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Total mineralization of 2-ethylhexyl nitrate by bacterial cocultures

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Abstract :

2-Ethylhexyl nitrate (2-EHN) is a widely-used chemical which is commonly added to diesel oil to boost its cetane index. The 2-EHN molecule is recalcitrant to biodegradation but still utilized as sole carbon source by Mycobacterium austroafricanum IFP 2173. The incomplete degradation of 2-EHN by this strain results in the accumulation of an intermediary metabolite i.e. 4-ethyldihydrofuran-2(3H)-one (4-EDF). The study aimed at isolating 4-EDF degraders in order to achieve total mineralization of 2-EHN in cocultures with M. austroafricanum IFP 2173. Bacterial isolates were obtained from diesel-contaminated soil by enrichment in serial cultures supplemented with 4-EDF, the degradation of which was monitored by CO2 measurements. Two strains were isolated and identified as Bacillus cereus and Burkholderia sp., respectively. Complete mineralization of 2-EHN was achieved by associating M. austroafricanum IFP 2173 with either bacterial isolate in cocultures. In the context of environmental acceptability, efficient degradation of a potentially persistent pollutant by a bacterial consortium is demonstrated.

Keywords : 2-EHN, Biodegradability, Cocultures, Cetane index

INTRODUCTION

2-Ethyhexyl nitrate (2-EHN) is added at 0.05 % to 0.4 % to diesel oil to improve ignition of fuel oil in diesel engines (Guibet 1999 ; Bornemann *et al*. 2002). The annual production of 2-EHN associated with the worldwide consumption of diesel oil is about 100 000 tons.

Considering the widespread market of 2-EHN and the risks of accidental release, the question of its biodegradability is relevant from an environmental standpoint. The degree of persistence

of contaminants in natural environment is influenced by various factors including their chemical structure, the presence of microorganisms able to degrade them, and environmental conditions suitable for microbial biodegradation activities (Philp *et al.* 2005).

The biodegradability of chemicals is usually assessed using tests conducted aerobically in liquid cultures (OECD 1993). A substance is considered readily biodegradable if the level of degradation reaches 70 % in terms of chemical oxygen demand or 60 % in terms of biological oxygen demand over a period not exceeding 28 days. A substance may be not readily but nevertheless inherently biodegradable, if complete degradation is obtained in a special-purpose "inherent test" of high degrading power (Pagga 1997).

2-EHN was found not readily biodegradable according to the American Chemistry Council Petroleum Additives Panel (Health, Environmental, and Regulatory Task Group, 2006). However, 2-EHN was recently shown to be degraded using particular microbial populations originating from a treatment plant supplied with wastes from petroleum refineries (Solano-Serena *et al*. 2009). Moreover, *Myco. austroafricanum* IFP 2173 was found to partially degrade 2-EHN with concomitant accumulation of 4-ethyldihydrofuran-2(3H)-one (4–EDF) (Nicolau *et al.* 2008). This six-carbon lactone was not further metabolized by strain IFP 2173. The aim of this study was to isolate microorganisms capable of utilizing 4-EDF as sole source of carbon, then associate such isolates with *Myco. austroafricanum* IFP 2173 as a means to achieve total mineralization of 2-EHN.

MATERIALS AND METHODS

Strain isolation and liquid cultures

The soil sample used for strain isolation originated from the upper layer of a diesel-polluted site. Its moisture content was 27% according to the norm ISO11465; pH was 6.0 according to the norm ISO10390; diesel oil content was 10 g kg^{-1} of soil (dry weight).

Cultures on 4-EDF were performed in 15-ml test tubes closed with Teflon-coated stoppers and sealed with aluminium caps. Unless otherwise indicated, 1 µl of 4-EDF was used as carbon source in 2 ml of mineral salts medium (Bouchez *et al.* 1995). Tubes were incubated at 30°C with shaking (150 rpm). Biodegradation was monitored at time intervals by analysis of the CO_2 evolved in headspace. Measurement of endogenous respiration was performed similarly in control tubes with no added 4-EDF. Experiments were carried out in duplicates and abiotic controls supplemented with mercuric chloride (0.2 g l^{-1}) were run under similar conditions.

Cultures on 2-EHN were performed in 120-ml serum bottles as previously described (Nicolau *et al.* 2008). At the end of the incubation period, 10 ml of MTBE (methyl *tert*-butyl ether) was introduced into the flasks. After overnight extraction at 4°C, residual 2-EHN was analysed. CO_2 production by the cultures was quantified after acidification to pH 2 with H_3PO_4. The net amount of CO_2 produced was calculated as the difference between the amount of CO_2 found in test flasks and that found in the control flasks without 2-EHN. The mineralization yield was calculated as the carbon ratio between the net CO_2 produced and the theoretical amount of CO_2 generated from complete oxidation of the consumed 2-EHN.

CO_2, 2-EHN and 4-EDF were quantified by gas chromatography (GC) as previously described (Nicolau *et al.* 2008).

Nucleic acid extraction and phylogenic analyses

Genomic DNA of isolates was extracted from the bacterial pellet of a 5-ml Tween 80-grown culture (Solano-Serena *et al.* 2004).

16S ribosomal DNA was PCR-amplified using primer F8 (5'-AGAGTTTGATYMTGGCTCAG-3'), 1492R (5'-CGGTTACCTTGTTACGACCT-3') (Grabowski et al. 2005). Amplicons were cloned in pCR2.1 TOPO vector (Invitrogen), then

sequenced on both strands. Strains were identified using Blast on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/).

16S DNA sequences were aligned with the CLUSTALX program. Trees were constructed with neighbour-joining algorithms, gaps being removed from the analysis.

Chemicals

2-EHN (CAS Number 27247-96-7) was obtained from Sigma Aldrich. Mineral salts were from VWR (France). 4-EDF was prepared by chemical synthesis from furane-2(5H)-one according to the procedure described by Alexakis *et al.* (1997).

RESULTS

Strain selection on 4-ethyl-dihydrofuran-2-(3H)-one

In order to isolate microorganisms endowed with ability to degrade 4-EDF , enrichments were performed in liquid cultures using a sample of diesel-contaminated soil as starting inoculum. Ten mg of soil were initially incubated in liquid medium with 0.5 g l^{-1} 4-EDF as substrate. Bacterial growth was monitored by CO_2 measurement in the headspace of the cultures (Figure 1). CO_2 evolution became detectable after six days of incubation and stopped after 15 days. Two subcultures were successively performed using a 21 day-old culture as inoculum (10 % v/v). Figure 1 shows that lag phase before start of growth was shortened in subcultures compared to the initial culture.

Bacterial isolation from subcultures was performed by plating diluted samples on agar plates. Two morphological types of isolates were obtained on agar plates, designated S1 and S2. In both cases, colonies were white in colour and showed round and regular shape. They were identified by their 16S ribosomal DNA sequence as members of *Bacillus cereus* (strain S1) and *Burkholderia* sp. (strain S2), respectively.

2-EHN biodegradation in cocultures of bacterial isolates and *Myco. austroafricanum* IFP 2173

Bacillus cereus S1 and *Burkholderia* sp. S2 were incubated either separately or in combination with *Myco. austroafricanum* IFP 2173 in mineral salts medium containing 2-EHN. CO_2 evolution in the culture headspace was monitored over 17 days (Figure 2). As expected, a moderate CO_2 production was observed with *Myco. austroafricanum* IFP 2173 alone, corresponding to the partial mineralization of the substrate. A much higher level of CO_2 evolution was observed in cocultures where strain IFP 2173 was associated with either *Bacillus cereus* S1 or *Burkholderia* sp. S2. Over the same period of time, pure cultures of *Bacillus cereus* S1 and *Burkholderia* sp. S2 were unable to produce significant amounts of CO_2 at the expense of 2-EHN suggesting that they did not utilize this compound as carbon source.

Quantitative determination of 2-EHN in cultures by the end of the incubation allowed to calculate mineralization yields (Table 1).

In abiotic controls, high recovery rates (> 85%) were obtained, indicating that substrate losses were low over the experimental time period. *Myco. austroafricanum* IFP 2173 utilized 2-EHN extensively but substrate conversion reached only 23%, due to accumulation of 4-EDF (data not shown). High biodegradation rates and mineralization levels were obtained in cocultures of strain IFP 2173 with *Bacillus cereus* S1 or *Burkholderia* sp. S2, and no accumulation of 4-EDF was observed. In pure cultures of *Bacillus cereus* S1 and *Burkholderia* sp. S2, 2-EHN was not significantly degraded and a low level of CO_2 probably arising from respiration on endogenous substrate was detected.

DICUSSION

We isolated two strains able to degrade 4-EDF, a by-product that is released by *Myco. austrofricanum* IFP 2173 when grown on 2-EHN. The isolation procedure was easy and yielded two phylogenetically unrelated bacterial strains suggesting that the microbial ability to utilize lactones like 4-EDF is widespread in soil. Since many lactones have negative impact on human health, the biodegradability of 4-EDF by *Bacillus cereus* S1 and *Burkholderia* sp. S2 may reduce health hazard associated with the use of 2-EHN and improve the environmental acceptability of this chemical.

The concerted attack on 2-EHN in cocultures exemplifies the extended degradation capabilities of bacterial associations compared to individual microorganisms. This finding has important consequences in terms of environmental safety. In case of accidental release of 2-EHN in the environment, it may be expected that the chemical be degraded through natural attenuation involving commensalism processes, provided that adequate indigenous bacterial species co-exist on polluted sites.

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TABLE

Table 1 : Mineralization rates of 2-EHN in separate cultures of *Myco. austroafricanum* IFP 2173, *B. cereus* S1 and *Burkholderia* sp. S2, and in combinations.

Strains	Substrate recovery in abiotic flasks (%)	Substrate degradation in test flasks (%)	Mineralization yield (%)*
B. cereus S1	97	9,1	2,1
Burkholderia sp. S2	90	2,1	7,2
Myco. austroafricanum IFP 2173	95	> 99.5	23.1
S1 + IFP 2173	85	99,4	100
S2 + IFP 2173	89	99,1	95,4

* Mineralization yields were calculated with respect to the substrate consumed

FIGURES

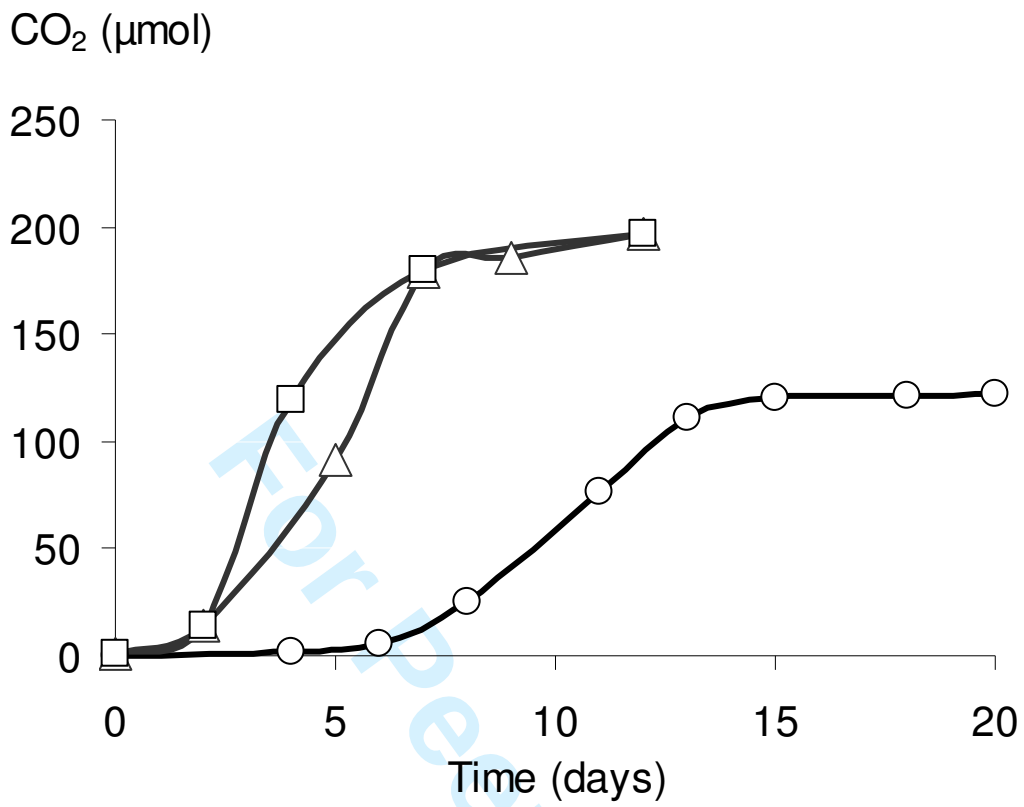

Figure 1: Mineralization curves of successive enrichment cultures grown on 4-EDF. ○ initial culture, Δ first subculture and □ second subculture. Strains S1 and S2 were isolated from the first and second subcultures , respectively.

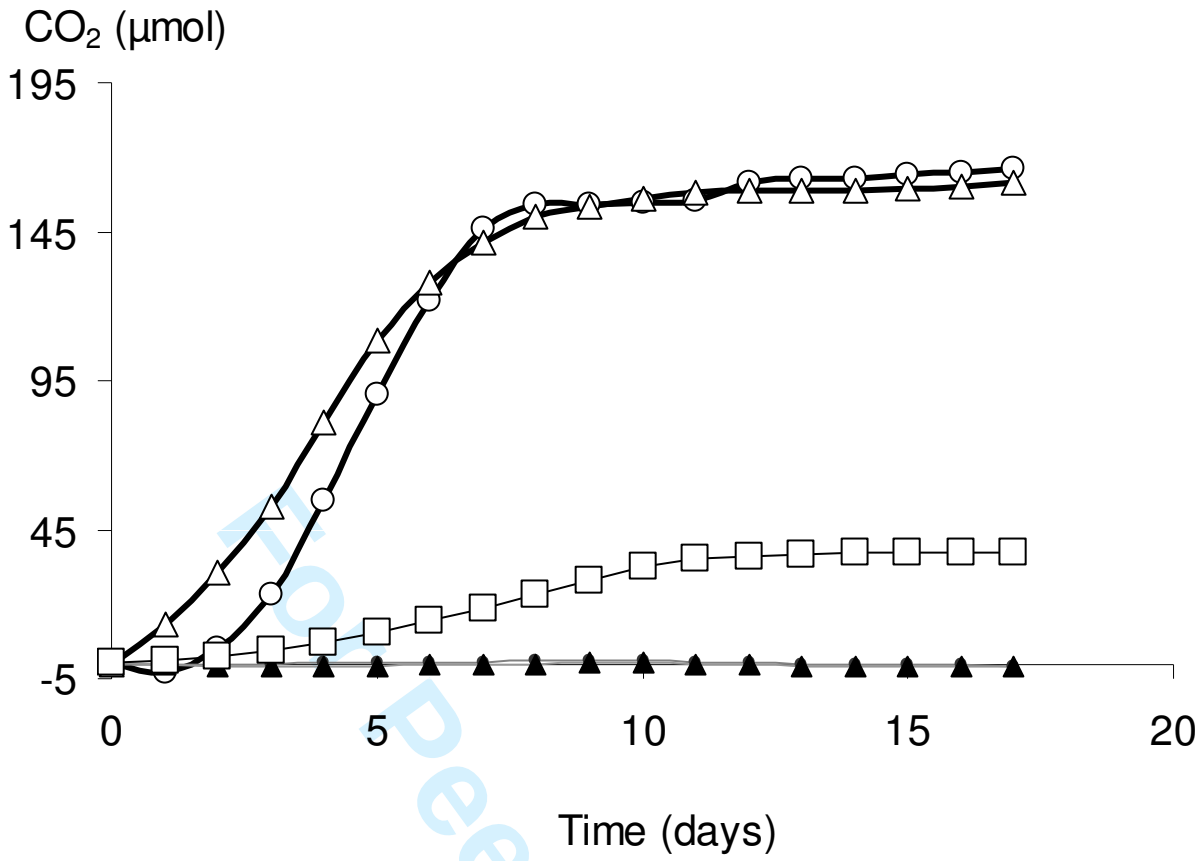

Figure 2 : Mineralization of 2-EHN in pure cultures and combinations with strain IFP2173.

○ Strain S1 with *Myco. austroafricanum* IFP 2173; Δ strain S2 with *Myco. austroafricanum* IFP 2173; □ *Myco. austroafricanum* IFP 2173 alone; controls of strains S1 ● and S2 ▲, alone.