



Enhancement of cometabolic biodegradation of 4-chlorophenol induced with phenol and glucose as carbon sources by *Comamonas testosteroni*

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ABSTRACT

The biological degradation of phenol and 4-chlorophenol (4-CP) by *Comamonas testosteroni* CECT 326T has been studied. Phenol and 4-CP were treated alone as a sole carbon and energy source, but only phenol was completely degraded by *C. testosteroni*. Since the presence of cosubstrates can enhance the toxic compounds removal by pure cultures, phenol and glucose were added as growth substrates for cometabolic transformation of 4-CP. High efficiencies were obtained in all the experiments carried out in presence of both cosubstrates. In spite of the fact that the addition of glucose reduced the lag phase of 4-CP removal, lower phenol concentrations were required to obtain the same degradation efficiencies. The cometabolic transformation of 4-CP was closely related with the extent of phenol removal. The values of the 4-CP/biomass concentration ratio (S/X) obtained for discriminating between complete ($S/X \leq 0.11$) and partial 4-CP ($S/X \geq 0.31$) transformation showed a narrower range than that reported in the literature. The extent of the cometabolic 4-CP transformation in the presence of phenol could be further enhanced by using glucose as an additional carbon and energy source. However, no significant influence of glucose concentration on 4-CP removal was observed over the concentration range studied.

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1. Introduction

Nowadays environmental contamination by toxic xenobiotic compounds is a serious worldwide problem. Among these toxic pollutants, phenol and chlorophenols have gained relevance due to their presence in the environment and biota because of the widespread use in many industrial processes such as the production of resins, nylon, plastics, antioxidants, lubricant additives, wood protectors, bleached pulp, pesticides, textile, dyes, explosives, disinfectants or biocides. The yearly industrial production of chlorophenols was estimated at 200,000 tons in 1989 (WHO, 1989; Field and Sierra-Alvarez, 2007). In 1999, 56,000 tons of waste phenol and 1900 tons of waste chlorophenols were generated by industries in the United States (Tarighian et al., 2003). Wastewaters from the industrial activities are characterized by variable concentrations of phenolic compounds (500–4000 mg/L) (Dojlido and Best, 1993; Park and Keane, 2003). Concentrations of monochlorophenols have been reported to range from non-detectable to 20 mg/L (WHO 1989). Some of the characteristics of chlorophenols are their acute toxicity and poor biodegradability (Armenante et al., 1999). Therefore, the treatment of the wastewaters, containing

variable concentrations of those pollutants, has generated a great interest in the last years.

Traditionally, those effluents have been treated by physical or chemical methods. Activated carbon adsorption or air stripping simply transfer the chlorinated organics from water into another medium (Prübe et al., 2008). Oxidation processes such as wet air oxidation, Fenton, or photochemical processes show several drawbacks like relatively high temperatures and/or pressures, large amounts of reagents and complex equipment, respectively (Santos et al., 2002; Pera-Titus et al., 2004). Catalytic hydrodechlorination shows a high efficiency for the removal of chlorophenols but so far it is still in an early stage (Diaz et al., 2008). Generally, chemical processes are much more energy-intensive than biological treatments due to severe reaction conditions, more expensive for higher contaminants loadings and might yield byproducts with similar or even higher toxicity than those of the starting pollutants (Prübe et al., 2008). In some cases these processes can be coupled to a biological treatment once the toxicity of the wastewater has been reduced by some previous treatment (Felis et al., 1999).

Novel biological processes based on aerobic, anaerobic and combined anaerobic–aerobic schemes have been postulated as emerging technologies for the degradation of halogenated organic compounds since they have the potential of mineralizing toxic compounds at relatively low cost. Several studies are available on the biological treatment of chlorinated phenols in aqueous effluents.

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Despite their recalcitrant nature, different microorganisms such as yeast (Hofrichter et al., 1994; Polnisch et al., 1992), bacteria, fungi and algae (Field and Sierra-Alvarez, 2007; Kim et al., 2002; Wu et al., 2004) have been used for biological degradation of phenolic and chlorophenolic compounds. In the present study, a pure culture of *Comamonas testosteroni* has been tested for the biological degradation of 4-chlorophenol (4-CP), which was selected as target toxic pollutant. *C. testosteroni* can be isolated from soil and wastewater by using phenolic and chlorophenolic compounds as a sole carbon and energy source (Chen et al., 2003; Hollender et al., 1994).

It is known that biodegradation of industrial wastewaters can be improved by using microorganisms previously adapted to the specific toxic compounds (González et al., 2001). Cometabolic removal of toxicants is also a well-established method to enhance their biodegradation (Sahinkaya and Dilek, 2006). Phenol has been claimed as a good growth substrate in the biodegradation of chlorophenolic compounds because of its similar chemical structure and lower toxicity. Although glucose has been a widely used conventional carbon source in biotransformation studies, it has never been used in the cometabolic transformation of 4-CP by *C. testosteroni*.

Previous works have studied the removal of phenolic and chlorophenolic compounds by *C. testosteroni* at temperatures between 25 and 32 °C (Hollender et al., 1994, 1997; Kim et al., 2002; Yap et al., 1999). However, lower temperatures can be commonly found in wastewater treatment plants. Therefore, the study of the influence of temperature on the degradation of phenolic compounds by *C. testosteroni* could elucidate if this species could be considered as a promising specialist degrader bacteria for the bio-augmentation of activated sludge systems.

A major part of the research on diauxic phenomena is concerned with cell growth on binary mixtures with sugars. Diauxic growth implies the inhibition of the consumption of one growth substrate by the presence of another, which requires a larger acclimation period for the utilization of the second substrate. In contrast to diauxic growth patterns, concurrent utilization of multiple substrates in natural ecosystems and in wastewater treatment systems is commonly observed. In general, the use of mixed substrates is desirable because of the enhancement of the removal rates and degradation efficiencies. In addition, the complete or partial biodegradation of 4-CP in presence of cosubstrates would depend on the 4-CP/biomass (S/X) concentration ratio. That dependence has been studied in the literature for *Pseudomonas putida* (Saez and Rittmann, 1993) but there is a lack of information in that respect relative to *C. testosteroni*. Although an enhancement of both the rate and extent of cometabolic 4-CP transformation by other microorganisms has been reported, the 4-CP removal by *C. testosteroni* in ternary systems has not been previously studied. The presence of a conventional carbon source mitigated the toxic effects of 4-CP while phenol induced the production of the enzymes needed for its cometabolic transformation by *P. putida* (Wang and Loh, 2000).

The aim of this work is to study the enhancement of 4-CP biodegradability by *C. testosteroni* in presence of cosubstrates. In the present work glucose has been used as a conventional carbon source, either as a sole growth substrate or in combination with phenol for the 4-CP degradation by *C. testosteroni*. Total Organic Carbon (TOC) removal efficiencies obtained by using both phenol and glucose in the transformation of 4-CP are compared.

2. Materials and methods

2.1. Microorganism and growth conditions

C. testosteroni strain CECT 326T used in this study was obtained from the Spanish Type Culture Collection (Colección Española de

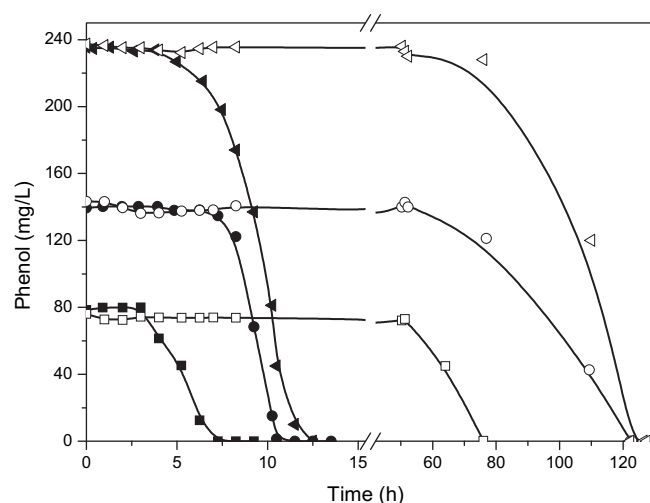


Fig. 1. Time-evolution of phenol concentration at 15 (open symbol) and 30 °C (filled symbol). Initial phenol concentration: (■, □) 80 mg/L, (●, ○) 150 mg/L and (▴, ▽) 240 mg/L.

Cultivos Tipo CECT, Valencia). The microorganism was maintained in frozen stock in microtubes at −40 °C in a nutrient medium with 15% (v/v) of glycerol. *C. testosteroni* was transferred to a nutrient medium containing 1 g beef extract, 2 g yeast extract, 5 g peptone and 5 g NaCl per liter of deionised water. The cell suspension resulting from the late exponential growth phase was subcultured in a mineral salts medium (Farrell and Quilty, 1999) with phenol (25 mg/L) as a sole carbon source and grown at 30 °C for 10–12 h in a thermostated orbital shaker (SW2L, Julabo). Agitation was maintained at an equivalent of 120 rpm. The resulting culture was inoculated at 2% (v/v) into conical flasks with a working volume of 150 ml containing mineral salts medium with either phenol or 4-CP as sole carbon sources or 4-CP/phenol, 4-CP/glucose and ternary mixtures. The aerobic batch cultures of *C. testosteroni* were carried out at 30 °C, 120 rpm and pH 7.2. Different concentrations of phenol (80–240 mg/L) and 4-CP (15 and 30 mg/L) were tested when they were treated alone. Studies of cometabolism were carried out at two 4-CP concentrations (20 and 40 mg/L) over a wide range of phenol (40–180 mg/L) and glucose (10–250 mg/L) concentrations. The results reported were the average values from duplicate runs. In all the cases, the standard errors were lower than 10%.

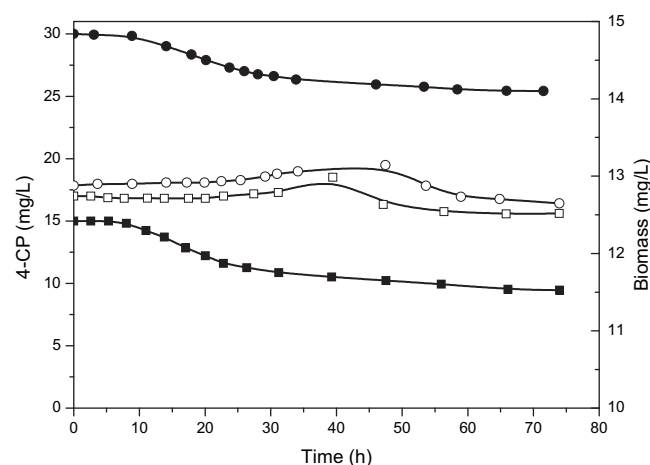


Fig. 2. Time-evolution of 4-CP (filled symbol) and biomass (open symbol) during the growth of *C. testosteroni* with 4-CP as a sole carbon source. Initial 4-CP concentration: (■, □) 15 mg/L and (●, ○) 30 mg/L.

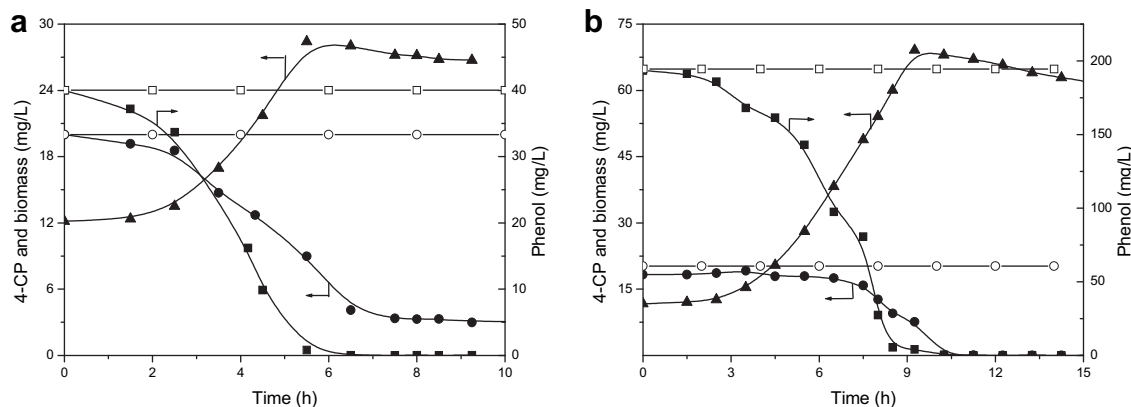


Fig. 3. Time-evolution of phenol (■), 4-CP (●) and biomass (▲) concentration working with mixtures of 4-CP (20 mg/L) and phenol: (a) 40 mg/L and (b) 200 mg/L. Abiotic blank experiments: phenol (□) and 4-CP (○).

Samples were periodically taken for biomass and substrate concentration measurements. Samples were centrifuged (Orto Alresa, mod. Digicen, Madrid, Spain) at $4300\times g$ for 10 min at room temperature. The supernatant fraction was then filtered (pore size $0.22\text{ }\mu\text{m}$; Whatman) and stored at $-40\text{ }^{\circ}\text{C}$ for subsequent analyses.

2.2. Analytical methods

Biomass concentration was determined by optical density measurements (Cary 50 conc, Varian) at 600 nm which were converted to cell dry weight using a previously obtained calibration curve. Aromatic compounds were analysed by HPLC/UV (Prostar, Varian) using a C_{18} column as stationary phase (Microsorb MW-100-5) and a mixture of acetonitrile and H_2O (40:60, vol.) as mobile phase. The flow rate was maintained at 1.0 ml/min and a wavelength of 280 nm was used. TOC was measured by an OI Analytical Model 1010 TOC apparatus.

Contribution of abiotic processes such as adsorption, volatilisation and photodegradation was measured. The adsorption assays were carried out at biomass concentrations and operating conditions comparable to those occurring during the biodegradation runs. Adsorption experiments were performed by using bacteria grown in different media (phenol, glucose and mixtures of 4-CP and both cosubstrates). Adsorption of phenol and 4-CP was determined on biomass samples after extraction with Soxhelt following the US-EPA method 8041. Volatilisation and photodegradation tests were performed under identical operating conditions to those

employed in the biodegradation experiments but in the absence of biomass. In order to avoid photodegradation and photosynthesis the flasks were protected from light.

3. Results and discussion

3.1. Phenol degradation

The time-evolution of phenol concentration at 15 and $30\text{ }^{\circ}\text{C}$ and different starting concentrations (80, 150 and 240 mg/L) when phenol was used alone is shown in Fig. 1. Although phenol exhaustion was achieved at both temperatures, a great influence of temperature on phenol removal rate can be observed. Thus, the values for degradation rates of phenol at $30\text{ }^{\circ}\text{C}$ ($20\text{--}36\text{ mg/L h}$) are nearly ten times of those obtained at $15\text{ }^{\circ}\text{C}$ ($2\text{--}3.3\text{ mg/L h}$) over the range of initial phenol concentrations studied. An increase of the temperature leads to a dramatic reduction of the lag time and to a much sharper decay of phenol. Therefore, in the following all the assays were carried out at $30\text{ }^{\circ}\text{C}$.

No significant influence of initial phenol concentration on the length of the lag phase was observed. However, phenol concentrations higher than those shown in Fig. 1 led to an increase of the length of this phase (data not shown). This phenomenon is related with the toxic action of phenol which affects the integrity of the cytoplasmic membrane (Keweloh et al., 1990; Heipieper et al., 1991).

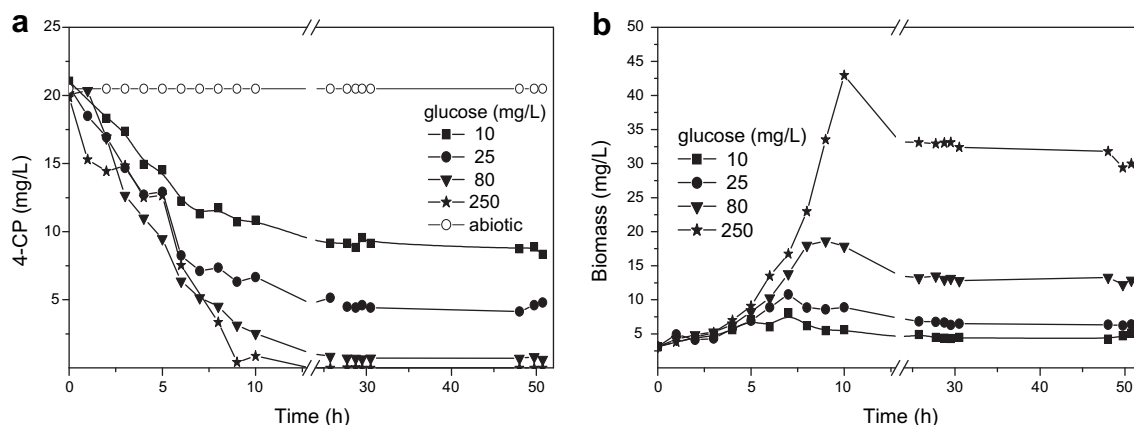


Fig. 4. Time-evolution of 4-CP (a) and biomass (b) concentration during the cometabolic removal of 4-CP (20 mg/L) with glucose as cosubstrate. Abiotic blank experiments (○).

3.2. 4-CP degradation

The degradation of 4-CP alone was studied at two different initial concentrations (15 and 30 mg/L). The time-evolution of 4-CP and biomass concentrations are shown in Fig. 2. As can be seen, low 4-CP transformation rates and conversions were achieved (36.9 and 15.3% for 15 and 30 mg 4-CP/L, respectively). This incomplete transformation was due to the toxicity of 4-CP on cell growth and consequently degradation ability. Similar results were found by Loh and Wang (1998). As the total amount of 4-CP removed is similar at both concentrations, the maximum biomass concentration reached was nearly the same. However, the higher toxic effect at 30 mg/L led to higher biomass decay during the final stage when the 4-CP concentration is stabilised.

3.3. Effect of phenol on 4-CP degradation

Fig. 3 shows the evolution of phenol, 4-CP and biomass concentrations along the degradation process. The results from the abiotic tests showed negligible effects of either stripping or adsorption, so any decrease of the target compounds concentration can be attributed exclusively to biological degradation. In all the experiments, shorter lag times in comparison with those observed when using phenol or 4-CP alone were found. Phenol, which was used as growth substrate, was transformed more rapidly than 4-CP over all the range of phenol concentrations tested. It was found that 4-CP was transformed rapidly only after phenol was almost fully depleted. Similar observations have been reported by Loh and Wang (1998) for phenol and 4-CP transformation by *P. putida*. Once phenol was exhausted, biomass started decaying. These results indicate that 4-CP cannot support cell growth, even in the presence of phenol.

In the cometabolic transformation of 4-CP, phenol is an excellent primary substrate since it not only easily induces the mono-oxygenase required for 4-CP transformation, but the phenol oxidation can also efficiently regenerates the consumed NADH (Bali and Sengül, 2002). Moreover, the addition of phenol greatly accelerated the degradation of 4-CP due to the increase of biomass production as reported by Bae et al. (1997).

The complete or partial biodegradation of 4-CP can be determined from the 4-CP/biomass (S/X) concentration ratio at the point where no further phenol removal is observed. In this work it was established that partial-removal was found for $S/X \geq 0.31$ (Fig. 3a), while complete 4-CP removal was achieved at $S/X \leq 0.11$ (Fig. 3b). Saez and Rittmann (1993) discriminated between complete ($S/X \leq 0.21$) and partial 4-CP ($S/X \geq 0.38$) removal by *P. putida*.

3.4. Effect of glucose on 4-CP degradation

In order to study the feasibility of using glucose as a growth substrate for 4-CP cometabolization, runs at two different 4-CP concentrations (20 and 40 mg/L) were carried out with glucose as the only added growth substrate over a wide range of initial concentrations (10–250 mg/L). Negligible adsorption of 4-CP onto biomass was found as can be seen in Fig. 4a. No lag phase in 4-CP biodegradation took place in any case, whereas complete removal of 4-CP was only achieved at the highest concentration of glucose tested. Although 4-CP retards the cell growth, its toxicity can be greatly attenuated by adding a primary substrate (Wang and Loh, 1999). Although the steep branch of the 4-CP decay curves is time-coincident with the growing region of the biomass curves (Fig. 4b), the biomass growth must be attributed to glucose consumption since *C. testosteroni* growth on 4-CP is limited as shown previously.

Fig. 5 compares both substrates, phenol and glucose, when used as the sole primary carbon and energy sources in the cometabolism of 4-CP. As can be seen, although the lag period of 4-CP removal

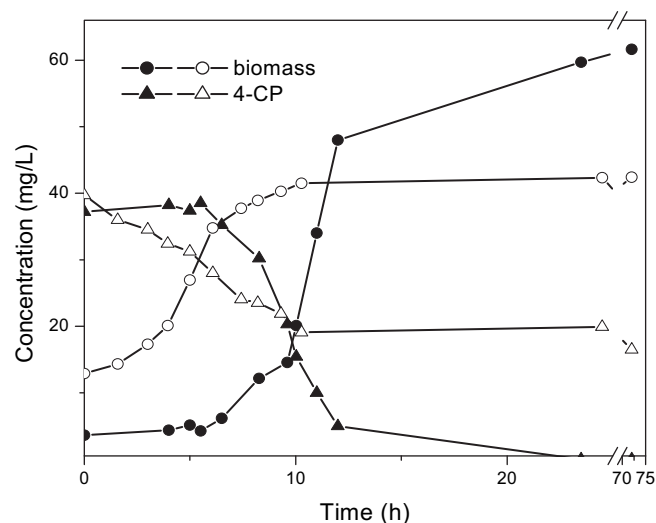


Fig. 5. Evolution of 4-CP and biomass concentration upon cometabolism of 4-CP (40 mg/L) with primary substrate (200 mg/L): phenol (filled symbol), glucose (open symbol).

disappears when using glucose (Tarighian et al., 2003) the presence of phenol leads to a higher removal rate once biodegradation starts. Complete 4-CP transformation was obtained using 200 mg/L of phenol, whereas only 58.4% was removed by adding the same glucose concentration.

Similarly, TOC removal efficiencies obtained with phenol as growth substrate were higher than those with glucose (Fig. 6). A comparison of the TOC removal efficiencies during 4-CP cometabolism showed no effect of the cosubstrate concentration on the final TOC values for concentrations higher than 80 and 250 mg/L of phenol and glucose, respectively. When phenol was added at concentrations higher than 80 mg/L, a residual TOC fraction of 20% was detected after the complete phenol depletion in all the experiments, regardless of the initial phenol concentration. This fact indicates the presence of unidentified refractory species derived from 4-CP biodegradation. Studies about the accumulation of chemical oxygen demand (COD) during phenol/4-CP degradation by *Acinetobacter* species have also indicated the presence of

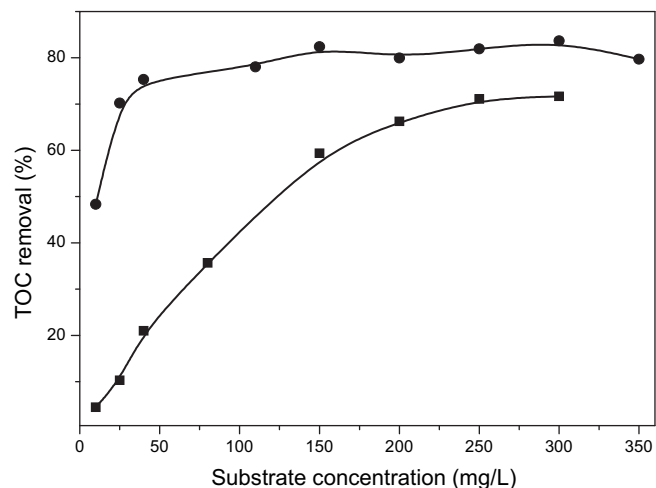


Fig. 6. TOC removal in cometabolism of 4-CP (20 mg/L) using glucose (■) and phenol (●) as the carbon substrates.

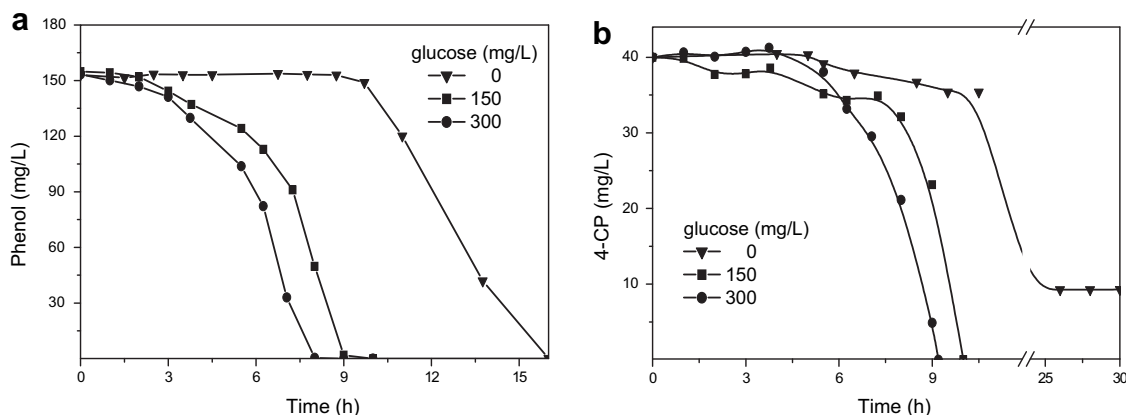


Fig. 7. Time-evolution of phenol (a) and 4-CP (b) treating mixtures of 4-CP (40 mg/L) and phenol (150 mg/L) and glucose (150 and 300 mg/L).

excreted products resulted from 4-CP transformation (Kim and Hao, 1999).

3.5. Effect of phenol and glucose on 4-CP degradation

Fig. 7 shows the degradation of phenol and 4-CP by *C. testosteroni* when glucose was added. As can be seen, the cometabolic transformation of 4-CP in the presence of phenol can be enhanced by adding a conventional carbon source like glucose. Although the use of sugars as a primary substrate does not require oxygenases for metabolism, it can support cometabolism of 4-CP through the generation of NADH (Wang and Loh, 1999). The addition of glucose reduced significantly the lag time observed for both phenol and 4-CP removal when phenol was used as a sole primary substrate. In these conditions the toxicity and inhibition of 4-CP can be attenuated and both cell growth and degradation rates can be significantly enhanced (Loh and Wang, 1998). No inhibition of degradation of either phenol or 4-CP by the presence of glucose was found. Increasing the glucose concentration from 150 to 300 mg/L did not reduce significantly the time required for complete removal of phenol and 4-CP.

4. Conclusions

C. testosteroni is capable of degrading phenol as a sole carbon and energy source within the range of concentrations tested (80–240 mg/L). However, results showed that the 4-CP transformation capacity was clearly deficient. Phenol and glucose acting as a primary growth substrate enhance 4-CP biodegradation. Nevertheless, higher 4-CP removal efficiencies can be obtained in presence of phenol than with glucose at the same initial concentrations. Whilst partial-removal was found for $S/X \geq 0.31$, complete 4-CP transformation was achieved at $S/X \leq 0.11$ when using phenol as cosubstrate. The simultaneous addition of phenol and glucose greatly reduces the minimum time required for complete 4-CP biodegradation. The complete removal of 4-CP and phenol is important for bioremediation purposes since both compounds are frequently found together in hazardous wastes.

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