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Comparison of p-toluenesulphonic acid degradation by two Comamonas strains

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p-Toluenesulphonic acid was degraded at equal rates by two Comamonas testosteroni T-2 and L-1 strains in shake flasks. However, immobilised cells of strain T-2 in a bioreactor had a degradation efficiency of up to 70% compared to 59% with strain L-1. In both open systems, initial added strains were the only p-toluenesulphonate consumers, but the composition of biofilm microbial population significantly varied.

Introduction

Aromatic hydrocarbons, halogenated and sulphonated aromatics and aliphatics, are widely used in chemical, surfactant, dyestuff, packaging and wood industries (Winter and Zimmerman, 1992). Many of these compounds are xenobiotic and pass through conventional treatment plants. The use of appropriate technology (e.g. advanced biofilm systems) coupled with suitable selection of specially adapted microbial communities has been shown to be fruitful in xenobiotic removal. In recent decades significant progress has been achieved in isolation and characterisation of xenobiotic degrading microorganisms. Two approaches are used in specialised biomass development (Horowitz and Vilker, 1994): the most frequently used is the development of an unidentified culture which can degrade specific compounds. The second approach is the development of a defined culture with proven capability of degrading target compounds. Screening allows the isolation of xenobiotic-degrading bacteria from soils, activated sludges or other systems already polluted by specific compounds. An extensive literature is available on enrichment, isolation and characterisation of microorganisms with degradation capacities (Egli, 1992; Kamely *et al.*, 1990; Neilson *et al.*, 1985). Microorganism selection is still one of the major problems to solve in biodegradation processes for waste treatment. Selected strains should meet the necessary requirements for xenobiotic degradation: to have high activity and affinity in the target compound degradation, to work with low concentration in the case of low aqueous compound solubility, to tolerate possible toxicity, high salinity, pH or temperature gradients of waste streams and to have low biomass yields within regard to sludge production minimisation.

In this article, we report on the biodegradation of *p*-toluenesulphonate by two bacterial strains in suspension and immobilised cultures. The comparison has been done with a view to find the criteria of strain selection for further applications.

Materials and methods

Microorganisms and culture medium

Comamonas testosteroni T-2 was obtained from Institute of Microbiology (ETH Zürich, Switzerland) was grown in a buffered (40 mM potassium phosphate buffer pH 7.2) mineral salts medium supplemented with trace elements and 6 mM *p*-toluenesulphonic acid (*p*-TS) as single carbon source (Thurnheer, 1988). The other *p*-TS degrading bacterium was isolated from activated sludge taken from the municipal sewage treatment plant Vidy (Lausanne, Switzerland). After 2 weeks of enrichment (incubation on the shaker at 30°C), samples of suspension culture were spread on agar plates with *p*-TS. Bacterial population was examined by plating and light microscopy. Individual colonies were transferred to agar plates with selective (mineral salts medium with *p*-TS) and non-selective rich media Tryptic Soy Agar (TSA) for following identification. The standard 96 well microplates (Biolog Inc., USA) preloaded with metabolic tests were applied for characterisation and identification of bacteria. Fatty acid analysis (Microbial Identification System Inc., USA) of newly isolated strain was carried out in LMG Culture Collection, Laboratotium voor Microbiology, University of Gent, Belgium.

Bacterial cultures were maintained in liquid mineral salts medium. Cells were harvested at mid-exponential phase by centrifugation (15 min at 10,000 g), washed,

and resuspended in the desired medium. Chemicals purchased from Fluka were of reagent grade. *p*-Sulfobenzoate (*p*-SB) was obtained from Aldrich.

Enumeration of bacteria in bioreactors was performed by means of standard plate count technique. Samples were diluted with sterile 0.9% NaCl solution and plated using a spiral plater D (Spiral systems, USA) on selective (sulphoaromatic compound as a carbon source) and non selective plate count agar (PCA) media. Results were expressed in colony formation units (CFU).

Preparation of cell suspensions

For short term experiments culture cell suspensions were prepared as follows. Cells grown on mineral medium were separated by centrifugation (15 min at 10,000 g), washed twice in 0.05 M Na-K-phosphate buffer (pH = 7.0) and resuspended in mineral medium without carbon and nitrogen sources. This suspension was incubated on the shaker at 30°C for 10 hours. Cells were then separated, washed and resuspended in fresh medium with 0.25% (w/v) NH_4Cl. Finally, *p*-TS was added to cell suspensions to attain concentrations of about 10 mM.

Analytical methods

All samples were filtered through 0.45 μm porosity membrane filter. Concentration of *p*-TS and *p*-sulphobenzoate (*p*-SB) were routinely determined by an HPLC at 264 nm. Mobile phase was 100 mM potassium phosphate buffer (pH = 7.0) and 40% (v/v) methanol in double distilled water. The detection limit was 0.1 mg L^{-1}. Dissolved organic carbon was determined using a Total Organic Carbon analyser TOC 500. 1 N HCl was added to all samples prior to analysis to remove CO_2 from samples. Sulphate concentration measured was by FIA analysis. Protein concentration was determined by the method of Lowry, with bovine serum albumin as the standard.

Bioreactors

Two identical fixed bed biofilm column reactors with 66 ml working volume were applied. Nylon fibers were used as a matrix for biofilm formation. Reactors were fed by peristaltic pumps, aerated with compressed air, and operated in batch, fed-batch and continuous modes in down flow co-current regime.

Results and discussion

Bacteria isolation and identification

Comamonas testosteroni T-2 is a Gram negative, motile rod described elsewhere (Locher *et al.*, 1989; Thurnheer *et al.*, 1986). The isolated strain was identified by means of the above mentioned methods. In both tests the best identification score was obtained for *Comamonas testosteroni*. The isolated strain was called *Comamonas testosteroni* L-1. This strain is also Gram negative, but non-motile, small (0.8 × 1.2–1.5 μm) aerobic rod forming on agar plates round (1.5–2.5 mm), transparent, uniform colonies. *C. testosteroni* L-1 was found in a mixture with another non *p*-TS degrading strain identified as *Ochrobactrum anthropi* by Biolog microplates. Microbes were distinguished by morphological characteristics: colonies on the solid medium were of different size and shape. Furthermore, *Ochrobactrum anthropi* could not grow on *p*-TS as sole carbon source in the absence of a *Comamonas* strain.

Biolog tests showed high biochemical similarity of both *p*-TS degrading strains. Locher and co-workers (Locher *et al.*, 1989) reported the ability of *C. testosteroni* T-2 to grow on *p*-sulphobenzoate, benzoate, *p*-phthalate and *p*-hydroxybenzoate. *C. testosteroni* L-1 utilised only *p*-phthalate as sole carbon source, whilst *C. testosteroni* T-2 utilised all above mentioned compounds. Neither strain grew on *p*-hydroxybenzaldehyde, *p*-sulphophenol and sulphanilic acid. It was found that *C. testosteroni* L-1 was more resistant to low pH levels, down to 5.

p-TS degradation in shake flasks

C. testosteroni T-2 completely degraded *p*-toluenesulfonate (*p*-TS) via oxidation of the methyl group to *p*-sulfobenzoate and further to cell material, CO_2 and sulphate (Locher *et al.*, 1989; Thurnheer *et al.*, 1986). *p*-Sulphobenzoate was excreted from cells and routinely detected in the culture broth in all experiments with this strain (Fig. 1). Sum of both sulphonated compounds represented 93–95% of total organic carbon (TOC) in *Comamonas testosteroni* T-2 culture medium (Fig. 2).

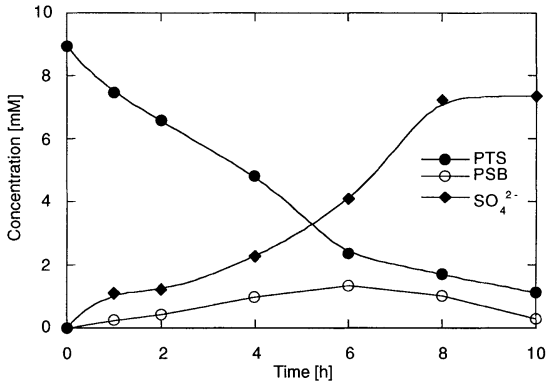

Figure 1 p-TS, p-SB and sulphate concentration profiles in suspension culture of Comamonas testosteroni T-2.

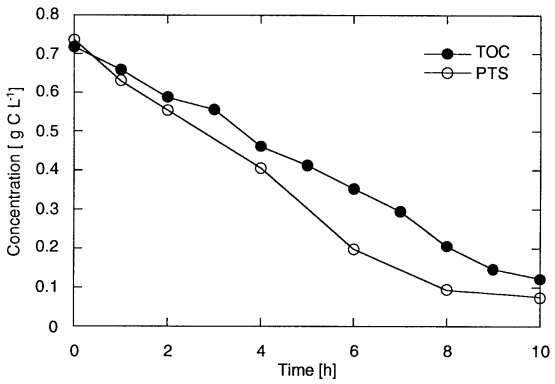

Figure 2 Total organic carbon and p-TS concentration profiles in short term experiments with Comamonas testosteroni T-2.

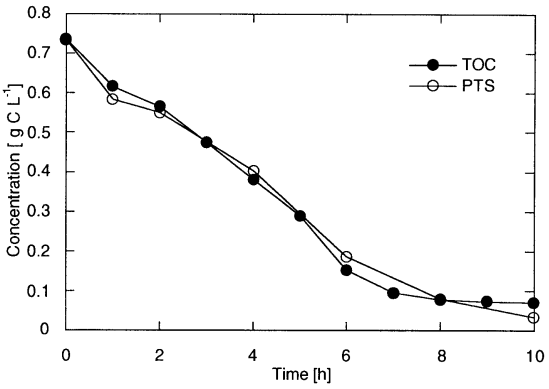

Figure 4 Total organic carbon and p-TS concentration profiles in short term experiments with Comamonas testosteroni L-1.

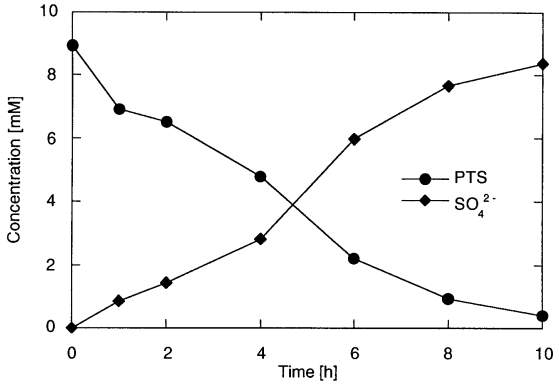

Figure 3 p-TS and sulphate concentration profiles in suspension culture of Comamonas testosteroni L-1.

In contrast, *C. testosteroni* L-1 degraded *p*-TS without detectable sulphonated intermediates (Fig. 3). *p*-TS represented practically 100% of total organic carbon available in *C. testosteroni* L-1 cultures (Fig. 4). In both cases sulfate appeared in the culture broth in quantities proportional to the degraded sulphonated aromatics. Results of these experiments summarised in Table 1.

Resuspension of washed cells in culture medium without *p*-TS gave at least 50 mg of carbon per liter which was considered as a basic residual TOC level. This level was practically constant throughout all cultivation. Even in cases of complete *p*-TS degradation, from 30 to 60 mg of dissolved organic carbon per liter were still detected in culture broth. In control flasks without cells, the *p*-TS concentration was constant during whole experimental period.

Either with suspension cultures pure *C. testosteroni* L-1 or in mixture with *Ochrobactrum anthropi*, TOC concentration decreased faster than with the *C. testosteroni* T-2 strain. Growth of *C. testosteroni* L-1 was directly proportional to the removed amount of *p*-TS. In contrast, in *C. testosteroni* T-2 suspensions, with formation of sulphonated intermediate, cell growth was not in proportion to target compound removal. The sum of both sulphonated compounds (*p*-TS and *p*-SB) should be taken for estimation of the degradation kinetics. From shake-flask experimental data, *C. testosteroni* L-1 strain degraded *p*-TS faster and without detectable intermediates than strain T-2, and was thus preferred for further application.

Bioreactor experiments

Two fixed bed biofilm bioreactors were operated in continuous mode preceded by several days of batch/fed-batch. Both reactors were inoculated with the same amount of pure cultures grown overnight. The pollutant concentration was kept at 6 mM. Biofilm development was tested under controlled conditions of temperature, medium pH, aeration and feeding rates.

Table 1 p-Toluenesulphonic acid degradation characteristics in short term cell suspension experiments

Bacterium	p-TS degradation rate		TOC degradation	SO_4^{2-} production	Growth yield
	mg p-TS L^{-1} h	mg C L^{-1} h	mg C L^{-1} h	mg L^{-1} h	mg protein (mg p-TS)$^{-1}$
C.t. T-2	135	66	59	71	0.38
C.t. L-1	138	68	66	80	0.46

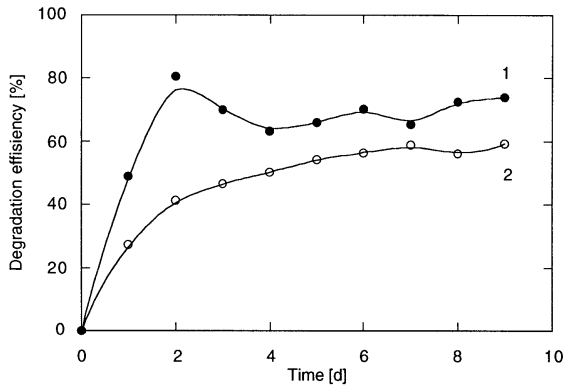

Figure 5 Total organic carbon degradation efficiency: (1) reactor inoculated with C. testosteroni T-2; (2) C. testosteroni L-1 at dilution rate 0.15 h^{-1}.

The reactor with immobilised *C. testosteroni* T-2 culture reached the maximum pollutant degradation efficiency on the second day (Fig. 5).

In the *C. testosteroni* L-1 inoculated reactor, the degradation efficiency reached the plateau only on the fifth cultivation day. At the tested loading rates, *C. testosteroni* T-2 was more efficient in removing the target pollutant and total organic carbon than strain L-1 (Table. 2).

Microscopic observations revealed a gradient in biofilm microorganism distribution through the column length. There was a higher content of xenobiotic degraders at the top of the columns than at the bottom, there were greater numbers of non-specific bacteria and protozoa. Microorganism diversity in the column inoculated with *C. testosteroni* T-2 was less than in the *C. testosteroni* L-1 inoculated reactor. In addition, the bacterial and protozoan species observed in the two reactors were different. Plating of samples on agar plates confirmed microscopic observations data. Differentiation between colonies on the solid medium was based on: colour, size and opacity. In the reactor inoculated with *C. testosteroni* T-2 only 2–3 microbial species and 1 type of protozoa were detected. With *C. testosteroni* L-1, 5–6 bacteria and several types of protozoa were observed. This could be ascribed to production of *p*-sulphobenzoate by *Comamonas testosteroni*. T-2, since benzoic acid and its derivatives are known to have a bacteriostatic effect. However, in tests on rich solid medium all bacteria from *Comamonas testosteroni* L-1 column were insensitive to concentrations of *p*-sulphobenzoate up to 4 mM. *p*-Sulphobenzoate concentrations detected in culture broth were lower than 2 mM, but local concentrations near cells could be higher. Microscopy and plating on non-selective and selective media demonstrated that initially added strains represented the major part of total microbial population and were single *p*-TS consumers in both reactors. *p*-TS degrading bacteria from *C.t.* T-2 reactor grew also on *p*-SB solid medium. In contrast, in the case of the *C. testosteroni* L-1 reactor no bacterium able to grow on *p*-SB solid medium was detected.

Table 2 p-Toluenesulphonic acid degradation rate in batch and continuous cultures

	Degradation rate mg C L^{-1} h		
	Batch	Continuous	
Reactor inoculated with		D = 0.15 h^{-1}	D = 0.30 h^{-1}
C. testosteroni T-2	11	72	84
C. testosteroni L-1	12	62	70

Xenobiotic removal tests by suspended and immobilised cultures are important for the evaluation of strain applicability. Our experiments showed that a high pollutant removal capacity of suspension cultures is not always a sufficient criterion for strain selection. Experiments in the model reactor system revealed that even two similar strains in controlled environmental conditions worked differently during short term runs. Very often xenobiotic degrading bacteria have a very narrow substrate range. Microorganisms with a wider substrate range have been shown for obvious reasons to be preferable for treatment of mixed wastes. Presence of other carbon sources (intermediates or products of degradation) allows development of non-target compound degrading microorganisms. High content of non specific bacteria leads to lower target pollutant removal, but it may enhance total organic carbon mineralisation. Spatial orientation of microorganisms presented in a biofilm also plays an important role on the film formation and may affect xenobiotic degradation efficiency. Xenobiotic uptake, the first step in its removal, may be inhibited by intermediates or other similar compounds. On the other hand, presence of such compounds may lead to more favorable conditions for the development of xenobiotic degrading microorganisms in biofilms.

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