Biodegradation (2008) 19:197–208
DOI 10.1007/s10532-007-9126-6

ORIGINAL PAPER

Toluene biodegradation by *Pseudomonas putida* F1: targeting culture stability in long-term operation

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Received: 26 October 2006 / Accepted: 11 April 2007 / Published online: 9 May 2007

Abstract The stability of *Pseudomonas putida* F1, a strain harbouring the genes responsible for toluene degradation in the chromosome was evaluated in a bioscrubber under high toluene loadings and nitrogen limiting conditions at two dilution rates (0.11 and 0.27 h^{-1}). Each experiment was run for 30 days, period long enough for microbial instability to occur considering previously reported studies carried out with bacterial strains encoding the catabolic genes in the TOL plasmid. At all tested conditions, *P. putida* F1 exhibited stable performance as shown by the constant values of the specific toluene degradation yield, CO_2 produced versus toluene degraded yield, and biomass concentration within each steady state. Benzyl alcohol, a curing agent causing TOL plasmid deletion in *Pseudomonas* strains, was present in the cultivation medium as a result of the monooxygenation of toluene by the diooxygenase system of *P. putida* F1. However, no mutant population growing at the expense of the extracellular excreted carbon or lysis products was established in the chemostat as confirmed by the constant dissolved total organic carbon (TOC) concentration and fraction of toluene degrading cells (approx. 100%). In addition, batch experiments conducted with both lysis substrate and toluene simultaneously confirmed that *P. putida* F1 preferentially consumed toluene rather than extracellular excreted carbon.

Keywords Biodegradation · Bioscrubber · Long-term operation · Process stability · Toluene · *Pseudomonas putida* F1

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Introduction

The extensive use of aromatic hydrocarbons in industrial and agricultural activities has increased substantially the number of emissions and accidental spills. Due to their high mobility, toxicity, and in some cases carcinogenic effects these compounds entail a potential risk for natural ecosystems and human health (Wang et al. 2000; US EPA 1994, 1996; Zilli et al. 2000). Toluene is a widespread aromatic contaminant emitted in the production of gasoline, synthetic rubber, paints or agricultural chemicals. In 1992, 191 million tons of toluene were released into the atmosphere in the United States alone (US EPA 1994). Public concern in this regard has tightened environmental regulations, which has promoted the rapid development of efficient technologies for the abatement of this contamination. Among these technologies, biological treatment methods constitute nowadays a well-established, low-cost alternative to conventional physical–chemical treatment methods for the destruction of organic

contaminants (Revah and Morgan-Sagastume 2005). Besides involving low construction and operation cost, biodegradation processes avoid the generation of secondary pollution as they are based on the ability of microorganisms to convert organic pollutants into CO_2, H_2O and biomass under mild conditions of temperature and pressure (Shareefdeen and Singh 2005). Within these biological processes, bioscrubbers constitute a cost-effective bioreactor configuration, being more reliable in terms of construction and process control than conventional biofilters (Koutinas et al. 2005). Bioscrubbers can be advantageous in processes operated at high pollutant loadings rates, where by-product accumulation is likely to occur, or during the degradation of chlorinated volatile organic contaminants, where medium acidification severely reduces process performance (Ensley and Kurisko 1994; Lee et al. 2000).

However, despite the above-mentioned advantages, biological technologies for the degradation of xenobiotic compounds still present serious limitations derived from the toxic nature of the target contaminants. Thus, irreversible or temporary looses of the degradation capacity of microorganisms can occur during short-term episodes of high inlet pollutant concentration, or as a result of the long-term exposure to mutagenic contaminants (Jones et al. 1997; Mirpuri et al. 1997; Oliveira and Livingston 2003; Tresse et al. 2003). For instance, Oliveira and Livingston (2003) demonstrated that high concentrations of monochlorobenzene induced rapid cell washout and process instability in a bioscrubber due to the high pollutant toxicity. Episodes of nitrogen deprivation during toluene removal dramatically decreased the activity of the dioxygenase involved in the first oxidation step of toluene biodegradation by *Pseudomonas putida* NCIMB 11767 (Jenkins and Heald 1996). Jones et al. (1997), Villaverde et al. (1997a, b) and Villaverde and Fernandez (1997) showed the prolonged exposition of *Pseudomonas putida* 54g to toluene vapours (toluene liquid concentrations of ≈ 7 mg l^{-1}) caused important fractions of the culture to die or irreversibly lose their capacity to duplicate whereas other cells lost their capacity to degrade toluene. In this context, Villaverde and Fernandez (1997) reported these phenomena were more pronounced the higher the toluene concentration and the longer the time of exposure was, and suggested that viable non-toluene degrading cells survive at the expenses of leakage and lysis products from injured and dead cells. Likewise, benzyl alcohol and benzoate, intermediates in the toluene degradation pathway of *P. putida* 54g, promoted the lost the TOL plasmid encoding toluene metabolism (Keshavarz et al. 1985; Leddy et al. 1995; Willians et al. 1988). Therefore, further research on process design and operation strategies is needed in order to overcome these limitations as well as to increase the elimination capacity (EC) and the stability of biological process in the long-term operation.

In this context, especial attention was given to the selection of the pollutant degrading community to enhance process stability. The stability of *Pseudomonas putida* F1, a strain harbouring the genes responsible for toluene degradation in the chromosome (Zylstra et al. 1988), was tested under sterile conditions. The influence of toluene concentration and dilution rate on culture stability (evaluated using process parameters such as specific toluene degradation yield, fraction of toluene degrading cells, CO_2 produced vs. toluene degraded yield and ATP cell content) in long-term operation was investigated at high toluene loadings. In addition, the kinetics of cell growth on both toluene and lysis substrate were evaluated.

Materials and methods

Microorganisms and culture conditions

A *Pseudomonas putida* F1 strain [DSMZ 6899] was selected for its well known capability for mineralising toluene and since its degradation pathways have been thoroughly described in literature (Gibson et al. 1970; Bordel et al. 2007). The culture was maintained at 4°C in Mineral Salt Medium (MSM) with toluene as the sole carbon and energy source. To furnish fresh inoculum, 500-ml E-flasks were supplied with 250 ml of MSM, 200 µl of toluene, closed with cotton plug, sealed with aluminium and parafilm paper, and incubated for 12 h in an orbital shaker at 200 rpm and 30°C.

The MSM used for bacterial cultivation was composed of (g l^{-1}): $NaHPO_4 \cdot 12H_2O$, 6.15; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.038; and 10 ml l^{-1} of a trace element solution containing (g l^{-1}): EDTA, 0.5; $FeSO_4 \cdot 7H_2O$, 0.2;

$ZnSO_4 \cdot 7H_2O$, 0.01; $MnCl_2 \cdot 4H_2O$, 0.003; H_3BO_3, 0.03; $CoCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.001; $NiCl_2 \cdot 6H_2O$, 0.002; $NaMoO_4 \cdot 2H_2O$, 0.003. The final pH of medium was 7.0.

Chemicals

All chemicals and reagents were purchased from PANREAC with a purity of +99% (Barcelona, Spain). Analytical grade benzyl alcohol (BA) was purchased from Sigma-Aldrich (USA). Synthetic air (Carburos Metálicos S.A., Spain) was used during the entire experimentation.

Experimental design

Stability tests in bioreactor

The influence of toluene concentration and dilution rate (D) on process stability (evaluated through the daily monitorization of the specific toluene degradation yield, fraction of toluene degrading cells, CO_2 production, and ATP cellular content,) was investigated under sterile conditions in a magnetically stirred 1-l glass bioreactor (Afora S.A, Spain) operated as a chemostat. The reactor was filled with 900 ml of sterile MSM and inoculated with 40 ml of *P. putida* F1 to attain an initial biomass concentration of approx. 4–7 mg Dry Weight l^{-1} (from now on mg DW l^{-1}). Temperature and agitation rate were maintained constant at 25°C and 500 rpm, respectively. Toluene was supplied in the gas phase through the aeration (1100 ml min^{-1} of synthetic air filtered through a 0.2 μm Millex®-FG membrane filter) by mixing a toluene-saturated stream with a toluene-free air stream at different proportions (Fig. 1). Two series of continuous experiments were carried out during 30 days at D of 0.11 and 0.27 h^{-1}, respectively. In the first series of experiments (D = 0.11 h^{-1}) toluene inlet concentration was set at 3.3 ± 0.2 g m^{-3} during 21 days and thereafter increased up to 6.2 ± 0.4 g m^{-3}. In the second series (D = 0.27 h^{-1}) the bioscrubber was fed with 11.7 ± 0.7 g toluene m^{-3} during the first 21 days and thereafter with 20.2 ± 1.9 g toluene m^{-3}. At D = 0.27 h^{-1} the MSM formulation resulted in nitrogen limiting conditions, as confirmed by experimental determinations of ammonia concentration. Gaseous toluene and CO_2/O_2 concentrations were daily monitored by simultaneous withdrawing 250 μl samples with Gas-Tight Hamilton syringes through valves A and C (Fig. 1). Excreted metabolites, dissolved total organic carbon (TOC) content, pH, ATP concentration, absorbance at 650 nm, and CFU plate counts in selective medium (toluene) and non-selective medium (peptone) were also daily recorded by withdrawing a 10 ml liquid sample through valve B under sterile conditions (Fig. 1). In addition, Dissolved Oxygen Concentration (DOC) and Temperature (T) were monitored on line.

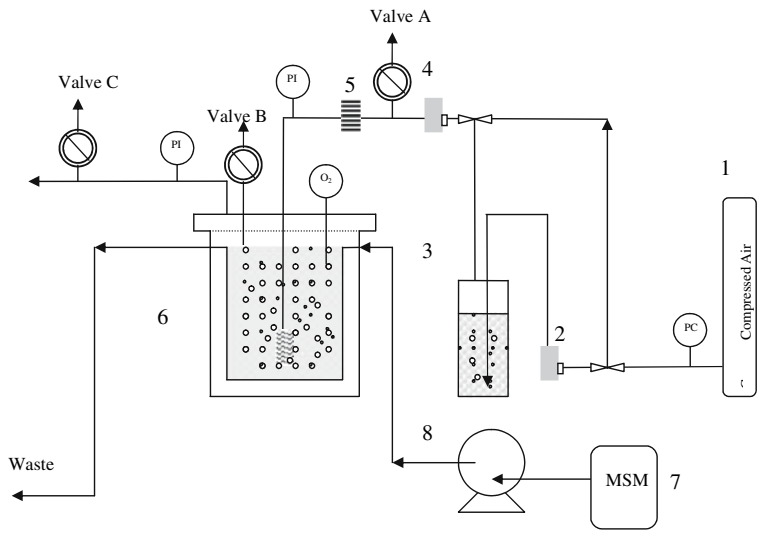

Fig. 1 Schematic representation of the experimental set up. 1, Compressed air; 2, Mass flow controller; 3, toluene evaporator; 4, mass flow controller; 5, air-filter; 6, stirred tank reactor, 7, MSM reservoir, 8, MSM pump. PC, pressure control; PI, pressure indicator

Growth kinetics in batch experiments

Growth kinetics of *P. putida* F1 in toluene (T), lysis substrate (L), and mixed substrate (T + L) were evaluated in batch cultivation mode. Lysis substrate was prepared from a *P. putida* F1 culture disrupted in a Vibra Cell Sonicator VCX 600 × 20 kHz (Sonic and Materials Inc., USA) coupled with a CV 26 Tip. The cell suspension was treated for 2 min (2 s On/3 s Off cycles) and cells debris removed by vacuum filtration through a 0.2 μm nitrocellulose membrane filter. Glass flasks of 125 ml were filled with 30 ml of MSM and inoculated with 0.4 ml of *P. putida* F1. Systems T and L were supplied with toluene at 9 g m^{-3} (headspace concentration) and lysis substrate at 50 mg TOC l^{-1}, respectively. Systems T + L were supplied with both toluene and lysis substrate at the above-mentioned concentrations. The flasks were finally closed with Teflon coated butyl septa, sealed with aluminum caps and cultivated under magnetic agitation (300 rpm) at 25°C. Toluene, ATP and CO_2 concentration were periodically monitored. Sterile control tests (in the absence of biomass) were carried out under similar conditions to record abiotic losses of toluene. The experiments were performed in duplicate.

Analytical procedures

Toluene analysis was performed in a Gas Chromatograph (Hewlett-Packard 5890, Palo Alto, USA) coupled with a Mass Spectrometer Detector (Hewlett-Packard 5973 MSD, Palo Alto, USA) and a HP-5MS fused silica capillary column (Agilent Technologies, USA). Oven Temperature was maintained at 50°C during 3 min and increased up to 90°C at 30°C min^{-1}. Injector and MS Quadrupole were maintained at 250 and 150°C, respectively. Helium was used as carrier gas at 0.9 ml min^{-1}. External standards prepared in volumetric bulbs (Sigma-Aldrich, USA) were used for toluene quantification.

CO_2 and O_2 concentrations were measured using a GC-TCD (Agilent Technologies 6890N, Palo Alto, CA, USA) equipped with a PORAPAK N, 80/100 3 m × 1.8 (Teknokroma, USA) and a Molecular sieve 13 × 45/60 0.9 m × 1/8 (Tecknokroma, USA). Helium was used as a carrier gas at a flow rate of 5.4 ml min^{-1}. The temperature of the detector and injector were maintained at 200 and 150°C, respectively. The oven temperature was initially maintained at 40°C during 3 min, increased at 40°C min^{-1} up to 100°C, and maintained at 100°C during 1.5 min. External standards enabled CO_2 and O_2 quantification.

The bioreaction metabolites (i.e. BA, Bordel et al. 2007) were quantified by HPLC-UV using a WATERS 515 HPLC pump coupled with a UV1000 Spectraseries Detector (Thermo separation Products, California, USA) and a Supelcosil LC-PAH column (Supelco, Bellefonte, USA). Samples were eluted isocratically using a mobile phase composed of methanol and water (60/40 v/v) at a flow rate of 0.5 ml min^{-1}. UV-detection was performed at 254 nm. Liquid samples of 1.5 ml were centrifuged at 4°C for 10 min at 14,000 rpm prior to analysis. External standards were used to enable quantitative determination.

Dissolved TOC in the aqueous phase was measured using a TOC analyser (Shimadzu TOC-5050A, Japan) according to the manufacturer. Liquid samples of 7 ml were centrifuged at 6,000 rpm during 20 min prior to analysis. Ammonia concentrations in the liquid phase were determined using an Ammonia Electrode, Orion 900/200 (Thermo Electron Corporation, Beverly, USA).

The DOC and the temperature in the bioreactor were determined using an O_2 transmitter 4100 (Meter Toledo GmbH, Urdolf, Germany). A CRISON micropH 2002 (Crison Instruments, Barcelona, Spain) was used for pH determination.

Absorbance at 650 nm was used as an indicator of microbial growth and measured using a HITACHI U200 UV/visible spectrophotometer (Hitachi Ltd, Tokyo, Japan). A correlation between absorbance at 650 nm and *P. putida* F1 dry weight was performed according to American Public Health Association (1995). ATP was measured using a Microbial ATP kit HS (Biothema, Stockholm, Sweden) and a Microtox 500 luminometer (Azur Environmental, Carlsbad, Germany).

The fraction of viable bacteria degrading toluene was determined by standard plate count of colony-forming units (CFU) on non-selective medium (casein peptone 15 g l^{-1}, soymeal peptone 5 g l^{-1}, NaCl 5 g l^{-1}, and agar 15 g l^{-1}) and selective medium (MSM supplied with toluene vapours). Cells were incubated in sealed containers for 24 h in triplicate at 30°C. Dilutions were made to obtain a number of CFU ranging between 30 and 300 colonies per plate (American Public Health Association 1995).

Calculations

The specific toluene degradation yield (Y_{SX}), the percentage of toluene degrading cells (ϕ), the CO_2 produced versus Toluene degraded yield coefficient (Y_{CO2TOL}), specific ATP content, EC and removal efficiency (RE) were calculated as follows:

$$Y_{SX} = \frac{\text{Toluene elimination rate}}{\text{Biomass production rate}} = \frac{\left(\left(C_g^{in} - C_g^{out}\right) \cdot \dot{V}_g / V_R\right)}{X \cdot D} [\,=\,] \text{ g g}^{-1} \quad (1)$$

$$\phi = \frac{\text{Toluene degrading cells}}{\text{Total viable cells}} \times 100 [\,=\,] \% \quad (2)$$

$$Y_{CO_2TOL} = \frac{\text{CO}_2 \text{ production rate}}{\text{Toluene elimination rate}} = \frac{\left(C_{CO_2}^{out} - C_{CO_2}^{in}\right) \cdot \dot{V}_g}{\left(\left(C_g^{in} - C_g^{out}\right) \cdot \dot{V}_g / V_R\right)} [\,=\,] \text{ g g}^{-1} \quad (3)$$

$$\text{Specific ATP content} = \frac{\text{ATP concentration}}{\text{Biomass concentration}} [\,=\,] \text{ mol ATP gDW}^{-1} \quad (4)$$

$$EC = \frac{\left(C_g^{in} - C_g^{out}\right) \cdot \dot{V}_g}{V_R} [\,=\,] \text{ g m}^{-3} \text{ h}^{-1} \quad (5)$$

$$RE = \frac{\left(C_g^{in} - C_g^{out}\right)}{C_g^{in}} \times 100 [\,=\,] \% \quad (6)$$

where C_g^{in}, C_g^{out}: Inlet and outlet toluene concentration, respectively; [=] g m^{-3}; $C_{CO_2}^{in}$, $C_{CO_2}^{out}$: Inlet and outlet CO_2 concentration, respectively; [=] g m^{-3}; [TOC]: Total Organic Carbon in liquid samples; [=] mg l^{-1}; V_R: Reactor volume; [=] l; D: Dilution rate; [=] h^{-1}; $\dot{V}_g$: Gas flow rate; [=] m^3 h^{-1}.

Statistical treatment

All the results are given as the average value with its corresponding error at 95% and the number of data points used for calculation (Table 1). Results were analysed using a one-way ANOVA with significance at $P \leq 0.05$. The Excel statistical package (Microsoft Corporation, USA) was used for data treatment. Plate count values were treated using the Grubb's test with significance at $P \leq 0.05$ to reject outliers.

Results

Stability tests in bioreactor

Toluene biodegradation by *P. putida* F1 was stable under continuous operation regardless pollutant concentration and dilution rate. The specific toluene degradation yield remained constant within each steady state established in the chemostat (Fig. 2). When operated at D of 0.11 h^{-1}, Y_{SX} of 1.47 ± 0.08 and 1.82 ± 0.12 g tol gDW^{-1} were obtained at 3.3 and 6.2 g tol m^{-3}, respectively, while these values decreased to 1.26 ± 0.10 and 1.21 ± 0.12 g tol gDW^{-1} at D of 0.27 h^{-1} for the first and second steady state, respectively. The percentage of toluene degrading cells (ϕ) in the culture fluctuated within an average steady state value of 100% (Fig. 2a, b). Likewise, biomass concentration, Y_{CO2TOL}, and the specific ATP content remained constant within each steady state (Fig. 3).

3-Methyl catechol (the first intermediate in the toluene degradation pathway in *P. putida* F1, Gibson et al. 1970) was never detected in the cultivation medium. Benzyl Alcohol (BA) was however detected at concentrations ranging from 10 to 20 mg l^{-1} when the bioreactor was operated at 6.2 g tol m^{-3} (D = 0.11 h^{-1}), and from 40 to 70 mg l^{-1} when supplied with 11.7 and 20.2 g tol m^{-3} (D = 0.27 h^{-1}) (Fig. 4a, b). There was no significant difference in the dissolved TOC concentration at the two steady states reached at D of 0.27 h^{-1} (269.9 ± 28.2 mg C l^{-1} at 11.7 g tol m^{-3} and 287.5 ± 29.4 mg C l^{-1} at 20.2 g tol m^{-3}). TOC concentration did however increase when toluene concentration was increased from 3.3 to 6.2 g m^{-3} at D = 0.11 h^{-1} (Table 1).

DOC and pH remained also constant within each steady state. pH and DOC values of 5.8 ± 0.1 and 2.6 ± 0.5 mg l^{-1} (n = 21), respectively, were obtained at D = 0.11 h^{-1} and 3.3 g tol m^{-3}. These values

Table 1 Steady state values of the target monitoring parameters

Dilution (h^{-1})	Toluene ($g\ m^{-3}$)	Y_{SX} ($g\ tol\ gDW^{-1}$)	Biomass dry weigh ($g\ DW\ l^{-1}$)	Y_{CO2X} ($g\ CO_2\ gDW^{-1}$)	ATP content ($mol\ gDW^{-1}$)	TOC ($mg\ C\ l^{-1}$)	EC ($g\ m^{-3}\ h^{-1}$)	RE (%)	ϕ (%)
0.11	3.3	1.36 ± 0.09 (n = 19)	1.31 ± 0.07 (n = 20)	2.77 ± 0.13 (n = 20)	$3.91 \pm 0.22 \times 10^{-6}$ (n = 20)	140.3 ± 12.8 (n = 20)	216.2 ± 15.1 (n = 20)	89 ± 2 (n = 20)	101 ± 4 (n = 20)
	6.2	1.66 ± 0.12 (n = 11)	1.85 ± 0.06 (n = 11)	3.61 ± 0.18 (n = 11)	$4.74 \pm 0.42 \times 10^{-6}$ (n = 11)	270.3 ± 23.0 (n = 12)	369.9 ± 19.6 (n = 11)	84 ± 2 (n = 11)	99 ± 5 (n = 11)
0.27	11.7	1.08 ± 0.10 (n = 19)	1.65 ± 0.07 (n = 19)	2.24 ± 0.09 (n = 19)	$3.83 \pm 0.22 \times 10^{-6}$ (n = 19)	269.9 ± 28.2 (n = 19)	552.9 ± 45.2 (n = 19)	67 ± 4 (n = 18)	101 ± 10 (n = 18)
	20.2	1.03 ± 0.13 (n = 9)	1.75 ± 0.07 (n = 9)	2.19 ± 0.06 (n = 9)	$4.03 \pm 0.29 \times 10^{-6}$ (n = 9)	287.5 ± 29.4 (n = 9)	595.0 ± 59.4 (n = 9)	44 ± 6 (n = 9)	97 ± 14 (n = 14)

The results are expressed as the average ± the error at 95% CI with the corresponding number of points the calculation was based on

decreased to 5.3 ± 0.1 and 1.8 ± 0.3 mg l^{-1} (n = 11) when toluene inlet concentration was increased up to 6.2 g m^{-3}. pH and DOC values of 5.66 ± 0.07 and 3.0 ± 0.5 mg l^{-1} (n = 28) were recorded at D = 0.27 h^{-1} regardless toluene concentration.

Toluene EC ranging from 216 ± 15 g $m^{-3}h^{-1}$ (RE = 89 ± 2%) to 595 ± 59 g $m^{-3}h^{-1}$ (RE = 44 ± 6%) were achieved in the experimental system (Table 1). At the lowest dilution rate, 58 ± 3% of the carbon was transformed to CO_2, 37 ± 2% to biomass, and 8 ± 1% to TOC (Total C recovery of 103 ± 5%). At D = 0.27 h^{-1}, 54 ± 3% of carbon was converted into CO_2, 15 ± 3% into TOC, and 46 ± 3% into biomass (Total C recovery of 115 ± 7%).

Growth kinetics in batch experiments

There was no significant effect of lysis substrate on toluene biodegradation (toluene removal rate, and ATP and CO_2 production) as shown in Fig. 5a. Specific growth rates of 0.48 ± 0.03, 0.42 ± 0.08, and 0.52 ± 0.1 h^{-1} (based on ATP measurements) were recorded in the systems supplied with toluene, lysis substrate and the mixed substrate (T + L), respectively. *P. putida* F1 exhibited however, a slightly longer lag phase when lysis substrate was provided as the sole carbon and energy source. CO_2 concentration increased concomitantly with biomass production. However, while CO_2 production slightly decreased after complete toluene depletion (≈5 h) in the systems supplied with toluene, it continued increasing linearly in the systems supplied with the mixed substrate.

Discussion

Several studies in literature have reported operational problems derived from microbial instability in processes treating toluene. However, these phenomena have not been studied in details and the mechanisms underlying these processes are not yet well understood. For instance, Song and Kinney (2005) reported a decline in the EC of biofilters subjected to high toluene loadings, likely due to the deterioration of the toluene degrading community. The process was maintained stable during 3 weeks at sub-critical conditions (RE = 100%) and then overloaded. The

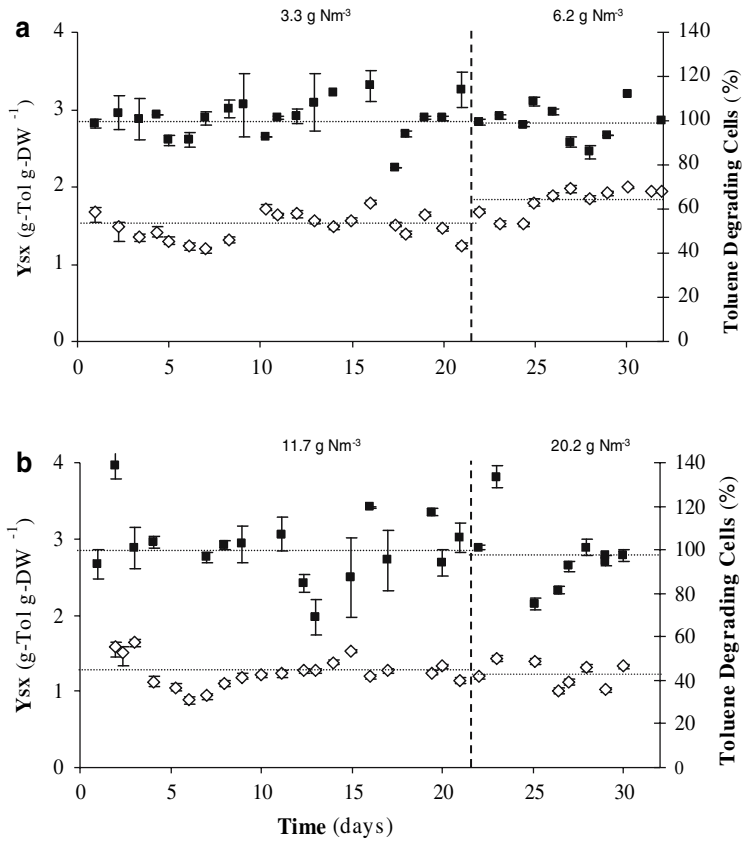

Fig. 2 Time course of the specific toluene degrading capacity (◇), and percentage of toluene degrading cells (■) during the continuous degradation of toluene by *P. putida* F1 at D of 0.11 h^{-1} (**a**) and 0.27 h^{-1} (**b**). Dotted lines represent the average values within each steady state

EC immediately increased (concomitantly with a decrease in RE) reaching a pseudo-stationary state and started to decrease approx. 10–12 days after process overload. However, the mechanisms responsible of microbial deterioration were not identified. Villaverde et al. (1997a, b) and Villaverde and Fernandez (1997) also reported a deterioration of the toluene degrading community in biofilm structures, which depended on the toluene concentration and time of exposure. Likewise, Duetz et al. (1991) observed that *Pseudomonas putida mt-2* was overcome by their own mutants (lacking the TOL plasmid pWWO) during continuous cultivation on benzoate (a reaction intermediate in toluene biodegradation) in less than a week as a result of the growth rate advantage of the mutants generated during the biodegradation process. Therefore, this deterioration in the pollutant degrading community in biological treatment processes will ultimately result in a poor process performance as process operation would be very restricted to low loading rates in order to avoid the above-mentioned phenomena.

In this context, *P. putida* F1, a toluene degrading bacteria harbouring the genes responsible for toluene degradation in the chromosome (Zylstra et al. 1988), was evaluated under high toluene loading rates (up to 1,480 g toluene m^{-3} reactor h^{-1}) and nitrogen limiting conditions. The experimental conditions were selected in order to trigger microbial instability. Each experiment was continuously operated under sterile conditions over 1 month (290 and 120 generations for D of 0.27 and 0.11 h^{-1}, respectively). This period was considered sufficient to ensure a stable operation as in all reported cases process instability occurred within 2 weeks of operation after process overload.

In the present study Y_{SX}, ϕ, Y_{CO2TOL} and the specific ATP content, were used as indicators of process stability. Y_{SX} did not decrease with the time course, which suggest that no mutant population established in the culture at the expense of the organic carbon excreted by the wild type cells (Fig. 2). This was confirmed by two facts: First, TOC and BA remained constant within each steady

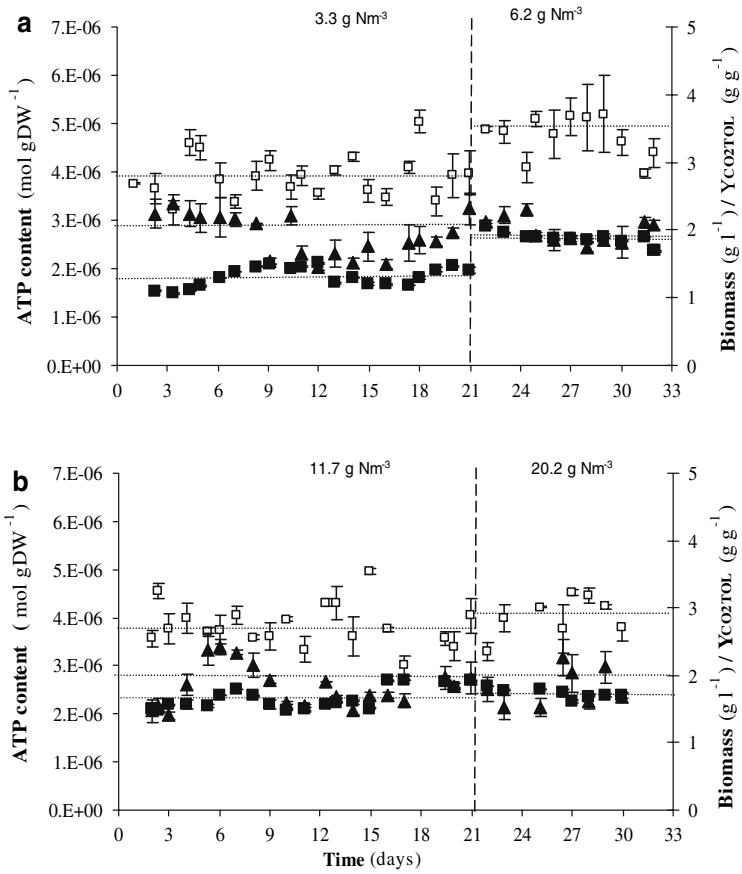

Fig. 3 Time course of biomass concentration (■), CO_2 produced versus toluene degraded yield (▲), and specific ATP content (□) during the continuous degradation of toluene by *P. putida* F1 at D of 0.11 h^{-1} (**a**) and 0.27 h^{-1} (**b**). Dotted lines represent the average values within each steady state

state (Fig. 4), and second, no significant differences in the number of CFU growing on selective and non-selective medium was observed throughout the experimentation (Fig. 2). The establishment of a mutant pollutant growing in the extracellular carbon excreted by *P. putida* F1 would lead to a decreasing Y_{SX} and ϕ. Indeed, the total depletion of the dissolved TOC would result in approx. a 20% decrease in Y_{XS} and ϕ (assuming a 50% C—conversion into biomass and a biomass composition with 50% in C). In addition, control experiments in the absence of toluene were performed. These tests were carried out when operating the system at toluene gas concentration of 7 g m^{-3} and D = 0.1 h^{-1}, by shutting down toluene supply during 8 h while maintaining continuous air bubbling (1,100 ml min^{-1}) and MSM input. The results indicated that 8 h of toluene deprivation resulted in a decrease of both the ATP specific content (from 3.34×10^{-6} to 0.7×10^{-6} mol ATP g DW^{-1}), TOC concentration (from 410 to 156 mg TOC l^{-1}), BA concentration (from 9 mg l^{-1} to below detection level) and the specific CO_2 production (from 4.11 to 0.39 g CO_2 g DW^{-1}).

The stability of *P. putida* F1 was also supported by the fact that Y_{CO2TOL}, the biomass concentration, and the specific ATP content did not increase with the time course within each steady state. The consumption of extracellular carbon by mutant cells would result in an increasing Y_{CO2TOL} and biomass concentration as more carbon would be assimilated and oxidize to CO_2. It should be stressed that contamination of the culture by other microorganisms different from *P. putida* F1 did not occur as confirmed by periodical culture plating in non-selective medium. In addition, the specific ATP content, which can be regarded as a measurement of microbial activity during toluene degradation (Bordel et al. 2007), also remained constant within each steady state.

The process was thus stable under high loading rates (up to 1,480 g toluene m^{-3} reactor h^{-1}) and

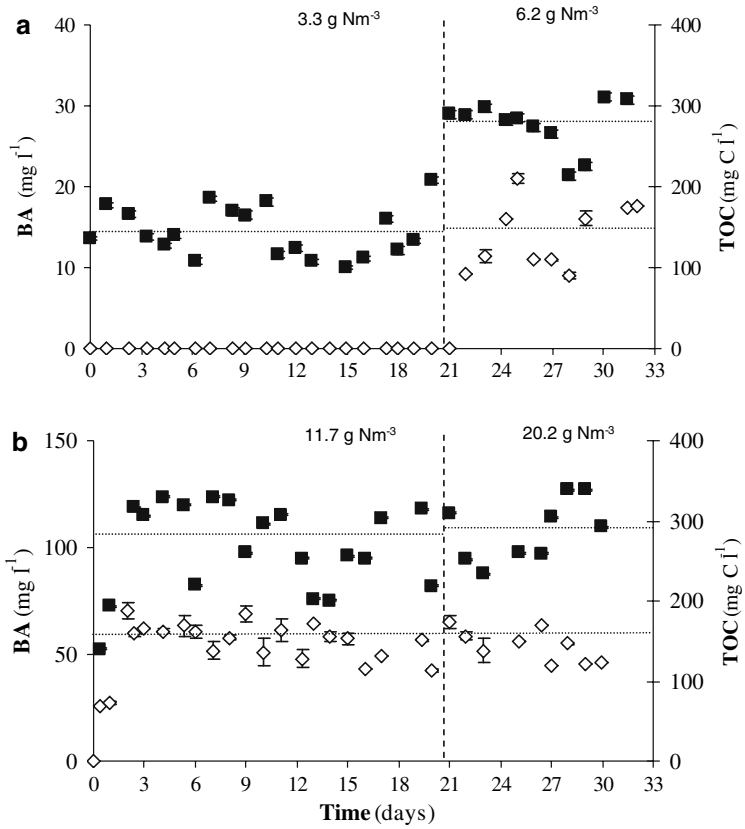

Fig. 4 Time course of Dissolved TOC (■), and benzyl alcohol (◇) during the continuous degradation of toluene by *P. putida* F1 at D of 0.11 h^{-1} (**a**) and 0.27 h^{-1} (**b**). Dotted lines represent the average values within each steady state

nitrogen limiting conditions (at D = 0.27 h^{-1}). In addition, the presence of BA at concentrations up to 70 mg l^{-1} (BA originated from toluene monoxygenation by the dioxygenase system of *P. putida* F1; Bordel et al. 2007) did not result in any significant loss in the toluene degrading capacity of the culture. In this regard, at the highest tested toluene inlet concentrations (11.7 and 20.2 g m^{-3}) the proportion of degraded toluene biotransformed into BA was 2.5 ± 0.7% and 2.2 ± 0.4%, respectively. Early studies on toluene biodegradation demonstrated that BA was responsible for the loss of the toluene degrading capacity in *Pseudomonas putida* 54g (Leddy et al. 1995). Leddy and co-workers (1995) reported that the prolonged exposure of *Pseudomonas putida* 54g to BA resulted in the loss of both plasmid and chromosomal encoded catabolic pathways with a loss of 13% of the toluene degrading population in 5 days. Under the tested conditions of our study, *P. putida* F1 maintained its toluene degrading capacity in the presence of BA as shown by the results herein presented. This confirms the importance of microbial selection on the long term stability of bioremediation processes. However, a direct comparison with the results obtained by Leddy et al. (1995) in terms of BA threshold concentration inducing a significant damage in bacterial genotype was not possible. This was due to the fact that *Pseudomonas putida* 54g was immobilized in 47-mm cellulose acetate filters and incubated in BA vapour rather than in liquid medium supplied with BA.

The above-mentioned results are in agreement with the observations made in growth kinetics experiments. Experiments with mixed substrate (T + L) showed that at the initial stages of the biodegradation process toluene-adapted wild cells of *P. putida* F1 preferentially consumed toluene rather than lysis substrate in the presence of both substrates, being the latter readily mineralised after toluene depletion (Fig. 5). The 5-h lag phase occurring when lysis substrate was supplied alone together with the similar patterns on toluene removal, biomass growth and CO_2 production in the systems supplied with toluene and toluene + lysis substrate showed that

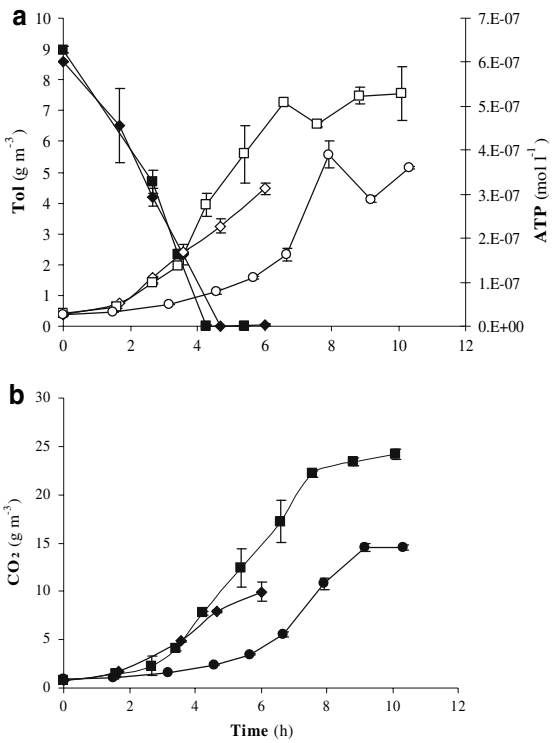

Fig. 5 Time course of toluene degradation (**a**, closed symbols) and ATP concentration (**a**, open symbols), and CO_2 concentration (**b**) in the headspace in systems supplied with toluene (diamonds), lysis substrate (circles), and a mixed substrate (squares)

toluene supported bacterial growth at the initial stage of the biodegradation process in systems supplied with mixed substrate (T + L). In addition, there was not dramatic decrease on microbial activity after toluene depletion, and according to the experimental results, both toluene and lysis substrate supported similar specific growth rates. The results herein presented indicate that toluene and lysis substrate consumption did not follow a diauxic growth model and suggests that in the presence of both substrates, toluene was preferentially consumed by toluene-adapted *P. putida* F1.

Extremely high degradation rates were recorded in our experimental system at the tested conditions. Thus, when the bioreactor was operated at 11.7 g m^{-3} EC of 553 ± 45 g m^{-3} h^{-1} and 67% RE were achieved. These ECs are much higher than those typically reported in bioscrubbers using bacterial cultures, although conventional bioscrubbers are operated at lower loading rates and higher RE. For instance, Harding et al. (2003) reported toluene EC of 35 g m^{-3} h^{-1} using an external loop airlift reactor fed with 15 g toluene m^{-3} and inoculated with a *Pseudomonas putida* strain. Likewise, Neal and Loehr (2000) recorded toluene EC of up to 29 g m^{-3} h^{-1} in a conventional bioscrubber fed with 0.8 g toluene m^{-3} inoculated with a mixed bacterial culture. The performance of the tested bioreactor is only comparable to the performance of fungal biofilters, membrane bioreactors and biphasic systems (addition of a second liquid phase of organic nature), which nowadays are the most efficient devices for off-gas treatment (van Groenestijn and Kraakman 2005). For instance, Davidson and Daugulis (2003) reported elimination capacities of up to 235 g toluene m^{-3} h^{-1} in a biphasic system supplied with hexadecane, 9 g toluene m^{-3}, and inoculated with an *Alcaligenes xylosoxidans* strain. Likewise, Garcia Peña et al. (2001) achieved toluene eliminations of 250 g m^{-3} h^{-1} at 6 g m^{-3} of toluene inlet concentration using a fungal biofilter packed with vermiculite.

The results reported in this study for *P. putida* F1 agree well with previously reported data. For instance, Bordel et al. (2007) reported cellular yields and specific CO_2 yields (Y_{CO2X}) of 1.0 ± 0.1 g DW g tol^{-1} and 1.91 ± 0.31 g CO_2 g DW^{-1}, respectively, at μ ranging from 0.41 to 0.72 h^{-1}. Cellular yields and Y_{CO2X} values ranging from 0.81 to 0.84 ± 0.1 g DW g tol^{-1}, and 2.2 ± 0.1 g CO_2 g DW^{-1} were obtained at $D = 0.27$ h^{-1}. Slightly lower cellular yields and higher Y_{CO2X} values were recorded at $D = 0.11$ h^{-1} (Table 1) likely due to the larger extent of the requirements for maintenance at low growth rates (Nielsen et al. 2003). Likewise, Bordel et al. (2007) reported an ATP concentration of 3.6 ± 0.2 × 10^{-6} mol ATP gDW^{-1} in *P. putida* F1 during the degradation of 2 mg tol l^{-1}, which agrees well with the values herein presented (Table 1).

In brief, *P. putida* F1 exhibited stable performance during toluene degradation under conditions of high toluene concentrations, nitrogen limitation and prolonged exposure to BA. This study confirms the key role of microbial selection in the long-term operation of biological treatment plants and suggests that chromosome-encoded catabolic pathways might exhibit a higher stability than those encoded in plasmids in the biodegradation of toxic contaminants. Therefore, process inoculation with highly resistant

microbial strains capable to withstand temporary (short term enzymatic inhibition) or irreversible (loss of the genetic material encoding toluene catabolism) losses of the toluene EC, is crucial in order to ensure a stable long term operation of the biodegradation process.

Acknowledgements This research was supported by the Spanish Ministry of Education and Science (PPQ2006-08230 and JCI-2005-1881-5 contracts). Dr. J. M. Bueno and Araceli Crespo are gratefully acknowledged for their practical assistance.

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