Hydrocarbon degradation by a soil microbial population with \( \beta \)-cyclodextrin as surfactant to enhance bioavailability

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Abstract

In general the biodegradation of nonchlorinated aliphatic and aromatic hydrocarbons is influenced by their bioavailability. Hydrocarbons are very poorly soluble in water. They are easily adsorbed to clay or humus fractions in the soil, and pass very slowly to the aqueous phase, where they are metabolised by microorganisms. Surfactants that increase their solubility and improve their bioavailability can thereby accelerate degradation. Cyclodextrins are natural compounds that form soluble complexes with hydrophobic molecules. They are widely used in medicine and harmless to microorganisms and enzymes. This paper describes their in vitro effect on the biodegradative activity of a microbial population isolated from a petroleum-polluted soil, as shown by the decrease of dodecane (C\(_{12}\)), tetracosane (C\(_{24}\)) anthracene and naphthalene added individually as the sole carbon source to mineral medium liquid cultures. \( \beta \)-cyclodextrin accelerated the degradation of all four hydrocarbons, particularly naphthalene, and influenced the growth kinetics as shown by a higher biomass yield and better utilization of hydrocarbon as a carbon and energy source. Its low cost, biocompatibility and effective acceleration of degradation make \( \beta \)-cyclodextrin an attractive option for bioremediation. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

The release of hydrocarbons into the environment, whether accidental or due to human activities, is a main cause of water and soil pollution. Many bioremediation technologies have been developed to remove these contaminants, as some biological treatments are cheaper than chemical and physical treatments and sometimes result in complete mineralization [1–3].

Several petroleum aliphatic and polycyclic aromatic hydrocarbons can act as source of carbon and energy for the growth of soil microorganisms [4]. One main factor that influences the extent of their biodegradation is their bioavailability and this is a priority research objective in the bioremediation field [3,5]. Their hydrophobicity and low water solubility mean that hydrocarbons pass very slowly from a non-aqueous to the aqueous phase liquid in which they are metabolised by microorganisms [5,6]. Moreover, in the soil they are adsorbed to clay or humus fractions [5,7].

Surfactants enhance solubilization and removal of contaminants [8,9]. Biodegradation is also enhanced by surfactants due to increased bioavailability of pollutants [10–13]. They are suitable for in situ bioremediation if microorganisms producing biosurfactants could be used to reduce costs, or if readily degradable biosurfactants could be used; otherwise, they could increase the risk of groundwater pollution. They are also useful in ex situ processes, as they reduce their duration and hence the cost and environmental impact of pollution events.

\( \alpha \), \( \beta \) and \( \gamma \)-cyclodextrins are cyclic oligosaccharides formed by 6, 7 or 8 \( \alpha \)-1,4-linked glucose units respectively [14]. Since they have toroidal hydrophobic cavities with a hydrophilic shell, they are water-soluble and form inclusion complexes with hydrophobic molecules of a size compatible with their hydrophobic core. In this way, the aqueous solubility of several compounds is increased by cyclodextrins through their dynamic equilibrium exchange with guest molecules that then dissociate from the cyclodextrin complex and become available for catabolism. Cyclodextrins are natural, non-toxic compounds that are harmless to mi-
croorganisms and free enzymes [15], and hence widely used in medicinal applications [16]. Their employment for microbial degradations, e.g. purification of wastewater from pesticides [17] or phenol [18], has also been investigated. Bacterial degradation of toluene, an aromatic solvent, and p-toluic acid, a water-soluble aromatic compound, is enhanced by β-cyclodextrin due to alleviated toxicity of substrates [19]. It has also been demonstrated that hydroxypropyl-β-cyclodextrin enhances biodegradation of phenanthrene, a polyaromatic hydrocarbon [20]. Cyclodextrins could thus be suitable for bioremediation of hydrocarbon-polluted soils or waters, where surfactants that would alter the equilibrium of natural habitats cannot be employed. This paper illustrates the way in which β-cyclodextrin enhances the biodegradation of aliphatic and polycyclic aromatic hydrocarbons present at concentrations in excess of their aqueous solubility limit. Its effect was investigated in vitro in a simplified system and in operating conditions as close as possible to those of a real application. The biodegradative activity of a natural microbial population isolated from a petroleum-polluted soil was examined on four representative petroleum hydrocarbons: a medium-chain (dodecane) and a long-chain (tetracosane) aliphatic hydrocarbon and two polyaromatic hydrocarbons (naphthalene and anthracene). β-cyclodextrin was used as the cheapest form.

2. Materials and methods

2.1. Microorganisms and growth conditions

Microbial colonies were isolated from a petroleum-polluted soil by dispersing a sterile-water suspension of a soil sample in MMA (Mineral Medium Agar: 0.8 g/l K2HPO4, 0.2 g/l KH2PO4, 0.05 g/l CaSO4 · 2H2O, 0.5 g/l MgSO4 · 7H2O, 0.09 g/l FeSO4 · 7H2O, 1 g/l (NH4)2SO4, 15 g/l agar) with 4% v/v dodecane as the sole carbon source in Petri dishes, and incubating it at 28°C for 5 days. A random pool of 54 colonies was transferred to 200 ml of LMM (Liquid Mineral Medium: as MMA, without agar) with 4% v/v dodecane in a conical flask and incubated on an oscillatory shaker at 180 rpm at 28°C. This mixed culture was stored at 4°C and used as inoculum for the assays. The morphology of microorganisms grown in it was observed in bright-field microscopy (400×) and by plating on Oatmeal Agar (60 g/l Oatmeal flower, 20 g/l agar) and YEME (Yeast Malt Extract Agar, Difco).

2.2. Degradation of hydrocarbons

The degradation rate of dodecane, tetracosane, naphthalene or anthracene in liquid cultures was determined by comparing their consumption in the presence and absence of β-cyclodextrin. 50 ml aliquotes of LMM in conical flasks were supplemented alternatively with 4% v/v dodecane, 4% w/v tetracosane, 2% w/v naphthalene or 2% w/v anthracene; 1% w/v β-cyclodextrin was added to half of the flasks. Each flask was inoculated with 1 ml of the liquid microbial culture. Control flasks were made without inoculum. The assays were executed in duplicate. The incubation was done on an oscillatory shaker at 180 rpm at 28°C and continued until degradation of the hydrocarbon had stopped. Microbial growth in the presence of dodecane and naphthalene was evaluated from the dry weight of the biomass produced.

2.3. Hydrocarbon extraction and quantitation

Hydrocarbons were extracted from the whole volume of each flask by shaking three times with 10 ml of toluene. The mixture was centrifuged at 4000 rpm for 5 min. The organic phase was transferred to a fresh tube and the three fractions were mixed in a final volume of 30 ml. 100 μl of a standard solution (1% w/v naphthalene in toluene for the determination of dodecane and tetracosane; 1% v/v dodecane in toluene for the determination of naphthalene and anthracene) were added to 1 ml of extract. The samples were analyzed with a gas chromatograph (GC HP 5890 series II) equipped with a flame ionization detector (FID) on HP1 Cross-Linked Methyl Silicone capillary column, 15 m long and 0.32 mm i.d., film thickness 1.0 μm. The operating condition were: temperature from 100 to 250°C at 20°C/min; detector temperature 250°C; gas helium, linear flow rate 5.2 ml/min; pressure 13 psi; injection volume 4 μl.

The extraction yield was previously evaluated for each hydrocarbon by adding it at different known concentrations to 50 ml aliquotes of LMM and following the entire procedure of extraction; the results were checked on the basis of the calibration curves.

3. Results and discussion

The microbial population of the petroleum-polluted soil originated 3.2 × 10^7 CFU/g (wet weight) on dodecane. The degradative activity of the microbial liquid culture from the random pool of 54 colonies was assessed on dodecane in LMM with the addition of 1% glucose to see whether co-metabolism with a different carbon source was useful or necessary. Degradation of contaminants due to co-metabolism, in fact, is dependent on the addition of an energy and carbon source (called primary substrate), and happens “en passant” due to catalysis of enzymes involved in the catabolism of this substrate [21]. The addition of glucose made no difference to the degradation rate (data not shown) and it was not used in the subsequent experiments.

Dodecane was fully degraded in 9 days compared with 4 days when β-cyclodextrin was added (Fig. 1a). Tetracosane degraded in 18 days. Addition of β-cyclodextrin reduced this to 16 days (Fig. 1b). Interestingly, the catabolism of tetracosane produced dodecane in the form of a new peak in the gas chromatogram, whose identity was established when...
addition of a known concentration of dodecane resulted in a proportional increase of the unknown peak. Both peaks disappeared at the end of the assay. This result is unexpected and requires confirmation by GC-mass spectrometry, since degradation of tetracosane originates dodecanoic acid or dodecanol by oxidation by subterminal attack [22], but not dodecane.

The full degradation time of both hydrocarbons was shortened by \(\beta\)-cyclodextrin, but the reduction was drastically influenced by chain length: to 50% for the medium-chain compared to 89% for the long-chain form.

Interesting results were obtained with naphthalene and anthracene (Fig. 1c and 1d). \(\beta\)-cyclodextrin had a more pronounced effect on their degradation than on the aliphatics. This effect is also evidenced for naphthalene by the increased degradation rate, expressed as the mass of hydrocarbon consumed in one hour (Fig. 2c). This result is very important as low bioavailability due to their strong insolvency is one of the main reasons why nonchlorinated polycyclic hydrocarbons are not degraded by microorganisms. Wang et al. (1998) found a similar result for phenanthrene, another polycyclic aromatic hydrocarbon: biodegradation was strongly enhanced by 1% hydroxypropyl-\(\beta\)-cyclodextrin. Moreover, soil and water pollution by volatile hydrocarbons such as naphthalene is always accompanied by their airborne diffusion. Earlier and faster degradation significantly reduces this secondary, hard-to-control pollution. Since \(\beta\)-cyclodextrin enhances degradation of solvent vapours and facilitates absorption of the gaseous substrate [19], it reduces the airborne diffusion of these hydrocarbons and allows the degradation of their vapours.

The full degradation time of both aromatic hydrocarbons was shortened by \(\beta\)-cyclodextrin, though to an extent determined by their chemical structure. Anthracene, composed of three aromatic rings, was fully degraded in 11 days compared with 9 days for naphthalene, which is formed of two rings. The degradation rates were also different. The rate for anthracene was similar to that for dodecane (Fig. 2d). The greater enhancement by \(\beta\)-cyclodextrin of the degradation of aromatic compared with long-chain aliphatic hydrocarbons must be taken into account in the bioremediation of petroleum-polluted soils.

The catabolism of aliphatic and aromatic hydrocarbons follows different metabolic pathways [22]. The biomass growth kinetics on dodecane and on naphthalene was therefore examined to see whether the microbial population used both as an energy and carbon source for growth. \(\beta\)-cyclodextrin greatly increased the total biomass produced (Fig. 3). No growth was observed on \(\beta\)-cyclodextrin as the sole carbon source (data not shown). Therefore, nonchlorinated hydrocarbons are easily used as carbon and energy sources by microorganisms, as expected, but their solubilization drastically favours growth. Moreover, the rate of hydrocarbon consumption for growth, calculated as the ratio between the

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Fig. 1. Percentage of remaining hydrocarbon during the microbial degradation of dodecane (1a), tetracosane (1b) naphthalene (1c) and anthracene (1d). Data are reported as media of two replicate determinations and standard errors are indicated with bars.
hydrocarbon decrease and the biomass increase (Table 1), is higher in the samples without β-cyclodextrin, indicating that the metabolic yield is lower.

Lastly, the degradation rate is higher in the presence of β-cyclodextrin, mainly in the early stages (Fig. 2a, b, c and d). The difference is evident for naphthalene, and less marked for tetracosane; the rate for dodecane and anthracene peaked at the start of degradation. These data demonstrate that the effect of β-cyclodextrin is maximum soon after its application. As the amount of remaining hydrocarbons is in excess of their aqueous solubility limit until the last phases of degradation, their smaller concentration due to biodegradation cannot be regarded as a limit for the reaction rate. One explanation could be that the metabolites produced are not easily degradable or are toxic.

1% of β-cyclodextrin seems to ensure a good result at a low cost. It is totally in solution as its solubility limit in water at 25°C is 1.85%. Wang et al. (1998) found that for phenanthrene degradation 10% as opposed to 1% hydroxypropyl-β-cyclodextrin displayed a longer lag phase, probably due to because degraders need to adapt to the higher availability of phenanthrene or to cyclodextrins themselves, since cyclodextrins may be toxic [23]. Reduction of degradation time was greater for 10% of hydroxypropyl-β-cyclodextrin, but a good result was also obtained with 1% [20].

Degradation is slower when β-cyclodextrin is not added and this difference is significant for dodecane, naphthalene

Fig. 2. Hydrocarbon degradation rate calculated from data (media of two replicate determinations) reported in Fig. 1 and expressed as mg of hydrocarbon degraded in one hour. 2a: dodecane; 2b: tetracosane; 2c: naphthalene; 2d: anthracene. Pearson product-moment correlation coefficient was calculated for each hydrocarbon between the two degradation rate series (with and without β-cyclodextrins).

Fig. 3. Biomass growth kinetics on dodecane (3a) and naphthalene (3b). Data are reported as media of two replicate determinations and standard errors are indicated with bars.
worked out. As the application of bioremediation of polluted soils by landfarming is being at a lower cost. A model for the application of this method to whether its percentage can be lowered to obtain the best results aromatic hydrocarbons and of petrol. We will also determine b...ment, it helps to reduce atmospheric pollution by accelerating reductions. It is a natural, biocompatible compound and does not introduce any element that alters natural habitats. Moreover, it is cost-effective and its considerable acceleration of biodegradation: implications for \textit{in situ} bioremediation. In: Biotechnology for soil remediation. \textit{Scientific bases and practical applications}. R. Serra. C.I.P.A. S.r.l., Milan, Italy, 1998, 67–80.


4. Conclusions

\(\beta\)-cyclodextrin enhances the degradation of aliphatic and polycyclic aromatic hydrocarbons by increasing their bioavailability. \(\beta\)-cyclodextrin, the cheapest cyclodextrin, was significantly effective at a 1% concentration and enhanced the degradative activity of a natural microbial soil population on both aliphatic and aromatic hydrocarbons. Reduction of degradation time was determined by the chemical structure of the contaminants and was greater for aromatic than long-chain aliphatic hydrocarbons. The maximum degradation rate was observed in the early stages of degradation.

\(\beta\)-cyclodextrin’s low cost and its considerable acceleration of degradation make it attractive for \textit{ex situ} applications. It is a natural, biocompatible compound and does not introduce any element that alters natural habitats. Moreover, when volatile hydrocarbons are released into the environment, it helps to reduce atmospheric pollution by accelerating their degradation.

Our next step will be to investigate the effectiveness of \(\beta\)-cyclodextrin in biodegradation of mixtures of aliphatic and aromatic hydrocarbons and of petrol. We will also determine whether its percentage can be lowered to obtain the best results at a lower cost. A model for the application of this method to bioremediation of polluted soils by landfarming is being worked out. As the application of \(\beta\)-cyclodextrin to \textit{in situ} soil bioremediation could be suitable, due to low cost, biocompatibility and biodegradability, we are also looking to see whether increased percolation of complexed contaminants results in increased groundwater pollution and how this can be reduced.

References


