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ORIGINAL PAPER

Kinetics of the biodegradation of phenol in wastewaters from the chemical industry by covalently immobilized *Trichosporon cutaneum* cells

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Abstract A simple method for the preparation of the biocatalyst with whole cells is presented, and the applicability of the technique for biodegradation of phenol in wastewater from the chemical industries using the basidomycetes yeast *Trichosporon cutaneum* is explored. Kinetic studies of the influence of other compounds contained in wastewater as naphthalene, benzene, toluene and pyridine indicate that apart from oil fraction, which is removed, the phenol concentration is the only major factor limiting the growth of immobilized cells. Mathematical models are applied to describe the kinetic behavior of immobilized yeast cells. From the analysis of the experimental curves was shown that the obtained values for the apparent rate parameters vary depending on the substrate concentration (μ_{maxapp} from 0.35 to 0.09 h^{-1} and K_{sapp} from 0.037 to 0.4 g dm^{-3}). The inhibitory effect of the phenol on the obtained yield coefficients was investigated too. It has been shown that covalent immobilization of *T. cutaneum* whole cells to plastic carrier beads is possible, and that cell viability and phenol degrading activity are maintained after the chemical modification of cell walls during the binding procedure. The results obtained indicate a possible future application of immobilized *T. cutaneum* for destroying phenol in industrial wastewaters.

Keywords Biodegradation · Phenol · Immobilized cells · Kinetic parameters

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Introduction

Wastewaters containing phenols are often problematic because of the toxicity and recalcitrance of phenol compounds. The coke industry is one of the main sources of phenol and its derivatives. The concentration of phenol and its derivatives in wastewaters from the coke industry depends on the actual process and the process conditions as well as the coke source used, but typically ranges from 0.1 to 7 g dm^{-3}.

Several microbial strains are able to use hydrocarbons as a primary source of carbon and energy. They are obtained through selection, mutation or genetic engineering approaches have the specific enzyme systems needed for the biotransformation of these compounds. Strains as *Pseudomonas stutzeri OX1* are isolated from activated sludge of a wastewater treatment plant [2], or from the soil collected from hydrocarbon contamination site was isolated microorganism identified as *Arthrobacter citreus* [14]. Some of the most widely used bacteria and fungi which are capable of using phenol compounds as a sole source of carbon and energy are the bacterium *Pseudomonas putida* [27] and the fungus *Trichosporon cutaneum* [7, 22]. Most of the technological investigations to date have been based on the use of bacteria [1, 21, 23].

A comparison study of kinetic parameters on phenol removal in suspended-growth batch experiments with mixed and bacterial cultures recently published [19]

demonstrated the substrate inhibition on the processes with different strains.

In our previous work a dielectric of the resistance of *T. cutaneum* against toxic chemicals was studied [24]. The results show that cells grown on phenol are more resistant to toxic chemical such as phenol and benzylalcohol, but less resistant against less polar non-aromatic compounds such as *n* octanol.

Some of the microorganisms were immobilized on different carriers [3, 4, 13]. The *T. cutaneum* cells were immobilized on commercial products such as polyacrylonitrile and polyamide [9–11]. The carriers were previously activated with several steps of chemical modification and the immobilization of cells was by means of widely used glutaraldehyde. In the studies mentioned above only experimental results were demonstrated without mathematical modeling and evaluation of kinetic parameters of the processes.

The purpose of this paper is to study the kinetics of viable covalently immobilized *T. cutaneum* cells to one-step activated copolymer of acrylonitrile and acrylamide on a defined media containing glucose or phenol as a carbon source, as well as the growth of *Trichosporon* on real wastewater from coke plant. Mathematical models are applied to describe the kinetic behavior of immobilized cells.

Materials and methods

Microbial strain and reagents

The *T. cutaneum* strain was obtained from the National Bank of Industrial Microorganisms and Cell Cultures, Bulgaria. The mutant basidomycete yeast strain of *T. cutaneum* with phenol degradation activity was registered by Ivanova et al. under N 2414. A copolymer of acrylonitrile with acrylamide, in which the acrylamide units amount to 15% of the total number of units, was obtained at the Department of Biotechnology in UCTM, Sofia. Granules were obtained by dropping the polymer under pressure into a water/methanol (7:3) mixture containing 0.8 g dm^{-3} NaCl according to reference [28]. Glucose, phenol, naphthalene, pyridine, toluene, salts for mineral medium and formaldehyde were obtained from Merck (Germany). Peptone, yeast extract and agar were obtained from Riedel de Haen (Germany). All chemicals used were of high purity. Wastewater from the Kremikovzi plant in Sofia was used as a real object for treatment.

Culturing conditions

The *T. cutaneum* strain N 2414 was cultured on a solid agar medium containing glucose, yeast extract and peptone at 28°C for 48 h at pH 6.0. After incubation colonies were picked and suspended in a mineral salt medium with a glucose concentration of 20 g dm^{-3}. The composition of the nutrient medium was: $(NH_4)_2SO_4$ 4 g dm^{-3}; Na_2HPO_4 0.75 g dm^{-3}; KH_2PO_4 1.7 g dm^{-3}; $MgSO_4 \cdot 7H_2O$ 0.02; g dm^{-3}, thiamine 0.0002 g dm^{-3} and trace mineral medium $FeSO_4 \cdot 2H_2O$ 0.001 g dm^{-3}; $MnSO_4 \cdot H_2O$ 0.001 g dm^{-3}, $CaCl_2$ 0.001 g dm^{-3}, according to [22].

After 24 h incubation in a bath shaker at 28°C, pH 6.0, the cells were resuspended in the same nutrient medium containing 0.35 g dm^{-3} phenol instead of glucose, under the same conditions.

Activation of the copolymer

The immobilization procedure used is based on a method for covalent binding of enzymes to synthetic carriers containing active *N* hydroxymethyl groups, which bind to the amino acid residues of proteins [12, 15, 28]. The granulated carrier of copolymer of acrylonitrile with acrylamide was activated according to procedures described in reference [28]; 40 g of copolymer was activated with 12% (v/v) formaldehyde (dissolved in 0.1 M phosphate buffer, pH 7.8) and stirred for 4 h at 45°C in a closed vessel. The activated carrier was then washed abundantly with distilled water until no more formaldehyde was observed in the rinsing waters.

Covalent binding of *Trichosporon* viable cells to activated carrier

The activation procedure of the carrier was followed by the immediate treatment with a cell suspension of *T. cutaneum* (concentration 80 mg ml^{-1}). The cells and synthetic carrier were suspended in a synthetic nutrient medium, containing 0.1 g dm^{-3} glucose in a 0.1 M acetate buffer. The binding was carried out at pH 5.0 at a temperature of 28°C under continuous stirring for a period of 4 h.

Kinetic experiments

The free and immobilized cells were grown in batch experiments. This was done in flask cultures at pH 6.0 at a temperature of 28°C under continuous agitation. The initial concentration of immobilized yeast cells was in the same range as for the free ones, 0.0167 g dm^{-3}. During the experiments the biomass and glucose concentrations were measured every 2 h. Phenol degradation was followed at different substrate concentrations in the range 0.1–1 g dm^{-3}. The influence of the addition of different substances such as naphthalene, toluene, ammonium thiocyanate, and pyridine, which are typical constituents of coke wastewaters was also studied. The kinetic studies in

real wastewaters from Kremikovzi plant were done after removal of suspended oils by filtration.

Analytical procedures

The biomass of suspended cells was measured spectrophotometrically at 600 nm. Cell growth of suspended and immobilized cells was also determined as dry cell weight, according to the method described by Mallette [17]. The samples were dried until they reached a constant weight at 105°C. The analysis was checked by the determination of the protein content using a modified Lowry's method according to Schacterlee and Pollack [20]. Phenol and other organic compound concentrations in the liquid phase were measured using an HPLC with an HP1050 column (LiChrosphere) and a variable wavelength monitor by measuring the absorbance at 256 nm. The formaldehyde in rinsing waters was determined by gas chromatography Thermo Quest TRASE GC 2000 using column AT-Wax Altech 30 m × 0.25 ID × 0.25 μm, flow 0.6 ml/min, nitrogen as a carrier gas.

Pictures of granules were taken using a Nikon Coolpix 900 digital camera and a Nikon E600 microscope.

Results and discussion

The amount of immobilized cells after immobilization was around 40 mg g^{-1} dry carrier. The microscopic pictures of granules with immobilized cells are shown in Fig. 1. The recovery of the phenol transformation ability of the covalently bound cells after immobilization treatment was studied. The cells were first grown in a defined medium with 20 g dm^{-3} glucose, and then subsequently in a defined medium only with phenol as the sole carbon and energy source at a concentration of 0.35 g dm^{-3}. Biomass growth and substrate consumption on a defined medium with 20 g dm^{-3} glucose and 0.35 g dm^{-3} phenol were compared. The results show that the cells grow less well on phenol as compared to glucose. Also, the lag phase on phenol is much longer (4 h on glucose and 6 h on phenol, respectively).

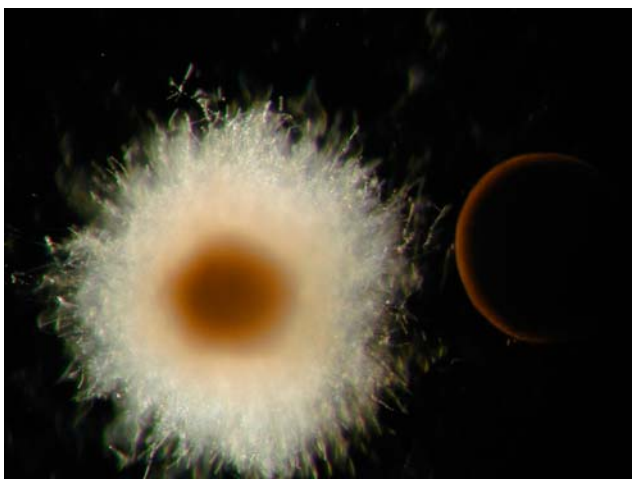

Fig. 1 Beads with immobilized *Trichosporon cutaneum* cells in 0.35 g dm^{-3} phenol

Microbial growth during incubation of immobilized cells in 24 h batch cultures was monitored for 20 cycles of phenol degradation. During this time cell viability and activity was maintained at high levels, showing the possibility for the prolonged proliferation and preservation of the stability and the activity of the immobilized cells.

Microbial growth in real and simulated wastewater

It proved to be impossible to grow *Trichosporon* directly in real wastewater. However, removal of suspended oils allowed growth of *Trichosporon* to occur at rates comparable to these in model waters.

Analysis of the composition of wastewater showed that it contained, apart from phenolic compounds, levels of naphthalene (0.05 g dm^{-3}), benzene (0.05 g dm^{-3}), toluene (0.05 g dm^{-3}) and pyridine. Addition of these compounds to cultures on defined media at concentrations comparable to those in the wastewaters showed that none of the compounds affected *Trichosporon* growth to a level comparable to that in real wastewater. Kinetic studies of the influence of these substances and kinetic parameters derived from the experiments using Monod kinetics are shown in Table 1 for all other, respectively. The results indicate that, apart from the oil fraction, which is removed, the phenol concentration is the only major factor limiting the growth of *Trichosporon* in wastewaters.

Kinetic studies

The kinetics of growth on phenol was investigated at different substrate concentrations in the range 0.1–1 g/l in model waters. At phenol concentrations of 0.6 and 1 g dm^{-3} the longer lag and exponential phases were observed. The analysis of the experimental curves using simple Monod kinetics [8] shows that the obtained values for the apparent rate parameters vary depending on the substrate concentration (μ_{maxapp} from 0.35 to 0.09 $h^{-1}$and K_{sapp} from 0.037 to 0.4 g dm^{-3}). The decrease of the maximum rate of cell growth with increasing phenol concentration, coupled with an increase in the Monod constant, indicates that the substrate has an inhibitory effect.

To find the values of the kinetic parameters, first apparent values of the parameters μ_{max} and K_s were obtained for the Monod equation [16]. The following approaches were used:

Table 1 Kinetic parameters describing the growth of immobilized *Trichosporon cutaneum* yeast cells in presence of different compounds in model waters

Compounds	μ (h^{-1})	μ_{max} (h^{-1})	K_s (g dm^{-3})
0.6 g dm^{-3} phenol	0.19	0.27	0.30
0.6 g dm^{-3} phenol + 0.6 g dm^{-3} pyridine	0.17	0.26	0.30
0.8 g dm^{-3} ammonium rhodanide + 0.6 g dm^{-3} phenol	0.16	0.24	0.30
0.05 g dm^{-3} toluene + 0.6 g dm^{-3} phenol	0.14	0.22	0.31
0.1 g dm^{-3} benzene + 0.6 g dm^{-3} phenol	0.14	0.22	0.29
0.05 g dm^{-3} naphthalene + 0.6 g dm^{-3}phenol	0.12	0.18	0.30

From the linear part of the logarithmic plot of $\ln(X/X_0)$ versus $(t - t_{lag})$, an initial estimation of the μ_{max} was made. Differentiation on the entire experimental kinetic curve (d ln X vs. dt) was applied to obtain the temporary values of the specific growth rate $\mu(t)$ and $\mu(S)$, respectively. Then linearization of these data (by plotting $1/\mu$ vs. $1/S$, or S/μ vs. S), as usually done for systems following Monod kinetics, was used to estimate apparent values for μ_{maxapp} and K_{sapp}. Because of the inhibitory effect, limiting values of μ_{max} and K_s for diminishing initial phenol concentrations S_0 were used in order to exclude the effect of the inhibitory constant K_i. Figure 2a, b, presenting the dependence μ_{maxapp} versus S_0 and K_{sapp} versus S_0, are an illustration of this approach. Their intercepts give $\mu_{max} = 0.457\ h^{-1}$ and $K_s = 0.011$ g dm^{-3}, respectively, which is similar to the results previously reported for phenol biotransformation: $\mu_{max} = 0.47\ h^{-1}$ [27] and $0.436\ h^{-2}$ [13]; $K_s = 0.02$ g dm^{-3} [27] and $K_s = 0.062$ g dm^{-3} [13].

Then models including different substrate inhibition terms were used to describe the effect of the inhibition. The following three models are between the most used for describing the substrate inhibition during phenol biodegradation [1, 5, 18, 27].

$$\mu = \frac{\mu_{max}}{(1 + K_s/S + S/K_i)} \quad \text{(Model 1)}$$

$$\mu = \frac{\mu_{max}}{\left(1 + K_s/S + (S/K_i)^2\right)} \quad \text{(Model 2)}$$

$$\mu = \frac{\mu_{max}}{(1 + K_s/S)} \exp(-S/K_i) \quad \text{(Model 3)}$$

In most publications, concerning the phenol biodegradation process by *T. cutaneum*, models of type 1 are used. Values reported in the literature for pure and mixed cultures degrading phenol are in the range of $K_i = 0.4$–0.6 g dm^{-3}. In some recent investigations with pure cultures of *Pseudomonas* strains a modified two-phase model was applied and the obtained results for μ_{max}, K_s and K_i were, respectively, 0.38 h^{-1}, 18 and 0.214 g dm^{-3} [5]. Model parameters determined from phenol degradation with Ca–alginate immobilized *Ralstonia eutropa* were μmax = 0.89 h^{-1}, $K_s = 0.055$ g dm^{-3}, $K_i = 0.257$ g dm^{-3} [6]. The obtained kinetic parameters from phenol bioconversion by *P. stutzeri OX1*, immobilized by adhesion on active biofilm were $\mu_{max} = 0.71\ h^{-1}$, $K_s = 0.310$ g dm^{-3}, $K_i = 0.130$ g dm^{-3} [26].

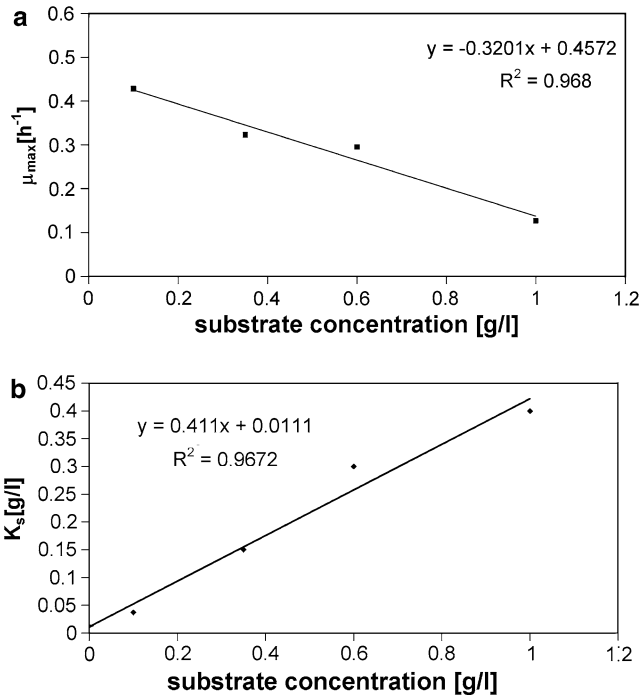

Fig. 2 **a** Effect of phenol concentration on μ_{max}; **b** effect of phenol concentration on K_s

The models 1–3 were used in this study in the range of initial phenol concentrations 0.1–1 g dm^{-3}. The inhibitory constant K_i was determined by fitting the experimental and calculated kinetic curves with the obtained $\mu_{max} = 0.457\ h^{-1}$ and $K_s = 0.011$ g dm^{-3} and with K_i as an adjustable parameter. The curves for cell growth and substrate consumption were simulated using a Runge–Kutta technique. The value of the yield coefficient $Y_{X/S}$ was determined from the experimental data with different initial substrate concentration. An illustration of the dependence of the yield coefficient on the substrate concentration is shown in Fig. 3.

As shown previously [25] the usefulness of the models in predicting the inhibitory effect of the phenol, is

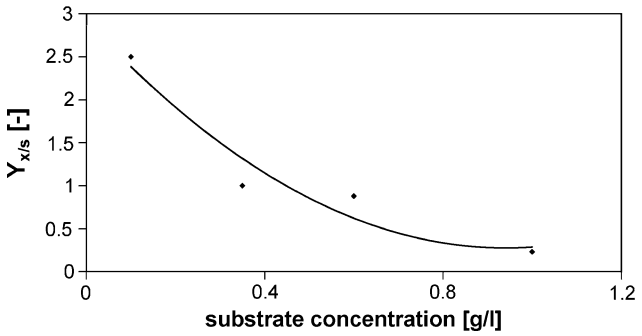

Fig. 3 Effect of phenol concentration on $Y_{X/S}$

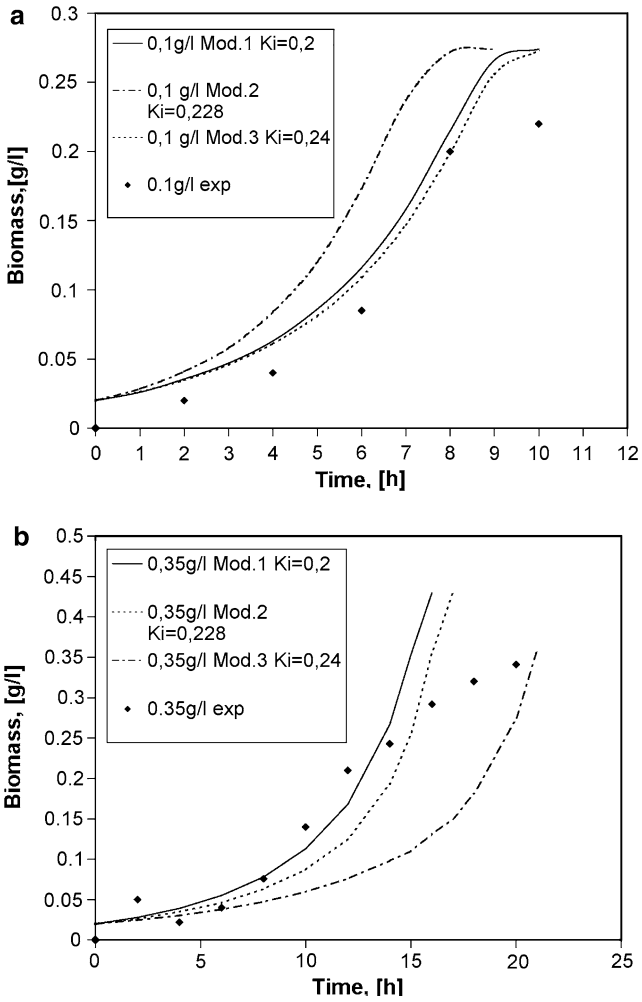

Fig. 4 **a** Calculated with different models (*lines*) and experimental (*points*) kinetic curves for biomass growth at start concentrations of phenol 0.1 g dm^{-3}. **b** Calculated with different models (*lines*) and experimental (*points*) kinetic curves for biomass growth at start concentrations of phenol 0.35 g dm^{-3}

dependent on the concentration range. The final choice of the appropriate model is made depending on its ability to describe the experimental curves in the whole concentration range (0.1–1 g/l phenol) with a fixed set of parameters

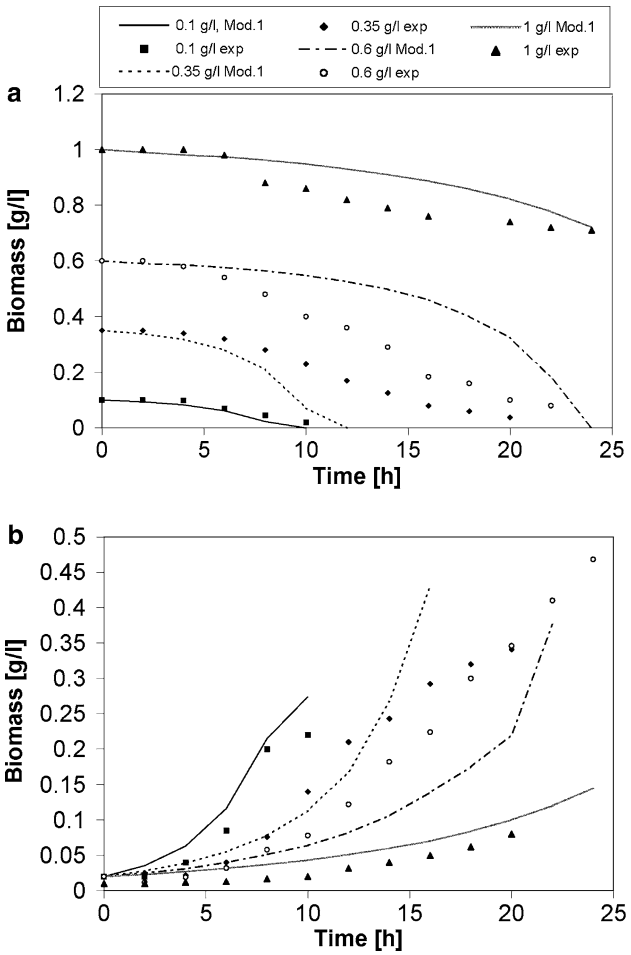

Fig. 5 **a** Calculated with model 1 and experimental kinetic curves for substrate consumption with different initial phenol concentrations ($S_0 = 0.1$; 0.35; 0.6; 1 g dm^{-3}) in real waters. **b** Calculated with model 1 and experimental kinetic curves for biomass growth with different initial phenol concentrations ($S_0 = 0.1$; 0.35; 0.6; 1 g dm^{-3}) in real waters

(μ_{max}, K_s and K_i). Figure 4a, b present the calculated with the three models curves in comparison with the experimental ones for initial phenol concentration 0.1 and 0.35 g dm^{-3}. As can be seen, the value of $K_i = 0.24$ g dm^{-3}, which gives the best fit of the experimental curve with model 3 for 0.1 g dm^{-3} phenol, is far from the satisfactory prediction with 0.35 g dm^{-3} phenol concentration. The latter is well described by model 2 with $K_i = 0.23$ g dm^{-3}, but for 0.1 g dm^{-3} the respective calculated curve is far from the experimentally obtained. With higher phenol concentrations (0.6 and 1 g dm^{-3}) models 2 and 3 predict a much stronger inhibitory effect, respectively; the values of K_i differ drastically. Model 1 was found able to describe satisfactorily the experimental curves in the whole concentration range with the same value of $K_i = 0.2$ g dm^{-3}. So it was concluded that this model gives the adequate description of the phenol inhibitory effect. The following Fig. 5a, b represent the

calculated curves with model 1 for substrate consumption and biomass growth in comparison to the experimental ones in the whole concentration region 0.1–1 g dm^{-3}.

Conclusions

It has been shown that covalent immobilization of *T. cutaneum* whole cells to polymer carrier beads is possible, and that cell viability and phenol degrading activity are maintained after the chemical modification of cell walls during the binding procedure. Mathematical models applied to describe the kinetic behavior of immobilized yeast cells show that the inhibitory effect of phenol is described satisfactory by model 1 in the whole concentration range with the same value of K_i so it can be concluded that this model gives the adequate description of the phenol inhibitory effect. The results obtained indicate a possible future application of immobilized *T. cutaneum* for destroying phenol in industrial wastewaters from coke, pulp and paper or pharmaceutical.

This kinetic study of immobilized yeast cells on polymer carrier will be used for the realization of continuous processes and the biofilm formation and models will be investigated.

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