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Growth inhibition of biomass adapted to the degradation of toluene and xylenes in mixture in a batch reactor with substrates supplied by pulses

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Abstract

A biomass adapted to degrade toluene and xylenes in mixture was grown in a batch reactor with substrates supplied by pulses. The inhibition of biomass growth in the course of substrate degradation was investigated. The maximal biomass concentration of 7 g l^{-1} was obtained using 150 μl of toluene and 15 μl of a mixture of xylenes in one litre of liquid medium, and the maximal biomass productivity and yield were 53 mg l^{-1} h^{-1} and 0.32 g_{DW} g_s^{-1}, respectively. Higher quantities of substrate added by pulses, that is 200 μl of toluene with 20 μl of xylenes and 300 μl of toluene with 30 μl of xylenes, caused an accumulation of metabolites. These higher quantities of substrates caused inhibition of microbial growth. Among the metabolites produced, 4-methyl catechol was found in large quantities in the culture medium and in the cells.

Introduction

The amount and variety of aromatic hydrocarbons generated by commercial and industrial activities has continued to increase over the years. Compounds such as toluene and xylenes are widely used as industrial solvents. In addition, these compounds provide the starting materials for the production of pharmaceuticals, agrochemicals, polymers, colorants, and other everyday products. Their emission to the environment is still escalating in spite of governmental intervention of many countries.

Biodegradation of contaminated waste gases using biofilters is of increasing interest. This is an attractive alternative when compared to chemical and physical treatments, because of its low cost, especially when rapid degradation of the compounds involved can be obtained.

Many bacteria are reported to degrade *m*-xylene and *p*-xylene, but only a few microorganisms are known to degrade *o*-xylene, like *Corynebacterium* (Schraa et al. 1987) and *Nocardia* (Gibson et al. 1984) among Gram-positives, and a strain of *Pseudomonas stutzeri* among Gram-negatives (Baggi et al. 1987, Barbieri et al. 1993). It seems that the relative position of methyl groups in the aromatic ring plays a significant role in the catabolic pathway.

Few authors have studied the biodegradation of three xylenes and toluene or/and benzene simultaneously. Worsey and Williams (1975) widely studied the pathways of toluene and xylenes degradation: A model of a catabolic route and its regulation by *P. putida* mt-2 (PaW1) were found for *m*-xylene, *p*-xylene and toluene, but not for *o*-xylene.

The interactions among benzene, toluene, and *p*-xylene during their biodegradation were studied by Oh et al. (1994). Their experiments confirmed that *p*-xylene in this mixture can not serve as a growth substrate, and its cometabolism reduces yields on benzene and toluene. Lee et al. (1995) observed that benzene, toluene, and *p*-xylene can be mineralized by natural strains only separately, but that an engineered strain could degrade the mixture simultaneously.

The degradation of three isomers of xylene and toluene and their metabolic pathway leads to the accumulation of metabolites inhibitory to the microbial growth. Duggleby and Williams (1986) identified different isomers of methylcatechol as the interme-

diates of the *m*-xylene and *p*-xylene degradation: 3-methylcatechol of *m*-xylene, and 4-methylcatechol of *p*-xylene. They are catabolized by different enzyme systems to 2-oxopental-4-enoate. Gibson et al. (1974) studied an alternative mode of attack of *p*-xylene and *m*-xylene via direct dioxygenase attack of the aromatic ring. This was shown to be via corresponding cis-dihydrodiol and subsequent conversion to substituted catechols (3,6-dimethylcatechol from *p*-xylene and 3,5-dimethylcatechol from *m*-xylene) by dehydrogenase type enzymes.

Preliminary experiments in our laboratory on toluene and xylenes (*p*-xylene, *m*-xylene, and *o*-xylene) degradation, using different techniques, showed that the best process to grow a biomass able to degrade these compounds was a batch culture.

The objective of the present work was to find out the causes of the growth inhibition of a biomass adapted to the biodegradation of toluene and xylenes in mixture in a batch culture with substrates supplied by pulses. We studied the accumulation of metabolites in the course of toluene and xylenes degradation, which caused a growth inhibition. We also studied biological parameters such as substrate and the oxygen consumption rates by adding different quantities of toluene and xylenes in mixture.

Material and methods

Microorganisms

Initial bacterial consortia were collected from an industrial wastewater treatment plant (Schweitzerhalle, Ciba-Geigy, Switzerland) and two industrial biofilters for purification of exhaust gas containing aromatic solvents.

Adaptation and enrichment

The bacterial cultures containing 1.5 g l^{-1} of biomass dry weight were fed with 30 μl of toluene and 3 μl of xylenes (*o*-xylene, *p*-xylene and *m*-xylene) manually, then automatically by pulses for 15 hours, before the introduction of appropriate quantity of substrates. Toluene and the xylenes were introduced into the medium simultaneously, and the time between two pulses changed according to the toluene, xylenes, and intermediates residual concentrations.

Media and culture conditions

The composition of the medium used for enriching and maintaining the culture was the following: 3.3 g l^{-1} K_2HPO_4, 1.9 g l^{-1} $NaH_2PO_4.2H_2O$, 4.5 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.5 g l^{-1} $CaCl_2.2H_2O$, 10 ml l^{-1}trace-elements and 2.5 ml l^{-1} vitamins. The trace-element solution contained 5 g l^{-1} $CaCl_2.2H_2O$, 1 g l^{-1} $EDTA.2H_2O$, 1 g l^{-1} $FeSO_4.7H_2O$, 160 mg l^{-1} $MnCl_2.4H_2O$, 40 mg l^{-1} $ZnSO_4.7H_2O$, 30 mg l^{-1} H_3BO_3, 40 mg l^{-1} $CoCl_2.2H_2O$, 40 mg l^{-1} $CuCl_2.2H_2O$, 4.6 mg l^{-1} $NiCl_2.H_2O$ and 40 mg l^{-1} $NaMoO_4.2H_2O$. The vitamin solution consisted of 8 mg l^{-1} biotin, 100 mg l^{-1} p-aminobenzoic acid, 0.2 gl^{-1} nicotinic acid, 0.2 g l^{-1} thiamin-Cl, 0.2 g l^{-1} Ca-pantothenate, 0.2 g l^{-1}pyridoxine-HCl, 20 mg l^{-1} cyanocobalamin, 80 mg l^{-1} riboflavine, 40 mg l^{-1} folic acid, 0.2 g l^{-1} choline-Cl and 0.8 g l^{-1} myo-inositol.

The only carbon and energy source was the toluene-xylenes mixture added to the reactor in the liquid phase. Toluene and the xylenes were supplied by Fluka, Buchs, Switzerland. In order to avoid the toluene-xylenes mixture being stripped out, due to its high volatility, a discontinuous oxygenation mode was employed instead of a continuous one. The cultivation started by oxygenation, until the medium was saturated by oxygen. Then the reactor was operated as a closed system. The substrate was added by pulses. During the first pulse, the substrate and the oxygen concentration in the medium decrease until the substrate was consumed totally. A new pulse of substrate addition followed. The next oxygenation cycle started when dissolved oxygen present in the medium was almost all consumed.

The number of substrate pulses during one oxygenation cycle was determined so that dissolved oxygen was always present. The time between two pulses was sufficiently long to complete degradation of toluene and xylenes, as well as the metabolites produced.

A production cycle was to the interval of time between the start and end of biomass production using the same concentration of substrate added by each pulse, and it was composed of several oxygenation cycles.

Reactor

Growth of biomass was carried out in a two-liter fermentor KLF 2000 (Bioengineering AG, Wald, Switzerland) (1000 rpm, 30 °C) containing one liter of medium. The regulation of the temperature, pH,

aeration rate, and substrate supply during cultivation was controlled automatically.

As oxygen consumption was faster than carbon dioxide production, a pressure reduction inside the reactor could have caused non-scheduled, unwanted substrate addition by aspiration. In order to solve this problem, a bag (Bag Tedlar, Gas sampling; Bag, 21, No41003, $9\times9''$) was connected to the reactor by a teflon tube. Solvents were introduced by means of two calibrated syringes (series 1000, Haminton, Reno, Nevada, USA) of 50 ml and 10 ml volume for toluene and the xylenes mixture, respectively, and which were controlled by a multi-syringe pump (multichannel-syringe pumps R74900-Series Cole Palmer, Vernon Hills, Illinois, USA).

Analytical methods

Concentrations of toluene and the xylenes mixture were determined by capillary gas chromatography (Varian Star 3400 Cx, Varian, Palo Alto, USA), with a flame ionization detector fitted and a DB-624 capillary column (30 m length, 0.53 mm diameter) (J & W Scientific, Folsom, USA). Samples were analyzed at a 5.8 ml min^{-1} nitrogen flow rate and at 580 ml min^{-1} split injection. Injection and detector temperatures were 220 °C and oven temperature was increased at a rate of 10 °C min^{-1} from 80 °C to a final temperature of 150 °C. The culture was sampled by means of a 100 μl sample syringe (Dinatech, series A-2, pressure lock, Baton Rouge, USA).

The accumulation of metabolites in the culture medium and cells extracts was analyzed by High Pressure Liquid Chromatography (Varian 910, Zug, Switzerland) with an ORH801 column (Interaction chromatography Inc., San Jose, USA). The mobile phase was sulfuric acid (0.01N) at a flow rate of 1 ml min^{-1}. The cell extract was prepared by passing cell suspension twice through a French press (Aminco, Urbana, USA) at 90 Mpa. After filtration of medium through a 0.2-μm filter (Schleicher and Schuell, Dassel, Germany) the metabolites were detected by measuring the UV-absorbance at 255 nm with a UV-spectrophotometer with double beam (Hitachi U-2000, Tokyo, Japan), and identified by comparing their retention times and absorption spectra with those of pure substances.

Culture growth was monitored by dry-weight duplicate determination from 15 ml of sample (24 h of drying at 105 °C) and by spectrophotometry at 650 nm after 15 s of ultrasonic disintegration (Bransonic, Danbury, USA). The absorbance was correlated to dry weight by an almost constant factor of 0.8 and was used to estimate biomass concentration.

Dissolved organic carbon (DOC) was determined by a TOC-5050A, ASI-5000A analyzer (Schimadzu, Japan). Before DOC analysis the sample was filtrated through a 0.2 μm filter (Schleicher & Schuell, Dassel Germany), acidified with HCl and gasified.

Oxygen uptake rate was determined by measuring the concentration of dissolved oxygen in the culture media using a polarographic probe (Ingold AG, Urdorf, Switzerland) after reducing the agitation rate to 300 rpm in order to limit oxygen transfer.

The biomass growth yield and productivity were determined and defined as increase in biomass dry weight per unit amount of substrate consumed (g_{dw} g_s^{-1}) and the increase in biomass dry weight per unit of medium volume and time (mg_{DW}^{-1} l^{-1} h^{-1}), respectively.

Results and discussion

We studied the ability of an adapted bacterial consortium to degrade simultaneously toluene and *o*-, *m*- and *p*-xylene in a mixture. The substrates were added to the medium by pulses. In the course of each experiment, the specific degradation rate decreased as the quantity of the substrates increased.

Degradation of toluene and xylenes in mixture

In order to investigate the degradation efficiency of the adapted mixed culture toward toluene and each of the xylenes, its mixture was added to the medium as the sole source of carbon and energy. Typical kinetics of degradation of toluene and xylenes in mixture during one pulse is presented in Figure 1. We observed that our adapted bacterial consortia were able to degrade *o*-xylene simultaneously with *m*- and *p*-xylene and toluene.

The effect of quantities of toluene and xylenes added to the medium on biomass yield was investigated. The following quantities were added to medium: 30, 70, 150, 200, and 300 μl of toluene with 3, 7, 15, 20, and 30 μl of xylenes in mixture. The data are presented in Table 1. The adapted bacterial consortia degraded 30 to 200 μl of toluene and 3 to 20 μl of xylenes in mixture per liter of medium. 150 μl of toluene and 15 μl xylenes in mixture for a pulse interval of 60 min was the optimal concentration for biomass productivity. With these quantities, the maximal bio-

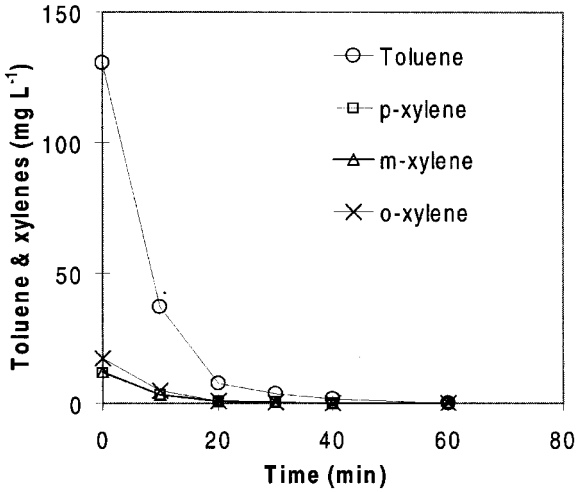

Figure 1. Degradation of 150 μl toluene and 15 μl xylenes by adapted bacterial consortium during one pulse.

Table 1. Growth yield and biomass productivity using different quantities of toluene and xylenes

Toluene and xylenes added by pulses (μl)	Growth yield ($g_{DW} g_S^{-1}$)	Productivity ($mg_{DW}\ l^{-1}\ h^{-1}$)
30 + 3	0.41	19
50 + 5	0.43	38
150 + 15	0.32	53
200 + 20	0.27	28
300 + 30	0	0

mass productivity was 53 mg l^{-1} h^{-1} and the biomass yield was 0.32 g_{DW} g_s^{-1}.

The growth was inhibited when the concentrations of substrates in the pulses increased up to 200 μl toluene and 20 μl of xylenes. At this concentration, the biomass production was low: The biomass productivity decreased from 53 mg $l^{-1}h^{-1}$ to 28 mg $l^{-1}h^{-1}$, and the yield was 0.27 $g_{DW}g_s^{-1}$. The inhibition was greater when substrate addition was increased to 300 μl of toluene and 30 μl of xylenes, for which biomass prouction was zero.

We measured the substrate consumption rate (Q_s) and the oxygen consumption rate (Q_{O2}) at the beginning ($Q_s\ t_o$, $Q_{O2}\ t_o$) and at the end ($Q_s\ t_f$, $Q_{O2}\ t_f$) of each production cycle for the different substrate concentrations. The results are presented in Table 2. We observed that both rates decreased in the course of each production cycle, regardless of the substrate concentration. It is possible that this decrease is related to metabolite accumulation.

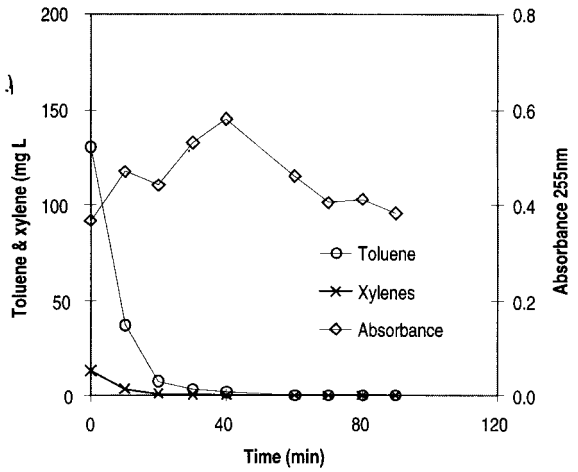

Figure 2. Evolution of the absorbance (A_{255}) and substrate degradation of 150 μl toluene and 15 μl xylenes during one pulse.

Metabolites

Figure 2 shows the evolution of the metabolites (A_{255}) and degradation of substrates using optimal substrate quantity of 150 μl of toluene and 15 μl of xylenes.

When the optimal quantities of 150 μl toluene and 15 μl xylenes were used, we observed that in the first phase of substrate degradation, the absorbance at 255 nm increased from an initial value of 0.35 to a maximal value of 0.60 and then decreased below 0.35. It suggests that the substrate and metabolites were totally degraded in 90 min (Figure 2). Toluene was degraded in 40 min, while xylenes in 20 min, due to the lower initial concentration. The absorbance increase from 0.5 to 0.8 occurred during the time of toluene removal and probably was due to the accumulation of metabolites containing aromatic or aliphatic double bonds which absorb UV light.

The accumulation of metabolites was observed by the UV-absorbance at 255 nm and DOC increase for all production cycles. Figure 3 presents the evolution absorbance, biomass concentration, and DOC during a production cycle at 150 μl toluene and 15 μl xylenes added by pulses. DOC increases from 62 mg l^{-1} to 98 mg l^{-1}. The absorbance stayed almost constant when compared to the DOC and biomass concentration increase. In one production cycle adding 200 μl of toluene and 20 μl of xylenes, the DOC increased from 69 mg l^{-1} to 152 mg l^{-1} and the absorbance from 0.4 to 1.8. This significant increase, relates to accumulation of aromatic metabolites, was confirmed by HPLC analysis. Many peaks at different retention times were observed. Although the cells were able to

Table 2. Specific substrate consumption rate and specific oxygen consumption rate using different quantities of toluene and xylenes in each production cycle

Toluene and xylene added by pulses μl	$Qs\ t_o$ ($mg_S\ g_{DW}^{-1}\ h^{-1}$)	$Qs\ t_f$ ($mg_S\ g_{DW}^{-1}\ h^{-1}$)	$Q_{O2}\ t_o$ (mmol O_2 $g_{DW}^{-1}\ h^{-1}$)	$Q_{O2}\ t_f$ (mmol O_2 $g_{DW}^{-1}\ h^{-1}$)
30 + 3	18	11	3.9	1.2
70 + 7	26	11	4.4	3.4
150 + 15	81	44	5.3	2.8
200 + 20	63	12	6.9	3.7

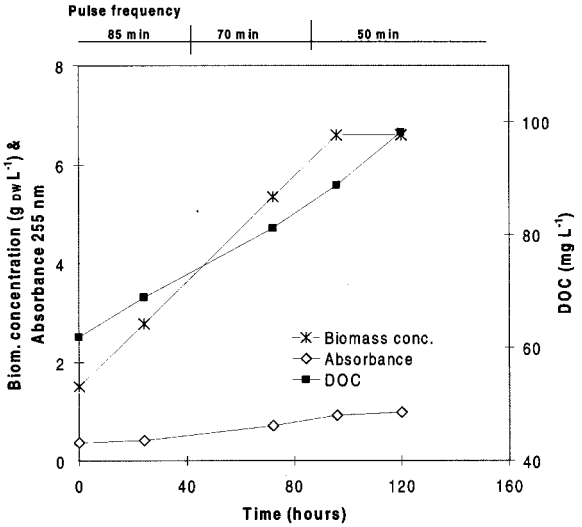

Figure 3. Absorbance (A_{255}), biomass and DOC concentration during a production cycle adding 150 μl toluene and 15 μl xylenes by pulses.

degrade 200 μl of toluene and 20 μl of xylenes, an important, progressive metabolites accumulation was observed.

The accumulation of detected metabolites in the medium and their influence on bacterial growth was investigated by adding 300 μl of toluene and 30 μl of xylenes by pulses. The cells were able to degrade the substrates in the first pulse, but already in the third pulse, cell growth was inhibited. Bacterial growth stopped if the absorbance at 255 nm exceed 1.8. The HPLC and gas chromatographic analyses of medium and cell extract revealed the presence of many peaks corresponding to different metabolites. However, no peak corresponded to the initial substrate peaks, that is, toluene or any of xylenes. That means that substrate was completely transformed and an accumulation of metabolites occurred. In these conditions, after growth inhibition, an important lysis of bacterial cells occurred and resulted in foaming because of intracellular protein release into the medium. In addition, the culture medium turned brown then black within 24 hours. This color was probably due to catechol accumulation. Di Lecce et al. (1997) observed that the cells turned brown during growth on *m*-xylene and *p*-xylene, suggesting the accumulation of partially oxidized metabolites. It is possible that bacterial activity was inhibited by the intermediate formation. Mirpuri et al. (1997) observed when cells degrading toluene at low concentration were suddenly exposed to high concentration, substrate uptake rates increased accordingly. This increase led then to the accumulation of intracellular intermediates up to the inhibitory level.

The HPLC spectra of the culture medium were compared with the spectra of the standard substances. The metabolite having the retention time of R_t= 2.7 min was identified as 4-methylcatechol. Duggleby and Williams (1986) identified the same metabolite in the course of toluene and xylenes degradation.

The HPLC analysis of the culture medium also revealed the presence of peak at R_t = 6.1 min and R_t= 6.6 min, which are attributed to the 2,4- and 2,5-dimetylphenol, respectively. Di Lecce et al. (1997) identified the same metabolites. The operating conditions we applied in HPLC analyses, were similar to the conditions described by these authors.

Optimization

The objective of these experiments was to increase the volumetric mass loading ($mg_S\ g_{DW}^{-1}h^{-1}$) to a maximum. We studied the frequency of pulses, the time between each pulse, and the influence of these parameters on biomass production.

Figure 4 shows the evolution of biomass concentration and biomass productivity during a production

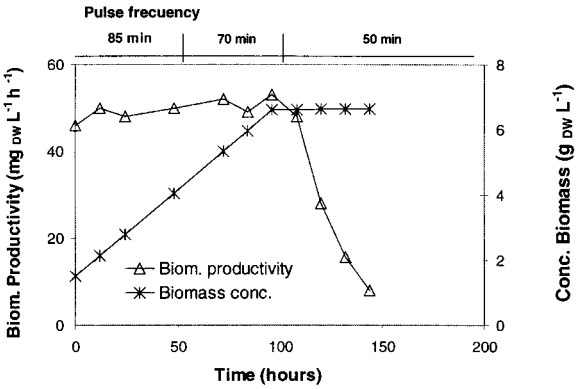

Figure 4. Biomass concentration and biomass productivity during a production cycle adding 150 μl toluene and 15 μl xylenes by pulses.

cycle using 150 μl toluene and 15 μl xylenes. The intervals of pulses studied were: 85, 70 and 50 min. The biomass productivity increased rapidly up to 53 mg l^{-1} h^{-1}, and remained constant to the point when biomass concentration attained its maximal value of 7 g l^{-1}. To increase the biomass production, it would be necessary to increase the substrate loading by increasing the quantities of substrates added by each pulse or the frequency of pulses.

The evolution of biomass concentration and substrate mass loading for each production cycle with the different quantities of substrates added by pulses was investigated. The highest mass loading and biomass concentration was obtained adding 150 μl toluene and 15 μl xylenes. Higher substrate quantities, that is 200 μl of toluene and 20 μl of xylenes mixture, caused the substrate mass loading and biomass concentration to decrease. The interval of time could not be reduced, because in this case the bacteria had not enough time to mineralize neither substrate nor metabolites.

In conclusion, the adapted bacterial consortium was able to degrade toluene, *m*-xylene, *p*-xylene, and *o*-xylene simultaneously in the batch culture. The highest substrate consumption rate and the biomass concentration were obtained when operating at 150 μl toluene and 15 μl xylenes added by pulses. The substrate consumption rate and the biomass concentration decreased when quantities of 200 μl of toluene and 20 μl of xylenes in mixture were used. Finally, bacterial growth was inhibited if 300 μl of toluene and 30 μl of xylenes mixture were added to the culture medium. The accumulation of metabolites was observed. Among the produced metabolites 4-metylcathecol, 2,4- and 2,5-dimethylphenol were detected in the culture medium.

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References

Baggi G, Barbieri P, Galli E & Tollari S (1987) Isolation of a *Pseudomonas stutzeri* strain that degrades *o*-xylene. Appl. Environ. Microbiol. 53: 2129–2132

Barbieri P, Palladino L, Di Gennaro P & Galli E (1993) Alternative pathways for *o*-xylene or *m*-xylene and *p*-xylene degradation in a *Pseudomonas stutzeri* strain. Biodegradation 4: 71–80

Dugglebely CJ & Williams PA (1986) Purification and some properties of the 2-hydroxy-6-oxohepta-2,4-dienoate (2-hydroxymuconic semialhyde hydrolase) encoded by the TOL plasmid pWW0 from *Pseudomonas putida* mt 2. J. Gen. Microbiol. 132: 717–726

Lee JY, Jung KH, Choi SH & Kim HS (1995) Combination of the tod and the tol pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralization of benzene, toluene and *p*-xylene mixture. Appl. Environ. Microbiol. 61: 2211–2217

Di Lecce C, Accarino M, Bolognese F, Galli E & Barbieri P (1997) Isolation and metabolic characterization of a *Pseudomonas stutzeri* mutant able to grow on the three isomers of xylene. Appl. Environ. Microbiol. 63: 3279–3281

Gibson DT, Mahadaven V & Davey JF (1974) Bacterial metabolism of *p*-xylene and *m*-xylene: oxidation of the aromatic ring. J. Bacteriol. 119: 930–936

Gibson DT & Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds (pp 181–252). Marcel Dekker, Inc., New York

Mirpuri RG, Jones WL, McFeters GA & Ridgway HF (1997) Physiological stress in batch cultures of *Pseudomonas putida* 54G during toluene degradation. J. Microb. Biotechnol. 18: 406–413

Oh YS, Shareefdeen Z, Baltzis BC & Bartha R (1994) Interactions between benzene, toluene and p-xylene (BTX) during their biodegradation. Biotechnol. Bioeng. 44: 533–538

Schraa G, Bethe BM, Van Neerven ARW, Van Den Tweel WWJ, Van Der Wende E & Zehnder AJB (1987) Degradation of 1,2-dimethylbenzene by *Corynebaterium* strain C125. Antonie van Leewenhoek 53: 159–170

Worsey MJ & Williams PA (1975) Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124: 7–13