toxic compound by the yeast culture.

Additionally, the system has the advantage that the *C. tropicalis* cells become concentrated in the foam formed during the aerated cultivation of the yeast, thus significantly reducing the biocatalyzer separation costs.

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