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Biodegradation of *p*-hydroxybenzoic acid by *Pseudomonas putida*

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ABSTRACT

Due to the toxicity of *p*-hydroxybenzoic acid and its presence in a wide range of industrial wastewaters, new developments are needed to efficiently remove this phenolic pollutant. In this study, and for first time, pure *Pseudomonas putida* (DSM4478) culture was used for biodegradation of *p*-hydroxybenzoic acid in shake flask experiments at 28°C and concentrations between 0 and 200 ppm, obtaining removal rates of approximately 48 mg/L h under optimal conditions. Increasing initial *p*-hydroxybenzoic acid concentrations resulted in higher specific growth rates, lower yields, and longer lag phases. From batch experiments carried out with *p*-hydroxybenzoic acid as limiting substrate, the kinetic constants of the Monod equation were $\mu_m = 0.47\ h^{-1}$ and $K_S = 19$ ppm and the length of the lag phase increased linearly with *p*-hydroxybenzoic acid concentration. Under oxygen-limiting conditions, biomass yield, duration of the lag phase and specific growth rate were considerably reduced. Finally, using the fitting parameters obtained for the Gompertz model, the best conditions for maximum growth of the *P. putida* (DSM4478) and for a maximum assimilation rate of *p*-hydroxybenzoic acid per bacterium were also evaluated.

Keywords: Biodegradation; Gompertz model; *p*-hydroxybenzoic acid; *Pseudomonas putida*; Oxygen limiting conditions

1. Introduction

The phenolic compound *p*-hydroxybenzoic acid appears very commonly in a great variety of wastewaters related to the agroindustrial sector (manufacturing of olive oil, wine, spirits, etc.), industrial synthetic processes (such as the synthesis of acetyl-salicylic acid), and also in cosmetics manufacture [1–3]. *p*-Hydroxybenzoic is also a common and typical phenolic allelochemical that is released into the environment by plants through foliar leachate and residue decomposition [4]. In addition, hydroxybenzoic acids are the most important intermediate metabolites in the microbial degradation pathways of various aromatic hydrocarbons, aromatic dicarboxylic acids, phenolic compounds, or dimeric lignin compounds [5] and frequently they are regarded as a sort of primitive model compound for humic acids [1,6].

Given the bactericidal nature of the phenolic compounds when found at high concentrations, the classic biological treatment cannot be applied [7–11]. Therefore, in order to remove the *p*-hydroxybenzoic acid, new developments are needed, which may be based on the study of alternative treatment techniques or the improvement of the existing biological treatments.

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In the first case, systems based on the generation of very reactive oxidizing free radicals have generated increasing interest [12,13]. These systems, commonly termed advanced oxidation processes, have been successfully used in the treatment of water contaminated with hydroxybenzoic acids [14,15]. However, these processes frequently involve high costs [16]. Besides, the sequence of oxidation intermediates formed during treatment tends to be increasingly resistant to complete chemical degradation [17].

The improvement of the biological treatments using pure cultures of micro-organisms specially adapted to metabolizing the contaminant appears to be an alternative to conventional processes. The pure cultures that are most active in biodegrading hazardous substances can be isolated by conventional microbiological methods, quickly identified by molecular biological methods, and tested for pathogenicity and biodegradation properties. Applications of defined pure starter cultures have the following theoretical advantages: greater control over desirable processes; lower risk of release of pathogenic or opportunistic micro-organisms during biotechnological treatment and lower risk of accumulation of harmful micro-organisms in the final biotreatment product [18].

In the literature, the degradation of phenolic compounds by bacterial strain *Pseudomonas putida* is very well documented [19–24]. However, research into the degradation potential of *p*-hydroxybenzoic acid by means of *P. putida* is scarce and its main aim is to study the microbial metabolism and the degradation pathway. To the best of our knowledge, the biodegradation kinetics and yields of *p*-hydroxybenzoic acid by means of a pure culture of *P. putida* have not been explored, even when this bacterium is the predominant species in activated sludge. This information can be significant in order to determine synergetic effects between species or the length of time required for acclimation and as a necessary previous step for investigating the potential benefits that may be obtained by the addition of a carefully selected pure culture to a mixed culture, for the degradation of xenobiotics. Mixed cultures which previously may not have been capable of successful degradation may be following bioaugmentation with a strain containing catabolic capabilities [25–27].

Taking into account these considerations, the aim of this study was to investigate the possibility of biodegradation of *p*-hydroxybenzoic acid by *P. putida* (DSM4478), paying special attention to those conditions that improve the yield and the rate of the biodegradation process.

2. Materials and methods

2.1. Micro-organisms and culture medium

Experiments were carried out with a culture of *P. putida* DSM4478 (German Resource Center for Biological Material), which contains the SAL plasmid that encodes genes for the degradation of salicylate. Stock cultures were maintained by periodic subculture on nutrient agar slants, supplemented with salicylic acid, which were stored at 4°C.

The nutrient medium contained (g/L): meat extract (3), peptone (5), and mineral salt medium (MSM) whose composition was KH_2PO_4 (0.422), K_2HPO_4 (0.375), $(NH_4)_2SO_4$ (0.244), $MgSO_4{\cdot}7H_2O$ (0.05), NH_3·Fe·Citrate (0.054), NaCl (0.015), $CaCl_2{\cdot}12H_2O$ (0.015), and tryptone (0.05), at pH 7.

Before the experiments, the nutrient medium, MSM, and materials were autoclaved at 121°C for 20 min.

After sterilization, the single substrate solution of *p*-hydroxybenzoic acid (Sigma-Aldrich) was added to MSM at the desired concentration.

2.2. Experimental procedure

2.2.1. Biodegradation experiments

P. putida (DSM4478) was activated in nutrient medium at 30° up to late exponential phase. Cells were harvested in inoculums by centrifugation for 10 min at 8,500 rpm. After centrifugation, cells were inoculated to an OD of 0.05 (corresponding to 1.5×10^7 cfu/mL) in 100 mL of MSM (250 Erlenmeyer flask) supplemented with *p*-hydroxybenzoic acid for enzyme adaptation. The different strategies of acclimation are described in detail during the first section of *Results*.

2.2.2. Analysis of cells and substrates

For measuring cell density, samples were centrifuged at 13,500 rpm for 5 min. The supernatant was used for substrate determination. Cell density was monitored spectrophotometrically by measuring the absorbance at a wavelength of 600 nm with a UV–vis spectrophotometer.

Phenol was determined by standard colorimetric assays at a wavelength of 510 nm [28]. *p*-Hydroxybenzoic acid was determined by HPLC on a C18 column. A mixture of methanol/water (60:40) was used as the mobile phase. Two-hundred and fifty microliters of trifluoroacetic acid was added to adjust the pH to 2.5. Flow rate was 1 mL/min. The sample was injected and analyzed using a UV detector (Waters 2690). The wavelength was set at 254 nm.

3. Results

3.1. Selection of the strategy of acclimation

It is a widely accepted fact that adequate acclimation is a key issue in achieving the correct biodegradation of a recalcitrant compound. It has been observed that the degree of adaptation varies depending on the acclimation procedure [29,30]. The objective of this section was to determine the best strategy of acclimation of *P. putida* (DSM4478) for the degradation of *p*-hydroxybenzoic acid.

(1) Without acclimation: initially, the adaptation of *P. putida* (DSM4478) to different concentrations of *p*-hydroxybenzoic acid was studied. With this objective, the micro-organisms were precultured in the growth medium, centrifuged and transferred to the minimum salt media (MSM) with three different concentrations of *p*-hydroxybenzoic acid (100, 200, and 300 ppm) under the following operational conditions: 150 rpm, 28°C, and an initial inoculum 2.2×10^{-2} g/L. As shown in Fig. 1 for the three initial concentrations, a low growth of the bacteria was achieved, which may be mainly due to the presence of a small amount of tryptone in the composition of the MSM. This is corroborated by the fact that the soluble COD remained approximately constant during the experimentation. Consequently, the direct inoculation of the bacteria without previous adaptation to the *p*-hydroxybenzoic acid in the medium was found to be inappropriate.

(2) Addition of a cosubstrate (phenol): substrate interactions in the biodegradation processes of phenolic compounds have been investigated in some previous studies using different micro-organisms such as *Pseudomonas* species [20,31,32]. In our case, the strategy of adaptation involved the simultaneous presence of phenol (25 ppm) and *p*-hydroxybenzoic acid (100 ppm) in the MSM (Fig. 2). The presence of phenol led to higher biomass production, although it only had a slight effect on the final COD achieved at the end of the experiment. These facts suggest that the growth of the *P. putida* (DSM4478) was mainly due to the phenol, this compound being more easily assimilable by the bacterium than the *p*-hydroxybenzoic acid.

(3) Sequential acclimation: two strategies of sequential adaptation (three cycles) of the *P. putida* (DSM4478) were also investigated, using: 1/constant concentrations (100 ppm *p*-hydroxybenzoic acid at the beginning of each cycle), or 2/progressive concentrations (50, 75, and 100 ppm for the first, second, and third cycles, respectively). In both cases, each cycle involved the culture for 24 h of an MSM with the corresponding initial concentration of *p*-hydroxybenzoic acid, which was inoculated with 10 mL of the medium obtained during the previous cycle. Inoculum size for the first cycle was 2.5×10^{-2} g/L.

In both strategies, at the end of the last cycle, 10 mL of that medium were again inoculated into a new MSM medium with 100 ppm of *p*-hydroxybenzoic acid, and the evolution of the biomass and pollutant concentration was measured (Fig. 3).

The results show that the sequential acclimation at a constant concentration per cycle is clearly more effective that the employment of progressive concentrations. For example, complete depletion of the *p*-hydroxybenzoic acid was achieved in less than 20 h using the *P. putida* (DSM4478) acclimated at constant concentrations, whereas only 10% removal was observed during the same time if the acclimation used progressive concentrations. Growth curves also show

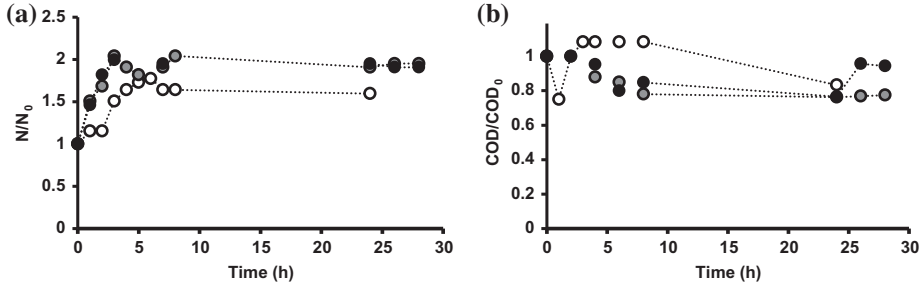

Fig. 1. Growth of *P. putida* (DSM4478) (a) and COD removal (b) in shake flasks without acclimation and under different *p*-hydroxybenzoic acid concentrations: 100 ppm (hollow symbols), 200 ppm (gray symbols), and 300 ppm (black symbols). In all cases: N_0: 2.5×10^{-2} g/L; T = 28°C; and 200 rpm.

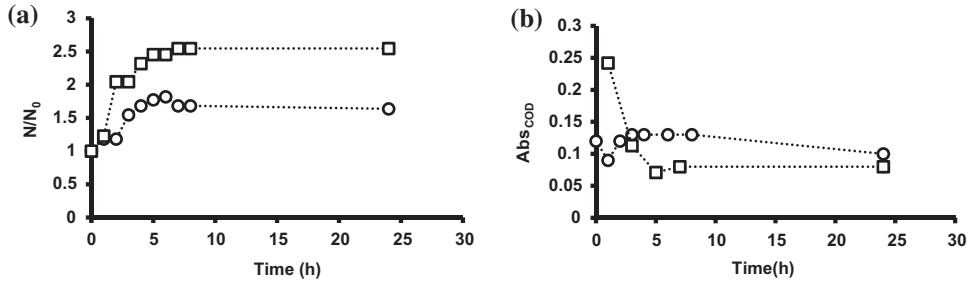

Fig. 2. Growth of *P. putida* (DSM4478) (a) and COD removal (b) in a medium with 100 ppm of *p*-hydroxybenzoic acid without (circles) or with addition (squares) of 25 ppm of phenol in all cases: N_0: 2.5×10^{-2} g/L; T = 28°C; and 200 rpm.

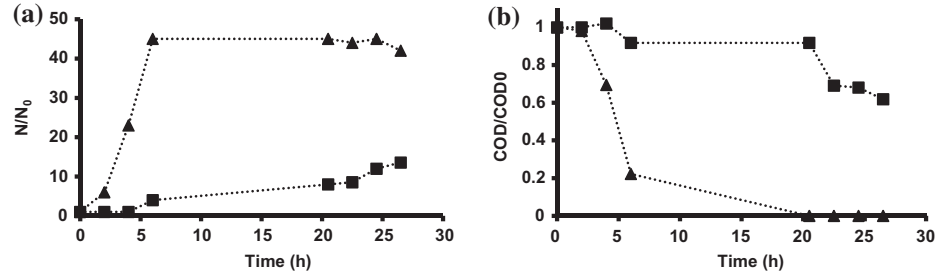

Fig. 3. Growth of *P. putida* (DSM4478) (a) and COD removal (b) in a medium with 100 ppm of *p*-hydroxybenzoic acid after a sequential acclimation (three passes) by means of constant (squares) or progressive concentrations (triangles). In all cases: N_0: 2.5×10^{-2} g/L; T = 28°C and 200 rpm.

the differences between the two strategies. A *P. putida* (DSM4478) culture sequentially acclimated at constant concentration for each cycle achieved the stationary phase in under 6 h. In this case, the specific growth rate (μ) was 0.30 h^{-1}, which was sevenfold higher that the μ value obtained by a sequential acclimation by means of progressive concentrations.

(4) One-cycle acclimation: in order to reduce the complexity and the time needed for the sequential acclimation, a new strategy based on only one cycle of 48 h and an initial concentration of 100 ppm was also evaluated. The results are shown as triangles in Fig. 4. This acclimation strategy achieved the complete depletion of the *p*-hydroxybenzoic in 8 h and a specific growth rate of 0.20 h^{-1}. If these results are compared with those obtained by sequential acclimation by progressive concentrations (6 h and 0.30 h^{-1}), it can be concluded that this one-pass acclimation is an attractive alternative, due to its simplicity and good results. As a result, this strategy was selected as the acclimation method for the biodegradation experiments described in the next sections.

3.2. Biodegradation of p-hydroxybenzoic acid

3.2.1. Effect of initial concentration

Fig. 4 shows the remaining concentration of *p*-hydroxybenzoic acid and the population density as functions of time for different initial *p*-hydroxybenzoic concentrations.

Shaken cultures exposed to 50, 100, and 200 ppm *p*-hydroxybenzoic acid were found to achieve the complete conversion of the *p*-hydroxybenzoic acid present in all cases. Times for total depletion of the pollutant were around 6 h for initial concentrations of 50 and 100 ppm, and 8 h when the initial concentration was 200 ppm. For an initial concentration of 200 ppm *p*-hydroxybenzoic acid, Chen et al. [33] also reported that *Phomopsis liquidambari* (strain B3), which is capable of growing on phenolic *p*-hydroxybenzoic acid as the sole carbon and energy source, degraded 99% of the compound within 24 h. Fairley et al. [34] confirm that *Haloarcula* sp. strain D1 was able to completely remove 550 ppm *p*-hydroxybenzoic acid after 86 h.

The *p*-hydroxybenzoic acid concentration in the medium clearly decreased when the micro-organism started to grow. The experimental growth curves showed a typical trend: after a lag phase, linear plots

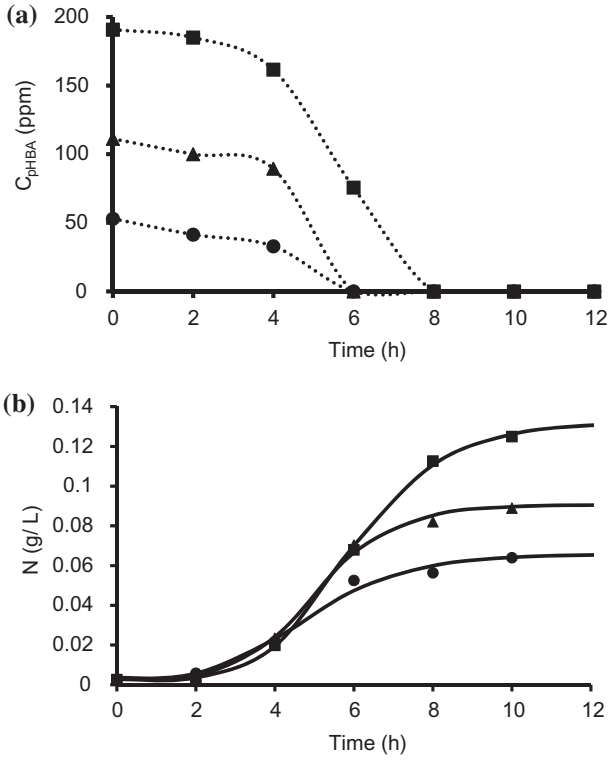

Fig. 4. Evolution of the *p*-hydroxybenzoic acid concentration (a) and growth of *P. putida* (DSM4478) (b) in shake flasks under different *p*-hydroxybenzoic acid concentrations: 50 ppm (●), 100 ppm (▲) and 200 ppm (■). In all cases: N_0: 2.5×10^{-2} g/L; T = 28°C; and 200 rpm.

were obtained at all initial concentrations, which indicated that the *p*-hydroxybenzoic acid was the limiting substrate in this part of the curve and the cultures were growing exponentially. The growth was again slow at the end of the experiment, reaching a stationary phase due to the depletion of the *p*-hydroxybenzoic acid.

3.2.2. Modeling

In order to better compare the effect of the initial concentration on each of the three phases, experimental data shown in Fig. 4 were fitted to the Gompertz model:

$$\log\left(\frac{N}{N_0}\right) = A\ \exp\left\{-\exp\left[\frac{\mu e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

Using this model, the three phases of the growth curve can be described by three parameters: the specific growth rate (μ) is defined as the tangent at the inflection point; the lag time (λ) is defined as the *x*-axis intercept of this tangent; and the asymptote ($A = \log(N_\infty/N_0)$) is the maximum value reached [35]. Table 1 resumes the values of the kinetic parameters obtained using nonlinear regression analysis in Micromath Scientist©.

According to the results, it can be concluded that the specific growth rate (μ) at 200 rpm initially increases with increasing *p*-hydroxybenzoic acid concentration and subsequently reaches a maximum value of around 0.42 h^{-1}. This behavior seems to correspond to Monod's model:

$$\mu = \frac{\mu_m S_0}{K_S + S_0} \quad (2)$$

where μ_m is the maximum specific growth rate and K_S is the substrate saturation constant. The values of the biokinetic parameters obtained from the fitting to Monod's model were $\mu_m = (0.47 \pm 0.04)\ h^{-1}$ and $K_S = (19 \pm 9)$ ppm ($r^2 = 0.997$).

3.2.3. The lag phase

Table 1 also shows that the length of the lag phase (λ) increased with the increase in the initial *p*-hydroxybenzoic acid concentration between 0 and 200 ppm, achieving a maximum value of around of 3 h for this initial concentration. The lag time increased linearly in the range 0–200 ppm, according to the following best-fit curve:

$$\lambda\ (\mathrm{h}) = (0.016 \pm 0.001) \cdot S_0\ (\mathrm{ppm})\ (r^2 = 0.98) \quad (3)$$

3.2.4. Final biomass concentration

Fig. 4 and Table 1 show a typical pattern for the final growth achieved. It is clear that higher concentrations of *p*-hydroxybenzoic acid resulted in higher biomass generation at the end of the exponential phase. A linear relationship between initial *p*-hydroxybenzoic concentration and the final biomass concentration was observed, a biomass yield ($Y_{X/S}$) of 0.66 g *P. putida*/g *p*-hydroxybenzoic acid being obtained. This value of the yield coefficient falls within the range reported in the literature for the degradation of phenol by *P. putida* [36], but slightly lower than that obtained during the biodegradation of toluene or benzene [37].

3.3. Effect of dissolved oxygen concentration

3.3.1. Dependence on agitation rate

Batch experiments were conducted to examine the effects of dissolved oxygen concentration on the growth of *P. putida* (Fig. 5).

Table 1
Kinetic parameters for Gompertz model obtained during the biodegradation of *p*-hydroxybenzoic acid by means of *P. putida* (DSM4478)

Agitation rate (rpm)	S_0 (ppm)	μ (h^{-1})	λ (h)	A	r^2	$Y_{X/S}$ (g/g)
200	50	0.33 ± 0.03	1.0 ± 0.2	1.37 ± 0.05	0.9992	1.192
	100	0.42 ± 0.02	2.0 ± 0.1	1.40 ± 0.03	0.9997	0.783
	200	0.41 ± 0.02	3.02 ± 0.06	1.70 ± 0.02	0.99994	0.679
100	50	0.20 ± 0.03	0.6 ± 0.2	0.69 ± 0.02	0.996	0.213
	100	0.21 ± 0.02	0.6 ± 0.2	0.92 ± 0.02	0.997	0.133
	200	0.167 ± 0.007	1.3 ± 0.1	0.842 ± 0.008	0.9997	0.083
50	50	0.16 ± 0.06	0.7 ± 0.2	0.69 ± 0.08	0.990	0.197
	100	0.16 ± 0.03	0.7 ± 0.4	0.64 ± 0.04	0.992	0.151
	200	0.153 ± 0.006	1.2 ± 0.1	0.617 ± 0.004	0.99998	0.139

It looks evident from the figures that the agitation speed, because of its effect on the dissolved oxygen concentration, plays a key role in the growth kinetics of *P. putida* (DSM4478). Regardless of the initial concentration selected, an increase in the agitation rate improved the growth rate of the *P. putida* (DSM4478). However, this improvement in the biomass production was slight for low agitation rates (50 and 100 rpm), being more significant for higher agitation rates (200 rpm). Thus, when the initial concentration was 100 ppm, the final biomass concentrations after 8 h at 50 and 100 rpm were 9.0×10^{-3} and 1.4×10^{-2} g/L, respectively; whereas the biomass concentration obtained on selecting an agitation rate of 200 ppm was more than five times higher than that obtained in the cultures agitated at 100 rpm.

3.3.2. Mechanisms

Previous results can be explained as a response to a change in the limiting substrate: dissolved oxygen or *p*-hydroxybenzoic acid. Therefore, experiments with agitation rates of 50 and 100 rpm were probably being conducted under oxygen-limiting conditions, with the mass transfer controlling the overall bioprocess rate. The k_La increased with the agitation rate increase, which caused a higher availability of oxygen in the media, thus speeding up the bioprocess rate. This can also explain why the initial *p*-hydroxybenzoic acid concentration has no significant effect on the growth curves at 50 or 100 rpm (see hollow or gray symbols, respectively, in Fig. 5). When the agitation rate provided an oxygen transfer rate that was higher than its uptake rate, *p*-hydroxybenzoic acid became the new limiting substrate. For this reason, an increase in the *p*-hydroxybenzoic acid concentration led to an increase in *P. putida* (DSM4478) growth when the agitation rate was 200 rpm (see Fig. 5).

3.3.3. Modeling

Again, the experimental data obtained under the different agitation rates were successfully fitted to the Gompertz model. The fitting parameters are summarized in Table 1. The effect of the agitation rate on the three Gompertz model parameters was low for 50 and 100 rpm, but it was more significant when the agitation rate was augmented to 200 rpm.

When the agitation rate was changed from 50 to 100 rpm, the specific growth rate (μ) showed a small increase, due to a higher dissolved oxygen concentration in the media, similar values being obtained for both rates, irrespective of the initial concentration. This corroborates the oxygen limiting conditions for agitation rates lower than 100 rpm. On the contrary, the high value of μ obtained at 200 rpm, which also depends on the initial concentration, is in agreement with a change in the kinetic control of the overall process and with *p*-hydroxybenzoic acid being the new limiting substrate.

However, the fastest acclimation of *P. putida* (DSM4478) was observed for an agitation rate of 100 rpm, whereas the highest values for the lengths of the lag phase (λ) were observed at 200 rpm. As was foreseeable, for all the agitation rates assayed, an increase in the *p*-hydroxybenzoic concentration causes a rise in the lag phase. It can be seen that the acclimation of *P. putida* (DSM4478) is favored by low concentrations of *p*-hydroxybenzoic acid and moderate concentrations of dissolved oxygen in the medium.

With regards to the maximum growth achieved, it can deduced from Table 1 that an increase in the

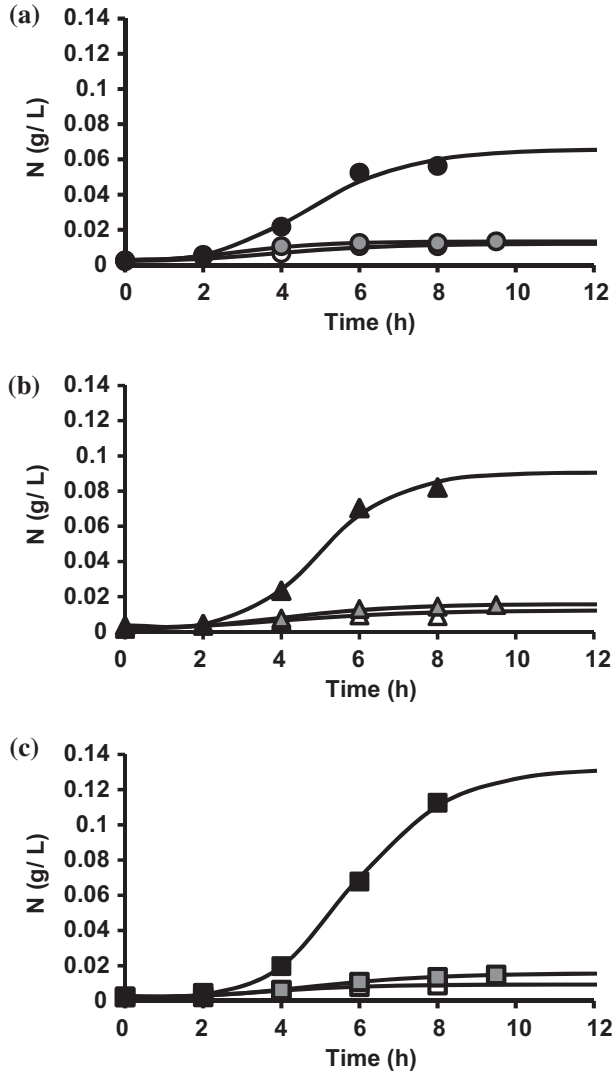

Fig. 5. Growth of *P. putida* (DSM4478) in shake flasks under different agitation speeds: 50 rpm (hollow symbols), 100 rpm (gray symbols) and 200 rpm (black symbols) for the next initial concentrations of *p*-hydroxybenzoic acid: (a) 50 ppm (circles), (b) 100 ppm (triangles), and (c) 200 ppm (squares). In all cases: N_0: 2.5×10^{-3} g/L; $T = 28$ °C.

agitation rate from 50 to 100 rpm produced a slight increase in the A value, the maximum growth being duplicated when the speed was raised to 200 rpm. It should also be noted that no effect of the initial concentration of *p*-hydroxybenzoic acid on the *A* value was observed at low agitation rates, while on the contrary, the biomass production increased with the initial concentration for a agitation rate of 200 rpm. It seems reasonable that the selection of *p*-hydroxybenzoic acid limiting conditions instead of oxygen limiting conditions allows higher biomass production, especially when a high *p*-hydroxybenzoic concentration is also chosen.

Finally, biomass yields of *P. putida* (DSM4478) during the assimilation of *p*-hydroxybenzoic acid under the different conditions were also calculated according to the following equation:

$$Y_{X/S} = \frac{N_{max} - N_0}{(C_{pHBA})_{initial} - (C_{pHBA})_{final}} \text{ being } N_{max} = N_0 \times 10^A \quad (4)$$

The agitation rate selected had a clear effect on the biomass yields (Table 1). An increase in the agitation rate, that is, in the dissolved oxygen availability, improves the ratio of the amount of biomass produced to the amount of substrate consumed, particularly when the agitation rate was changed from 100 to 200 rpm. In fact, biomass yields obtained at 200 rpm were approximately six times higher than those obtained when the agitation rate was reduced by half. These results could be due to the fact that under oxygen limiting conditions the *P. putida* (DSM4478) reduced its anabolic routes, using most of the *p*-hydroxybenzoic acid for the generation of energy. However, when the availability of dissolved oxygen is ensured by a sufficiently high agitation rate, the energy requirements of the bacterium are covered and it is able to produce new cells, thus increasing the biomass yield. As can be seen from Table 1, low concentrations of *p*-hydroxybenzoic acid also improved the biomass yield, particularly when the concentration was reduced from 100 to 50 ppm.

3.4. Kinetics of p-hydroxybenzoic Acid Bioremoval

3.4.1. Modeling

In order to deal with the kinetics of *p*-hydroxybenzoic acid biodegradation by means of *P. putida* (DSM4478), two biodegradation rates based on the Gompertz parameters can been defined as:

$$-r_{pHBA} \approx \frac{(C_{pHBA})_{initial} - (C_{pHBA})_{final}}{\left(\frac{A}{\mu}\right)} \quad (5)$$

$$-r'_{pHBA} \approx \frac{(C_{pHBA})_{initial} - (C_{pHBA})_{final}}{\left(\lambda + \frac{A}{\mu}\right)} \quad (6)$$

where $(C_{pHBA})_{initial}$ and $(C_{pHBA})_{final}$ are, respectively, the *p*-hydroxybenzoic acid concentrations at the beginning of the experiment and when the stationary phase

is achieved. λ is the duration of the lag phase and (A/μ) is the duration of the log phase. Thus, for the calculation of $(-r_{pHBA})$, only the time required for the log phase is considered, whereas the length of the lag phase was also included in the $(-r'_{pHBA})$ definition.

Fig. 6 shows the values obtained for each condition. Both agitation rate and initial concentration had a positive effect on the removal rate when length of lag phase is not considered. In this case, removal rates of around 48 mg/L h can be achieved by *P. putida* (DSM4478) under the optimal conditions: 200 ppm and 200 rpm. It is worth noting that this value falls within the expected range of bioremoval rates (5–50 mg/L h) of phenolic compounds by different micro-organisms based on the bibliographic information [38]. However, when the length of the lag phase is taken into account, the removal rates $(-r'_{pHBA})$ showed a considerable decrease, particularly for high concentrations. Although high initial concentrations of *p*-hydroxybenzoic acid gave rise to an acceleration of the rate during log phase, they also involved the need of a longer lag phase in order to acclimate the bacteria, thus exerting an overall negative effect on the global removal rate. Similar behavior was observed when the agitation rate was changed from 100 to 200 rpm. Both factors explain why similar global removal rates $(-r'_{pHBA})$ were observed at 100 and 200 rpm (Fig. 6(b)).

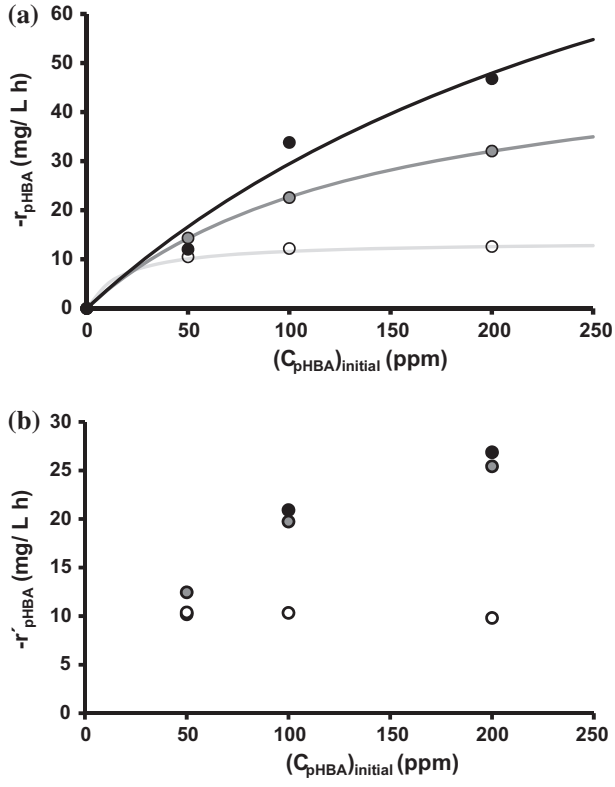

Fig. 6. Effect of the initial concentration of *p*-hydroxybenzoic acid on its biodegradation rate by means of *P. putida* (DSM4478) not considering (a) or considering (b) the length of the lag phase under different agitation speeds: 50 rpm (hollow symbols), 100 rpm (gray symbols), and 200 rpm (black symbols). In all cases: N_0: 2.5×10^{-3} g/L; $T = 28$ °C.

From Fig. 6(a), it can also be seen that bioremoval rates seem to tend towards a maximum, constant value when the initial concentration is increased. A possible explanation for this behavior is the approximation of the system to oxygen-limiting conditions as the initial concentration increases. Thus, the bioremoval rate under these conditions would be controlled only by the oxygen transfer rate, which depends on the agitation rate. These maximum biodegradation rates were determined by the fitting of the experimental data to the following equation:

$$(-r'_{pHBA}) = \frac{(-r'_{pHBA})_{max} \cdot (C_{pHBA})_{initial}}{K + (C_{pHBA})_{initial}} \quad (7)$$

Table 2 summarizes the values of $(-r'_{pHBA})_{max}$ and K obtained using nonlinear regression analysis in Micromath Scientist©. As was expected, the theoretical maximum biodegradation rate $(-r'_{pHBA})_{max}$ increased with the agitation rate. Additionally, a linear relationship between the agitation rate and $(-r'_{pHBA})_{max}$ was also observed within the agitation rate and initial concentration ranges of 0–200 rpm and 0–200 ppm, respectively:

$$(-r'_{pHBA})_{max} = 0.76 \cdot n(\text{rpm}) - 23.14 \quad r^2 = 0.9993 \quad (8)$$

3.5. Evaluation of the metabolic activity

The metabolism of bacteria has an energy-generating component, catabolism, and an energy-consuming, biosynthetic component, anabolism [38]. In this section, the most favorable conditions of agitation rate and initial concentration of *p*-hydroxybenzoic acid for each of the two components were studied. In this way, the best conditions for maximum growth of *P. putida* (DSM4478) and for the maximum assimilation rate of *p*-hydroxybenzoic acid per bacterium were evaluated.

3.5.1. Best growing conditions

In order to determine the best conditions for culture of *P. putida* (DSM4478), the parameter ω was defined, using the Gompertz model parameters obtained after the fitting of the experimental data as:

Table 2
Kinetic parameters obtained during the biodegradation of *p*-hydroxybenzoic acid by means of *P. putida* (DSM4478)

Agitation rate (rpm)	$(-r'_{pHBA})_{max}$ (mg/L h)	K (mg/L)	r^2
50	13.69 ± 0.15	18.22 ± 0.98	0.9997
100	54.70 ± 0.43	141.24 ± 2.14	0.99998
200	128.37 ± 20	335.724 ± 80	0.98

$$\omega = \frac{A}{\lambda + A/\mu} \tag{9}$$

As can be deduced from the previous equation, high values of ω can be due to a large increase in the final amount of biomass (A), a low time for the acclimation of the *P. putida* (DSM4478) to the media conditions (λ), and/or rapid multiplication of the bacteria (μ). In this manner, high ω values are associated with better growing conditions. Fig. 7(a) shows the values of ω obtained for the different conditions of agitation and initial concentration.

According to the figure, it seems reasonable to propose that the growth of *P. putida* (DSM4478) is favored by media with high availability of dissolved oxygen and low concentrations of *p*-hydroxybenzoic acid present. Thus, the maximum ω value, which was obtained at 200 rpm and 50 ppm of *p*-hydroxybenzoic acid, was two times higher than that observed under the worst conditions (50 rpm and 200 ppm). It is also noticeable that the effect of the agitation rate on growth is more marked than the effect of the initial concentration. In fact, it can be assumed that ω values obtained for biodegradation experiments with 100 or 200 ppm can be considered equal when the agitation rate is the same.

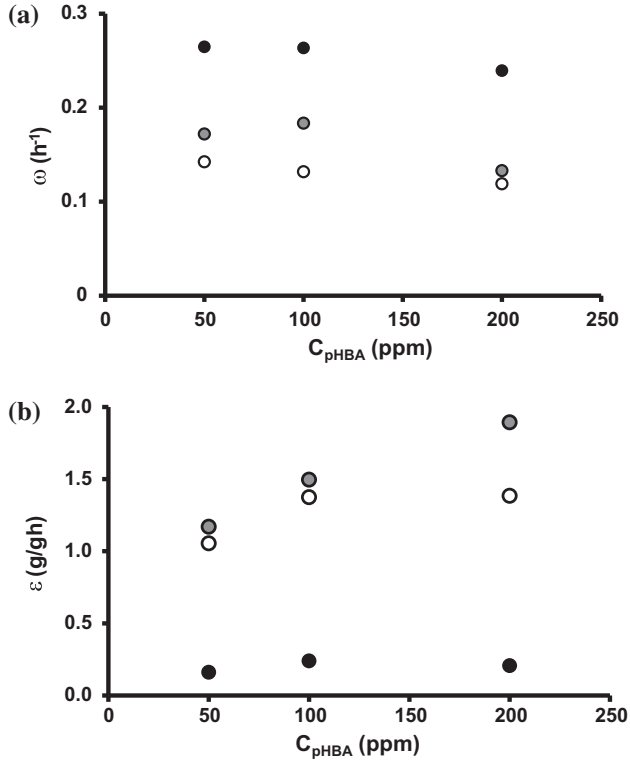

Fig. 7. Effect of the initial concentration of *p*-hydroxybenzoic acid on the ω and ε values under different agitation speeds: 50 rpm (hollow symbols), 100 rpm (gray symbols), and 200 rpm (black symbols). In all cases: N_0: 2.5×10^{-3} g/L; T = 28°C.

3.5.2. Best metabolization conditions

With the aim of trying to quantify the degree of metabolic assimilation of the *p*-hydroxybenzoic acid by *P. putida* (DSM4478) under the different conditions employed in this work, a parameter ε is introduced, according to the next equation, based on the Gompertz model parameters:

$$\varepsilon = \frac{(C_{pHBA})_{initial} - (C_{pHBA})_{final}}{(N_{max} - N_0) \cdot (\lambda + A/\mu)} = \frac{1}{Y_{X/S}(\lambda + A/\mu)} \tag{10}$$

This new parameter relates the amount of *p*-hydroxybenzoic acid removed ($(C_{pHBA})_{initial} - (C_{pHBA})_{final}$), the biomass production ($N_{max} - N_0$) and the time required for the degradation ($\lambda + A/\mu$). Thus, this ε parameter can be considered as a measurement of the assimilation rate of substrate per bacterium. Conditions for maximum ε value indicate when the *P. putida* (DSM4478) is metabolizing the *p*-hydroxybenzoic acid faster, with low lag times. Under these conditions, the *p*-hydroxybenzoic acid assimilated by *P. putida* (DSM4478) is mainly employed for energy generation and not in anabolic routes, that is, in the growth of new bacteria.

From an industrial point of view, a low ε value would indicate that the conditions selected for the biotreatment are unsuitable, due to a high generation of sludge ($Y_{X/S}$) and/or an excessive period of

acclimation (λ). Fig. 7(b) shows the ε values obtained under the different conditions used. The best results were observed with an agitation rate of 100 rpm, at which the bacteria showed the highest level of metabolic activity. On the other hand, an agitation rate of 200 rpm significantly reduced the capacity of the bacteria to metabolize the *p*-hydroxybenzoic acid. For example, for 100 ppm, ε values at 200 rpm were approximately 0.20 g/gh, whereas this value was 1.50 g/gh when the agitation rate was reduced by half. In the case of an agitation rate of 50 rpm, the reduction in ε values with respect to those obtained at 100 rpm was small; being more marked when the initial concentration of *p*-hydroxybenzoic acid was 200 rpm.

Regarding the effect of the initial concentration on the ε value, it becomes more important as the agitation rate approaches 100 rpm. In fact, for 200 rpm, the value of ε can be considered constant, independently of the initial concentration of *p*-hydroxybenzoic selected.

The maximum ε value corresponded to an agitation rate of 100 rpm and an initial concentration of 200 ppm (Fig. 7(b)). Selecting these conditions, the capacity of *P. putida* (DSM4478) for metabolizing the *p*-hydroxybenzoic acid reaches a maximum. However, it must be taken into account that a maximum value of ε does not imply a maximum for the global biodegradation rate of the *p*-hydroxybenzoic acid, because the ε value is defined per unit of dried cell weight, whereas the biodegradation rate depends on the biomass concentration:

$$-r'_{\mathrm{pHBA}} = \frac{(C_{\mathrm{pHBA}})_{\mathrm{initial}} - (C_{\mathrm{pHBA}})_{\mathrm{final}}}{(\lambda + A/\mu)} = \varepsilon \cdot (N_{\max} - N_0) \tag{11}$$

4. Conclusions

Biodegradation of *p*-hydroxybenzoic acid by a pure of *P. putida* (DSM4478) culture was investigated using batch shake flask experiments. The initial *p*-hydroxybenzoic acid concentration and the agitation rate were varied between 50 and 200 ppm and 50 and 200 rpm, respectively. Different strategies of activation were investigated, finally selecting a one-cycle acclimation of the bacteria during 48 h under 28°C and 100 ppm of *p*-hydroxybenzoic acid. Acclimated cultures had the ability to completely degrade *p*-hydroxybenzoic acid at concentrations of up to 200 ppm within 10 h. Under *p*-hydroxybenzoic acid limiting conditions, the Monod equation adequately described cell growth with kinetic constants $\mu_m = 0.47\ h^{-1}$, $K_S = 19$ ppm. The acclimation of the *P. putida* is favored by low concentrations of *p*-hydroxybenzoic acid and dissolved oxygen. An increase in the agitation rate also improves the ratio of the amount of biomass produced to the amount of substrate consumed.

Removal rates of around 48 mg/L h can be achieved by *P. putida* under the optimal conditions: 200 ppm and 200 rpm. The best growing conditions have been obtained at low *p*-hydroxybenzoic concentrations and a high agitation rate, whereas the highest specific rates for assimilation of *p*-hydroxybenzoic acid corresponded to an agitation rate of 100 rpm and an initial concentration of 200 ppm.

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