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Biodegradation kinetics of 1,4-benzoquinone in batch and continuous systems

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Abstract Combining chemical and biological treatments is a potentially economic approach to remove high concentration of recalcitrant compounds from wastewaters. In the present study, the biodegradation of 1,4-benzoquinone, an intermediate compound formed during phenol oxidation by chlorine dioxide, was investigated using *Pseudomonas putida* (ATCC 17484) in batch and continuous bioreactors. Batch experiments were conducted to determine the effects of 1,4-benzoquinone concentration and temperature on the microbial activity and biodegradation kinetics. Using the generated data, the maximum specific growth rate and biodegradation rate were determined as 0.94 h^{-1} and 6.71 mg of 1,4-benzoquinone l^{-1} h^{-1}. Biodegradation in a continuous bioreactor indicated a linear relationship between substrate loading and biodegradation rates prior to wash out of the cells, with a maximum biodegradation rate of 246 mg l^{-1} h^{-1} observed at a loading rate of 275 mg l^{-1} h^{-1} (residence time: 1.82 h). Biokinetic parameters were also determined using the steady state substrate and biomass concentrations at various dilution rates and compared to those obtained in batch cultures.

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Introduction

Phenols and phenol like compounds are widely occurring compounds in wastewaters produced from all the major chemical industries (Benitez et al. 1997; Beltra et al. 2005, and Prpich and Daugulis 2005). Biological treatments alone are not effective for the removal of phenolic compounds because of their bactericidal nature at high concentrations (Cao et al. 2009). Therefore, advanced oxidation treatments have been applied for the removal of high concentrations of phenolic and other toxic compounds from various industrial wastewaters (Herrmann et al. 1993; Kowalska et al. 2004; Pignatello et al. 2006). The high amounts of chemical oxidants required to completely mineralize the organic contaminants make the chemical treatment processes uneconomical (Zazo et al. 2005). Integrating chemical and biological treatments is an attractive alternative to minimize treatment costs for the removal of high concentrations of toxic compounds from wastewater (Edalatmanesh et al. 2008).

We have reported the steady state oxidation of phenol in a bioremediation medium using chlorine dioxide in our earlier work and have determined the optimum concentration of ClO_2, temperature and pH using the CCRD (central composite rotatable design)

approach (Kumar et al. 2010b). Based on the result of that study, 2,000 mg l^{-1} of phenol were completely oxidized by 2,000 mg l^{-1} chlorine dioxide. This quantity of chlorine dioxide was half of Fenton's reagent (4,000 mg l^{-1} H_2O_2 and 1,500 mg l^{-1} of $FeSO_4{\cdot}7H_2O$) that was needed to accomplish the same task (Kumar et al. 2010a). With chlorine dioxide, the main oxidation products were identified as 1,4-benzoquinone and 2-chloro-1,4-benzoquinone. Figure 1 shows the reaction mechanism of phenol oxidation by chlorine dioxide. It was also observed that these two intermediates persist even when much higher concentrations of chlorine dioxide were applied (Kumar et al. 2010b).

The inability of chlorine dioxide to oxidize 1,4-benzoquinone and 2-chloro-1,4-benzoquinone, and the result of a preliminary experiment which confirmed biodegradability of 1,4-benzoquinone, inferred that an integrated chemical and biological treatment may be a suitable alternative to exclusive chemical oxidation, and highlighted the necessity to study the biodegradation of these intermediate compounds. The present work focuses on biodegradation of 1,4-benzoquinone, one of the major intermediate compounds of phenol oxidation by chlorine dioxide, using *P. putida* (ATCC 17484).

1,4-benzoquinone is one of the most toxic xenobiotics and is generated during the oxidation of benzene and a wide variety of its derivatives (Li et al. 2007). A few studies have been reported on photochemical degradation (Shevchuk and Kirsho 1981) and electrochemical detoxification (Pulgarin et al. 1994) of 1,4-benzoquinone. Pulgarin et al. (1994) studied the electrochemical detoxification of 1,4-benzoquinone using Ti/IrO_2 and Ti/SnO_2 electrodes and found that the nature of the electrode is the most important parameter. No prior study has been published on the biodegradation kinetics of 1,4-benzoquinone. Spain and Gibson (1991) studied the biodegradation pathway of *p*-nitrophenol using *Moraxella* sp. and found that reaction proceeds via 1,4-benzoquinone formation which is further reduced to hydroquinone. Hydroquinone was finally converted to β-ketoadipate acid. The objective of this study was to investigate the biodegradation kinetics of 1,4-benzoquinone in both batch and continuous stirred tank bioreactors, with the focus being on the effects of 1,4-benzoquinone concentration and loading rate, as well as temperature.

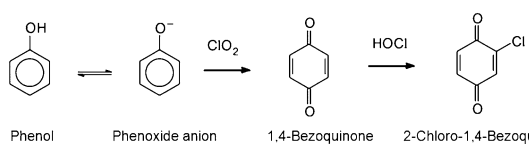

Fig. 1 Reaction mechanism of phenol oxidation by chlorine dioxide based on the identified oxidation products (Kumar et al. 2010b)

Materials and methods

Culture and medium

Pseudomonas putida (ATCC 17484) was used in biodegradation experiments in both batch and CSTR systems. *P. putida* was initially grown in 500 ml Erlenmeyer (shake) flasks containing 200 ml sterilized McKinney's medium with 200 mg l^{-1} of phenol. When the optical density (OD) reached the maximum value (0.42), 20 ml (10% v/v) of the culture was transferred in a second shake flask containing McKinney's medium and a mixture of 100 mg l^{-1} of phenol and 50 mg l^{-1} of 1,4-benzoquinone. Following the complete removal of phenol and 1,4-benzoquinone, 20 ml (10% v/v) of this second culture was used to inoculate double concentrated McKinney's medium containing only 100 mg l^{-1} 1,4-benzoquinone. This procedure ensured *P. putida* was acclimatised to utilize 1,4-benzoquinone. Reverse osmosis water was used for preparing all solutions. The growth medium (double concentrated McKinney's medium) used in all experiments consisted of (mg in 1 l reverse osmosis water): K_2HPO_4, 750; KH_2PO_4, 849; $(NH_4)_2SO_4$, 474; NaCl, 60; $CaCl_2$, 60; $MgSO_4$, 60; $Fe(NH_4)SO_4$, 20; and 1 ml of trace mineral solution. The trace mineral solution consisted of (mg in 1 l reverse osmosis water): $ZnSO_4{\cdot}7H_2O$, 200; $MnCl_2$, 60; H_3BO_3, 600; $CoCl_2$, 400; $CuCl_2$, 20; $NiCl_2$, 40; Na_2MoO_4, 60. Subculturing was carried out every 2 days using the acclimatised culture (10% v/v inoculum) and double concentrated McKinney's medium containing 100 mg l^{-1} 1,4-benzoquinone. The cultures were maintained at room temperature (23°C).

Experimental procedures

Batch experiments were conducted to study the kinetics of microbial growth and biodegradation of

1,4-benzoquinone at a pH of 7. The effects of initial concentration of 1,4-benzoquinone and temperature were investigated using 500 ml Erlenmeyer (shake) flasks as batch reactors. Each flask was loaded with 200 ml sterile double concentrated McKinney's medium containing either 25, 50, 100, 150 or 200 mg l^{-1} 1,4-benzoquinone. These were then inoculated with freshly grown *P. putida* (10% v/v) on 100 mg l^{-1} 1,4-benzoquinone. Samples were taken at regular intervals using stainless steel needles and hypodermic syringes. Samples used for 1,4-benzoquinone concentration measurement were filtered through 0.22 μm nylon microfilters to remove biomass prior to analysis by HPLC. For the measurement of biomass concentration, a 2 ml sample was taken for cell counting. All shake flasks were completely covered with aluminum foil and placed on the gyratory shaker (New Brunswick Scientific Co., Inc, Edison, NJ, USA) operated at 100 rpm. The effect of temperature was determined by conducting biodegradation of 1,4-benzoquinone experiments at three different temperatures (10, 15 and 25°C). All the biodegradation experiments were performed in the temperature controlled chambers to maintain the desired temperatures. Control experiments were conducted under similar conditions without inoculation.

A BIOFLOW III reactor (New Brunswick Scientific, Inc., Edison, NJ, USA) was used as the continuous flow reactor (CSTR). The CSTR was vigorously stirred with a mechanical agitator equipped with Rushton turbine impeller. Filtered air, required for bacterial activity, was supplied through a ring sparger. The working volume of the reactor was 750 ml. Initially the CSTR was operated batchwise for 2 days using 100 mg l^{-1} of 1,4-benzoquinone dissolved in double concentrated McKinney's medium. The reactor was inoculated with 10% (v/v) freshly grown *P. putida* culture. The bioreactor was switched to continuous mode when 1,4-benzoquinone was removed completely. The feed consisted of double concentrated McKinney's medium with 500 mg l^{-1} of 1,4-benzoquinone that was pumped continuously into the bioreactor, at an initial flow rate of 45 ml h^{-1}, using a peristaltic pump. The bioreactor was covered with aluminum foil to prevent exposure to light. The bioreactor temperature was maintained at 15°C by circulating cold water through the jacket. This temperature was selected based on batch experiments. In the batch experiments, it was found that 1,4-benzoquinone starts to spontaneously degrade at 25°C and slows down the biodegradation of 1,4-benzoquinone. The flow rate of the feed was increased stepwise. At each flow rate sufficient time was given for establishment of steady state which was verified by a relatively constant residual substrate concentration. Once steady state was confirmed, a sample was taken from the bioreactor for measuring cell number by the plate counting technique. The flow rate of the feed was increased until cell wash out occurred. The applied dilution rates were in the range 0.06–0.75 h^{-1}.

Analytical methods

Optical density has been used as an indication of biomass concentration when *P. putida* is grown on phenol (Hill and Robinson 1975). However, with 1,4-benzoquinone, progress of biodegradation results in a change of colour (yellowish to brown) and thus optical density could not be used. So biomass concentration was measured using the plate counting technique. Agar plates were prepared by pouring the agar mixture, 3 g Difco Bacto agar and 3 g tryptose phosphate in 100 ml sterilized RO water, on Petri dishes. Following the serial dilutions, 100 μl of diluted sample was used to inoculate the agar plates. The number of developed colonies were then determined and converted to cell concentration.

The substrate concentration (1,4-benzoquinone) was determined using an HPLC (Agilent HPLC 1100, CA, USA) equipped with a C_{18} column (Nova pack: 4.6 × 150 mm: 4 μm) and a diode array detector. The mobile phase was a mixture of acetonitrile and water (10/90 v/v) with a flow rate of 2.1 ml min^{-1}. Detection was carried out using the diode array detector at a wavelength of 254 nm. The retention time for 1,4-benzoquinone was 2.1 min.

Results and discussion

Effect of initial concentration of 1,4-benzoquinone

Figure 2 (a–d) shows the results of microbial growth and substrate removal as a function of time in the batch reactors containing different initial concentrations of 1,4-benzoquinone at 15°C. No investigation of intermediate metabolites was made in this study.

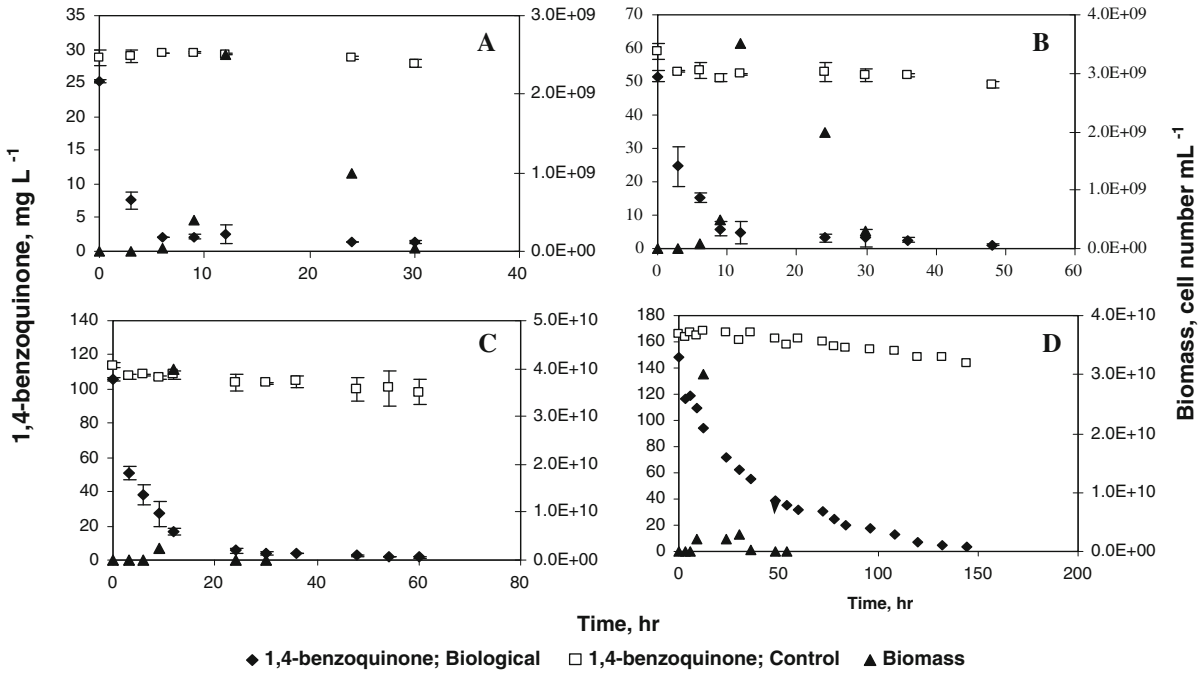

Fig. 2 Substrate and biomass concentrations as a function of time in batch reactors at various initial substrate concentrations at a fixed temperature of 15°C. **A** initial substrate concentration of 25 mg l^{-1}; **B** 50 mg l^{-1}; **C** 100 mg l^{-1}; **D** 150 mg l^{-1}. *Error bars* represent one standard deviation

However, examination of the samples taken at the end of each batch run by HPLC indicated complete removal of 1,4-benzoquinone and no other peaks were observed on the HPLC chromatogram. Control experiments, using sterilized 1,4-benzoquinone solutions without inoculation, were run at each tested concentration to show that removal of 1,4-benzoquinone was due to bacterial activity and not by spontaneous degradation. In all biodegradation experiments a direct relationship between microbial growth and substrate utilization were observed and the specific growth rates and biodegradation rates were determined using the exponential growth phase data. Table 1 shows the values of specific growth and biodegradation rates observed at different initial concentrations of 1,4-benzoquinone. As shown in Table 1, both specific growth rate and biodegradation rate increase with increase in substrate concentration and reach maximum values at a 1,4-benzoquinone concentrations of 100 mg l^{-1}. With further increase in substrate concentration, specific growth rate and biodegradation rate start to decline, indicating substrate inhibition (toxicity). The maximum values of specific growth rate and biodegradation rate were 0.91 ± 0.04 h^{-1} and 6.71 ± 0.27 mg l^{-1} h^{-1} and observed at an initial concentration of 100 mg l^{-1}. A similar trend was observed with the overall yield of biomass; increasing with increase in initial substrate concentration, reaching a maximum at a substrate concentration of 50 mg l^{-1} and decreasing with further increase in initial substrate concentration. The maximum value of the overall yield was 6×10^6 cell mg^{-1} substrate, and was observed with 50 mg l^{-1} substrate.

Table 1 Summary of specific growth and biodegradation rates determined at various initial substrate concentrations at 15°C (batch reactors)

Initial substrate (mg l^{-1})	Specific growth rate (h^{-1})	Biodegradation rate (mg l^{-1} h^{-1})
25	0.77 ± 0.03[a]	2.48 ± 0.04[a]
50	0.83 ± 0.06[a]	3.74 ± 0.21[a]
100	0.91 ± 0.04[a]	6.71 ± 0.27[a]
150	0.85	1.13
200	0.39	0.91

[a] Average and standard deviation based on the duplicate experiments

Effect of temperature on biodegradation of 1,4-benzoquinone

Figure 3 shows the results of microbial growth and substrate removal at an initial concentration of 100 mg l^{-1} as a function of time at different temperatures of 10 and 25°C. The values of specific growth rate and biodegradation rate at various temperatures for 1,4-benzoquinone at initial concentration of 100 mg l^{-1} and pH 7 are shown in Table 2. The value of specific growth rate was found to increase with increase of temperature in the range 10–15°C and then decreased slightly at 25°C. Spontaneous degradation of 1,4-benzoquinone was observed at 25°C that might have generated some unidentified toxic intermediates which inhibit the growth of *P. putida*. The temperature dependence toxicity of substrate has been reported by other researchers and could be another reason for the lower specific growth rate (Bao et al. 2008; Roma et al. 1990).

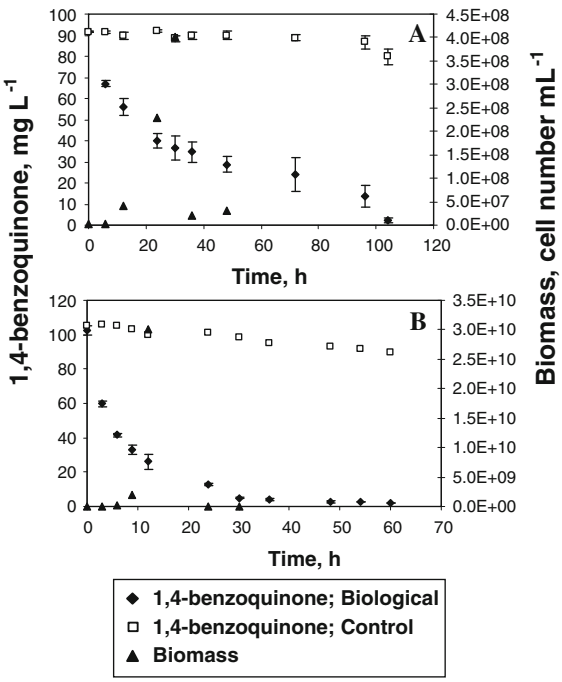

Fig. 3 Substrate and biomass concentration profiles as a function of time in a batch reactor at different temperatures for 100 mg l^{-1} of 1,4-benzoquinone concentration **a** 10°C; **b** 25°C. *Error bars* represent one standard deviation

Table 2 Summary of specific growth rate and biodegradation rate at different temperatures for 100 mg l^{-1} of substrate (batch reactors)

Temperature (°C)	Specific growth rate (h^{-1})	Biodegradation rate (mg l^{-1} h^{-1})
10	0.19	0.65
15	0.91 ± 0.04[a]	6.71 ± 0.27[a]
25	0.90	3.15

[a] Average and standard deviation based on the duplicate experiments

Biodegradation of 1,4-benzoquinone in continuous stirred tank reactor

Figure 4 shows the steady state profiles of biomass and substrate concentrations obtained at different dilution rates ranging from 0.06 to 0.75 h^{-1}. It can be seen from this figure that the biomass concentration increases with increasing dilution rate from 0.06 to 0.25 h^{-1}, while 1,4-benzoquinone was completely consumed. Further increase in dilution rate resulted in a decrease in biomass concentration and increase in residual 1,4-benzoquinone concentration. The maximum biomass concentration (1×10^{11} cell numbers ml^{-1}) was found at a dilution rate of 0.25 h^{-1} at which complete removal of substrate was also achieved. The residual 1,4-benzoquinone concentrations approached to the feed concentration at a dilution rate of 0.75 h^{-1} and wash out in form of significant decrease in biomass concentration was observed.

To determine the kinetic parameters, Monod model was used and the maximum specific growth rate and saturation constant were determined by fitting the steady state experimental data to Eq. 1 (Shuler and Kargi 2002):

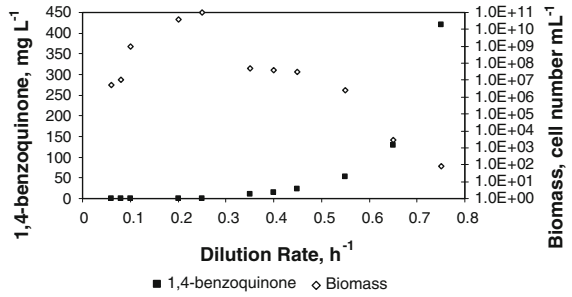

Fig. 4 Steady state profiles of biomass and substrate concentrations as a function of dilution rate for 500 mg l^{-1} 1,4-benzoquinone

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad (1)$$

The Lineweaver–Burk, Eadie–Hofstee and Hanes–Woolf plots were used to analyze the data. The values of maximum specific growth rate and saturation constant were determined by these three plots. The average values of maximum specific growth rate and saturation constant were $0.74 \pm 0.03\ h^{-1}$ and $14.2 \pm 3.2\ mg\ l^{-1}$, respectively.

The rate of biodegradation of 1,4-benzoquinone as a function of its loading rate obtained in the CSTR operated with an initial substrate concentration of $500\ mg\ l^{-1}$ is shown in Fig. 5. The loading and biodegradation rates were calculated using the concentration of 1,4-benzoquinone in the feed, steady state residual concentration of 1,4-benzoquinone and dilution rate. It could be seen from this figure that biodegradation rate increases with increase in loading rate up to $300\ mg\ l^{-1}\ h^{-1}$, after that biodegradation starts to decline with increase in loading rate. The maximum biodegradation rate obtained in the CSTR was $246\ mg\ l^{-1}\ h^{-1}$ at a loading rate of $275\ mg\ l^{-1}\ h^{-1}$ (residence time: 1.82 h). In the CSTR, biomass yield was found to vary with change in dilution rate; it first increased with increase in dilution rate (0.06–0.25 h^{-1}) and then declined with further increase in dilution rate (0.35 to 0.75 h^{-1}). The maximum biomass yield obtained was 2×10^8 cell mg^{-1} substrate. Comparing the data obtained in batch and CSTR, the value of maximum specific growth rate was found to be higher in batch reactors but at a lower substrate concentration. However, the CSTR is very efficient in terms of biodegradation rate of 1,4-benzoquinone, with the rate being 36 times higher than the biodegradation rate obtained in the batch reactor.

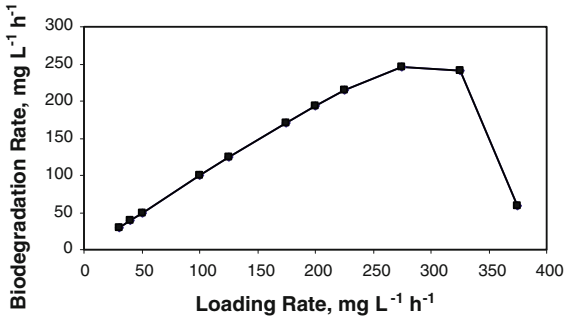

Fig. 5 Biodegradation rate as a function of loading rate in CTSR operated with an initial substrate concentration of $500\ mg\ l^{-1}$ at 15°C

As mentioned earlier, 1,4-benzoquinone is one of the main intermediate compounds of phenol oxidation by chlorine dioxide and it is not oxidized even when high concentrations of chlorine dioxide are used. So in this work, the biodegradation of 1,4-benzoquinone was successfully demonstrated in both batch and continuous stirred tank reactors. No study has been reported in the past on the biodegradation kinetics of 1,4-benzoquinone in the literature, so biokinetic parameters of 1,4-benzoquinone could not be compared with literature data. However, for comparison purposes, the biokinetic parameters of 1,4-benzoquinone was compared with phenol and are presented in Table 3. Data shown in Table 3 indicates that the value of maximum specific growth rate ($0.91 \pm 0.04\ h^{-1}$) obtained in this study is significantly higher than the maximum specific growth rate of *P. putida* on phenol. The value of saturation constant obtained is comparable to those reported in the literature but using phenol as the substrate.

Conclusions

The biodegradation of 1,4-benzoquinone, one of the intermediate of phenol oxidation by chlorine dioxide,

Table 3 Comparison of biokinetic parameters of 1,4-benzoquinone and phenol

Reference	Microbial culture	Substrate	Maximum specific growth rate (h^{-1})	Saturation constant ($mg\ l^{-1}$)	Biodegradation rate ($mg\ l^{-1}\ h^{-1}$)
Hill and Robinson (1975)	*P. putida* ATCC *17484*	Phenol	0.534	0.015	
Kumar et al. (2005)	*P. putida* MTCC *1194*	Phenol	0.216	20.59	
Nikakhtari and Hill (2006)	*P. putida* ATCC *17484*	Phenol	0.175	<1	
Present work (batch)	*P. putida* ATCC *17484*	1,4-benzoquinone	0.91 ± 0.04		6.71 ± 0.2
Present work (continuous)	*P. putida* ATCC *17484*	1,4-benzoquinone	0.74 ± 0.03	14.2 ± 3.2	$246\ mg\ l^{-1}\ h^{-1}$

was studied in batch and continuous reactors. Complete biodegradation up to 150 mg l^{-1} of 1,4-benzoquinone was achieved in batch reactors. The values of maximum specific growth rate and biodegradation rate obtained in the batch reactor were 0.91 ± 0.04 h^{-1} and 6.71 ± 0.27 mg l^{-1} h^{-1}, respectively. Over the tested temperature range (10–25°C) the optimum temperature for microbial growth and biodegradation of 1,4-benzoquinone was 15°C. In the continuous bioreactor, increase of loading rate up to a value of 275 mg l^{-1} h^{-1} (residence time: 1.82 h) caused a linear increase in biodegradation rate with a maximum value of 246 mg l^{-1} h^{-1} obtained at this loading rate. Using the experimental data generated in the CSTR, the specific growth rate and saturation constant were determined as 0.74 ± 0.03 h^{-1} and 14.2 ± 3.2 mg l^{-1}, respectively. Finally an integrated chemical and biological approach has been shown to be a successful strategy for the removal of phenol.

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