Biodegradation **8:** 329–338, 1998.

Enhancement of biodegradation of phenol and a nongrowth substrate 4-chlorophenol by medium augmentation with conventional carbon sources

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Accepted 16 October 1997

Key words: biodegradation, cometabolism, nongrowth substrate, conventional carbon sources, inhibition, pH regulation, toxicity

Abstract

The enhancement of biodegradation of phenol and 4-chlorophenol (4-cp) as a cometabolised compound by *Pseudomonas putida* ATCC 49451 was accomplished by augmenting the medium with conventional carbon sources such as sodium glutamate and glucose. Compared with phenol as the sole carbon source, the addition of 1 gl^{-1} sodium glutamate increased the toxicity tolerance of cells toward 4-cp and significantly improved the biodegradation rates of both phenol and 4-cp even when the initial concentration of 4-cp was as high as 200 mgl^{-1}. On the other hand, supplementation of glucose caused a significant drop in the medium pH from 7.2 to 4.3 resulting in a reduction of degradation rate, leaving a considerable amount of 4-cp undegraded when the initial concentration of 4-cp was higher than 100 mgl^{-1}. By regulating the pH of the medium, however, enhancement of degradation rates of phenol and 4-cp in the presence of glucose was achieved with a concomitant complete degradation of phenol and 4-cp.

Abbreviations: 4-cp – 4-chlorophenol; GC – gas chromatography; FID – flame-ionization detector; Glu – glucose; SG – sodium glutamate; SDR – specific degradation rate; OD – optical density; NADPH – reduced nicotinamide adenine dinucleotide phosphate

Introduction

Biodegradable organic pollutants can be categorised into primary substrates (growth substrates) and cometabolised compounds (nongrowth substrates) (Saéz and Rittmann 1991). Biotransformation of the latter is generally more complicated because the nongrowth substrates cannot support cell growth and can only be transformed in the presence of a primary substrate. The primary substrate not only serves to sustain biomass production but also acts as an electron donor for degradation of the nongrowth substrate. However, nongrowth substrates have been shown to inhibit the oxidation of the primary substrate (Saéz and Rittmann 1993; Hyman et al. 1995). As such, the rate and efficiency of cometabolism are always dependent on a complex interaction between the primary substrate and nongrowth substrate. Nevertheless, because some of the most common chlorinated solvents are known to be biodegraded through cometabolic pathways (Alexander 1994; Hyman et al. 1995), the biodegradation behaviour of cometabolised compounds is of great importance to the biological treatment of polluted groundwater, industrial effluent, hazardous waste sites, and so on. It is therefore necessary to devote attention to study the interaction between the primary substrates and the nongrowth substrates in order to enhance the rate of the cometabolism.

Previous studies have shown that the degradation rate of some xenobiotic compounds can be improved by augmenting with additional carbon sources or other nutrient compounds such as nitrogen, phosphate as well as mineral constituents (Papanastasiou and Maier 1982; Topp et al. 1988; Boiesen et al. 1993; Chaud-

huri and Wiesmann 1995; Fava et al. 1995; Steffensen et al. 1995). Some conventional carbon sources, such as glucose, sodium glutamate, and yeast extract have been used to facilitate the degradation of toxic chemicals (Topp et al. 1988; Yu and Ward 1994; Fava et al. 1995). Yu and Ward (1994) reported that the rate and extent of pentachlorophenol (pcp) degradation were dramatically increased by the addition of glucose and peptone. Topp and Hanson (1990) found that glucose stimulated cell viability and pcp degradation. These findings suggest that the addition of some conventional carbon sources may aid in reducing the toxicity and growth inhibition of xenobiotics on cells, thereby increasing the transformation rate of xenobiotics. Conventional carbon sources may also provide reducing power for degradation of recalcitrant organic compounds (Perkins et al. 1994) or, in some cases, act as inducing agents for biodegradative enzymes (Chaudhuri & Wiesmann 1995). Potentially, this has greater significance in conversion of nongrowth substrates since cometabolism generally leads to a slow conversion of the substrate (Alexander 1994). However, scarce attention has focused on the effects of supplementing alternative conventional substrates on the biodegradation of the cometabolised compound as well as its primary substrate (Jacobson and Alexander, 1981).

The objective of this work is to study the influence of supplementary conventional carbon sources on enhancing the biotransformation rates of phenol as the primary substrate and 4-chlorophenol (4-cp) as a nongrowth substrate. 4-cp is known to be an excellent typical nongrowth substrate which can be degraded by phenol-induced bacterial cells (Saéz and Rittmann 1993). Since glucose (Glu) and sodium glutamate (SG), two common conventional carbon sources, are known to facilitate degradation of some chlorinated pollutants (Papanastasiou and Maier 1982; Topp et al. 1988), they are separately tested as the alternative conventional carbon sources.

Materials and Methods

Organism and culture conditions

The organism used throughout this work was *Pseudomonas putida* ATCC 49451. Stock cultures of *P. putida* were maintained by periodic subtransfer on nutrient agar (Oxoid, Hampshire, UK) slants and stored at 4 °C. All batch cultures were performed in 500 ml Erlenmeyer flasks with cotton plug at 50% medium volume. The mineral salt medium contained (gl^{-1}):K_2HPO_4, 0.65; KH_2PO_4, 0.19; $NaNO_3$, 0.5; $mgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.00556; $(NH_4)_2SO_4$ (absent if sodium glutamate was used), 0.5; and 10 ml trace mineral solution per liter medium. The trace mineral solution contained (gl^{-1}): nitrilotriacetic acid, 1.5; $MnSO_4.H_2O$, 0.5; $CoCl_2.6H_2O$, 0.1; $CaCl_2$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4.2H_2O$, 0.01; and $AlK(SO_4)_2.12H_2O$, 0.01. The concentrations of Glu and SG were variable.

All media (except phenol and 4-cp), pipette tips, and Erlenmeyer flasks fitted with cotton plugs were autoclaved at 121 °C for 20 min before using. Culture transfers and sampling were conducted aseptically around a bunsen flame to minimise contamination.

Prior to inoculation for each experiment, cells were induced by transferring a loop of stock culture maintained on the nutrient agar slant to the mineral medium and adding 200 mgl^{-1} of phenol as the sole carbon source. Two ml inoculum from the late exponential growth phase was then transferred to each flask. After inoculation, phenol and 4-cp were added directly from stock solutions to give the desired initial concentrations. Cells were grown in flasks on a New Brunswick rotary shaker at 30 °C and 200 rpm.

Chemicals

All the chemicals used in this study were of analytical grade. D-Glucose and sodium glutamate were purchased from BHD (England) and Sigma (Missouri, USA), respectively. Both phenol and 4-cp were obtained from Merck (Darmstadt, Germany). Phenol and 4-cp were dissolved in 0.02 N NaOH solution to make 10,000 mgl^{-1} stock solutions.

Analytical methods

Samples were withdrawn periodically for analysis. Cell density, medium pH and concentrations of phenol and 4-cp were monitored.

Cell density and pH analysis. A 6 ml sample from each flask was taken for determination of pH and biomass. pH was measured using a small pH electrode and pH meter (Model HI 9021, Hanna Instruments, U.S.A.) suitable for small volume samples. Cell growth was monitored spectrophotometrically by measuring the absorbance at 600 nm. The measurement was made such that the optical density (OD) of the sample was

less than 0.70 by diluting the samples. This is to ensure that Beer-Lambert law applies.

Phenol and 4-cp analysis. For the analysis of phenol and 4-cp, a further 3 ml sample was taken and immediately acidified to pH 2 with 6N sulphuric acid to quench the biodegradation reaction. The acidified samples were extracted with 3 ml of methylene chloride (Merck, Darmstadt, Germany), which contained 100 mgl^{-1} o-cresol (Merck, Darmstadt, Germany) as internal standard. The extract was analysed for phenol and 4-cp by gas chromatography (GC). GC analysis was carried out using a capillary GC (Perkin Elmer, Model 8700) equipped with a split injector and flame ionization detector (FID). The injector and detector temperatures were both 300 °C. Injection volume was 2 μl and sample split ratio was 20:1. The oven-temperature profile started with maintaining at 100 °C for 1 minute before ramping at 10 °C/min to 160 °C after which the program was halted.

TOC Measurement. 5 ml of culture medium was taken from the flask and diluted with deionized water and then filtered through 0.45 μm filter (Millipore, MA) to remove suspended solids for determination of total organic carbon (TOC) concentration in the medium. TOC was measured by a TOC analyzer (Shimadzu, Model TOC-5000A) which measures total carbon (TC) and inorganic carbon (IC) concentrations. Sample TOC was obtained by subtracting IC from TC. The operation is based on the combustion/non-dispersive infrared gas analysis method.

Evaluation of average degradation rate. In calculating the average degradation rates of phenol and 4-cp, two important issues were accounted for. Firstly, it was found that experimental duplicates were nearly identical for the biodegradation phases except for the lengths of the lag phase. Because of this, average degradation rates cannot be obtained consistently and real differences in degradation rates may be erroneous when the length of the lag phase was included. Secondly, it was difficult to ascertain exactly the time when complete degradation was achieved or when the degradation had stopped. Consequently, the average biotransformation rates of phenol and 4-cp were calculated by dividing the net amount of transformed phenol and 4-cp for the time period when phenol and 4-cp were about 10% and 90% of the initial phenol and 4-cp, respectively (or that percentage which cannot be further degraded), by the corresponding elapsed time.

Estimation of the specific degradation rate. In order to estimate the specific degradation rate (SDR), data for phenol and 4-cp concentrations versus culture time were correlated respectively using the multivariable regression technique in Microsoft Excel to obtain the equations of best fit of the degradation curves. The correlations were differentiated with respect to time and then divided by the cell mass at distinct times to determine the SDR's of phenol and 4-cp.

Table 1. Summary of batch biotransformation experiments

Test No.	Initial nominal concentration (mgl^{-1})			
	Phenol	4-cp	SG	Glu
F1	200	100	0	0
F2	200	100	1000	0
F3	200	100	0	1000
S1	200	200	0	0
S2	200	200	1000	0
S3	200	200	0	1000
T1	200	200	0	1000
T2	200	200	0	1000

Results

A series of biodegradation experiments was performed with different phenol/4-cp concentrations with the addition of 1 gl^{-1} SG or 1 gl^{-1} Glu, respectively. For comparison, degradation tests with phenol and 4-cp as the sole carbon sources were carried out. In addition, degradation of phenol and 4-cp in the presence of glucose under pH regulations was also monitored to elucidate the effect of pH on the biodegradation process. All biodegradation experiments in this work are summarised in Table I.

Supplementation of glucose or sodium glutamate

The first set of experiments (F1-F3) was performed with a concentration ratio of phenol to 4-cp of 2. Figures 1a and 1b show the effect of the additional carbon source (Glu or SG) on the biodegradation of phenol and 4-cp, respectively. No effect of the additional carbon source on biodegradation of phenol was found. Phenol was completely degraded within 13 hours in all three cases: without additional carbon source and with 1 gl^{-1} of SG or Glu, respectively. Figure 1b shows that 4-cp was quickly degraded after a lag of about 11 hours. However, the difference in degradation rates of 4-cp for the three cases is quite discernible. The average degradation rates were calculated to be 45 $mgl^{-1}h^{-1}$ (without SG or Glu), 60 $mgl^{-1}h^{-1}$ (with SG), and

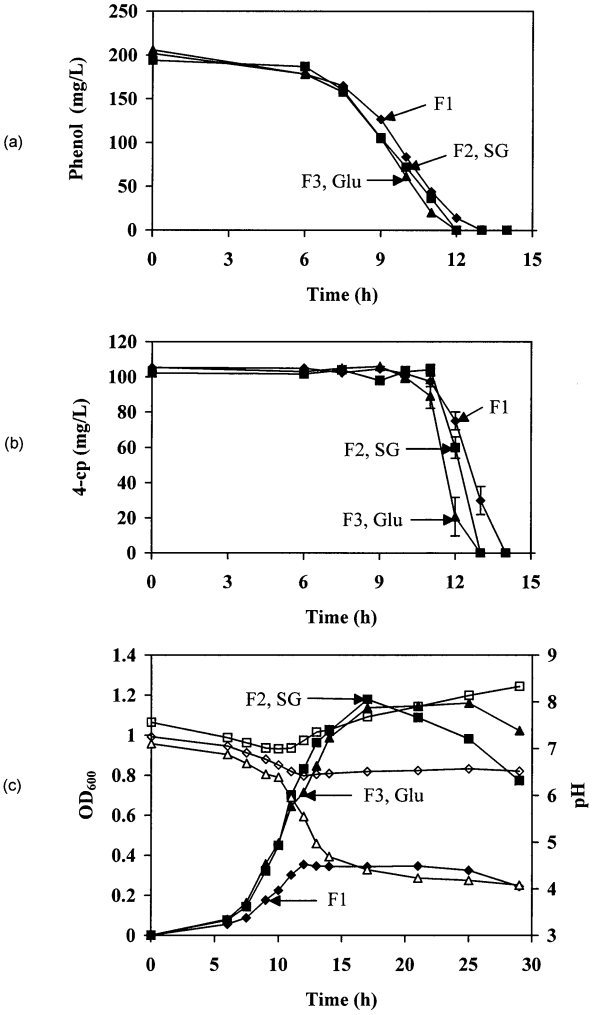

Figure 1. Effects of sodium glutamate and glucose at initial concentrations of 200 mgl^{-1} of phenol and 100 mgl^{-1} of 4-cp. (a) phenol concentration; (b) 4-cp concentration. Error bars indicating the deviation of duplicate experiments are reported here because of the few data points on the steep sections; (c) biomass concentration (expressed as OD_{600}) and medium pH. Symbols: ♦, ◊, test F1; ■, □, test F2; ▲, △, test F3. The corresponding open symbols represent pH.

69 $mgl^{-1}h^{-1}$ (with Glu), clearly indicating that the biodegradation rate of 4-cp can be enhanced by augmentation with SG or Glu.

Figure 1c shows the cell growth and pH time profiles for the three experiments F1-F3. The addition of SG or Glu greatly increased the cell mass. The cultures containing a supplementary carbon source grew faster and accumulated much more biomass than that containing only phenol and 4-cp as the only carbon sources. The maximum specific growth rates during

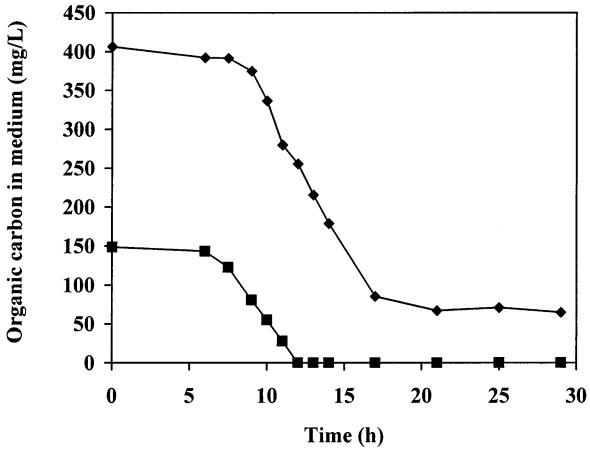

Figure 2. Substrate concentration indicated by TOC determinations for test F2. Symbols: ♦, TOC^*; ■, OC_{ph}. (TOC^* = TOC – OC_{ph}). TOC: total organic carbon in medim; OC_{ph}: organic carbon associated with phenol in medium.

exponential growth were calculated to be 0.35 h^{-1} (without SG or Glu), 0.42 h^{-1} (with SG) and 0.42 h^{-1} (with Glu). The highest cell densities (OD_{600}) recorded were 0.36 (without SG or Glu), 1.18 (with SG) and 1.15 (with Glu). This resulted in the faster conversion rates of 4-cp (Figure 1b). Figure 1c also shows that when phenol was the sole carbon source (F1), the pH of the culture decreased slightly from 7.3 to 6.5 during the initial 11 hours of cultivation and remained nearly constant thereafter. On the other hand, pH decreased drastically from 7.1 to 4.2 with cell growth in experiment F3. It is interesting to note that phenol and 4-cp were completely degraded before the pH dipped below pH 5. In the experiment supplemented with SG, pH only decreased slightly during complete degradation of phenol and 4-cp after which the pH increased marginally to pH 8.3.

It can be ascertained from the biomass concentration profile in Figure 1 that SG or Glu was simultaneously utilized with phenol. As soon as the lag phase was over, the biomass density in experiments F2 and F3 showed greater increase than that of experiment F1 with phenol as the only primary substrate. To further confirm this, the total organic carbon (TOC) in the culture broth was monitored over time for test F2. The results of this are presented in Figure 2. The TOC measured provides an indication of the concentration of SG in the culture broth. This can easily be rationalized by performing a material balance for the organic carbon in the broth (Appendix). As shown in Figure 2, both SG and phenol were utilized concurrently. After the

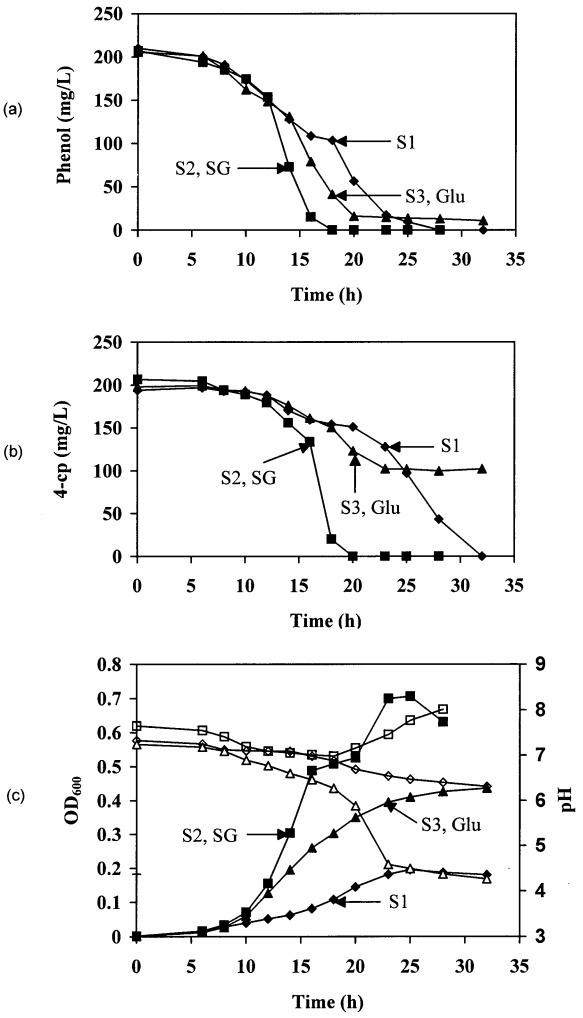

Figure 3. Effects of sodium glutamate and glucose at initial concentrations of 200 mgl^{-1} of phenol and 200 mgl^{-1} of 4-cp. (a) phenol concentration; (b) 4-cp concentration; (c) biomass concentration (expressed as OD_{600}) and medium pH. Symbols: ♦, ◊, test S1; ■, □, test S2; ▲, △, test S3. The corresponding open symbols represent pH.

complete degradation of phenol, the bacteria continued to metabolize SG with a concomitant increase in the biomass (Figure 1c). This simultaneous utilization of the toxic substrate and an added carbon source has been reported by previous investigators (Papanastasiou & Maier 1982; Fava et al. 1995). The residual concentration of TOC* at the end of the experiment can be attributed mainly to the organic carbon associated with 2-hydroxy-5-chloromuconic semialdehyde (HCMSA) (Saéz and Rittmann 1991), which again confirms that 4-cp is a nongrowth substrate in this system.

Higher concentration of 4-cp

The growth substrate, phenol, and nongrowth substrate, 4-cp, may exert substrate toxicity on oxygenase expressing cultures (cultures which produce oxygenase enzymes) at elevated concentrations (Chang & Alvarez-Cohen 1995), and the resultant toxicity most likely reduces degradation rates of both phenol and 4-cp. Saèz and Rittmann (1993) reported that 4-cp will, not only inhibit oxidation of the cell's primary substrate, phenol, but also its own degradation. The toxicity of 4-cp to cell growth and its inhibition to oxidation of phenol (competitive inhibition) as well as itself are expected to increase with an increase in 4-cp concentration in the medium. The next set of experiments (S1-S3) was performed with an increase in the concentration of 4-cp to about 200 mgl^{-1}in order to elucidate the effects of conventional carbon sources at high 4-cp concentrations.

Figure 3a shows that the degradation rate of phenol with addition of 1 gl^{-1} SG was the highest (21 $mgl^{-1}h^{-1}$), and the phenol was completely degraded in 18 hours, while the complete disappearance of phenol occurred in 28 hours without the supplementation of additional carbon sources at a rate of 12 $mgl^{-1}h^{-1}$. With the addition of 1 gl^{-1} Glu, it was found that oxidation of phenol only proceeded for 20 hours (the average biodegradation rate was 14 $mgl^{-1}h^{-1}$) after which a residual phenol of about 10 mgl^{-1} remained in the culture broth.

In examining the biotransformation of the nongrowth substrate, 4-cp, Figure 3b shows that the addition of 1 gl^{-1} SG (S2) resulted in the highest degradation rate of 4-cp compared with no additional carbon source and the addition of Glu. Complete transformation of 4-cp was achieved in 20 hours at an average degradation rate of 27 $mgl^{-1}h^{-1}$. Augmenting the medium with 1 gl^{-1} Glu, the degradation of 4-cp continued until about 23 hours into the experiment. The average degradation rate was only 8 $mgl^{-1}h^{-1}$. Then, the degradation of 4-cp stopped and a substantial concentration of 4-cp (about 100 mgl^{-1}) remained undegraded in the medium. In the case of phenol as the sole primary substrate, 4-cp was completely transformed in 32 hours, substantially longer than the degradation time required with the addition of SG. The average degradation rate in this case was 11 $mgl^{-1}h^{-1}$.

Figure 3c plots the time profiles for cell growth and medium pH for experiments S1-S3. As shown in Figure 3c, both the rate of cell growth and the maximum cell density achieved were higher in the cases where the

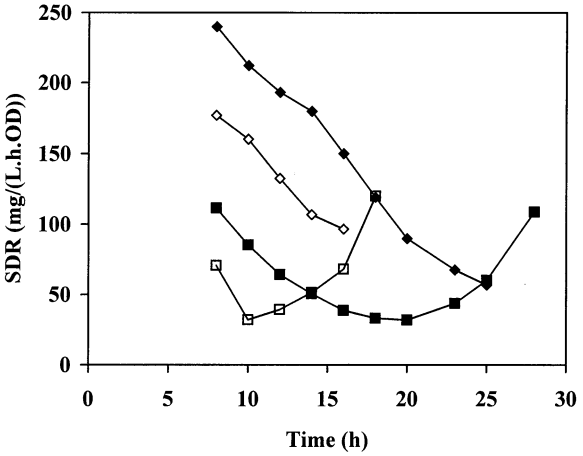

Figure 4. Time profile of specific degradation rates of phenol and 4-cp for Tests S1 and S2. Symbols: ♦, ◊, SDR of phenol; ■, □, SDR of 4-cp. The filled and open symbols represents tests S1 and S2, respectively.

medium was supplemented with SG or Glu. The maximum specific growth rates during exponential growth were found to be 0.13 h^{-1} (without SG or Glu), 0.34 h^{-1} (with SG) and 0.24 h^{-1} (with Glu), while the highest cell densities (OD_{600}) attained were 0.20 (without SG or Glu), 0.71 (with SG) and 0.43 (with Glu). In the case of pH profiles, with phenol as the sole primary substrate (S1), the pH decreased slightly from 7.3 to 6.3 albeit gradually. When the medium was supplemented with SG, during the time when phenol and 4-cp were being transformed, pH dropped from 7.6 to 7.0. After phenol and 4-cp were completely degraded, the pH increased from 7.0 to 8.0. Supplementing the medium with Glu exhibited the most dramatic change in medium pH. During the 20 hours when phenol and 4-cp were being degraded, the medium recorded a drop in pH from 7.2 to 5.9. This was followed by a drastic drop from 5.9 to 4.3. With this decreased pH, the biodegradation of phenol and 4-cp stopped and an increase in the amount of suspended solids was observed in the culture broth.

The above results clearly indicate that both SG and glucose can support cell growth and the addition of these conventional carbon sources substantially increased cell density. In order to clarify the function of the added conventional carbon sources on degradation process, the specific degradation rates (SDR) of phenol and 4-cp for S1 and S2 were calculated and compared in Figure 4 (for simplicity, analysis for S3 was not included due to the complication of effect of pH).

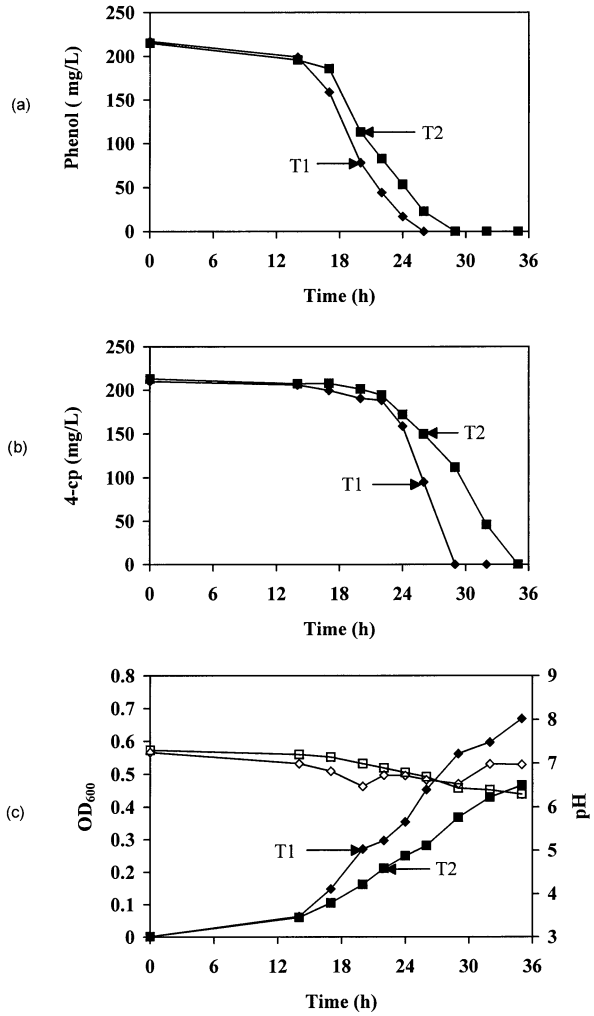

Figure 5. Degradation of phenol and 4-chlorophenol at the initial concentrations of both at 200 mgl^{-1} with pH regulation. (a) phenol concentration; (b) 4-cp concentration; (c) biomass concentration (expressed as OD_{600}) and medium pH. Symbols: ♦, ◊, test T1; ■, □, test T2. The corresponding open symbols represent pH.

For test S1, it can be seen from Figure 4 that the SDR of phenol decreased monotonically with culture time (i.e. with cell growth). This resulted from the decrease in enzymatic degradation rate of phenol with the decrease in the phenol concentration in the medium according to enzyme kinetics (Bailey and Ollis 1986). For the degradation of 4-cp, the SDR of 4-cp decreased until about 20 hours, at which time phenol was almost exhausted, and then increased to about 110 mg/(L.h.OD). Similarly, in the presence of SG in the medium (S2), Figure 4 also shows that the SDR of phenol decreased monotonically with culture time.

Table 2. Effects of additional carbon sources and 4-cp on maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/ph}$), and the average biotransformation rates of phenol (V_{ph}) and 4-cp (V_{cp})

Test No.	μ_{max} (h^{-1})	$Y_{x/ph}$ (OD/mgl^{-1})	V_{ph} ($mgl^{-1}h^{-1}$)	V_{cp} ($mgl^{-1}h^{-1}$)
F1	0.35	0.0018	27	45
F2	0.42	0.0042	34	60
F3	0.42	0.0035	32	69
S1	0.13	0.0009	12	11
S2	0.34	0.0025	21	27
S3	0.24	0.0019	14	8
T1	0.20	0.0015	19	21
T2	0.14	0.0012	18	15

* $Y_{x/ph}$ was calculated based on phenol consumption. The linear relationship between biomass and the consumption of phenol suggests that phenol and the additional carbon sources were simultaneously utilized.

However, the SDR of phenol in this case was at all times lower than that of S1. This was due to the higher cell density resulting from simultaneous utilization of SG. With the addition of SG in the medium (S2), the concurrent utilization of both growth substrates (SG and phenol) greatly increased the cell mass. Since the specific degradation rate of phenol was normalized against the total cell density (other than that derived solely from phenol consumption), the SDR of phenol for S2 was consequently lower compared to that for S1. For the degradation of 4-cp, compared to that of S1, the SDR of 4-cp in experiment S2 also decreased and was lower than that of S1 initially. However, with nearer depletion of phenol in the medium, the specific degradation rate quickly increased to about 121 mg/(L.h.OD) and became higher than that of S1 subsequently. A possible explanation and relevant discussion on this observation are presented in Discussion.

Biodegradation with pH regulation

Results from experiment S3 suggest that pH could play an important role in the biotransformation of phenol and 4-cp in the presence of glucose. In an effort to better understand the effect of pH on the degradation of phenol and 4-cp with the supplementation of additional carbon sources especially at high 4-cp concentrations, the third set of experiments (T1 and T2) was carried out. For Test T1, pH was regulated using 2 N NaOH solution to maintain pH in the range of 6.5-7.5; Test T2 was carried out by replacing the phosphate salt content of the medium with a phosphate buffer containing 1.74 gl^{-1} $K_2HPO_4$and 0.24 gl^{-1} KH_2PO_4. As shown in Figure 5, when the pH was maintained between 7.3 and 6.3, both phenol and 4-cp were completely degraded in both experiments. The effect of using different pH regulation methods was not significant. The average degradation rates of phenol and 4-cp were calculated to be 19 $mgl^{-1}h^{-1}$ and 21 $mgl^{-1}h^{-1}$ for T1, respectively, and 18 $mgl^{-1}h^{-1}$ and 15 $mgl^{-1}h^{-1}$ for T2, respectively. The relevant data pertaining to all the experiments performed in this investigation are summarised in Table II.

Discussion

In this work, we investigated the effect of adding supplementary carbon sources, sodium glutamate and glucose, on the relative rate and efficiency of cometabolism of phenol and 4-cp. Not only was there an increase in the maximum specific growth rate of the cells, the volumetric biodegradation rates of phenol and 4-cp were improved with supplementation.

Based on the degradation mechanism proposed by Saéz and Rittmann (1991), the initial step in the transformation of 4-cp is hydroxylation facilitated by an NADPH-dependent monooxygenase to form 4-chlorocatechol, and the NADPH consumed in the monooxygenase reaction can be regenerated by the oxidation of phenol or phenol-induced biomass (Saéz & Rittmann 1991 and 1993). Similarly, the oxidation of phenol is also initiated by an NADPH-dependent hydroxylase to form catechol (Yang & Humphrey 1975). Therefore, phenol and 4-cp are expected to inhibit oxidation of each other competitively. When the ratio of phenol and 4-cp was high (200/100), the competition was dominated by the oxidation of phenol, and 4-cp cannot effectively bind with the monooxygenase. As a result, almost no transformation of 4-cp took place until phenol was nearly depleted (Figure 1). When the concentration of 4-cp was increased to 200 mgl^{-1}, 4-cp could compete better for the monooxygenase, and a slow transformation of 4-cp occurred before complete removal of phenol (Figure 3). However, Figure 4 shows that the competitive inhibition was not just between phenol and 4-cp. As culture time proceeded, even with a decrease in phenol concentration, the SDR of 4-cp did not increase but showed an initial decline. This can be explained using an earlier hypothesis proposed by Saéz and Rittmann (1993). They proposed that, in the cometabolism of phenol and 4-cp, phenol and 4-cp compete for the monooxygenase, and an oxidized

intermediate of phenol may compete with 4-cp for a site of regulatory binding. With the fast oxidation of phenol, the presence of the oxidized intermediate of phenol resulted in reduction in the proportion of 4-cp's binding with the enzyme. As a result, the SDR of 4-cp initially decreased with cell growth. After phenol was nearly exhausted in the medium, the competitive inhibition of phenol on degradation of 4-cp was lifted and the SDR quickly increased. At this time, the biomass in test S2 continued to grow on SG (Figure 3c). This suggests that the oxidation of SG did not inhibit the degradation of 4-cp, contrary to the action of phenol as the primary substrate. This finding may have important implications in practical applications. In cometabolism, a primary substrate must be provided to support cell growth; however, the structurally analogous primary substrate usually inhibits the degradation of the nongrowth substrate and vice versa (competitive inhibition). From this study, fortunately, it was found that some conventional carbon sources such as SG could maintain cell growth and in addition, may not inhibit the oxidation of the nongrowth substrate.

4-cp cannot support cell growth and is well documented to be inhibitory to the oxidation of phenol and toxic to bacterial cells (Saéz and Rittmann 1993; Hale et al 1994). As shown in Table II, by comparing cultures performed under correspondingly identical conditions except for the concentration of 4-cp, with an increase of 4-cp concentration from 100 mgl^{-1} to 200 mgl^{-1}, there is a marked decrease in the maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/ph}$), and the average biotransformation rates of phenol and 4-cp. These imply that the nongrowth substrate, 4-cp, not only severely inhibited cell growth, reduced biomass yield but adversely slowed down degradation of phenol as well as itself. Our results indicate that these adverse effects can be reduced by supplementation of some kind of conventional carbon sources, such as SG and Glu (except for test S3 due to pH drop). It is possible that the simultaneous utilization of conventional carbon sources (e.g. SG and Glu) and phenol enabled the cells to overcome the growth inhibition effects of both phenol and 4-cp. When the concentration of 4-cp was relatively low (about 100 mgl^{-1}), the supplementation of either Glu or SG can facilitate the conversion of the cometabolised compound, 4-cp. When the toxicity is increased by increasing the concentration of 4-cp to about 200 mgl^{-1}, compared with phenol as the sole carbon source, the presence of SG significantly enhanced the oxidation of both phenol and 4-cp. This is in correlation to previous investigations which have reported that the toxicity and inhibition of target compounds on cells can be attenuated by the uptake of nontoxic nutrients and the presence of more confluent biomass (Topp et al. 1988; Saéz and Rittmann 1991). Topp et al. (1988) reported that the degradation of pentachlorophenol (pcp) could be greatly facilitated by adding alternative carbon sources, such as SG, Glu, and the like. The addition of these nontoxic compounds stimulated the viability of cells and enhanced the degradation rate of pcp (Topp et al. 1988; Topp and Hanson 1990). In another study, Saéz and Rittmannn (1991) reported that the 4-cp transformation rate was controlled by the concentration ratio of biomass to 4-cp. In their study, the high biomass was obtained by cell growth using phenol as the sole carbon source. In our investigation, the high biomass required was achieved by the supplementation of additional non-toxic carbon sources.

It has often been noted that pH plays an important role in biodegradation processes (Valo and Salkinoja-Salonen 1985; Omori et al. 1987; Overmeyer and Rehm 1995). When phenol or phenol plus SG were the carbon sources, the initial slight decrease of the pH might be attributed to the formation of 2-hydroxy muconic semialdelyde and 2-hydroxy-5-chloromuconic semialdehyde (Yang and Humphrey 1975; Mörsen and Rehm 1990; Saéz and Rittmann 1991). Further utilization of SG led to an increase in pH after both phenol and 4-chlorophenol were completely transformed. This may be due to the formation of ammonia during glutamine metabolism. With glucose metabolism, the sharp drop in medium pH greatly reduced or even stopped the biodegradation activity, leaving a substantial amount of 4-cp in the culture especially when the concentration of 4-cp was high at 200 mgl^{-1}. The drastic drop in pH may be attributed to the formation of acidic end-products such as acetic and lactic acids during glucose metabolism. The complete transformation of 200 mgl^{-1} phenol and 4-cp in the presence of glucose by regulating the pH of the medium therefore confirms the important role of pH on the biodegradation. These results affirm similar findings reported by Overmeyer and Rehm (1995). They found that complete degradation of up to 40 mM of 2-chloroethanol by *Pseudomonas putida* US2 could be obtained only when the pH was regulated.

Cometabolism is considered a special class of biological transformation and such transformations have considerable importance in nature. It has been proposed that the cometabolic transformation rate of nongrowth substrates may be promoted by increasing the

concentration of its primary substrate because a greater population of cometabolizing cells is produced (Chang et al. 1993; Mu and Scow 1994). However, the adoption of this method requires careful consideration. In the case of biodegradation of chlorinated phenol, the primary substrate, phenol, is by itself, a toxic contaminant. The response of adding more phenol can be unpredictable. For example, for bioremediation of contaminated soil, some part of the supplemented phenol may escape into the aqueous system which subsequently finds its way to the groundwater or the atmosphere through volatilization since the availability of the contaminants in soils for biological conversion is affected by many physical, chemical and structural properties of both the contaminant and the soil matrix, as well as by the relative importance of a number of possible transport mechanisms. In this case, supplementation of some suitable conventional carbon sources can help eradicate or reduce such risks. Furthermore, the toxic primary substrate phenol may be completely substituted by some suitable conventional carbon sources, which study is being undertaken.

Acknowledgements

The authors wish to acknowledge the financial support from the National University of Singapore for its scholarship (to S-J Wang).

Appendix

A material balance for the organic carbon present in the culture broth can be performed. Defining, for component x, the organic carbon derived from x as [x], initially, the total organic carbon, TOC, is the sum of all organic carbon attributable to phenol, 4-cp and SG, i.e.,

$$\mathrm{TOC} = [\mathrm{Phenol}] + [4-\mathrm{cp}] + [\mathrm{SG}]$$

At any time t, after centrifuging the sample,

$$\mathrm{TOC} - [\mathrm{Phenol}] = [4-\mathrm{cp} + 4-\mathrm{cp} - \mathrm{metabolite}] + [\mathrm{phenol} - \mathrm{metabolite}] + [\mathrm{SG}]$$

In the course of biooxidation of phenol, the concentration of the intermediates of phenol excreted into the medium can be assumed to be negligible (Hill and Robinson 1975; Mörsen and Rehm 1990). On the other hand, 4-cp is transformed to 2-hydroxy-5-chloromuconic semialdehyde (HCMSA) in a 1:1 stoichiometry (Saéz and Rittmann 1991) and therefore [4-cp + 4-cp-metabolite] remains constant during biodegradation. The change of TOC* = TOC-[Phenol] consequently indicates directly, changes in the concentration of SG in the culture broth with time.

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