# Upgraded conventional cultivation techniques for the mass production of bacterial communities adapted to the degradation of volatile organic compounds (TEX)

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ABSTRACT. This study focuses on an automated Substrate Pulse Batch (SPB) technique used for the mass cultivation of bacteria adapted to the degradation of a mixture composed of toluene, ethylbenzene, *o*-, *m*- and *p*-xylenes (TEX). A small-scale prototype reactor was designed. A computer-based monitoring program was also developed. The key parameters to be monitored were handled by LabVIEW including, temperature, pH, dissolved oxygen and turbidity. Other parameters, such as biomass, ammonium or residual substrate concentrations needed offline measurements.
SPB technique has been successfully tested experimentally on TEX. The dynamic behavior of the mixed bacterial population was observed under different operational conditions. Average productivity and yield values obtained were 0.45 $kg_{DW}$ $m^{-3}$ $d^{-1}$ and 0.59 $g_{DW}$ $g_C^{-1}$, respectively. These data come up to the industrial specifications and confirm the benefits of such an improved technology. Adapted microorganisms obtained in this way can play a major role in the removal of hydrocarbons from industrial off-gases, degradation of substitute organic solvents, reduction in odors from wastewater plants and the food industry or even the removal of pollutant mixtures containing chlorinated solvents.

## 1 INTRODUCTION

Emission of aromatic hydrocarbons to the environment is still escalating in spite of governmental intervention in many countries. Volatile compounds such as toluene, ethylbenzene and xylenes (TEX) are known to be toxic towards bacteria, even for species able to withstand them (De Bont, 1998).
Many studies devoted to biodegradation of contaminated waste gases clearly proved their benefits. In particular, the SPB technique is an attractive process for the production of a specialized biomass used to inoculate biosystems which treat volatile organic compounds (VOCs). SPB can be considered as a special limit case of a conventional, semi-continuous extended culture operation: total volume remains virtually constant because substrate is fed in a gaseous, solid or very concentrated liquid form, according to an intermittent profile (i.e. substrate injected by repeated pulses in the bioreactor). This process is recommended to achieve a more stable and efficient biomass culture. However, its industrial implementation is limited because of its rules of thumb.

This study was aimed to upgrade this state-of-the-art technique in context and in the framework of the BIOMAC project (Bioreactor For Innovative Mass Bacteria Culture, Eureka Project E!2497). In particular, gathering enough information was necessary to design an on-line control strategy in order to optimize the productivity of cell-mass, using SPB technique.
The ability of an adapted bacterial consortium to degrade TEX in an automated bioreactor was studied. The main objective has been to produce active biomass with interesting degradation yield and productivity corresponding to industrial specifications.

## 2 MATERIAL AND METHODS

### 2.1 *Inoculum preparation*

The bacterial consortium used to inoculate the bioreactor was selected in our laboratory in a previous work (León *et al*., 1999). The bacteria were originally issued from the sludge of a wastewater treatment plant (Novartis and Rohner AG, Basel, Switzerland) and from the biotrickling filters (Rohner AG, Basel, Switzerland), adapted to the TEX degradation and cultivated on TEX or toluene as sole source of carbon then preserved at -80°C. A sample of this adapted mixed culture was cultivated in flasks containing nutrient medium (CM67, Oxoid LTD, Basingtoke, England) at 30°C for 1 day. Then the obtained biomass was collected by centrifugation and transferred into the batch reactor. The nutrient medium consisted of inorganic salts and vitamins necessary for the growth of microorganisms, the same composition as previously reported (León *et al*., 1999) with ammonium as the nitrogen source. The sole carbon and energy source consisted in 90 % (v/v) toluene (Tol), 2.5 % ethylbenzene (EB), 6.5 % m- and p-xylenes (X), 1.3 % o-xylene. This mixture is called "TEX". Compounds were supplied by Fluka, Buchs, Switzerland. Experiments were made under non-sterile conditions. The volatile organic carbons (VOCs) i.e., TEX were supplied by a precision feeding pump (SpectraPhysics, San José, Calif.).

### 2.2 *Bioreactor and automation program*

The experimental setup used in this study is presented in Figure 1. The bioreactor is made of stainless steel and Pyrex glass; with a total volume of 14.5 l. The working liquid volume was 10 l. Numerous ports along its height and at the bottom allow feeding, sampling and connecting the measuring probes. The characteristics of the reactor and the operating conditions for the experiments are described in Table 1.

Table 1. Operational data for the bioreactor.

| Parameter | Value | Control | *Regulation* |
|---|---|---|---|
| Temperature | 35 °C | PT 100 | *Bain-marie, digital thermostat* |
| pH | 6.5-7 | Electrode, Liquisys S | *LabVIEW (Threshold contact)* |
| Dissolved Oxygen (DO) | 0.8-98 % L=31.7 cm | Oximetric probe Ingold Messtecknick | *LabVIEW (100 %=7.14 mg/l)* |
| Agitation | 170-800 RPM | Motor : Lust | *LabVIEW* |
| Aeration | 0.5-0.7 l/min | Brooks Instrument | *LabVIEW ($K_{La}$=3-9 $h^{-1}$)* |

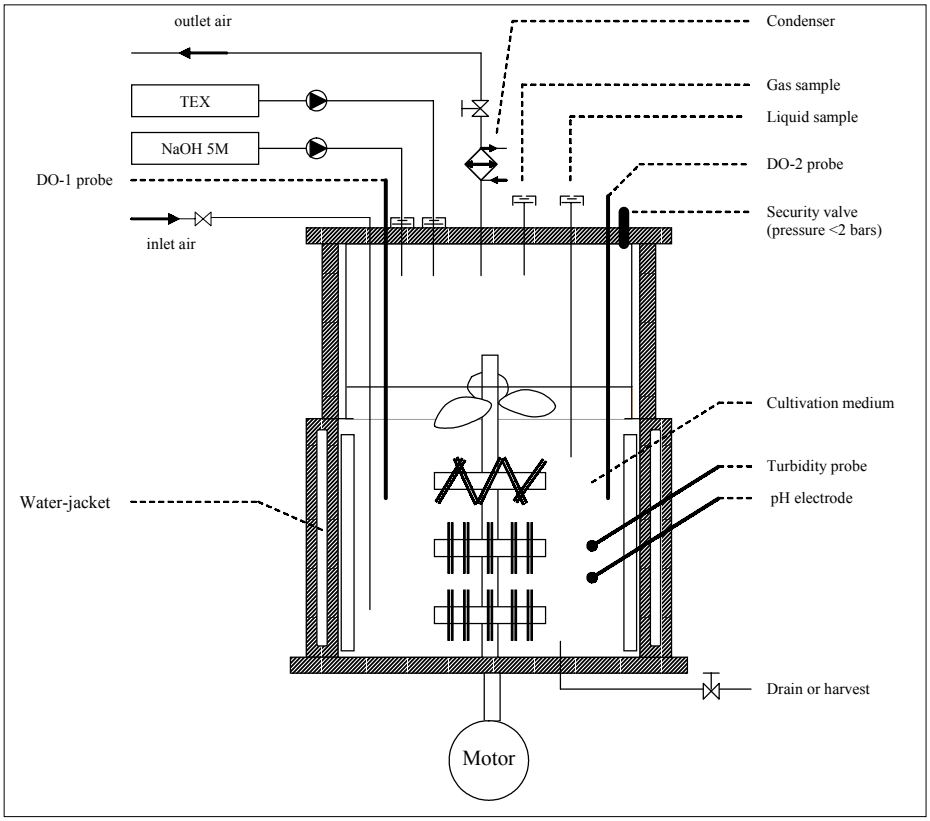


Figure 1. Schematic presentation of the laboratory bioreactor.

The overall setup was controlled by the so-called BioOPT program. This LabVIEW computer-based monitoring program allows data acquisition and was used to optimize the biodegradation process. The advantages of the BioOPT program leans more on its flexibility than on its analytical properties. The program records the instrumental data (inputs) and at the same time commands the output terminals. Up to now, command and data acquisition are still partially separated, but the aim is to reinforce the interaction between both. It would provide a completely autonomous system.
Modulable FieldPoints (National Instruments) were the connecting tools between computer and instrumental systems. The main electrical components are listed in Table 2.

Table 2. List of the National Instruments modules.

| | |
|---|---|
| **FP-2000** | Ethernet network module capable of running LabVIEW Real-Time embedded code and 3 Mb Flash Memory for user |
| **FP-AI-110** | 8 analog inputs 0-20 /4-20 mA, 16 bits, 50/60 Hz filtering |
| **FP-AO-200** | 8 analog outputs 0-20 /4-20 mA, 12 bits |
| **FP-RLY-420** | 8 relays, SPST (form A), 3 A at 250 VAC or 35 VDC |
| **FP-RTD-PT100** | Dual Channel I/O: 4-wire RTD input modules (-50 to 350 °C) |
| **FP-TB-10** | Dual-Channel terminal base for 6 dual-channel I/O modules |

2.3 *Analytical methods*

Concentrations of TEX mixture were determined by capillary gas chromatography (GC -14B, Integrator C-R65A, Shimadzu, France), with a flame ionization detector fitted and a DB-624 capillary column (30 m length, 0.53 mm diameter) (J&W Scientific, Folsom, California). Samples were analyzed at a 32 ml/min nitrogen flow rate and at 30 ml/min split injection. Injection and detector temperatures were 220 and 300°C, resp. Oven temperature was increased at a rate of 4°C/min from 85°C to a final temperature of 110°C. The culture was sampled by means of a 250 □l Gas-tight syringe (Dynatech, Serie A2, Bâton-Rouge, USA).

Dissolved organic carbon concentration was measured by an IR detector of Shimadzu TOC-500 (Burkard Instrumente, Switzerland). The reactor sample was filtrated at 0.45 mm (Schleicher&Schuell, Germany), acidified by HCl 2 M and the $CO_2$ was purged before analysis.

Metabolites estimation was made by measuring the optical density of the filtered sample at 255 nm in an UV spectrophotometer (Hitachi U-2000, Tokyo, Japan). Ammonium and nitrate in the liquid phase of the batch reactor were determined by the enzymatic method (Boehringer, Manheim, Germany) to control the nitrogen measurement for VOC degradation.

The biomass concentration was monitored by dry-weight measurement and by absorbance at 650 nm.

## 3 RESULTS AND DISCUSSION

To study the ability of an adapted bacterial consortium to degrade TEX in an automated batch bioreactor first the cultivation of a mixed bacteria was carried out under different operational conditions, then biological and kinetics parameters were controlled. The aim was to produce active biomass with interesting degradation yield and productivity according to industrial specifications.

3.1 *Biomass cultivation – SPB technique*

SPB technique aims to save both overall heterogeneity and kinetic dynamics of the mixed population thanks to the continual variation of substrate concentration in the medium. Short contacts due to rapid degradation also avoid toxic damages which may result from the solvent effects onto bacterial cells (De Bont, 1998). Therefore such a process allows a better stability to the culture and improved biomass productivity. However it is mainly built on empiricism. In this context, the operator plays a crucial role, which limits the industrial implementation. This is the reason why this study focuses on the automation of the process.

The cultivation of the mixed bacteria in this study was handled in a batch culture with the pulse feeding technique. The overall process can be categorized into two periods. First, microorganisms are progressively fed with increasing but still low amounts of TEX. This step allows the induction of the enzymatic pool and strongly influences the further TEX degradation (Grady *et al*., 1996). Second, the most efficient substrate loading is investigated in order to improve both productivity and degradation yields.

During the cultivation, four parameters are checked to have the best conditions for microbial growth:

• TEX concentrations in the gas phase;
• Intermediate concentration in the liquid phase;

• Oxygen concentration in the liquid phase. It should be at least 20 % of the saturation concentration. It is measured in two places in the bioreactor (Dissolved Oxygen probes DO-1 and DO-2);
• Nitrogen concentration (ammonia) in the batch medium shall be higher than 100 mg/l to avoid limitation of the bacterial growth.

In this study, volatile substrates (TEX) are injected by repeated pulses in the gaseous phase. 0.15 m above the liquid surface is the selected height to perform that crucial step: it favors and regulates the injection of the convenient amount of solvents (no overpressure) and limits any uncontrolled volatilization losses (stripping process). The equilibrium between gas and liquid phases is reached in 2 minutes after the end of the TEX injection. The stripping process depends on both aeration and agitation.

Three steps are successively performed. Phase 1 – aeration for about 5 minutes to saturate the liquid phase. Air flow was set at 7 to 10 l/min and 80 % of the substrate would be stripped if it were injected at the beginning of this phase. Phase 2 – injection of TEX – within15 seconds. The substrate amounts provided to the culture can be modified according to the magnitude and/or the frequency of the injected pulses. Phase 3 – reaction – allows the biodegradation of the parent molecules and their intermediate metabolites. The delay depends on the microbial activity. During phases 2 and 3, the air flow was set at 0.5 l/min to minimize the stripping (evaluated at resp. 2 % and 7 to 23 % of the initial TEX amount).

### 3.2 *Automation parameters*

Each of the three phases can be precisely defined at the beginning of the cultivation process. Experimental data are automatically recorded and the instrumental system is completely autonomous, except for the decision to increase the substrate loading. Indeed, it depends on the behavior of the biomass which should consume the solvents as expected. It means for the bacteria to incorporate the parent molecules, degrade them and their metabolites. Aerobic degradation of TEX is correlated with oxygen consumption. In particular, minimum of dissolved oxygen concentration ($DO_{min}$) is recorded whenever no residual concentration of TEX is measured in the gaseous phase (manual GC analyses). Therefore it is possible to indirectly control the convenient degradation of TEX by the automatic and continuously recorded DO evolution. In fact, two parameters are used to characterize the bacterial cultivation: (1) time necessary to degrade TEX during phase 3 and (2) decrease of DO. First, time delay of phase 3 can be forecasted according to the cellular yield of biomass production $Y_{X/S}$. They allow the calculation of the global rate of substrate degradation $q_s$ ($q_s = \mu/Y_{X/S}$). Then frequency of the pulses is determined in a way that the charge of TEX is inferior to the maximum degradative capacity of consortium ($= q_s.[X]$). Unfortunately, this method is limited by the evolution of the consortium characteristics during the cultivation. Moreover, concentration of biomass still needs to be determined (manual operation).

Second, as previously reported, concomitant consumption of oxygen and TEX allows a DO-based regulation process. In particular, an extra-delay appears necessary after having reached $DO_{min}$. Time corresponds approximately to 1/3 of the delay necessary for the TEX to disappear from the aqueous phase; i.e. 1/3 of the time necessary to reach $DO_{min}$. It is attributed to the degradation of the intermediate by-products inside the bacterial cells. Therefore the succession of pulses can be automatically monitored and adapted to the real progression of the cultivation. However, such a method expects the reliability of the DO measurements which can be faltered in two cases. The first case happens when biomass growth implies an increasing $O_2$ demand: $O_2$ transfer becomes limiting into the liquid phase if the agitation remains the same. Thus DO variation is

attenuated and no $DO_{min}$ is clearly noted (Figure 2a). It outlines the need to link DO variation and agitation. For eg., □DO > 50 % can be specified in BioOPT program. Underneath this value, agitation would be automatically increased. The second case happens when microorganisms become unhealthy: $O_2$ consumption decreases but shortening the cycles would favor the bacterial inhibition. On the contrary, phase 3 should be lengthened (fig 2b). In both cases, it is supposed that DO probes are cleaned regularly (no bacterial film).

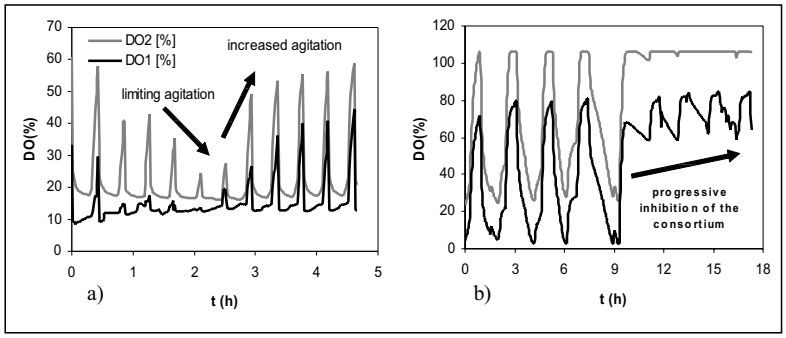


Figure 2. Evolution of DO during automatic runs of biomass cultivation.

3.3 *Performance of the bioreactor*

3.3.1 *Microbial growth and biological parameters*

The evolution of biomass concentration and substrate mass loading during the overall production process was investigated. Figure 3 illustrates the global behavior of the biological system.

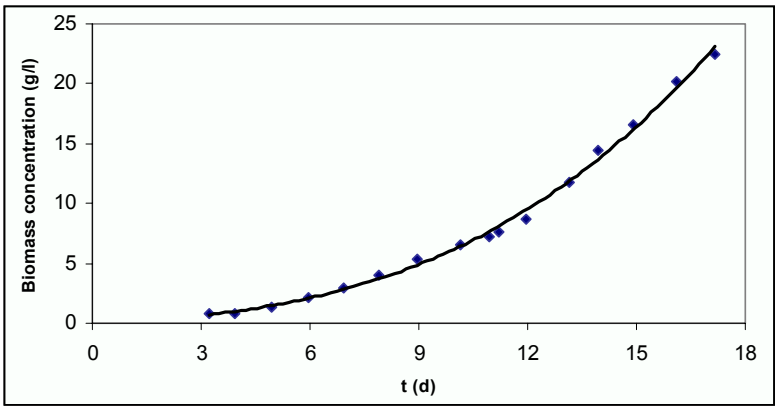


Figure 3. Evolution of the biomass concentration during the cultivation process.

Specific growth rates ($\mu_{max}$) were calculated to be ranged between 0.003 and 0.011 $h^{-1}$. These values are comparable with other reports (Shim and Yang, 1999). Indeed, they obtained $\mu_{max}$ of [0.0012 ; 0.002 $h^{-1}$] with toluene of [25 ; 60 mg/l] and $\mu_{max}$< 0.016 $h^{-1}$ with o-xylene concentration inferior to 50 mg/l.

During 30 days of cultivation, interval of time between successive pulses was reduced from 1.5 h until 0.5 h. Besides and alternatively to the higher frequency of pulses, the amounts of TEX were also increased from an initial concentration in the liquid phase of

24 $mg_c$/l until a final loading of 67 $mg_c$/l (stripping losses already subtracted). Therefore average productivity and yield values reached 0.45 $kg_{DW}$ $m^{-3}$ $d^{-1}$ and 0.59 $g_{DW}$ $g_c^{-1}$, resp. These data come up to the industrial specifications and confirm the benefits of such an improved technology.

3.3.2 *Kinetics*

Typical kinetics of degradation of TEX during one pulse is presented in Figure 4.

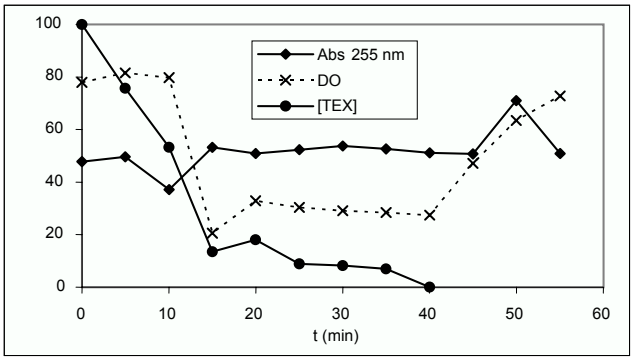


Figure 4. Disappearance ratio (%) of TEX, oxygen uptake and $A_{255}$ evolution during a representative pulse, after 4 days of cultivation.
$[TEX]_{l0}$ = 25 $mg_C$/l, [X] = 0.85 g/l, Agitation = 200 RPM, $Q_{air}$ = 0.5 l/min

TEX disappear immediately after their injection into the liquid phase. Three steps can be distinguished: (1) fast disappearance of TEX, concomitant with a high oxygen consumption during the first 15 minutes, (2) slower disappearance of TEX and stabilization of DO (lower bacterial consumption), (3) no residual concentration of TEX, significant increase of both DO and $A_{255}$ (absorbance at 255 nm). This step should correspond to the exportation of intermediate metabolites (León, 1999).

TEX degradation rates were investigated during the overall process. Two methods are available: dynamic $q_s$ correspond to the slope of the TEX disappearance from the gas phase (GC analyzes) during the first 15 minutes after injection of TEX, whereas global $q_s$ is calculated with the loading of TEX. Thus it takes into account the degradation of both TEX and intermediate metabolites, and so, it is lower than the dynamic rate. Figure 5 shows the evolution of the degradation rates during the cultivation process.

Whichever used methodology to calculate $q_s$, maximum values are obtained at the beginning of the process and come up to a mean value of 0.02 $g_c/g_{DW}$ h. It is unlikely that the above-mentioned rates correspond to proper degradation of the initial compounds. At least, it gives the rate of cellular absorption. Therefore, decreasing data outline more an increasing difficulty for the bacteria to incorporate the substrate than a metabolic inhibition. Bielefeldt and Stensel (1998) observed the same behavior: the longer the cultivation process, the lower the degradation rates. A progressive deterioration of the bacterial walls is prospected to explain this evolution. It is due to the well-known toxicological characteristics of the lipophilic substrates. This remark reinforces the need to select the most efficient conditions to accelerate the adaptation phase, in order to obtain a stable process and improve the biomass productivity.

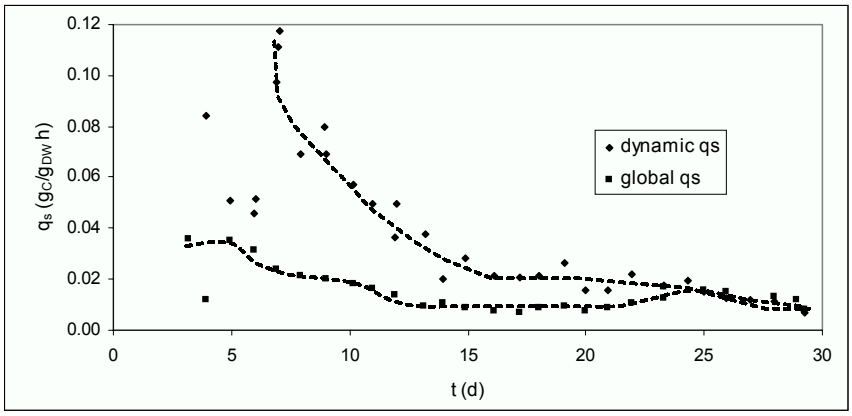


Figure 5. Evolution of the degradation rates $q_s$ during the cultivation process.

## 4 CONCLUSIONS

In this study, SPB technique has been adopted for the cultivation of bacteria for degradation of TEX. Obtained results indicated that relatively higher yields and productivity can be achieved using an automated process, in comparison with previous reports. In particular, this new generation of bioreactors can be used to produce large quantities of cell mass. They could be conditioned as granules or lyophilized. Microorganisms produced in this way can play a major role for the removal of hydrocarbons from industrial off-gases, degradation of substitute organic solvents, reduction in odors from wastewater plants and the food industry or even the removal of pollutant mixtures containing chlorinated solvents.

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