Enrichment of microbial cultures able to degrade 1,3-dichloro-2-propanol: A comparison between batch and continuous methods

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Abstract

Microbial cultures able to degrade xenobiotic compounds are the key element for biological treatment of waste effluents and are obtained from enrichment processes. In this study, two common enrichment methods, suspension batch and immobilized continuous, were compared. The main selection factor was the presence of 1,3-dichloro-2-propanol (1,3-DCP) as the single carbon source. Both methods have successfully enriched microbial consortia able to degrade 1,3-DCP. When tested in batch culture, the degradation rates of 1,3-DCP by the two consortia were different, with the consortia obtained by batch enrichment presenting slightly higher rates. A preliminary morphological and biochemical analysis of the predominant colonial types present in each degrading consortia revealed the presence of different constituting strains. Three bacterial isolates capable of degrading 1,3-DCP as single strains were obtained from the batch enrichments. These strains were classified by 16S rRNA analysis as belonging to the Rhizobiaceae group. Degradation rates of 1,3-DCP were lower when single species were used, reaching 45 mg l^{-1} d^{-1}, as compared to 74 mg l^{-1} d^{-1} of the consortia enriched on the batch method. Mutualistic interactions may explain the better performance of the enriched consortia.

Introduction

The industrial development needed to sustain the population growth over the last two centuries has led to a new type of environmental pollution: the production and widespread dispersion of chemicals not previously present in the biosphere. These chemicals are often referred to as xenobiotics ("stranger to life"). Biological Treatment Technologies (BTT) have been used as end-of-pipe treatment technologies for treatment of waste effluents, e.g., sludge treatment and biofiltration. However, many xenobiotic compounds present in industrial effluents pass through biological end-of-pipe treatment processes unaltered. Legislation has been introduced to regulate the discharge of specific xenobiotic compounds, so called "priority pollutants" (Eckenfelder 1995). This has created a demand for new technologies to reduce discharges of these compounds to a sufficiently low level to meet these regulations (Arcangeli & Arvin 1995; Christiansen et al. 1995; Klecka et al. 1996; Livingston et al. 1998). Point-source BTT can be a feasible treatment solution should specialised microbial cultures be selected.

Selection of appropriate microbial cultures is the first problem to solve towards the implementation of a BBT and it can last for several months. Suitable microbial cultures are often derived from polluted sites. Isolation of the specialised microbial culture is often preceded by an acclimation period, generally accomplished by classical cultivation techniques: batch and continuous enrichment methods. Although many microbial cultures are enriched in batch cultures, others may require more intensive selection. In such cases,

gradual adjustments to a chemostatic or continuous culture may be used to slowly enrich for a specific organism or degrading consortia (Splendianni et al. 2000; Steele & Stowers 1991). During enrichment processes, microbial cultures are exposed to xenobiotic compounds as the only source of carbon and energy, and a selective pressure is therefore introduced. A successful enrichment process will result in the isolation of microbial cultures able to degrade the target xenobiotics.

Epichlorohydrin and related compounds, such as 1,3-dichloro-2-propanol (1,3-DCP) and 2,3-dichloro-1-propanol (2,3-DCP), are halohydrins that are used widely as reagents in chemical manufacture, as solvents, as rodent chemosterilants and as precursors for the synthesis of optically active compounds. Halogenated aliphatic hydrocarbons are volatile and frequently presented as contaminants in industrial waste gases (van den Wijngaard et al. 1993). 1,3-DCP and 2,3-DCP are carcinogenic, mutagenic and genotoxic, having a high risk factor for animal and human toxicity with regards to the environment (Omura et al. 1995). Bacterial strains capable of utilising DCP and related compounds have been reported (Armfield et al. 1995; Assis et al. 1998; Fauzi et al. 1996; van den Wijngaard 1989) and fewer capable of degrading 2,3-DCP (Effendi et al. 2000) have been isolated from soil samples. In this study, a comparison between the two most commonly used enrichment methods, batch and continuous, was carried out for obtaining 1,3-DCP degrading microbial consortia. 1,3-DCP was used as the only source of carbon and energy. Microbial strains from each enriched consortium were purified and characterised, and their degradation capacities as single species were compared to the original consortium.

Material and methods

Soil and isolating media

Soil samples were collected from a 1,3-DCP potentially contaminated site in the vicinity of a paint plant in northern Portugal. Samples were well-mixed and undesirable materials (stones, garbage, roots, etc) removed. Samples of this mixture were inoculated in mineral media containing 50 mg l^{-1} of 1,3-DCP as the sole carbon and energy source. The mineral medium (MM) used on these experiments contained the following compounds per litre: $Na_2HPO_4 \cdot 2H_2O$ (2.67 g); KH_2PO_4 (1.4 g); $MgSO_4 \cdot 7H_2O$ (0.8 g); $(NH_4)_2SO_4$ (0.5 g); and 10 ml of a trace elements solution. The trace elements solution contained the following compounds per litre: NaOH (2.0 g); $(Na_2)EDTA \cdot 2H_2O$ (12 g); $FeSO_4 \cdot 7H_2O$, (2 g); $CaCl_2$ (1 g); Na_2SO_4 (10 g); $ZnSO_4 \cdot 7H_2O$ (0.4 g); $MnSO_4 \cdot 4H_2O$ (0.4 g); $CuSO_4 \cdot 5H_2O$ (0.1 g); $Na_2MnO_4 \cdot 2H_2O$ (0.1 g); H_2SO_4 concentrated, 0.5 ml). Sodium azide was used as an antimicrobial compound when required. After enrichment, microbial cultures were plated onto nutrient agar (NA, Oxoid) and minimal agar plates containing 1,3-DCP at a concentration of 50 mg l^{-1}. Enriched microbial cultures were maintained on slopes of NA at 5 °C and also frozen in 20% glycerol (v/v) and stored at −70 °C for further studies.

Batch enrichment method

Each batch reactor consisted of a 250 ml perfectly sealed flask containing 100 ml of MM supplemented with 1,3-DCP. All flasks were incubated at 25 °C with gentle agitation. During the monitorisation of the degradation process, samples were collected in duplicate and chloride liberation and biomass evolution were measured. The following nomenclature was used to enumerate the different batch reactors: B*.#, with B for Batch, * for the number of the reactor and # for the number of transfers referred to that reactor. E.g., assay B3.2 refers to batch assay number three and transfer number two. The first inoculation of a batch assay is referred to as B*.0.

Five different batch reactors were initially inoculated with soil samples (B1 to B5). Reactors B.4 and B.5 were used as control reactors with the addition of sodium azide. Transfers were carried out after stabilization of chloride concentration in the sample reactors and were performed by adding 5 ml of the running reactor volume to 100 ml of fresh MM supplemented with 50 mg l^{-1} of 1,3-DCP. Subsequent transfers were performed accordingly. Batch reactors containing either sterilized soil inoculum and sterilized medium with 1,3-DCP were run in parallel as control cultures. Investigations into growth of the enriched consortia at different 1,3-DCP concentrations were also carried out. Three flasks containing different 1,3-DCP concentrations of 20, 50 and 80 mg l^{-1} (B20, B50 and B80, respectively) were similarly prepared to investigate substrate growth inhibition.

Continuous enrichment method

A schematic diagram of the enrichment reactor used in this work is shown in Figure 1. Two identical

continuous reactors (C1 and C2) were assembled. Reactor C1 was used as the enrichment reactor whilst reactor C2 was used as the control reactor. Each reactor consisted of two tubular modules (A and B) connected together: in module A medium aeration was performed, and 40 ml of growth support material (glass beads, 1 mm diameter) and 23 g of soil inocula were introduced into module B. Norprene tubing impermeable to organic compounds was used throughout the experiments (Norton 6404-16, Cole Parmer).

MM containing 50 mg l^{-1} of 1,3-DCP was fed to module A at a flow rate of approximately 9 ml h^{-1} (± 1). The hydraulic residence time in module B was approximately 10 h. Samples were collected, in triplicate, from the input ($C1_{inlet}$ and $C2_{inlet}$) and from the output ($C1_{outlet}$ and $C2_{outlet}$) of reactors C1 and C2.

Chemicals

All chemicals used in the preparation of media were obtained from BDH and were of AnalaR grade. All the organic solvents were obtained from Sigma.

Analytical methods

Chloride concentration was assayed colorimetrically (Iwasaky et al. 1956). Biomass was previously removed by centrifugation at 14,000 rpm for 5 min. The chloride concentration was calculated from a standard calibration curve from 0 to 100 mg l^{-1}. The uncertainty in this assay was $\pm 5\%$ at the 10 mg l^{-1} level. Chloride liberation was used as an indirect assay for quantifying 1,3-DCP degradation. Total organic carbon analysis of centrifuged samples taken at the end of biodegradation tests confirmed that no partially metabolised intermediates were produced.

Microbiological Characterisation

At the end of the enrichment processes (batch and continuous), samples were spread plated onto NA and MM agar containing 1,3-DCP in order to characterise the consortia. From the continuous reactor, solid samples were removed and washed with sterile water in order to recover the attached biomass. Microorganisms recovered in solid media were purified by repeated subculturing on NA medium. A preliminary characterisation was based on colony and cell morphology, presence or absence of cytrochrome c oxidase and Gram staining.

The isolated microorganisms were analysed for their individual capacities to degrade 1,3-DCP. Each colonial type was suspended in 10 ml of MM supplemented with 1,3-DCP as the sole carbon and energy source and were incubated at 25 °C on a rotary shaker at 100 rpm (Julabo-SW-20C). Growth was monitored by measuring the optical density of the cultures at 600 nm and biodegradation was measured through chloride release into the medium. When growth on 1,3-DCP was evidenced by an increase in optical density and by liberation of chloride, samples of culture were plated onto NA in order to verify whether single species were present. When pure colonies were obtained, this procedure was repeated several times. For the single strain degraders, 16S rRNA sequence analysis was carried out, as described below. A comparison of the 1,3-DCP removal kinetics of the strains as single strain and of the enriched consortia was carried out.

16S rRNA sequence analysis

The 16S rRNA genes of the three strains were amplified by PCR using the primer set f27 and r1492 (Lane 1991) under standard PCR conditions (30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C) with Taq DNA polymerase (Promega). The template DNA was obtained boiling washed cell suspensions for 5 minutes and using 1–2 μl of the supernatant. The amplified fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced by STAB Genomica, Lisbon, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and ABI PRISM 310 capillary apparatus, Applied Biosystems) using 16S-specific primers f27 and f357 (Lane 1991) and universal *lacZ* primers M13fwd or M13rev. The 16S rRNA gene sequences were aligned and edited with the BioEdit program (version 4.8.8) (Hall 1999) and analysed using the programs SEQBOOT (100 iterations), DNADIST (Kimura 2-parameter), NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein 1995). BLAST searches (Altschul et al. 1997) and 16S rRNA sequence download were performed using the National Center for Biotechnology Information facilities (http://www.ncbi.nlm.nih.gov). Two alignments of 30 sequences by 540 nt or 28 sequences by 1280 nt nucleotides were used. Sequences have been deposited under GenBank Accession numbers AY064412, AY064413 and AY064414.

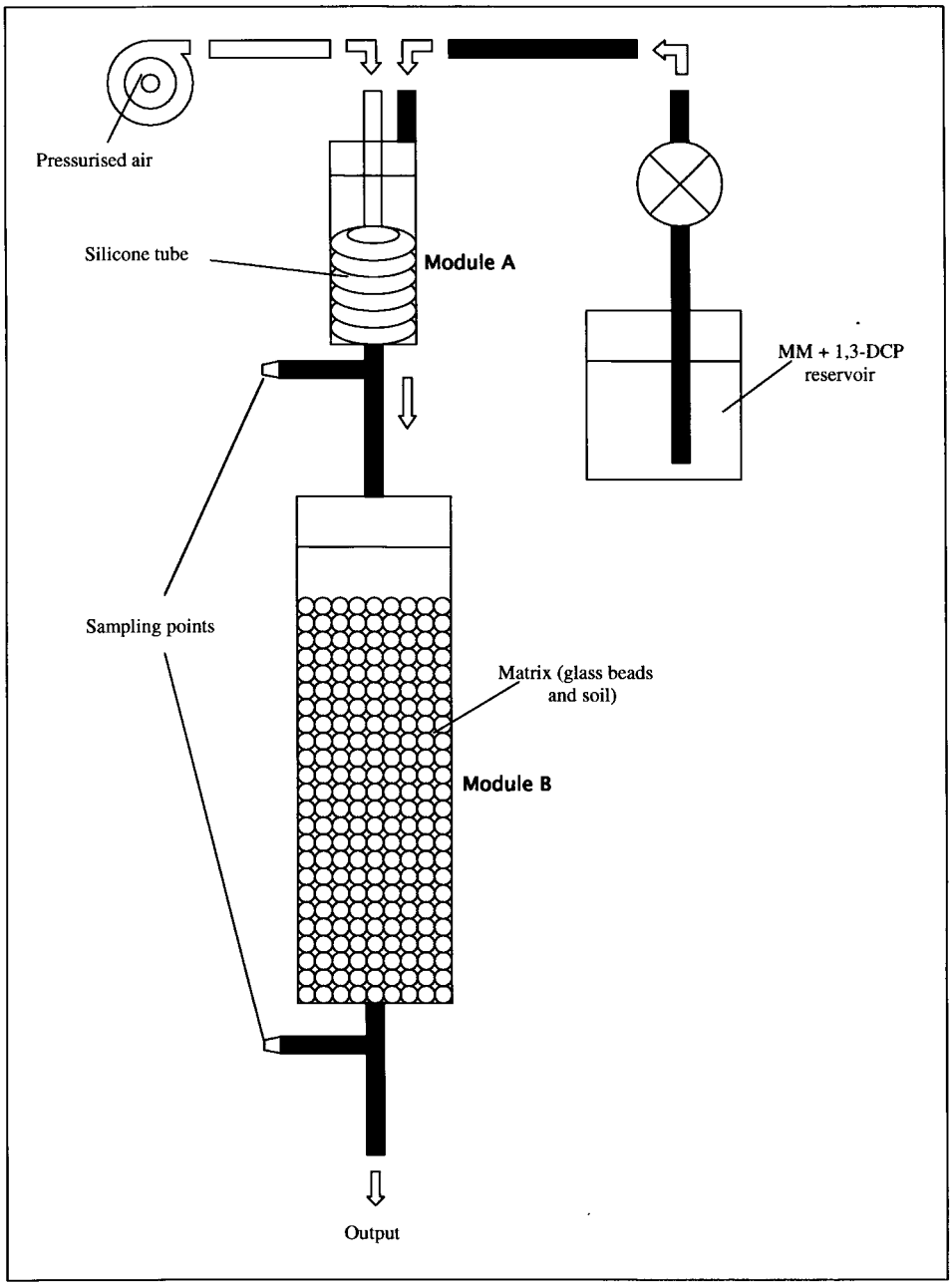

Figure 1. Schematic representation of an enrichment reactor.

Results and discussion

Batch enrichment method

In the batch reactors, due to the high turbidity provided by the soil inoculum, the initial readings of suspended biomass were not possible. The chloride concentration found in the reactors was also higher than the expected concentration existing in the mineral medium. This was probably due to a high chloride content of the soil sample used. After four days from inoculation, the chloride concentration increased on all batch reactors (B1.0–B3.0). The transfers prepared from these reactors (B1.1–B3.1) stabilised at chloride concentrations that corresponded to 30 mg l^{-1} of 1,3-DCP degraded; physical losses of the compound during sampling may

have occurred. The chloride level (5 mg l^{-1}) on the control reactors (B4.1 and B5.1) was maintained, supporting 1,3-DCP degradation in the enrichment flasks. No evolution of chloride was noticed on the control reactors. These reactors were run for 20 days.

Figure 2 shows the evolution of degraded 1,3-DCP in reactors B1.2–B5.2. In reactors B1.1 to B1.3 chloride evolution was observed from the time of inoculation and stabilised after the eleventh day, corresponding to a degraded 1,3-DCP concentration of 35 mg l^{-1}. In the control reactors (B4.2 and B5.2) no significant liberation of chloride was found. The initial 1,3-DCP degradation rate observed in reactors B1.2, B2.2 and B3.2 was ca. 4 mg l^{-1} d^{-1}. Further transfers were carried out.

Batch reactors containing mineral media with different 1,3-DCP concentrations were inoculated with samples from a previously prepared batch reactor, after 2 months of enrichment. Growth was observed on all reactors, which suggested that 80 mg l^{-1} did not completely inhibit biomass growth. From chloride concentration, total degradation of the 1,3-DCP initially supplied did not occur and there was a decrease on the percentage of 1,3-DCP degraded at higher 1,3-DCP concentrations: 93% 1,3-DCP degradation was observed in the B20 reactor, 68% in the B50 reactor and 59% in the B80 reactor. Physical losses of 1,3-DCP, due to volatilisation occurring during sampling, may have contributed to the results observed. No evidence of residual organic compound was detected.

Continuous enrichment method

Degraded 1,3-DCP evolution on the outlet of reactors C1 and C2 after the start-up of the continuous experiment is shown in Figure 3. The initial high value observed at the outlet of both reactors may be explained by the chloride content of the soil, which is in accordance with observations from the batch enrichment experiments. After two days of operation, the outlet chloride concentration on both reactors was negligible. After the fifteenth day, chloride concentration started to increase in the outlet stream of reactor C1, corresponding to a degraded 1,3-DCP concentration of 5 mg l^{-1}, which remained stable until the thirty-fifth day. From this period, the chloride concentration in the outlet started to increase. This slow evolution of biodegradation capabilities may be explained by an acclimatisation period of the microbial culture. Degraded 1,3-DCP stabilised at 33 mg l^{-1} on the forty-ninth day. After that stage, small variations in the chloride liberation were noticed, probably caused by slight fluctuations in the feeding medium flow. The 1,3-DCP degradation rate was 79 mg l^{-1} d^{-1}. The control reactor C2 always showed similar chloride concentrations between the inlet and outlet streams, which suggested no significant 1,3-DCP degradation.

The degradation capacity of the consortium enriched on the continuous method was confirmed in a batch assay (BC1) prepared using as inocula samples from reactor C1 taken at the fifty- eighth day of operation. Chloride liberation from 50 mg l^{-1} 1,3-DCP supplemented cultures followed the same pattern as in batch enrichment assays (see Figure 5).

Microbiological characterisation

In order to characterise the different strains present in the degrading consortia, samples from the enrichment process were spread plated onto NA and mineral agar plates containing 50 mg l^{-1} of 1,3-DCP. From the consortium enriched in the batch reactors, five different bacterial strains were recovered on NA medium and three were recovered on 1,3-DCP mineral medium. Among the strains isolated on NA, four were Gram-negative and one was Gram-positive. The strains recovered on solid mineral medium supplied with 1,3-DCP were all Gram-negative, and after transferring these to NA, one strain was found to be morphologically and biochemically (cytochrome c oxidase and catalase) similar to one previously recovered in NA. After purification by repeated subculturing on NA medium, each isolate was subsequently inoculated in batch reactors containing MM with 50 mg l^{-1} 1,3-DCP to test the capabilities of degradation as single strains. Only one strain recovered on NA plates (DCP1) and two recovered on 1,3-DCP MM (DCP2, DCP3) were able to grow and degrade 1,3-DCP as single species in the liquid cultures.

From the consortium enriched on the continuous method seven strains were recovered on NA media and three on 1,3-DCP mineral media and most were morphologically different from the ones recovered from the batch enrichments. They were all Gram-negative isolates. After transferring the bacterial types recovered in 1,3-DCP mineral solid medium onto NA, it was found that two of those strains were morphologically and biochemically (citochrome c oxidase and catalase) similar to one strain recovered in NA. Incubation of each of the purified strains in batch reactors containing 1,3-DCP did not lead to bacterial growth or 1,3-DCP degradation, as indicated by chloride re-

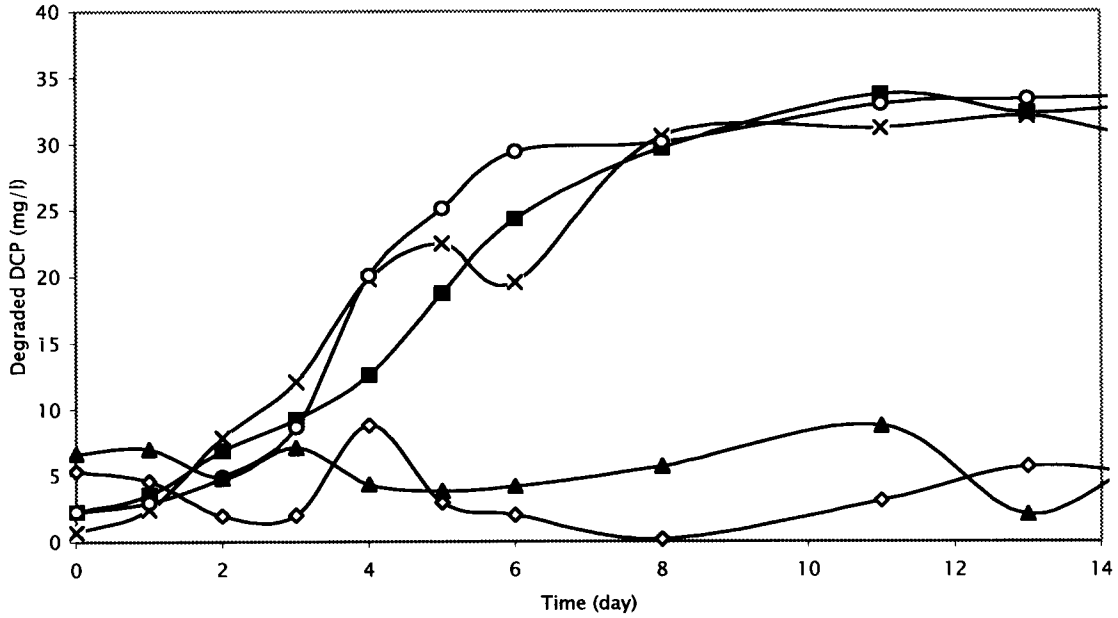

Figure 2. Evolution of 1,3-DCP degradation on batch enrichment reactors: B1.2 (○), B2.2 (×), B3.2 (■), B4.2 (▲), B5.2 (◇).

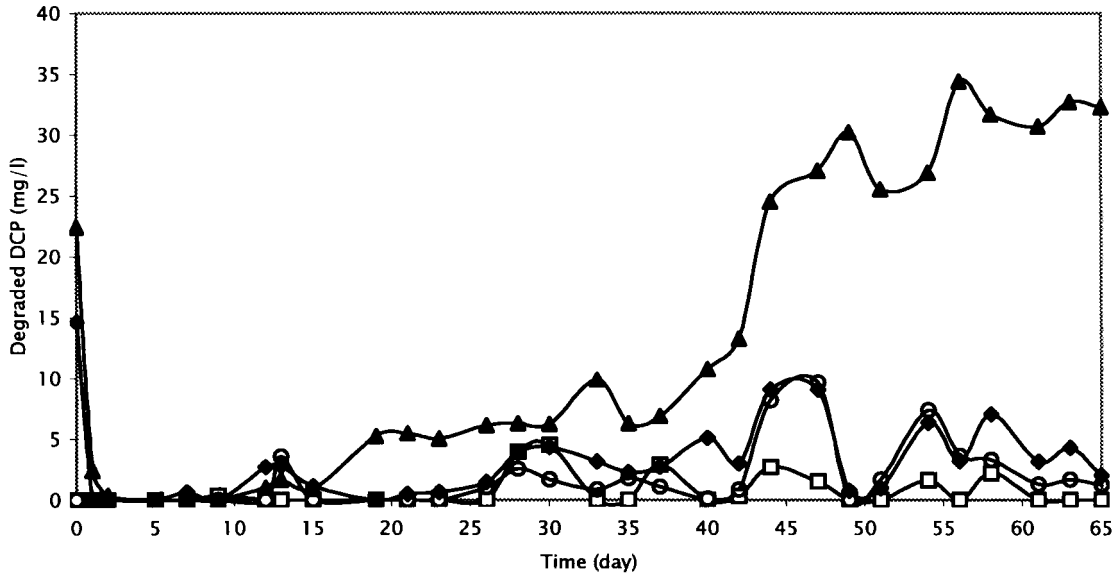

Figure 3. Evolution of 1,3-DCP degradation on continuous enrichment reactors: $C1_{inlet}$ (□), $C1_{outlet}$ (▲), $C2_{inlet}$ (○), $C2_{outlet}$ (♦).

lease. This may be due to biodegradation of 1,3-DCP being performed in a cooperative fashion by the recovered bacteria, and thus growth of the isolates as single species in liquid cultures was not obtained.

16S rDNA analysis of degrading strains DCP1, DCP2 and DCP3 (corresponding to AY064414, AY064412 and AY064413, respectively), revealed that all three belonged to the Rhizobiaceae, showing high identity (91–95%) to *Rhizobium giardinii*. Two of the sequences (DCP1 and DCP2) were very identical to each other (99% identity), while DCP3 was only 95–96% identical to the other two. This supports the results obtained by a similar phenotypical characterisation mentioned above for strains DCP1 and DCP2. Bacteria described previously to degrade 1,3-DCP include *Arthrobacter erithii* (Assis et al. 1998; Bull et

al. 1992), strains of *Corynebacterium* sp (Nakamura et al. 1992) and *Agrobacterium* sp. (Fauzi et al. 1996). Recently, 2,3-DCP degrading bacteria belonging to the *Rhizobia* group, which also had the capacity to dechlorinate 1,3-DCP, have been isolated (Effendi et al. 2000). The strains isolated in the present study were included in the Rhizobia group, but show identity values of 93 to 96% to the ones of Effendi et al. (2000) and do not group together with them in the phylogenetic analysis. In order to further investigate this comparison, we calculated two slightly different phylogenetic trees: one was obtained by including the partial sequences by Effendi et al. (2000), but the span of the alignment had to be reduced down to 540 nt; the other was obtained excluding from the alignment these two sequences so that the whole length of the sequences available could be used (1280 nt) and a more robust analysis performed (Figure 4). The topology of the two resulting trees is very similar: in both cases the sequences of the three strains described in this work fall in one group together with strains of *Sinorhizobium* and *Rhizobium*, but in the tree obtained from the longer alignment our DCP1, DCP2 and DCP3 strains cluster together in one clade supported by an 87% bootstrap value (Figure 4). In the other tree (data not shown) the sequences of Effendi et al. (2000) clearly group with different strains, one (AJ276433) in a tight cluster (boostrap value 87%) with *Ochrobactrum* strains, the other (AJ276434) within the two *Agrobacterium tumefaciens* strains (boostrap value 96%).

Overall, a great predominance of gram negative bacteria was observed on the strains isolated. Both methods successfully enriched microbial consortia able to degrade 1,3-DCP, but different bacterial composition was obtained from those, as indicated by the bacteria recovered by the cultivation on solid media. However, it is possible that the cultivation approach used for the recovery might not have targeted the majority of organisms present in both enrichments, which can explain differences in the bacterial composition. Despite that, bacterial isolates degrading 1,3-DCP as single strains were only obtained from the batch enrichments, and none were recovered from the established biofilm, and this also suggests that environmental conditions specific to each enrichment method could have influenced the selection process. On batch enrichment a selection pressure for strains that are able to grow faster is present. During a batch assay, the microbial strains that are able to grow faster will be the predominant strains on the inocula for the next transfer. Thus, a selection for a faster growing process is evident. Accordingly, during a continuous assay, the capability to grow as attached cultures is the first selection element. Microbial strains that are not able to grow in the attached form will be washed-out from the reactor, independently of their degradation capabilities. Furthermore, the continuous reactor is normally operated at a constant dilution rate. Subsequently, this will select for the microbial strains that in suspended growth present the required capabilities, i.e., strains that have a maximum growth rate lower than the dilution rate will be washed-out from the reactor.

Inoculation of the isolated strains into batch reactors to test 1,3-DCP degradation capabilities showed that most of the strains were not able to degrade 1,3-DCP as single species. This evidences the importance of microbial interactions on 1,3-DCP degradation (Atlas & Bartha 1997). Therefore, it is possible that most of these strains had a role on the degradation of 1,3-DCP. Other interactions, like co-metabolism could also play an important role. More detailed studies should follow in order to investigate this degradation pathway.

The consortium enriched on the batch method exhibited faster 1,3-DCP degradation on the batch reactors experiments than the consortium enriched on the continuous method (Figure 5), with microbial growth following the same pattern of chloride liberation. This may be explained by a higher affinity of this consortium to grow under such circumstances since it has been enriched under similar environments. The initial degradation rate (4 mg l^{-1} d^{-1}) of the batch enriched consortia was increased to 74 mg l^{-1} d^{-1} through subsequent transfers. A proper acclimatisation and a continuous selection for the best fitted cultures may explain these results. The degradation rate of the consortia enriched on the continuous method was not altered significantly on the batch assay. Overall similar maximum degradation rates were obtained in both enrichment methods.

The capability of the purified strains to degrade 1,3-DCP on batch reactors was compared to those of their original consortia. The consortia showed faster 1,3-DCP degradation rates than the isolated strains (ca 45 mg l^{-1} d^{-1}, Figure 5). This behaviour evidences the importance of microbial interactions on the overall degradation performance. In fact, mutualistic interactions between microbial strains that degrade xenobiotics are a common occurrence (Bull & Slater 1982). No single species able to degrade 1,3-DCP

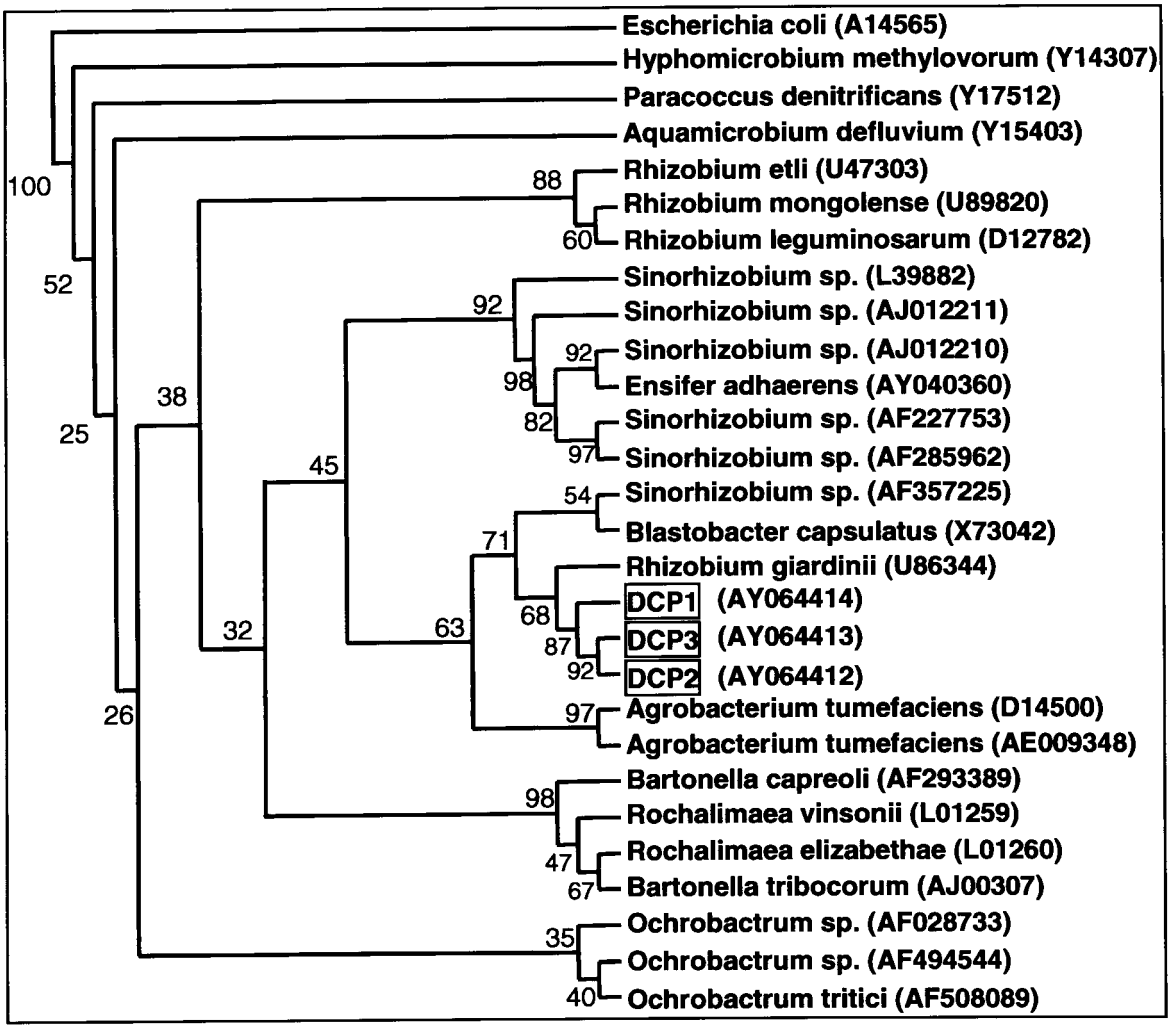

Figure 4. Neighbor-joining tree of 16S rDNA sequences. The numbers are bootstrap values over 100 replicates.

were obtained from the continuous method. A high degree and more complex microbial interactions may occur in the continuous method. Therefore, more robust microbial inocula, based on microbial culture with several species, may be obtained on continuous enrichment.

Conclusions

The two most commonly used enrichment methods have successfully enriched microbial consortia able to degrade 1,3-DCP from similar soil samples. Each consortia contained different microbial strains that were enriched under different environmental pressures specific to an enrichment method, as indicated by the bacteria recovered on solid media. From the microbial strains isolated from the batch degrading consortium three have shown capabilities to degrade 1,3-DCP as single strains. The degradation rates of both consortia were higher than the isolated strains. This may be due to microbial interactions that are known to improve the degradation capacities. The continuous method seemed to favour microbial interactions. However, not all 1,3-DCP degraders might have been culturable under the conditions used during the isolation and recovery. Overall, batch enrichments were shown to be a faster process than continuous enrichments.

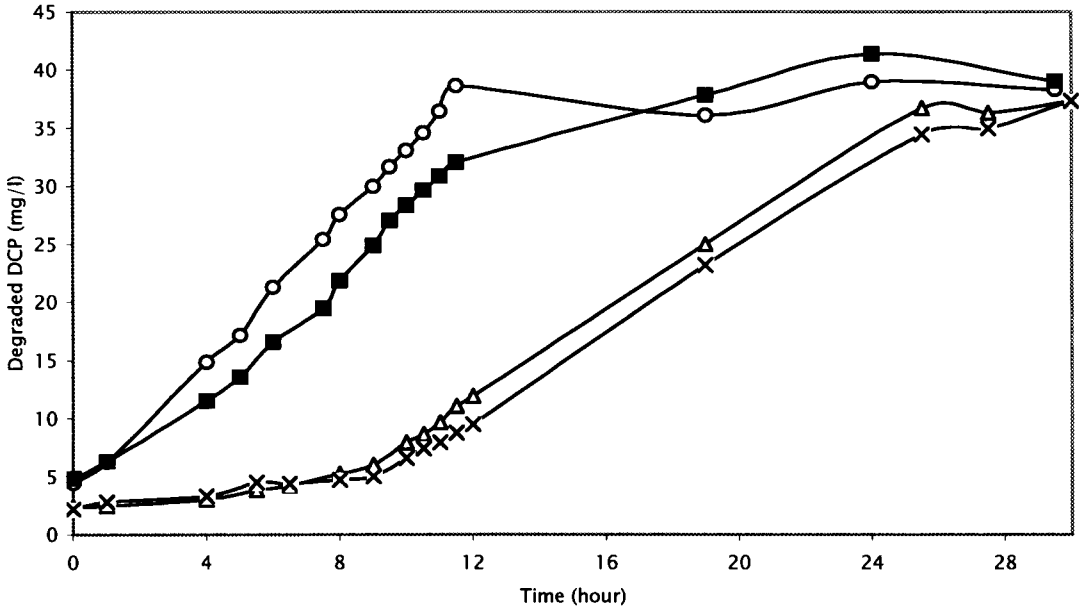

Figure 5. Comparison of degradation rates between microbial consortia enriched and single species able to degrade 1,3-DCP. Batch consortia (○), continuous consortia (*blacksquare*), strain DCP1 (×) and strain DCP3 (△).

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