Effect of pH and inoculum size on pentachlorophenol degradation by *Pseudomonas* sp.

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Abstract

Pentachlorophenol (PCP) is a toxic compound which is used as a fungicide, bactericide, herbicide and chemical intermediate. Because of its toxicity, there is a need to decontaminate the PCP-laden soils and bioremediation is a very useful alternative to conventional clean-up methods. The success of this depends on finding strains able to degrade PCP in a changeable environment.

The aim of this work was to study the influence of pH of the medium and the effect of inoculum size on pentachlorophenol degradation by *Pseudomonas* sp. A study of PCP degradation kinetics was performed to assess such effects.

PCP was degraded rapidly at pH values from 6.3 to 8, but the maximum rate of PCP degradation by *Pseudomonas* sp. was at pH 6.3. In contrast, the PCP degradation kinetics at pH 5.5 were significantly lower, although PCP was totally depleted. These results show the broad range of pHs for PCP degradation for this strain.

PCP was degraded at every inoculum size tested and PCP degradation increased with the increasing inoculum size, but cultures inoculated with the lowest inoculum showed the highest specific consumption rate. This reveals a lower consumption of PCP per CFU at a high population density.

These results are useful to understand the physiological and biochemical properties of *Pseudomonas* sp. before its optimum use in environmental application and these data will assist in choosing the right PCP-degrader for a changeable environment.

Keywords: biodegradation, bioremediation, inoculum size, pentachlorophenol, pH, *Pseudomonas* sp.

Introduction

Pentachlorophenol (PCP) has been used as a wood preservative, insecticide, and herbicide and was introduced into the environment by waste streams of several industrial operations. A large amount of PCP is used by the wood-preserving industry (Hoos, 1978; Cirelli, 1978) and PCP contamination in soil represents a serious problem to the environment that surrounds several wood treatment plants and sawmills (Rao, 1978; Valo et al., 1985). PCP is considered a priority pollutant by various regulatory agencies. This polychlorinated aromatic compound is toxic to numerous aquatic organisms at a concentration of as low as 0.5 $mg \cdot \ell^{-1}$ (Borthwick and Schimmel et al., 1978) and adversely affects flora and fauna (Chu and Kirsch, 1973; Liu et al., 1982). It is also suspected to be a human carcinogen (Mc Allister et al., 1996). Because of its toxicity, there is a need to decontaminate the PCP-laden soils.

Several decontamination techniques are available for the removal of contaminants from water, although not all are efficient enough to destroy the contaminant. The use of micro-organisms for bioremediation of PCP-contaminated sites may prove to be a viable alternative to conventional clean-up methods. Biodegradation is a technique which could potentially degrade these contaminants to innocuous products (mainly CO_2 and H_2O; also Cl^- in the case of chlorinated phenols). Biological treatment of chlorophenols attracts more attention than physical and chemical methods, because a variety of micro-organisms are known to utilise chlorophenols as their sole carbon or energy source and because the reaction products are Cl^- ions, CO_2 and biomass. Many species of soil bacteria have been isolated from contaminated soil samples. PCP-degrading bacteria include species of *Arthrobacter* (Stanlake and Finn, 1982), *Flavobacterium* (Saber and Crawford, 1985), *Pseudomonas* (Radehaus and Schmidt, 1992), *Rhodococcus* (Apajalahti and Salkinoja-Salonen, 1986) and *Corynebactrium* (Chu and Kirsch, 1972). Although several of them do not degrade PCP completely, others produce toxic metabolites and some do not tolerate changes of physical and chemical environmental factors.

In previous work published (Murialdo et al., 2003) a *Pseudomonas* sp. strain was isolated from a consortium that degrades PCP. It was shown that glucose and glutamate have positive effects on its population density. This micro-organism could be used very effectively for *in situ* bioremediation in an environment which is highly contaminated with PCP, other chlorinated phenols and hexadecane (Murialdo et al., 2003).

The success of bioremediation may depend on the availability of microbial strains that can mineralise high levels of PCP and withstand adverse conditions to compete under *in situ* conditions. An effective bacterial inoculum should be able to tolerate high levels of PCP while maintaining a level of activity to provide efficient mineralisation (Shaw et al., 1997). Understanding the physiological and biochemical properties of PCP-degrading bacteria is required before optimum use of bacteria in environmental applications.

In order to find a strain able to degrade PCP in a changeable environment, we studied the effect of inoculum size and the influence of the pH of the medium on PCP degradation by a PCP-degrading bacterium, *Pseudomonas* sp., isolated from soils contaminated with PCP.

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Experimental

Strain

P. aeruginosa was isolated from a soil with a history of phenol contamination, wood chunks that had been exposed to formaldehyde and chlorophenol solutions, and a soil containing pentachlorophenol near a wastewater discharge site (Murialdo et al., 2003).

Chemicals and reagents

Pentachlorophenol (PCP 99% pure) was obtained from Sigma Chemical Co. (St. Louis MO 63178 USA). All other chemicals used were of the highest purity commercially available.

Culture conditions and media

Minimal salts medium (MS) consisted of (in g·ℓ$^{-1}$): $NaNO_3$, 2.25; K_2HPO_4, 2.83; KH_2PO_4, 0.507; Mg SO_4.7 H2O, 0.45; $FeSO_4$, 0.135 and $CaCl_2$, 0.45. Where indicated, the pH of MS was adjusted to pH values of 5.5, 6.3, 7.1 and 8, changing the potassium-phosphate buffer system; 2 g glucose·ℓ$^{-1}$ were added to the MS and designated MSG when indicated. Pentachlorophenol was first converted to its sodium salt by dissolving it in 0.2 N NaOH and then autoclaved. This stock solution of pentachlorophenate (5 000 mg· ℓ$^{-1}$) was used to prepare the MS or MSG media supplemented with the different concentrations of PCP.

Influence of pH of the medium on PCP degradation

Pseudomonas sp. cells were grown in 50 mℓ MSG medium with 25 mg·ℓ$^{-1}$ of PCP at different pH values (5.5, 6.3, 7.1 and 8). This mixture was contained in a 125 mℓ Erlenmeyer flask. The cultures were placed on a shaker (100 r·min^{-1}) at 25° C in the dark, to avoid photodecomposition of PCP.

Growth was monitored by measuring the turbidity of the culture at 600 nm, using a Shimadzu uV-160 spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, MD, USA). Cells were harvested at 20 h during mid-log phase. Cultures (5 mℓ) were centrifuged at 10 000 r·min^{-1} for 5 min, the pellet was washed once with MS medium according to the different pHs used and resuspended in the same MS medium. This was used immediately as inoculum for the next batch. *Pseudomonas* sp. cells were inoculated into 50 mℓ of MS medium with 25 mg·ℓ$^{-1}$ of PCP, as the only source of carbon and energy, at different pH values. Cultures were maintained at 25° C on a shaker operated at 100 r·min^{-1}. Sample aliquots of 1 mℓ were removed at 2h intervals and the cells pelleted by centrifugation at 10 000 r·min^{-1} for 5 min. PCP degradation was assessed by measuring A_{320nm} of the supernatant.

Controls included cells in MS without PCP added and MS medium supplemented with PCP without cells.

Viability was measured 2 h after the addition of the inoculum and when the degradation of PCP started. Serial dilutions of cells at each time were plated on MSG agar plus PCP (25 mg·ℓ$^{-1}$) and colony-forming units (CFU) enumerated following 48 h incubation at 30°C.

PCP degradation

PCP degradation was established by measuring the PCP aromatic ring cleavage using a Shimadzu UV-160 spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, MD, USA) at A_{320} and compared with a standard curve. PCP A_{320} = 1 corresponding to 50 mg·ℓ$^{-1}$.

Effect of inoculum size on PCP degradation

The effect of inoculum size on PCP degradation was tested. Cells were grown as shake cultures at 25ºC in 50 mℓ of MSG medium supplemented with 20 mg·ℓ$^{-1}$ PCP at pH 7.1 in 125 mℓ Erlenmeyer flasks at 100 r·min^{-1}. Growth was monitored by measuring the turbidity of the culture at A_{600}. During mid-log phase (at 20 h) the viability was measured and different volumes of the MSG culture (0.2 mℓ, 1 mℓ and 5 mℓ) were harvested by centrifugation at 10 000 r·min^{-1} for 5 min. Thereafter, the pellets were washed once with MS medium and resuspended in the MS medium. The different inoculum sizes were transferred into 50 mℓ of MS medium supplemented with 20 mg· ℓ$^{-1}$of PCP, as the only source of carbon and energy. At different times, PCP degradation was measured. The final concentration of the inoculum at the initial time in each treatment were: $1.8x10^6$, $9.04x10^6$ and $4.5x10^7$ CFU/mℓ. These inoculum sizes were confirmed at the start of the experiment by plate count.

Controls included cells in MS without PCP and MS medium supplemented with PCP but without cells at pH 7.1.

The viability was measured at 24 h after addition of the inoculum and when the degradation started. Serial dilutions of cells were plated on MSG agar plus PCP (25 mg·ℓ$^{-1}$) and CFU enumerated following 48 h incubation at 30ºC.

Specific rate of PCP degradation

In order to establish how many mg·ℓ$^{-1}$ of PCP had been degraded for each CFU, the specific rate of PCP degradation was calculated as follows:

$$\frac{(\partial \text{ PCP}/\partial t)}{\text{CFU}}$$

where:

PCP is the concentration of PCP in mg·ℓ$^{-1}$

CFU (colony-forming units per mℓ)

t is the time in h.

Results

Influence of pH of the medium on PCP degradation

Four pH values from 5.5 to 8 were investigated (Fig. 1). PCP was degraded rapidly at pH 6.3, 7.1 and 8. At these pH values, PCP degradation was almost the same. However, the PCP degradation at pH 5.5 was slower and the PCP concentration decreased rapidly after 80 h post-inoculation. In control cultures no aromatic ring cleavage occurred.

Pseudomonas sp. cells grew better at pH values within a range of 6 to 8 in MSG-medium than at pH 5.5 (data not shown).

The specific consumption of PCP was analysed (Fig. 2). At pH values of 6.3 the specific rate of PCP degradation was greater than that obtained at pH 5.5, 7.1 or 8. These results showed that each CFU of *Pseudomonas* sp. degraded more PCP per hour at pH 6.3 than at any other pH value.

Effect of inoculum size on PCP degradation

PCP was degraded by *Pseudomonas* sp. during all the initial cell densities tested (Fig. 3). In cultures inoculated with

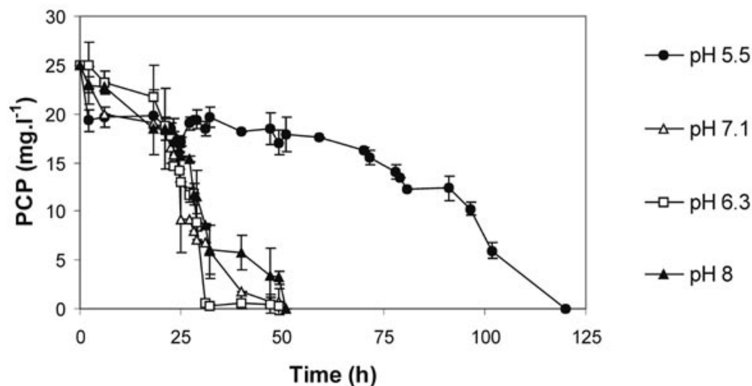
the highest cell density ($4.5x10^7$ CFU·mℓ$^{-1}$) the PCP concentration began to decrease rapidly after 20 h and reached 5 mg·ℓ$^{-1}$ after approximately 70 h. Degradation was complete after 80 h. However, in cultures receiving lower inoculum densities, there was a progressive decrease of PCP concentration. The PCP content began to decrease rapidly after 55 h and degradation was complete after 100 h with $9.04x10^6$ and $1.8x10^6$ CFU·mℓ$^{-1}$. In control cultures no aromatic ring cleavage occurred.

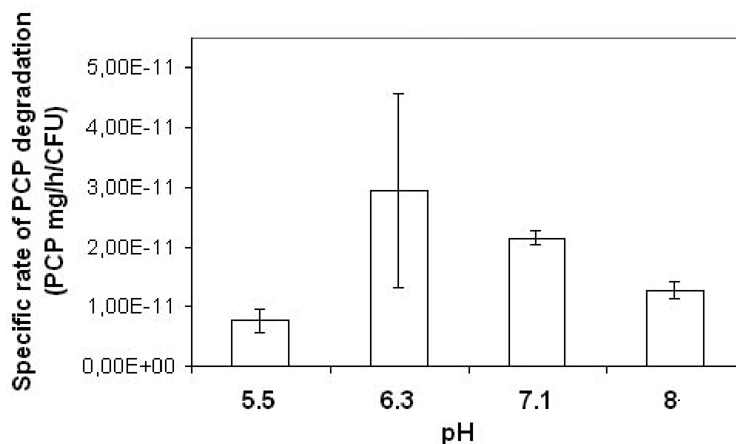
In addition, the specific rate of PCP degradation was tested (Fig. 4). Cultures inoculated with $1.8x10^6$ CFU·mℓ$^{-1}$ (lower inoculum size) showed the highest specific rate of PCP degradation, while the cultures inoculated with a higher inoculum size showed a decrease in specific consumption.

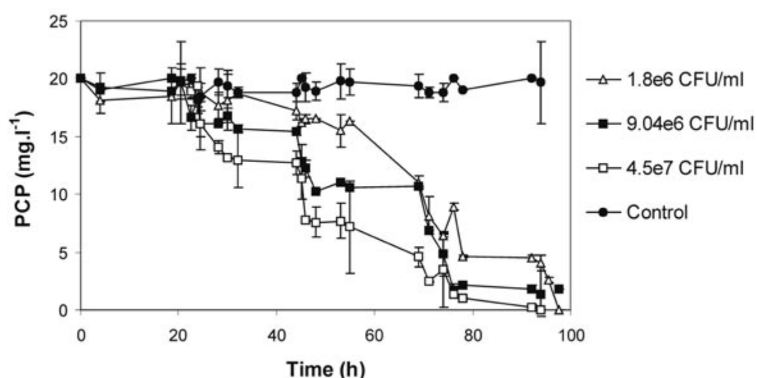
Discussion

Environmental factors such as pH, temperature and other substrates in the environment may affect the growth of micro-organisms and their degradational abilities. In the present study *Pseudomonas* sp. could degrade PCP at a wide range of pH, from 5.5 to 8, with an optimum pH of 6.3. Maximum degradation of all population and maximum specific consumption was at pH 6.3. It is possible that the enzymes for PCP degradation have their optimum enzymatic activity at pH 6.3. At pH values either higher or lower than 6.3, the rate of degradation was low.

The range of pH at which other strains degrade PCP was considerably narrower than the strain analysed in this work. Premalatha and Rajakumar (1994) reported a strain of *Pseudomonas aerugi-*







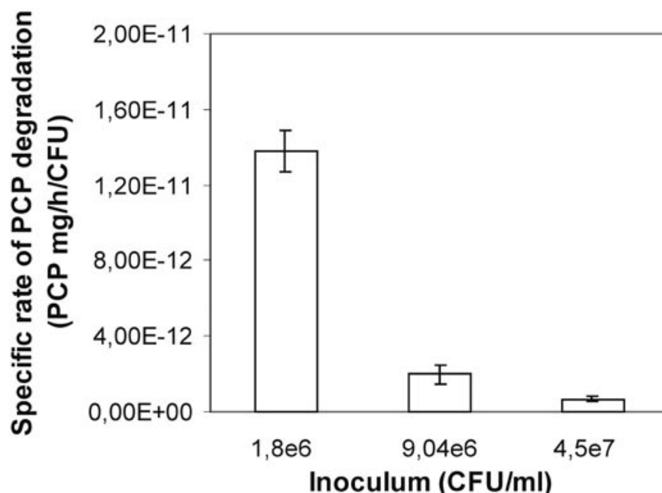


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Figure 1
PCP biodegradation by Pseudomonas *sp. cells at different pH values. The initial PCP concentration was 25 mg·ℓ$^{-1}$. PCP degradation was assessed by measuring the cleavage of the aromatic ring at A_{320nm} (PCP A_{320}=1 correspond to 50 mg·ℓ$^{-1}$). Results are means ± SD (n=4).*

Figure 2
Influence of pH of the medium on the specific rate of PCP degradation. The specific rate of PCP degradation is expressed as PCP mg·h^{-1}·CFU^{-1}. Results are means ± SD (n=4).

Figure 3
Effect of inoculum size on PCP degradation. Three different amount of inoculum were tested. The inoculum size is expressed as CFU·mℓ$^{-1}$in the final medium. The initial PCP concentration was 20 mg·ℓ$^{-1}$. MS medium without cells was used as a control. Results are means ± SD (n=3).

Figure 4
Relation between the specific rate of PCP degradation by Pseudomonas *sp. and inoculum size. The inoculum size is expressed as CFU·mℓ$^{-1}$in the final medium. The specific rate of PCP degradation is expressed as PCP mg·h^{-1}·CFU^{-1}. Results are means ± SD (n=3).*

nosa that degraded PCP at pH of between 7 and 8. Degradation decreased slowly above pH 8, and below pH 7 PCP was toxic to *Pseudomonas aeruginosa*. In addition, PCP degradation by *Flavobacterium* sp. (González and Hu, 1991) decreased considerably below pH 6.9. The acidification of natural environments may enhance the toxicity of PCP (Rutgers et al., 1998). Therefore, it is very important to find strains that degrade PCP at low pH. The strain of *Pseudomonas* studied in this work was able to degrade the PCP of the medium completely at pH 5.5, after a lag time. In addition, the lag phase of batch cultures was found to depend on PCP concentration and prior adaptation of the inoculum (unpublished data).

Interaction between micro-organisms responsible for degradation and transport of phenols/chlorophenols in the environment has been difficult to understand. This interaction is relevant, not only because it occurs before the metabolic reaction, but also because it is involved in the movement of phenol and chlorophenol in the environment. Some reports describe the biosorptive uptake of PCP on suspended biomass as being primarily influenced by pH. Brandt et al. (1997) corroborated the pH effect on the biosorptive uptake of PCP by *Mycobacterium chlorophenolicum* PCP-1. Antizar-Ladislao and Galil (2004) reported that an increase in pH value decreased the equilibrium sorption. This observation may be because as the pH increased, the overall surface charge on the cells became negative and this led to a lower electrostatic attraction between negatively charged phenol/chlorophenol and binding sites of the biomass surface. Daughney and Fein (1998) showed that an increase in pH from 5 to 9 showed a decrease in the percentage of 2, 4, 6 -TCP sorbed by *Bacillus subtilis*. It is known that there is some relationship between biosorption and biodegradation. Our results are in agreement with reports mentioned above and are correlated with the fact that there would be an increase in biosorption and in the specific rate of PCP degradation at pH 6.3 in *Pseudomonas* sp.

On the other hand, *Pseudomonas* sp. cells grew better at pH values within a range of 6 to 8 in MSG-medium than at pH 5.5 (data not shown). The same behaviour was described by Tsuji et al. (1982). The dependence of pH on growth rate may be explained as described by Tyler and Finn (1974) and Rutgers et al. (1998); at a given pH there is a certain amount of substrate present in dissociated and undissociated form. The undissociated form is the species that is believed to penetrate the cell membrane and to be responsible for both metabolic and inhibitory activity. Antizar-Ladislao and Galil (2004) reported that the pH primarily affects the degree of ionisation of phenol/chlorophenol and the surface properties of the biomass. At the pH range 6 to 9 the overall surface charge of the biomass was negative and this led to a lower electrostatic attraction between negatively charged PCP and binding sites of the biomass surface. These authors report a pK_a value of 6.59 for PCP. This could explain the lower growth rate of *Pseudomonas* sp. at pH 5.5 than at higher pHs in our work.

PCP degradation by *Pseudomonas* sp. increases linearly with the increase of inoculum size (Fig. 3), such as described by Shaw et al. (1997) for a PCP mineralising *Pseudomonas* sp. UG 30 and by Bidlan and Manonmani (2002) for a strain of *Serratia marcescens* DT-1P that degrades dichlorophenyltrichloroethane.

An effective bacterial inoculum able to tolerate and degrade high levels of PCP was $4.5x10^7$ CFU·mℓ$^{-1}$. However, our results show that an increase in the amount of inoculum did not improve the specific rate of PCP degradation. More research is needed to prove the possible existence of self-regulation in the *Pseudomonas* sp. population.

Conclusions

Our study showed that changes in the pH values of the culture mediums, and changes in the inoculum size affected the specific rate of PCP degradation by *Pseudomonas* sp. A pH value of 6.3 and an inoculum size of $4.5x10^7$ CFU·mℓ$^{-1}$were the optimal conditions to obtain the maximal degradation of PCP in this batch experimental system.

The strain studied in the work reported here was able to degrade PCP over a wide range of pHs, particularly at pH as low as 5.5. This is an important characteristic for the bioremediation of changeable environments.

Another feature worth mentioning is that this particular strain had shown that it was capable of degrading phenol, lower substituted chlorophenols and hexadecane (Murialdo et al., 2003). This final compound is very important because it is co-substrate of PCP and produces an increase of biomass.

The information provided here can be used to optimise degradation conditions in the field, by adjusting the pH to provide the inoculum with a competitive advantage over the natural flora. The inoculum size is another tool to achieve a good rate of PCP degradation, since usually, when remediating contaminated soils, the cost of growing the inoculum is lower than the cost of soil movement.

Preliminary identification of this strain has been carried out and further work continues on their characterisation. However, more research is necessary to understand the mechanisms of microbial degradation and to improve the conditions for *in situ* bioremediation of PCP-contaminated soils.

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