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ORIGINAL PAPER

Aerobic degradation of 3-chlorobenzoic acid by an indigenous strain isolated from a polluted river

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Abstract An indigenous strain of *Pseudomonas putida* capable of degrading 3-chlorobenzoic acid as the sole carbon source was isolated from the Riachuelo, a polluted river in Buenos Aires. Aerobic biodegradation assays were performed using a 2-l microfermentor. Biodegradation was evaluated by spectrophotometry, chloride release, gas chromatography and microbial growth. Detoxification was evaluated by using *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Lactuca sativa* as test organisms. The indigenous bacterial strain degrades 100 mg l^{-1} 3-chlorobenzoic acid in 14 h with a removal efficiency of 92.0 and 86.1% expressed as compound and chemical oxygen demand removal, respectively. The strain was capable of degrading up to 1,000 mg of the compound l^{-1}. Toxicity was not detected at the end of the biodegradation process. Besides initial concentration, the effect of different factors, such as initial pH, initial inoculum, adaptation to the compound and presence of other substrates and toxic related compounds, was studied.

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Keywords 3-Chlorobenzoic acid · Biodegradation · Detoxification · Indigenous strain

Introduction

Chlorinated aromatic compounds pose one of the most serious contemporary environmental problems worldwide, because they have been used in large quantities as herbicides, pesticides, and solvents (Ogawa and Miyashita 1995; Field and Sierra-Alvarez 2008). 3-Chlorobenzoic acid, specifically, is employed as raw material in the formulation of dyes, pharmaceuticals, fungicides and as a preserving agent for adhesives and paints; like other chlorobenzoates it can originate as dead-end product of the cometabolism of PCBs and chlorotoluenes (Baggi and Zangrossi 2001; Zhang et al. 2009) and in the process of water chlorination. Because of its widespread use, 3-chlorobenzoic acid can be found as a contaminant in industrial effluents, rivers and groundwaters. 3-Chlorobenzoic acid has been studied as a model chemical for biodegradation of halogenated aromatic compounds (Haby and Crowley 1996; Ledger et al. 2009). A half-life of 38, 75 and 340 days was estimated for the compound in water, soil and sediments, respectively, using EPA's PBT profiler (EPA 2006). Despite this, several microorganisms able to degrade the compound have been isolated (Hernández et al. 1991; Fulthorpe et al. 1998; Krooneman et al. 2000; Qi et al. 2009).

Developing countries are more likely to suffer the consequences of contamination, due to the lack of regulations or for the inobservance of them. In Argentina it is not unusual that industrial effluents are discharged into streams only slightly treated or even untreated. The selection of indigenous strains able to degrade xenobiotic compounds is therefore an area of interest, since biological remediation

methods are often cheaper and more environmentally friendly than their physical or chemical counterparts (Tamer et al. 2006). Unfortunately effluents and polluted sites can have variable conditions that may affect the survival of the strains employed in bioaugmentation processes. Moreover in some cases a partial transformation of the parent compound can occur, giving rise to the formation of more toxic intermediates (Zhang and Bennett 2005).

The purpose of the present investigation was the isolation of an indigenous bacterial strain able to use 3-chlorobenzoic acid as the sole carbon source and the study of the biodegradation process in aerobic reactors. In particular, the biodegradation study addressed the influence of concentration of compound, pH, cell inoculum, adaptation to the compound and presence of other substrates and related compounds. These parameters were chosen for their significance in any application of biodegradation to the removal of chlorinated compounds from wastewater or other polluted environments. Bioassays of toxicity were likewise performed to evaluate detoxification.

Materials and methods

Selection and identification of 3-chlorobenzoic-acid-degrading bacteria

An aerobic feed batch reactor was inoculated with samples from a polluted Buenos Aires river. The reactor had an effective volume of 1 l and was operated under continuous aeration. The culture medium employed for the enrichment was a synthetic minimal medium (Korol et al. 1989) containing 3-chlorobenzoic acid (50 mg l^{-1}) as the sole carbon source. The carbon source was added periodically to the reactor in order to restore the initial concentration of 50 mg l^{-1}.

The mixed cultures resulting from this enrichment were streaked onto tryptone soy agar medium (Merck, Darmstadt, Germany) supplemented with 20 mg of 3-chlorobenzoic acid l^{-1} with the purpose of maintaining a selective pressure on bacterial strain and avoiding the loss of genes responsible for compound catabolism.

An isolated pure culture capable of degrading 3-chlorobenzoic acid was examined for reaction to Gram staining and identified using the API system (Bio Mérieux, L' Étoile, France) and methods based on 16S rRNA gene sequence.

Gene sequence of the strain was determined by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA was extracted by conventional protocols (Reilly and Attwood 1998). PCR amplification of 16S rRNA gene fragment was performed as described by Greisen et al. (1994) in a GeneAmp 9600 (Perkin-Elmer, USA.) thermal cycler. The amplified products were purified using a PCR purification kit (QIAquick®, Qiagen Hilden, Germany). The amplicons were sequenced using a Big Dye Terminator System kit (Applied Biosystems, Foster City, USA). The 16S rRNA sequence was compared against the GenBank database using the National Center for Biotechnology Information (NCBI) BLAST program.

In order to evaluate the ability of the strain to use different organic compounds as the sole carbon source, assays were carried out using synthetic minimal medium supplemented with 50 mg of the corresponding organic compound l^{-1}.

Chemicals

3-Chlorobenzoic acid was of analytical grade and purchased from Fluka (Buchs, Switzerland). All the other chemicals were of analytical reagent grade and purchased from Mallinckrodt Chemical Co. (St. Louis, USA), and Merck (Darmstadt, Germany). 3-Chlorobenzoic acid was prepared aseptically by dissolving the necessary amount in sterile 0.1 M NaOH. Stock solutions of phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol and 4-chlorobenzoic acid were prepared in the same way.

Biodegradation test

Bacteria were adapted by inoculation in synthetic minimal medium supplemented with 100 mg of 3-chlorobenzoic acid l^{-1} as the sole carbon source, and incubated in a rotatory shaker at 28°C overnight (stock culture).

Biodegradation assays were performed in New Brunswick Multigen TA microfermentors, aerobically operated at 28°C with effective volumes of 1,250 ml of the same medium supplemented with the corresponding amount of 3-chlorobenzoic acid as carbon source according to the specific assay. Initial pH was 7. The system was inoculated with 5 ml of the stock culture (final concentration 10^6 cells ml^{-1}). During incubation, 10 ml samples were removed from the system at appropriate intervals in order to determine the amount of residual 3-chlorobenzoic acid and evaluate microbial growth. To estimate the abiotic loss of the compound, controls in similar microfermentors, without inoculation, were carried out.

To assess the effect of different factors on the biodegradation process, assays were carried out changing the initial conditions and always comparing results with the assay at pH 7 with 100 mg of 3-chlorobenzoic acid l^{-1} as the sole carbon source.

In the experiments at different values of initial concentration, the corresponding amount of the compound was added to minimal medium to obtain the desired concentration.

In the same manner, in assays at different initial pH values, phosphoric acid or 10% NaOH was employed to adjust the pH of the minimal medium. A ten-fold dilution of the stock culture in sterile synthetic minimal medium was employed in the low-inoculum assay. The adaptation step was eliminated in the experiment to prove its influence. In the corresponding assays, glucose and sodium acetate were added from filter-sterilized stock solutions as additional carbon sources at final concentrations of 100 mg l^{-1}. Phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol or 4-chlorobenzoic acid were added from stock solutions as toxic related compounds at final concentrations of 10 mg l^{-1}.

Analytical methods and control parameters

To determine the amount of residual 3-chlorobenzoic acid, bacterial cells were separated by centrifugation, and the filtered supernatant fluid was submitted to spectrophotometrical analysis (Metrolab UV 1700 Spectrophotometer); the absorbance was measured at 230 nm. Chemical oxygen demand (COD) and chloride were measured in samples taken at the beginning and at the end of the process according to APHA (2005).

To assess mineralization, selected samples were analysed by gas chromatography. A Hewlett-Packard model 6890 gas chromatograph equipped with a Model 5972 mass selective detector was used. Separations were obtained using a capillary column (HP-5MS, 30 m × 0.25 mm, 0.25 μm film) with He as carrier gas and a temperature gradient of 120–290°C, increasing at 15°C min^{-1}.

Determination of cell viability was performed by spreading sample dilutions on the surface of tryptone soy agar plates (APHA 2005).

Toxicity tests

Detoxification was evaluated by *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Lactuca sativa* toxicity tests. Toxicity tests were performed in samples taken at the beginning and at the end of the biodegradation process.

Luminescent bacteria test

The Microtox® acute toxicity test uses *Vibrio fischeri*, a marine luminescent bacterium, (strain NRRL B-11177) as the test organism. The bacterial strain was purchased from Strategic Diagnostic Inc. (Carlsbad, CA, USA) as a freeze-dried form. The Microtox® Model 500 Toxicity Analyzer (Azur Environmental, Carlsbad, CA, USA) was used to make the Microtox® test. Toxicity was assessed by measuring the inhibition of light emission of *Vibrio fischeri*. The bacteria were exposed to a series of sample dilutions for 15 min at 15 ± 1°C, according to ISO 11348–3 (1998). Results are expressed in effective concentration 50 (EC_{50}), concentration which provokes a 50% reduction in bacterial light emission relative to the test control after a period of exposure of 15 min. EC_{50} was determined using MicrotoxOmni® software. Before assays were performed, the organism sensitivity was evaluated using phenol as the reference toxic compound.

Alga test

Toxicity was assessed by measuring growth inhibition of the alga *Pseudokirchneriella subcapitata*, formerly *Selenastrum capricornutum* exposed for 72 h, according to ISO 8692 (2004).

Pseudokirchneriella subcapitata growth inhibition tests were performed at 23 ± 2°C, under continuous white light conditions. Results are expressed in effective concentration 50 (EC_{50}), concentration which provokes a 50% reduction in alga growth relative to control after a period of exposure of 72 h. Algal cell concentration was measured by direct enumeration, EC_{50} was estimated by graphical method. Before assays were performed, the organism sensitivity was evaluated; potassium dichromate was used as the reference toxic compound.

Seed test

Toxicity was assessed by measuring reduction in root elongation of *Lactuca sativa* according to EPA/600/3-88 (1989). Lettuce seeds were exposed to different sample concentrations. Ten lettuce seeds were placed in Petri dishes with one dish for each concentration on wet filter papers for 120 h at 24 ± 2°C in the dark. Results are expressed in effective concentration 50 (EC_{50}), concentration which provokes a 50% inhibition of lettuce root elongation compared to control after the 120 h period of exposure. Before assays were performed, the organism sensitivity was evaluated using zinc sulfate as the reference toxic compound.

Results and discussion

Identification of 3-chlorobenzoic-acid-degrading bacteria

An indigenous bacterial strain capable of degrading 3-chlorobenzoic acid was isolated from the Riachuelo, a polluted Buenos Aires river. The isolated strain was gram-negative, oxidase-positive, motile, did not ferment glucose and was unable to reduce nitrate to nitrite or nitrogen. This indigenous bacterial strain was identified as *Pseudomonas*

putida with a 99.7% probability using the API system. The partial 16S rRNA gene sequence determined confirmed this result, showing a 100% similarity to the prototype strain of *Pseudomonas putida*.

Diverse organic compounds were tested as the sole carbon source for the strain. The strain was able to grow on glucose, acetate, pyruvate, citrate, glutamate, catechol and benzoate, but it was not able to grow on other aromatic compounds such as: phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-tricholophenol, pentachlorophenol and 4-nitrophenol. Also 4-chlorobenzoate was not used as the sole carbon source by the strain. The specific degradation of only some chlorobenzoic acid isomers has already been reported. Baggi and Zangrossi (2001) described an *Alcaligenes denitrificans* strain that grew only on 2- or 4-chlorobenzoate, but not on 3-chlorobenzoate. A bacterial strain isolated by Kim and Picardal (2000) degraded only the isomer in position 4. This behavior was attributed to the different specificity of the oxygenases involved in the initial steps of the metabolism of chlorobenzoates (Brenner et al. 1993).

Biodegradation test

Biodegradation assays were carried out with initial inocula of 10^6 cells ml^{-1}. The assays showed that the indigenous strain of *Pseudomonas putida* was capable of degrading 100 mg of 3-chlorobenzoic acid l^{-1} within 14 h, with a specific growth rate (μ) of 0.41 h^{-1} and a lag phase of 4 h (Fig. 1). The removal of the compound reached 92.0% of the initial concentration, and an 86.1% removal of COD. The degradation of 3-chlorobenzoic acid was associated with stoichiometric liberation of chloride (Table 1). The control assay showed the absence of abiotic loss in the system. The absence of metabolites was proved by gas chromatography performed at the end of the process

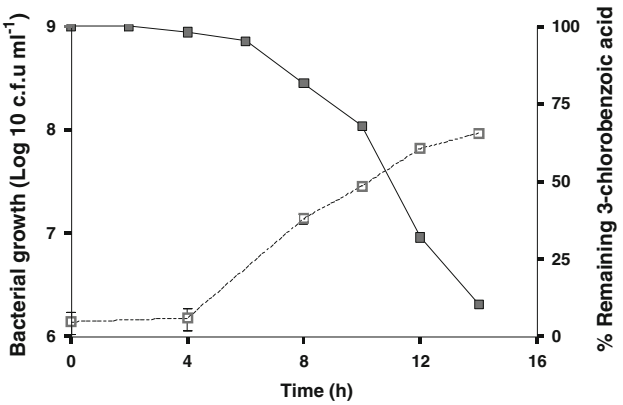

Fig. 1 Degradation of 3-chlorobenzoic acid (100 mg l^{-1}) by *Pseudomonas putida* in synthetic minimal medium. Growth kinetics of the strain (*open square*). Residual 3-chlorobenzoic acid (*filled square*)

Table 1 Biodegradation of 3-chlorobenzoic acid (100 mg l^{-1}) by *Pseudomonas putida*

Parameter (mg l^{-1})	Initial	Final	% Remotion
3-Chlorobenzoic acid	102.5	8.2	92.0
COD	210.0	29.2	86.1
Chloride	ND	20.9	–

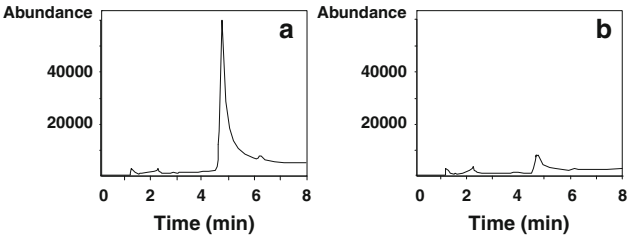

Fig. 2 GC-MS chromatograms 3-chlorobenzoic acid 100 mg l^{-1} **a** initial; **b** final

(Fig. 2). The extension of the incubation time after 14 h did not show changes in bacterial growth and residual compound concentration.

Factors affecting the biodegradation process

In order to assess the effect of different factors on the biodegradation process, assays were carried out changing initial conditions and always comparing results with the assay at pH 7 with 100 mg of 3-chlorobenzoic acid l^{-1} as the sole carbon source.

Initial concentration

The specific growth rate decreased when the concentration increased, as expected for a toxic substrate (Alexander 1999). When the concentration of the compound was increased to 200 and 400 mg l^{-1} the process of biodegradation was accomplished in 22 and 27 h with specific growth rates (μ) of 0.28 and 0.23 h^{-1}, respectively (Fig. 3a). Initial concentrations of 600 and 800 mg l^{-1} were degraded within 54 and 120 h, respectively. The indigenous strain of *Pseudomonas putida* was capable of degrading up to 1,000 mg of 3-chlorobenzoic acid l^{-1}. The process took place in 156 h (data not shown). In all cases the time reported represents the time after which no subsequent changes in bacterial growth and residual compound concentration occured.

Initial pH

Variation of the initial pH between 6 and 9 did not affect the biodegradation process (Fig. 3b). However the degradation was inhibited at an initial pH of 5. Bearing in mind

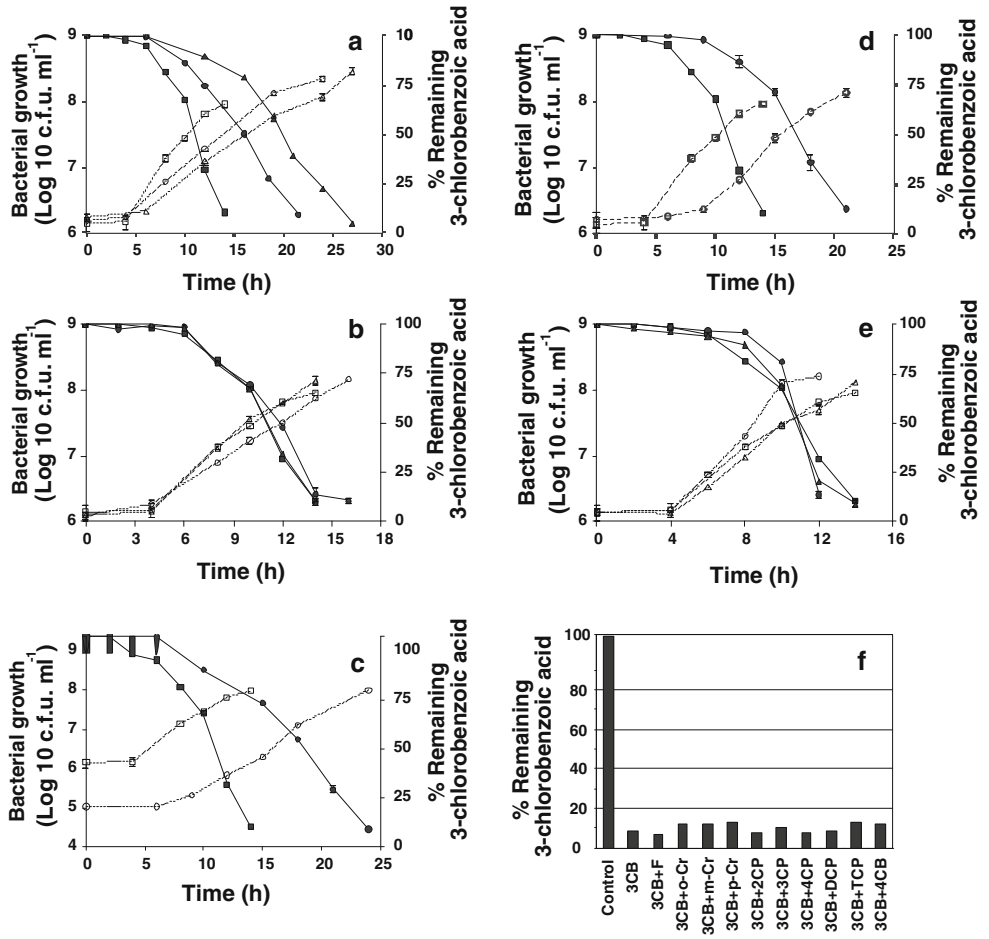

Fig. 3 Factors affecting the degradation process **a**. *Initial concentration*: Growth kinetics with a concentration of: 100 mg l^{-1} (*open square*); 200 mg l^{-1} (*open circle*) and 400 mg l^{-1} (*open triangle*). Residual 3-chlorobenzoic acid in minimal medium with a concentration of 100 mg l^{-1} (*filled square*); 200 mg l^{-1} (*filled circle*) and 400 mg l^{-1} (*filled triangle*) **b**. *Initial pH*: Growth kinetics with initial pH 7 (*open square*); 6 (*open circle*) and 9 (*open triangle*). Residual 3-chlorobenzoic acid in minimal medium with an initial pH of 7 (*filled square*); 6 (*filled circle*) and 9 (*filled triangle*) **c**. *Initial inoculum*: Growth kinetics with initial inoculum 1.1×10^6 (*open square*) and 1.4×10^5 (*open circle*). Residual 3-chlorobenzoic acid in minimal medium with an initial inoculum of 1.1×10^6 (*filled square*) and 1.4×10^5 (*filled circle*) **d**. *Adaptation*: Growth kinetics with adapted (*open square*) and unadapted inoculum (*open circle*). Residual 3-chlorobenzoic acid in minimal medium with adapted (*filled square*) and unadapted inoculum (*filled circle*) **e**. *Simultaneous presence of sodium acetate or glucose*: Growth kinetics with 3-chlorobenzoic acid 100 mg l^{-1}(*open square*); 3-chlorobenzoic acid and glucose 100 mg l^{-1} (*open circle*) and 3-chlorobenzoic acid and sodium acetate 100 mg l^{-1} (*open triangle*). Residual 3-chlorobenzoic acid in minimal medium with 3-chlorobenzoic acid 100 mg l^{-1} (*filled square*); 3-chlorobenzoic acid and glucose 100 mg l^{-1} (*filled circle*) and 3-chlorobenzoic acid and sodium acetate 100 mg l^{-1} (*filled triangle*) **f**. *Simultaneous presence of toxic related compounds*: Residual 3-chlorobenzoic acid at 24 h without inoculum (Control); with 3-chlorobenzoic acid 100 mg l^{-1} (3CB), with 3-chlorobenzoic acid 100 mg l^{-1} and 10 mg l^{-1} of phenol (3CB + F), *o*-cresol (3CB + *o*-Cr), *m*-cresol (3CB + *m*-Cr), *p*-cresol (3CB + *p*-Cr), 2-chlorophenol (3CB + 2CP), 3-chlorophenol (3CB + 3CP), 4-chlorophenol (3CB + 4CP), 2,4-dichlorophenol (3CB + DCP), 2,4,6-trichlorophenol (3CB + TCP) and 4-chlorobenzoic acid (3CB + 4CB)

that the compound is a weak acid, at low pH values an increase of the non-ionized form, usually more toxic, is expected, since it can better move across membranes and enter the cell (Stanlake and Finn 1982).

Initial inoculum

With an initial inoculum of 1.4×10^5 cells, the specific growth rate (μ) was of the same order of that obtained with 1.1×10^6 cells ($\mu = 0.36\ h^{-1}$). The lag time increased to 6 h and the process took place in 24 h (Fig. 3c).

Adaptation

The absence of the adaptation step resulted in a duplication of the lag phase, which increased to a value of 8 h. Simultaneously, the specific growth rate (μ) decreased to 0.25 h^{-1} (Fig. 3d). Despite this delay, the indigenous strain

was able to metabolize 100 mg of 3-chlorobenzoic acid l^{-1} within 22 h, proving the stability of the genes involved in the catabolism. Bacterial adaptation to a compound is reflected not only in its specific growth rate, but also in its stability (Hoffmann et al. 2003).

Presence of other substrates and related compounds

The simultaneous presence of glucose (100 mg l^{-1}) or sodium acetate (100 mg l^{-1}) in the culture medium did not affect the efficiency of the biodegradation process (Fig. 3e). Nor did the presence of toxic related compounds like phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol or 4-chlorobenzoic acid in concentrations of 10 mg l^{-1} inhibit the degradation of 3-chlorobenzoic acid (100 mg l^{-1}) (Fig. 3f).

Therefore the behavior of the indigenous strain supports the possibility of its utilization in effluent treatment or bioremediation processes, since in wastewaters and contaminated sites the target compound is usually present as a complex mix with other easily degradable organic compounds and toxic xenobiotics that may affect their degradation (Wang and Loh 2000; Schmidt et al. 1987).

Despite the high 3-chlorobenzoic acid removal rates, a final residual concentration of 8 mg l^{-1} was obtained in all assays. It has been described that the accumulation of chlorocatechols may have an inhibitory effect when 3-clorobenzoic acid is degraded via the catechol pathway (Perez Pantoja et al. 2003). Although metabolites were not detected by gas chromatography, two additional experiments were carried out in order to reject the hypothesis of the accumulation of a toxic product. In the first of them, in the same culture medium, a new amount of 100 mg of 3-chlorobenzoic acid l^{-1} was added to stationary phase cells from a culture of *Pseudomonas putida* which had grown at an initial concentration of 100 mg of the compound l^{-1}. The cells started to grow again and the same residual amount of 8 mg l^{-1} was obtained. In the second experiment, the initial concentration of the substrate was reduced to 20 mg l^{-1}. In this case, the same final concentration of 8 mg l^{-1} was also obtained (data not shown).

Another explanation for the residual substrate amount could be that the indigenous strain of *Pseudomonas putida* has a high threshold concentration for the compound. Threshold concentration was defined as the lower concentration necessary to support the growth of a bacterium (Alexander 1999). Tros et al. (1996) have described in *Pseudomonas* sp. B13 two systems for the uptake and utilization of 3-chlorobenzoic acid. The first of them, with high capacity and low affinity, operates at high substrate concentrations. The other, with low capacity but high affinity, was triggered at low concentrations. The absence or loss of a high affinity system resulting from the high concentrations present during the selection stage could be the cause of the high threshold concentration for the compound.

Table 2 EC_{50} (% v/v) values obtained in toxicity tests of samples taken at the beginning and the end of the biodegradation of 3-chlorobenzoic acid (400 mg l^{-1}) by *Pseudomonas putida*

Bioassay (% v/v)	Initial	Final
Vibrio fischeri EC_{50} 15 min	59.6	>90
Pseudokirchneriella supcapitata EC_{50} 72 h	22.0	>90
Lactuca sativa EC_{50} 120 h	15.6	>90

Toxicity test

Monitoring the loss of compounds initially present does not assure that complete detoxification has occurred, because of the appearance and potential toxicity of by-products of incomplete degradation and unknown interaction effects (Renoux et al. 1999). Biological assays complemented the physicochemical methods employed to evaluate wastewater quality and were recommended for the monitoring of treatment processes (EPA 1994). Whole Effluent Toxicity refers to the aggregate toxic effect from all pollutants contained in an effluent. In this kind of assay the effluent, or in this case, samples taken at the end of the batch process, are diluted sequentially in order to establish the dilution that provokes a 50% inhibitory effect. A standard test organism is employed and the dilution is expressed in terms of % volume/volume. The higher the EC50%, the less the toxicity. A value of EC50 higher than 100% shows the lack of toxicity of the sample. It should be mentioned that by means of experimental design certain dilution is always carried out, for example in order to add the inoculum of the standard test organism to the sample, and then the higher concentration in the assay is usually below 100%. In the assays detailed below the higher concentration tested was of 90%. A value of EC50 higher than 90% refers then to a non-toxic sample. Toxicity was not detected at the end of the biodegradation process demonstrating the ability the indigenous bacterial strain has to detoxify the compound. Table 2 shows result obtained with an initial concentration of 400 mg of 3-chlorobenzoic-acid l^{-1}. Our toxicity test results corroborated the analytical data of 3-chlorobenzoic acid disappearance and the absence of toxic metabolites. The utilization of organisms from different trophic levels in toxicity tests helps to better evaluate an effluent, since each organism may differ in its sensibility to different toxic compounds. (Hernando et al. 2005).

Conclusions

Despite the high extent of removal of the compound, over 90%, a final residual concentration of 8 mg l^{-1} was obtained in all the assays. Threshold concentrations are frequently in the order of μg l^{-1} but higher concentrations have also been reported. For example threshold concentrations of 40 mg l^{-1} were obtained for the biodegradation of PCB (Cho et al. 2003). Toxic compounds like 3-chlorobenzoic acid may be inhibitory even to strains capable of degrading them (Uysal and Türkman 2005). The expression and induction of catabolic genes for the metabolism of such compounds may be understood not just as a way to obtain energy but also as a defense mechanism (Benndorf and Babel 2002). Taken this into account, it is not surprising that the process was not affected by the simultaneous presence of other easily degradable organic compounds or that biodegradation ceased when detoxification was achieved. The biodegradation of 3-chlorobenzoic acid by *Pseudomonas putida* should not then be considered as an inefficient catabolic pathway because some nondegraded substrate remains, on the contrary, it should be looked upon as a sophisticated mechanism of detoxification which simultaneously serves to produce energy.

Effluents and polluted sites have variable conditions that may affect the survival of the strains employed in bioaugmentation processes. In such environments the pH and concentrations of the target compound may vary. Moreover, the pollutant can often be spilled in a discontinuous way or as part of a complex mix. Thus the ability of the isolated indigenous *Pseudomonas putida* strain to tolerate the different factors assayed represents a promising feature for its possible use to improve the treatment of wastewaters containing 3-chlorobenzoic acid and to bioremediate 3-chlorobenzoic acid-polluted sites.

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References

Alexander M (1999) Biodegradation and bioremediation, 2nd edn. Academic Press, London

APHA (American Public Health Association) AWWA (American Water Works Association) and WEF (Water Environmental Federation (2005). Standard methods for the examination of water and wastewater, 21th edn. Washington DC

Baggi G, Zangrossi M (2001) Assessment of the biodegradative potential versus chlorobenzoates as single or mixed compounds in a stable microbial consortium. Annu Rev Microbiol 51: 179–188

Benndorf D, Babel W (2002) Assimilatory detoxification of herbicides by *Delftia acidovorans* MC1: induction of two chlorocatechol 1, 2-dioxigenases as a response to chemostress. Microbiology 148:2883–2888

Brenner V, Hernández BS, Focht DD (1993) Variation in Chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. Appl Environ Microbiol 59:2790–2794

Cho YC, Sokol RC, Frohnhoefer RC, Rhee GY (2003) Reductive dechlorination of polychlorinated biphenyls: threshold concentration and dechlorination kinetics of individual congeners in Aroclor 1248. Environ Sci Technol 37:5651–5656

EPA (US Environmental Protection Agency) (1989) Protocols for short term toxicity screening of hazardous waste sites. A.8.7. Lettuce root elongation (*Lactuca sativa*). EPA 600/3-88/029

EPA (US Environmental Protection Agency) (1994) Using toxicity test in ecological risk assessment. ECO Update, vol 2. Number 1. Office of solid waste and emergency response. Office of emergency remedial response. Hazardous site evaluation division (5204G) Publication 9345.0-051

EPA (US Environmental Protection Agency) (2006) Office of pollution prevention and toxics. PBT profiler. Available at http://pbtprofiler.net. Last updated 21 Sep 2006

Field J, Sierra-Alvarez R (2008) Microbial degradation of chlorinated phenols. Rev Environ Sci Biotechnol 7:211–241

Fulthorpe RR, Rhodes AN, Tiedje JM (1998) High level of endemicity of 3-chlorobenzoate-degrading soil bacteria. Appl Environ Microbiol 64:1620–1627

Greisen K, Loeffelholz M, Purohit A, Leong D (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32:335–351

Haby PA, Crowley DE (1996) Biodegradation of 3-chlorobenzoate as affected by rhizodeposition and selected carbon substrates. J Environ Qual 25:304–310

Hernández BS, Higson FK, Kondrat R, Focht DD (1991) Metabolism and inhibition by Chlorobenzoates in *Pseudomonas putida* P111. Appl Environ Microbiol 57:3361–3366

Hernando MD, Fernández-Alba AR, Tauler R, Barceló D (2005) Toxicity assays applied to wastewater treatment. Talanta 65:358–366

Hoffmann D, Kleinsteuber S, Müller RH, Babel W (2003) A transposon encoding the complete 2, 4-dichlorophenoxyacetic acid degradation pathway in the alkalitolerant strain *Delftia acidovorans* P4a. Microbiology 149:2545–2556

ISO (International Organization for Standardization) (1998) 11348-3 Water quality. Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)

ISO (International Organization for Standardization) 8692 (2004) Water quality. Freshwater algal growth inhibition test with unicellular green algae

Kim S, Picardal FW (2000) A novel bacterium that utilizes monochlorobiphenyls and 4-chlorobenzoate as growth substrates. FEMS Microbiol Lett 185:225–229

Korol S, Orsingher M, Santini P, Moretton J, D'Aquino M (1989) Biodegradation of phenolic compounds II. Effects of inoculum, xenobiotic concentration and adaptation on *Acinetobacter* and *Pseudomonas* phenol degradation. Rev Lat Am Microbiol 31:117–120

Krooneman J, Sliekers O, Teresa M, Gomes P, Forney LJ, Gottschall JC (2000) Characterization of 3-chlorobenzoate degrading aerobic bacteria isolated under various environmental conditions. FEMS Microbiol Ecol 32:53–59

Ledger T, Aceituno F, Gonzáles B (2009) 3-Chlorobenzoate is taken up by a chromosomally encoded transport system in *Cupriavidus necator* JMP134. Microbiology 155:2757–2765

Ogawa N, Miyashita K (1995) Recombination of a 3-chlorobenzoate catabolic plasmid from *Alcaligenes eutrophus* NH9 mediated by direct repeat elements. Appl Environ Microbiol 61:3788–3795

Perez Pantoja D, Ledger T, Pieper DH, González B (2003) Efficient turnover of chlorocatechols is essential for growth of *Ralstonia eutropha* JMP134 (pJP4) in 3-chlorobenzoic acid. J Bacteriol 185:1534–1542

Qi Y, Zhao L, Tan X (2009) Cometabolism and immobilized degradation of monochlorobenzoate by *Rhodococcus erythropolis*. Afr J Microbiol Res 3:482–486

Reilly K, Attwood GT (1998) Detection of *Clostridium proteoclasticum* and closely related strains in the rumen by competitive PCR. Appl Environ Microbiol 64:907–913

Renoux AY, Millette D, Tyagi RD, Samson R (1999) Detoxification of fluorene, phenanthrene, carbazole and p-cresol in columns of aquifer sand as studied by the microtox® assay. Water Res 33:2045–2552

Schmidt SK, Scow KM, Alexander M (1987) Kinetics of p-nitrophenol mineralization by a *Pseudomonas* sp.: effects of second substrates. Appl Environ Microbiol 53:2617–2623

Stanlake GJ, Finn RK (1982) Isolation and characterization of a pentachlorophenol degrading bacterium. Appl Environ Microbiol 44:1421–1427

Tamer E, Hamid Z, Aly MA, Ossama ET, Bo M, Benoit G (2006) Sequential UV-biological degradation of chlorophenols. Chemosphere 63:277–284

Tros ME, Schraa O, Zehnder JB (1996) Transformation of low concentrations of 3-chlorbenzoate by *Pseudomonas* sp. Strain B13: kinetics and residual concentrations. Appl Environ Microbiol 62:437–442

Uysal A, Türkman A (2005) Effect of biosurfactant on 2, 4-dichlorophenol biodegradation in an activated sludge bioreactor. Process Biochem 40:2745–2749

Wang SJ, Loh KC (2000) Growth kinetics of *Pseudomonas putida* in cometabolism of phenol and 4-chlorophenol in the presence of a conventional carbon source. Biotechnol Bioeng 68:437–447

Zhang C, Bennett GN (2005) Biodegradation of xenobiotics by anaerobic bacteria. Appl Microbiol Biotechnol 67:600–618

Zhang G, Yang X, Xie F, Chao Y, Qian S (2009) Cometabolic degradation of mono-chloro benzoic acids by *Rhodococcus* sp. R04 grown on organic carbon sources. World J Microbiol Biotechnol 25:1169–1174