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A procedure for production of adapted bacteria to degrade chlorinated aromatics

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Abstract

Production of biomass adapted to the degradation of a mixture of chlorobenzene (CB) and 1,2-dichlorobenzene (DCB) was investigated in a batch culture with substrates supplied by pulses. CB and *o*-DCB concentrations which gave the best adapted biomass productivity were determined and found to be 150 and 30 μl l^{-1}, respectively. The biomass productivity was 51 mg l^{-1} h^{-1}. The biomass yield was 0.38 g of biomass dry weight per gram of substrate. The pulses of 200 μl CB and 40 μl *o*-DCB, were inhibitory to the bacterial culture. Among the metabolites, muconic acid was found in large quantities in the medium and in the cells.

At a time between two pulses of 60 min, adding 150 μl CB and 30 μl *o*-DCB per each pulse, 7.6 g l^{-1} of biomass was obtained.

The produced biomass served as an inoculum for the biotrickling filter which treated industrial waste gases contaminated by CBs.

The method of adapted biomass production was described using CBs, but the degradation of any other toxic volatile pollutant can be improved using this technique.

Keywords: Adapted biomass production; Batch culture; Chlorobenzenes; Biotrickling filter

1. Introduction

The extensive use of chlorinated benzenes as organic solvents, heat transfer agents and insecticides, and their production as intermediates in the synthesis of chemicals, such as pesticides and dyes, has led to release of these compounds into the environment. Even if the concentrations observed are too low to cause immediate toxicity, little information exists

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about long exposure to and bioaccumulation of chlorinated aromatics [1]. Chlorobenzene (CB) and 1,2-dichlorobenzene (DCB) have been identified among the others as priority pollutants by the US Environmental Protection Agency [2].

Biological treatment of waste gases, especially for those containing chlorinated organics, is of increasing interest in replacing chemical and physical methods.

The biofiltration process is a simple, practical and cost effective technology for the treatment of large volumes of air contaminated with low concentrations of biologically degradable compounds. It is a valuable alternative to the traditional volatile organic carbon (VOC) elimination technologies, such as incineration and adsorption. The low operating cost is mainly due to the use of microbial aerobic degradation at ambient conditions, rather than the more expensive oxidation by thermal or chemical means [3]. For a good working of a biotrickling filter specialized biomass able to degrade pollutants is needed.

The chlorinated aromatics can be used as sole source of carbon and energy by many strains of *Pseudomonas* [4–7], but in limited concentrations, due to their toxicity. The final degradation products, as well as the intermediates, can inhibit bacterial growth. One group of toxic metabolites has been identified as chlorocatechols [4,7,8].

From CBs biodegradation studies published recently [6,9,10], it can be seen that the biomass concentration did not exceed significantly $0.5\,g\,l^{-1}$ in a liquid media. However, the aim of research of the majority of the authors were focused on the increase of CBs biodegradation rate, and not on biomass productivity.

The purpose of our work was to develop a procedure for production of the starter culture to inoculate the biotrickling filter which has to degrade CB and DCB in the industrial waste gases.

The objective of the present study was to optimise biomass production for the biodegradation of CB and DCB mixture in a batch culture with substrates supplied by pulses. Different substrate concentrations were studied to define the conditions to obtain the highest biomass productivity. In the second part of this study, the maximal substrate concentration that can be used without affecting the growth inhibition was determined, and the biomass production was optimised by decreasing the time interval between each pulse.

2. Material and methods

2.1. Microorganisms

Initial bacterial consortia were collected from an industrial wastewater treatment plant and from two industrial biofilters treating chlorinated aromatic solvents (Schweizerhalle, Novartis, Switzerland and Rohner AG, Switzerland).

The individual members of the consortia were isolated by streaking on Plate Count Agar. The isolated strains were identified using the Biolog® Microplate™ system (Biolog Inc., Hayward, USA) and their characteristics were compared with the classification scheme in Bergeys' manual [11].

In the culture, a Live/Dead BacLight Bacterial Viability™ Kit (Molecular Probes Inc., Eugene, USA) was used to distinguish live bacteria with intact plasma membranes from dead bacteria with compromised membranes.

2.2. Adaptation and enrichment

The bacterial cultures containing 1.5–1.8 $g_{DW}\,l^{-1}$ (gram of dry weight per litre) were fed manually, first with 50 μl CB and 10 μl DCB pulses, then automatically for 16 h before the introduction of appropriate quantity of CBs. All experiments with different charges were inoculated with the same inoculum and were performed in a one-run experiment, as in toluene and xylenes degradation [12]. The CB and DCB were supplied by Fluka, Buchs, Switzerland. Both pollutants were introduced into the medium simultaneously and the time between two pulses changed according to the CB and DCB consumption rates and the concentrations added.

2.3. Media and culture conditions

Cultures were grown at 30°C in a mineral salt medium consisting of, per litre, 3.3 g K_2HPO_4, 1.9 g $NaH_2PO_4{\cdot}2H_2O$, 4.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.5 g $CaCl_2{\cdot}2H_2O$, 10 ml trace-elements and 2.5 ml vitamin solution. The trace-element solution contained (per litre) 5 g $CaCl_2{\cdot}2H_2O$, 1 g EDTA$\cdot 2H_2O$, 1 g $FeSO_4{\cdot}7H_2O$, 160 mg $MnCl_2{\cdot}4H_2O$, 40 mg $ZnSO_4{\cdot}7H_2O$, 30 mg H_3BO_3, 40 mg $CoCl_2{\cdot}2H_2O$, 40 mg $CuCl_2{\cdot}2H_2O$, 4.6 mg $NiCl_2{\cdot}H_2O$ and 40 mg $NaMoO_4{\cdot}2H_2O$. The vitamin solution consisted of 8 mg biotin, 100 mg *p*-aminobenzoic acid, 0.2 g nicotinic acid, 0.2 g thiamin-Cl, 0.2 g Ca-pantothenate, 0.2 g pyridoxine-HCl, 20 mg cyanocobalamin, 80 mg riboflavine, 40 mg folic acid, 0.2 g choline-Cl and 0.8 g myo-inositol. CB and DCB served as the sole carbon and energy source and was supplied manually in the liquid phase by a 250 μl syringe (series 1000, Hamilton, Reno, USA) or automatically by two syringes, of 50 and 10 ml for CB and DCB, respectively, connected to a multi-syringe pump (R74900-Series, Cole Parmer, Vernon Hills, USA).

Separate experiments were carried out for each mixture of CB and DCB applied, using the same source and the same concentration of inoculum. All experiments were repeated three times in order to assure the reproducibility of the results.

2.4. Reactor

Growth of biomass was carried out in a 2 l fermentor KLF 2000 (Bioengineering AG, Wald, Switzerland) with 400 rpm, at 30°C containing 1 l of medium (Fig. 1). The pH was maintained at 7.0 with NaOH (2 M) with a pH controller. The ports were closed with teflon-wrapped rubber stoppers. The culture was aerated with oxygen up to saturation. The reactor was connected to a 2 l tedlar bag (Alltech Associates Inc., Deerfield, USA) to compensate depression for the oxygen consumption during CB degradation. Solvents, CB and DCB, were introduced together at 10 $ml\,h^{-1}$ with the multi-syringe pumps. The quantities of CB and DCB added per litre were: 50 and 10, 100 and 20, 150 and 30 then 200 and 40 μl. The volumetric ratio of 5:1 corresponds roughly to industrial gas effluents emission of two CBs from Rohner SA chemical industry (Basel, Switzerland).

2.5. Analytical methods

Growth of the cultures was monitored by dry-weight (DW) measurements (duplicate from samples of 15 ml, dried during 24 h at 105°C and by spectrophotometry at 650 nm

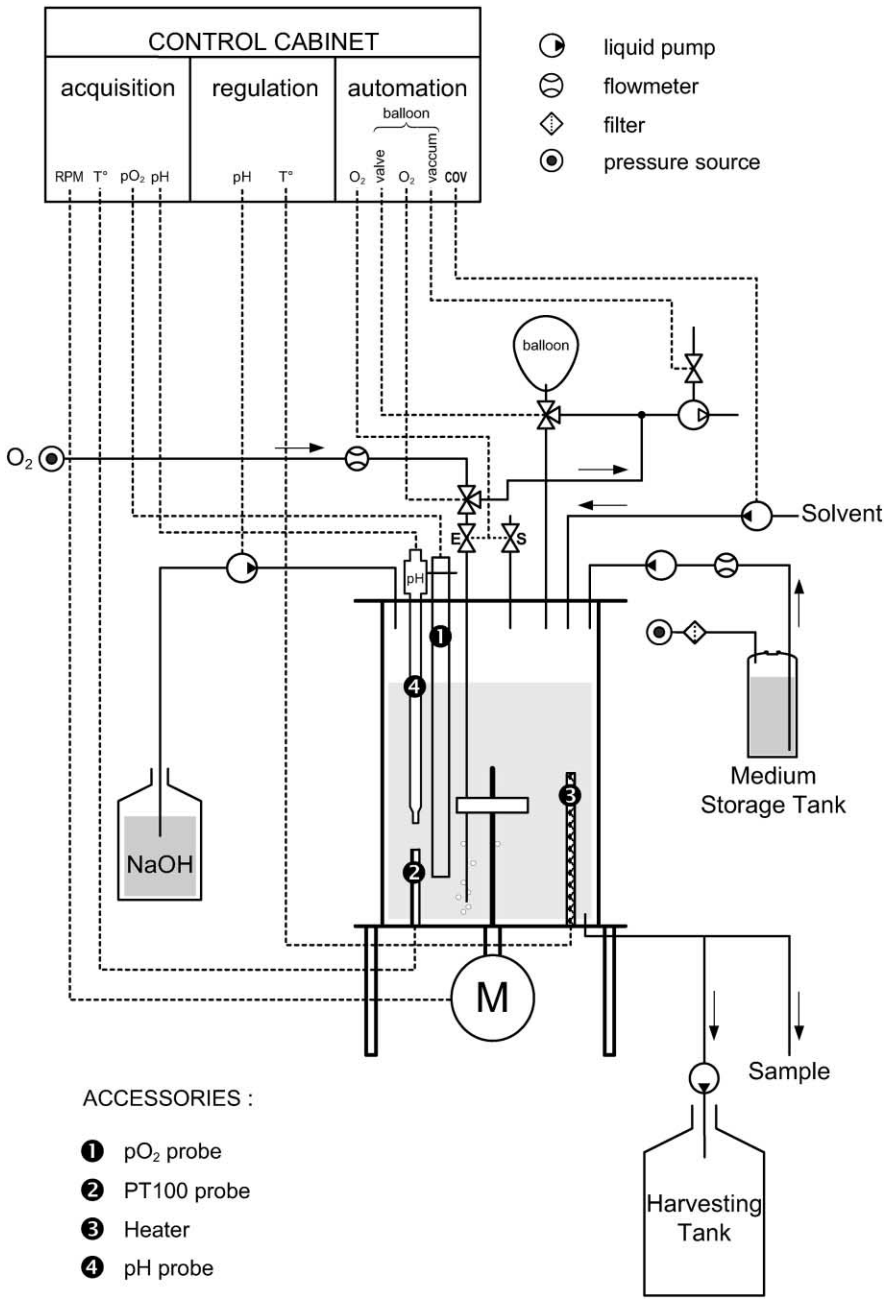

Fig. 1. Schematic of experimental setup.

after 15 s of ultrasonic disintegration (Bransonic, Danbury, USA). The absorbance was correlated to dry weight and was used to estimate biomass concentration. The CB and DCB concentrations were determined using a gas chromatograph (Varian Star 3400 Cx, Palo Alto, USA), equipped with a flame ionisation detector and a DB-624 capillary column (30 m length, i.d. 0.53 mm) (J & W Scientific, Folsom, USA). Gas samples were analyzed using a 5.8 ml min^{-1} nitrogen flow rate. The injection and detector temperatures were 220°C and the oven temperature was increased at a rate of 10°C min^{-1} from 80°C to a final temperature of 145°C, which was maintained for 2.5 min. Retention times were 4.5 and 7.8 min for CB and DCB, respectively. Utilisation of CB and DCB was monitored by taking 1 ml samples of the reactor headspace with a gastight syringe (series A-2 pressure lock, Dynatech, Baton rouge, USA). Standard curves were generated by completely evaporating measured amounts CB and DCB in a known volume of air. Chloride and ammonium ions concentrations were determined photometrically with a flow injection analyser (FIA, Tecator, Höganas, Sweden) at 463 and 590 nm, respectively. Conductivity was determined directly in the culture medium with a conductimeter (WTW, Weiheim, Germany). Concentrations of water-soluble metabolites were measured by high performance liquid chromatography (HPLC) (Varian 910, Palo Alto, USA), on a ORH801 column (Interaction chromatography Inc., San Jose, USA) with sulfuric acid (0.01 N) as the mobile phase at a flow rate of 1 ml min^{-1}. Compounds were detected by the IR refractivity index detector (Varian 9065 Polychrom, Palo Alto, USA) and their retention times were compared with *trans–trans* muconic acid p.a. quality, supplied by Fluka, Buchs, Switzerland.

The accumulation of UV-absorbing metabolites was monitored by UV-spectrophotometer (U-2000, Hitachi, Tokyo, Japan) at 255 nm after culture medium filtration through 0.22 μm filter (Schleicher and Schuell, Dassel, Germany). The dissolved organic carbon (DOC) concentration was measured after filtration, HCl-acidification and CO_2 elimination, using a TOC analyzer (Shimadzu, Tokyo, Japan). The 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.

3. Results

3.1. Substrate feeding

A principle of the substrate feeding by pulse with the purpose of producing specialized biomass able to degrade volatile organic pollutants is presented in Fig. 2.

The cultivation started by oxygenation, until the medium is saturated by oxygen (maximum of the oxygen concentration curve after about 2 h in Fig. 2). To avoid CB and DCB stripping and maintain the aerobic conditions, the reactor is used as a closed system during one oxygenation cycle (minimum of the oxygenation curve, up to about 26 h in Fig. 2). In the same time the substrate is added by several pulses. During the first pulse, substrate and the oxygen concentration in the medium decrease, until total substrate consumption. A new substrate addition follows. The substrate added is again degraded as long as there is still enough dissolved oxygen in the medium. The next oxygenation cycle started when dissolved oxygen in the medium is almost all consumed.

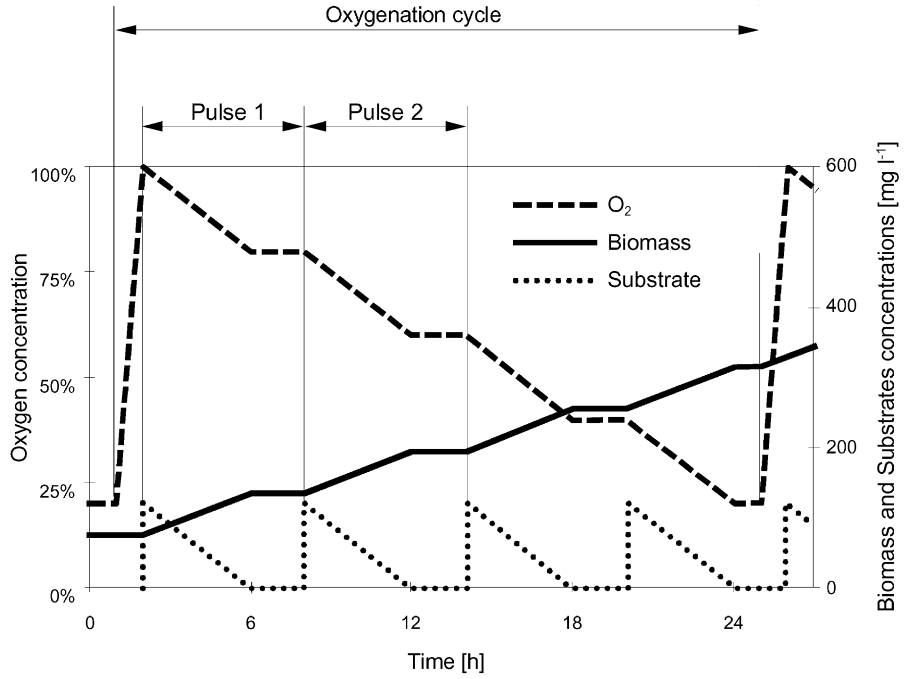

Fig. 2. Principle of batch culture with substrate addition by pulses.

The number of substrate pulses during one oxygenation cycle is determined by oxygen consumption. The pulse duration is the time between two substrate feeding by pulses, it should be sufficiently long to degrade the substrate and its metabolites to avoid their accumulation and possible growth inhibition.

A production cycle corresponding to the interval of time between the start and the end of biomass production.

To obtain the optimal biomass growth yield and productivity, two experimental runs were carried out. The first one was conducted at a different CB and DCB concentrations, from 50 to 200 and 10 to 40 μl of CB and DCB, respectively. The second run was conducted to decrease duration between two substrate feeding at the optimal CB and DCB concentrations found in the first run.

3.2. Degradation of CB and DCB

The results of a typical degradation experiment during one pulse are presented in Fig. 3. The example demonstrates the ability of the adapted bacterial culture to degrade 150 μl of CB and 30 μl of DCB in mixture for the pulse interval of 110 min. The bacteria started to degrade CB almost immediately, without a lag phase, and then DCB much slowly.

In Fig. 3 the evolution of intermediates (A_{255}) and DOC concentrations during one cycle of 110 min is also presented. We observed that in the first phase of substrate degradation,

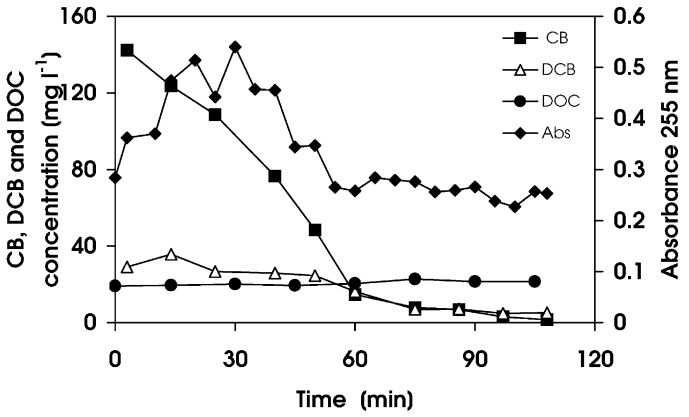

Fig. 3. Progress of CB and 1,2-DCB degradation by the adapted bacterial consortium, DOC and metabolites concentration (Abs) during one pulse of 150 $\mu l\,l^{-1}$ CB and 30 $\mu l\,l^{-1}$ DCB.

A_{255} increased from 0.3 to 0.55 and then decreased to the initial value when the substrate and metabolites were totally degraded (curve A_{255}). This was the criteria to stop a pulse. It is evident that there was no need to extend the pulse time longer than 90 min, while at that time the CB and DCB were degraded. The oxygen concentration decreased with CBs uptake, and it followed the evolution of substrate consumption.

The slow increase of DCB concentration at the beginning of the pulse could be explained by the fact that DCB needs about 12 min to attain an equilibrium between gaseous and liquid phase, while CB, due to its higher solubility and volatility, requires only 3 min.

3.3. Biomass yield and productivity

The effect of CB and DCB concentrations on biomass productivity and growth yield was investigated. The following amounts were added to the medium: 50, 100, 150 and 200 μl of CB in mixture with 10, 20, 30 and 40 μl of DCB per litre, respectively. The specific oxygen consumption rate was 4.5 mmol $g_{DW}{}^{-1}\,h^{-1}$and did not varied significantly during these experiments. The maximal specific substrate degradation rates are presented in Table 1. The maximal value attained was 121 mg $g_{DW}{}^{-1}\,h^{-1}$ at the concentration of 150 μl of CB.

Biomass productivity increased with augmentation of CB and DCB concentrations in the medium supplied by pulses. The pulses of 150 μl of CB and 30 μl of DCB per litre of medium were optimal for the production of adapted biomass; the productivity was 51 $mg_{DW}{}^{-1}\,l^{-1}\,h^{-1}$ and the yield of 0.38 $g_{DW}\,g_S{}^{-1}$ (gram of dry weight per gram of substrate CB and DCB). The maximal CB degradation rate was also attained at the 150 μl CB and 30 μl DCB in the medium, which was 121 mg $g_{DW}{}^{-1}\,h^{-1}$, while the DCB maximal degradation rate of 19 mg $g_{DW}{}^{-1}\,h^{-1}$ was highest when 100 μl CB and 20 μl DCB were added. The growth was inhibited if the addition of substrates in the pulse increased to 200 μl of CB and 40 μl of DCB per litre. At these concentrations there was no biomass production, due to growth inhibition and significant bacterial lysis. We observed that the inhibition

Table 1
Biomass productivity, growth yield and maximum specific consumption rate of CB and DCB in batch cultures

CB and DCB added by pulses ($\mu l\,l^{-1}$)	Productivity ($mg_{DW}\,l^{-1}\,h^{-1}$)	Growth yield ($g_{DW}\,g_{CB+DCB}{}^{-1}$)	Q_S max	
			CB ($mg_S\,g_{DW}{}^{-1}\,h^{-1}$)	DCB ($mg_S\,g_{DW}{}^{-1}\,h^{-1}$)
50 + 10	18	0.42	94	13
100 + 20	41	0.43	88	19
150 + 30	51	0.38	121	16
200 + 40	0	0	92	13

depended only on substrate concentration and not on the specific substrate mass loading. Substrate mass loading was 55 $mg_S\,g_{DW}{}^{-1}\,h^{-1}$, when concentrations were 200 μl CB and 40 μl of DCB and was less than the maximal value when the substrate quantity of 150 μl CB and 30 μl of DCB was added to the medium (66 $mg_S\,g_{DW}{}^{-1}\,h^{-1}$).

The biomass yield was equal from 0.43 to 0.38 $g_{DW}\,g_S{}^{-1}$ for concentrations up to 150 μl of CB and 40 μl of DCB which was similar to the value reported by Zaitsev et al. and Dang et al. [10,13] who studied the degradation of CB with *Rhodococcus opacus*.

3.4. Chloride concentration

Chloride was released into the medium in stoichiometric amounts during CB and DCB degradation. The chloride ion concentration was determined by FIA and the conductivity of the medium. Both methods were satisfactory and correlated well with the quantity of substrate added to the medium. No significant differences were observed between the theoretical and experimental chloride concentrations when the degradation was complete. Oh and Bartha [2] have reported that 0.2 M of chloride ions could inhibit bacterial growth. Chloride concentration was always kept under 0.2 M during our experiments.

3.5. Metabolites

The accumulation of metabolites in the culture medium was monitored by UV-absorbance at 255 nm (A_{255}) and the HPLC peak area having retention time of 7.4 min, which was the only peak appeared in the course of degradation under the conditions used in this analysis. Klecka and Gibson [14] have reported an accumulation of metabolite which had the UV peak at 255 nm and that the peak corresponded to that of muconic acid. During a pulse, the evolution of the A_{255} value was similar to the evolution of the HPLC peak area having a retention time of 7.4 min. Both measurements correlated well in the course of degradation. We concluded that it could be the same compound, which appeared as a peak by both methods and has been identified by HPLC as a derivative of muconic acid.

An accumulation of detected intermediate in the medium was investigated by adding 200 μl CB and 40 μl DCB by pulses. It was found that the ability of the bacterial population to grow and to degrade CB and DCB was not the same from first to the last pulse. The

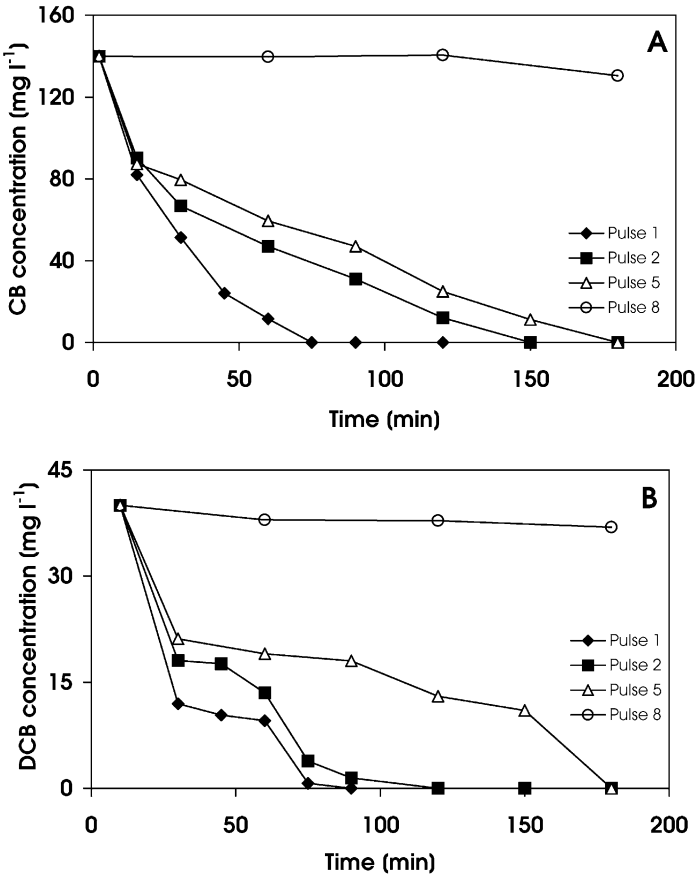

Fig. 4. Progress of CB (A) and DCB (B) degradation in the course of the first, second, fifth and eighth pulse of 200 μl l^{-1} CB and 40 μl l^{-1} DCB.

bacteria were able to degrade 200 μl of CB and 40 μl of DCB in the first pulse, but after the eighth pulse, they were not able to metabolise CBs. Fig. 4 illustrates the differences in CB (A) and DCB (B) degradation between pulses during the biomass production.

The specific substrate degradation rate (Q_S) decreased radically from 92 to 10 and 13 to 2 $mg_{DCB}\ g_{DW}{}^{-1}\ h^{-1}$ between first pulse and eighth pulse. The A_{255} value increased: 0.13, 0.36, 2.0, 2.5 at the end of the first, the second, the fifth and the eighth pulse, respectively. The increase of A_{255} values as well as decrease of Q_S suggested the accumulation of metabolites which inhibited bacteria activity.

As a consequence of inhibition at the end of biomass production, especially during the eighth pulse, a lysis of bacterial cells occured, which resulted in the appearance of foam, corresponding to the release of intracellular proteins into the medium, and the pH increase due to the cytoplasm release. Moreover, the culture medium turned brown and then black within 48 h. This colour was probably due to the intermediates accumulation.

The metabolites have been discussed by several authors to be toxic during degradation of CBs [4,7].

3.6. Pulse duration

To optimise the biomass production it was necessary to know the CB and DCB concentration at which the maximum production of adapted biomass could be obtained. They were found to be 150 μl of CB and 30 μl of DCB l^{-1} in each pulse (Table 1).

To avoid growth inhibition it was necessary to maintain the residual concentration of metabolites in the liquid medium at a minimum. The non-degraded metabolites were monitored by measuring the A_{255}. As the number of pulses increased, a slight increase of the absorbance was observed, but did not exceed the value of 0.3 when all CBs present in the medium were consumed. The biomass productivity was studied varying duration of each pulse. At the beginning of the biomass production 90 min was necessary to degrade all CB and DCB added by pulse (Fig. 3) and this time decreased progressively to 75 and 60 min (Fig. 5). The CBs concentration added by each pulse was constant. It was evident that a decrease in time of pulse duration, increases the volumetric mass loading, ($g_S\,l^{-1}\,h^{-1}$) and consequently the biomass productivity.

The purpose of this series of the experiments was to increase the volumetric mass loading to a maximum, which increased from 118 to 178 $mg\,l^{-1}\,h^{-1}$ of CB and DCB mixture. The evolution of the biomass productivity and biomass concentration during a production cycle is shown in Fig. 5.

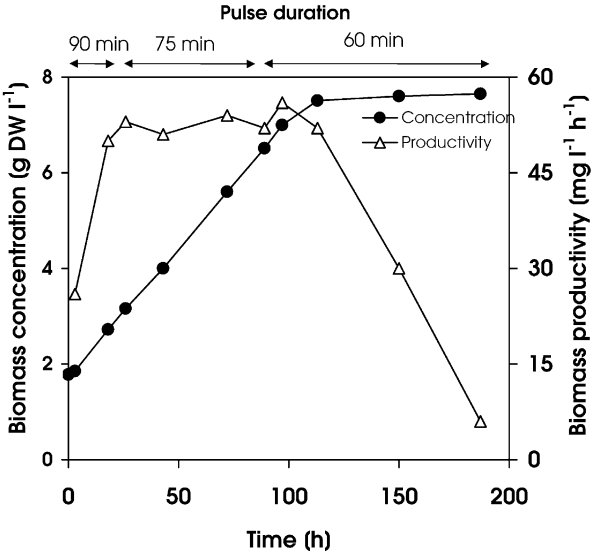

Fig. 5. Evolution of biomass concentration and biomass productivity during a production period. The interval between each pulse of 150 $\mu l\,l^{-1}$ CB and 30 $\mu l\,l^{-1}$ DCB were 90, 75 and 60 min.

The biomass concentration increased in the course of the experiment up to 7.6 $g_{DW}\,l^{-1}$, and then stayed constant. The productivity was about 51 $mg_{DW}\,l^{-1}\,h^{-1}$ and was constant to the point where the biomass concentration attained the maximum value of 7.6 $g_{DW}\,l^{-1}$, after about 110 h of the production cycle, in 85 repeated pulses of substrate addition.

To increase further the biomass production, it would be necessary (i) to increase the substrate loading by increasing the quantities of substrates added by each pulse or (ii) to reduce duration of each pulse.

(i) The introduction of a higher concentration of substrate, adding 200 μl of CB and 40 μl of DCB instead of 150 μl of CB and 30 μl of DCB in each pulse, provoked an inhibition of bacterial growth.

(ii) The time between two pulses could not be reduced, because neither CBs, nor metabolites can be mineralized in less than 60 min. If the time shortened, the CBs and metabolites would accumulate in the medium.

Therefore, it was better to stop the cultivation when there was about 7.6 $g_{DW}\,l^{-1}$ of biomass and then start a new production cycle (Fig. 5).

The possible biomass growth inhibition by chloride ions released into the medium due to degradation of CBs was also monitored during the production cycle, as well as the nitrogen concentration. The DOC was controlled and maintained at about 20 mg of carbon per litre of medium, to ensure that the substrate was consumed and there was no metabolite accumulation. (Fig. 6).

The new batch was started after about 75 h of run with a fresh medium inoculated with the biomass previously separated by centrifugation, in order to eliminate accumulated chloride ions and to avoid possible growth inhibition. The maximum chloride concentration of about 0.1 M (Fig. 6) is half of the inhibition concentration of 0.2 M reported by Oh and Bartha [2]. The ammonium concentration monitoring assured that there was always a sufficient quantity of nitrogen in the medium for biomass growth.

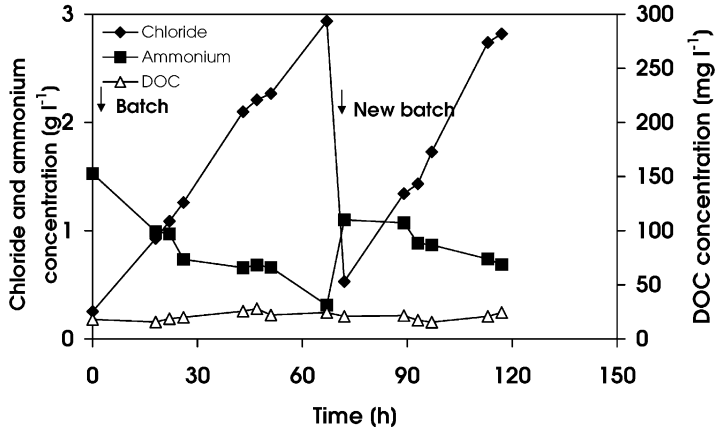

Fig. 6. Evolution of chloride, ammonium and DOC concentrations during biomass production. CB and DCB were added by pulses of 150 and 30 $\mu l\,l^{-1}$, respectively.

Viable cells were continuously monitored during the degradation experiments, the mixed bacterial consortia contained 90% living cell at the beginning and 60% of living cells at the end of the production cycle after about 120 h. The time of cell exposure to CBs, formation and accumulation of intermediates increased bacterial injury and consequently, the CB and DCB consumption rate decreased.

Pure bacterial strains were isolated from the mixed adapted consortia degrading CBs. They belonged to the family Pseudomonadaceae, to the genus *Stenotrophomonas*, *Comamonas*, *Rimerella* and *Pseudomonas*. The strains were identified as *Stenotrophomonas maltophilia*, *Comamonas acidovorans* and *Pseudomonas putida* and *Escherichia hermanii* [15].

In conclusion, the technique can be applied for industrial purpose to produce larger amount of adapted biomass able to degrade volatile pollutants, such as CBs. The produced biomass can serve for industrial biotricling filters as inoculum to increase the degradation capacity, or if the waste gases change in composition. In general, the capacity of biotrickling filters treating industrial waste gases containing any other volatile organic pollutant not easily mineralised can be improved by this method. However, for each particulate pollutant optimal conditions have to be found.

In industrial biotrickling filtration waste gases contain usually one type of pollutant and microbial consortia are adapted to these contaminants. If the composition of waste gas change, the bacteria can not degrade new xenobiotics and can even die. The inoculation of biotrickling filter with the adapted bacteria to the new pollutant, produced separately, can help. For example, if biotrickling filter remove successfully toluene and xylenes from waste gases and if in these gases CBs are introduced, the bacterial consortia will not eliminate CBs, and inhibition of growth or inactivation of microorganisms will occur. In this case inoculation with adapted biomass to the CB utilization, proposed in our paper will enable good continuous biofiltration.

We applied the method for adapted biomass production in 2 l fermentor depicted in this paper and transmitted to the fermentor of 20 l and than to 1 m^3. With the adapted biomass obtained in this way, the Rohner SA (Basel, Switzerland) inoculated their biotrickling filter after introducing CBs instead of toluene and xylenes, as the main volatile pollutants with a removal efficiency of more than 95%.

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