Biodegradation of fuel oxygenates: isolation and characterization of a novel ether-degrading bacterial strain, *Mycolicibacterium* sp. CH28

Theses of the PhD Thesis

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Introduction

The rapid increase in the number of motor vehicles results in increasing fuel consumption worldwide. In order to improve the physical and chemical properties of gasoline, it is necessary to add various fuel additives. Owing to the replacement of lead tetraethyl, which was banned by law, and in order to increase the octane number and to ensure a more complete combustion of fuels, the use of fuel oxygenates as an additive has become widespread.

Due to their numerous advantages, these compounds have rapidly become market leaders. However, it has soon become clear that due to their specific chemical structure, gasoline ether oxygenates are highly resistant to both natural physicochemical and biodegradation processes when released into the environment. Furthermore, measured on an evolutionary scale, microorganisms have had only a relatively short time to adapt to them. As they are highly soluble in water, they can easily cause extensive pollution, thereby endangering drinking water supplies. Although methyl *tert*-butyl ether (MTBE) is currently the most common one, other fuel oxygenates have become increasingly widespread in recent years, in particular ethyl *tert*-butyl ether (ETBE), which is a biofuel and in Hungary this oxygenate is currently used in the largest amounts in petrol. Furthermore, *tert*-amyl methyl ether (TAME) is also widely used today, and a more widespread use of diisopropyl ether (DIPE) and *tert*-amyl ethyl ether (TAEE) is also highly likely.

As the demand for fuel oxygenates is expected to increase further in the coming decades, the number of areas polluted with gasoline ether oxygenates is likely to increase further. Bioremediation is the most reasonable, efficient, cost-effective and environmentally sound choice for remediation of polluted areas. Due to the global use of MTBE and the high number of polluted sites, the biodegradation of MTBE has been extensively studied. However, as the extent and number of sites polluted with ETBE and DIPE are exponentially smaller today, the isolation and characterization of microorganisms that degrade these compounds still pose serious challenges for researchers. In this present research I aimed to isolate and to fully characterize a bacterial strain capable of efficiently degrading ETBE and DIPE.

Objectives

The aim of my doctoral dissertation was the isolation and environmentally relevant characterization of an individual bacterial strain that is capable of efficiently degrading DIPE and ETBE.

Based on literature data, I set the following objectives:

1. Isolation of DIPE- and ETBE-degrading bacterial strains using groundwater samples from a Hungarian DIPE-polluted area.
2. Routine laboratory maintenance of the isolated, individual strain, as well as investigation of its substrate spectrum and its key parameters in terms of the environment and bioremediation. My aim was to propose the phylogenetic classification of the isolated microorganism capable of degrading DIPE and ETBE as well as to identify the gene(s) important for bioremediation in the genetic stock of the strain which may allow for field monitoring of the strain in future *in situ* remediation processes.
3. Comparison of the DIPE-degrading potential of strain CH28 with bacteria capable of degrading DIPE described in the literature. Verification of the DIPE-degrading capacity of the isolated strain by bioaugmented microcosm experiments using groundwater from a pollution plume in a polluted area.
4. Investigation of the biodegradation pathway of DIPE.
5. Investigation of the potential for cometabolic biodegradation of gasoline ether oxygenates.
6. Plan and model a bioremediation technology

Methods

1. Isolation of an individual bacterial strain capable of degrading DIPE as the sole carbon and energy source using DM mineral salt medium and DMA mineral salt agar.
2. Analysis of the sequencing data of the DIPE-degrading microorganism was accomplished. Identification and the phylogenetic placement of the strain based on the 16S rDNA and *de novo* genome sequence of the bacterial strain was performed.
3. Substrate spectrum of the isolated bacterium was determined using mineral salt medium.
4. Properties of the isolate which are relevant from an environmental and remediation perspective were revealed. Among others temperature and pH tolerance, optimal temperature and pH were examined and experiments were performed to determine salt tolerance.
5. Intermediates formed during the biodegradation of DIPE were monitored using a gas chromatograph coupled with a mass spectrometer (GC-MS).
6. The rate of DIPE-decomposition was determined by resting cell experiments.
7. The DIPE-degrading potential of the strain was confirmed by microcosm experiments which were set up using groundwater samples supplemented with DIPE in different concentrations. Samples were continuously taken in the course of the experiments and the intermediates formed during the degradation of DIPE were continuously monitored by GC-MS.
8. As strain CH28 is capable of degrading ETBE to TBA, an artificial consortium with the previously isolated TBA-degrading *Hydrogenophaga* sp. strain T4 was created to achieve complete mineralization.
9. Relying on the cometabolic properties of the strain, I investigated the capacity of the isolate to degrade other ethers in microcosm experiments.
10. Column experiments were performed to elucidate DIPE degradation of the strain as well as the ETBE mineralization potential of the artificial consortium.
11. In the column experiments, the continuous presence of strains CH28 and T4 in the columns was monitored by plating, denaturing gradient gel electrophoresis (DGGE) and colony polymerase chain reaction (PCR).

Results

1. A microorganism capable of degrading DIPE and ETBE was successfully isolated from a groundwater sample collected from a monitoring well at a pharmaceutical production facility in Budapest, Hungary using laboratory enrichment procedures.
2. I identified the *ethRABCD* gene cluster in the genome of the strain. The *ethB* gene shows 99% similarity to the corresponding genes of *Rhodococcus ruber* IFP 2001 and *Aquincola tertiaricarbonis* L108. This gene encodes a cytochrome P450 monooxygenase which was experimentally proved to be responsible for the biodegradation of DIPE, ETBE, and other dialkyl ethers.
3. The 16S rDNA sequence of the isolated strain, as well as the results of comparative analyses of the genome, suggest that the isolated strain can be considered a new species.
4. The strain has been registered in the National Collection of Agricultural and Industrial Microorganisms under the name *Mycolicibacterium* sp. strain CH28 with ID: NCAIM B.02558.
5. The spectrum of substrates utilized by strain CH28 was examined in detail and it was revealed that the strain has an extremely broad substrate specificity. Strain CH28 is capable of utilizing normal alkanes, monoaromatic compounds, branched-chain hydrocarbons, dialkyl ethers, C_1 compounds, several short-chain alcohols, and organic acids as sole carbon and energy sources.
6. It was observed that the strain is able to grow in the temperature range of 10-35 °C, and the optimum growth temperature for the bacterium is between 30-32 °C.
7. It was revealed that strain CH28 is able to grow and reproduce in the pH range of 3.5-8.5, but typically grows better under acidic conditions. The optimal pH range for the strain is pH 5.0-6.0.
8. Based on the results of antibiotic tolerance and resistance tests of the strain, it can be stated that it shows a slight tolerance to tobramycin and is also resistant to carbenicillin.
9. I identified 2-propanol, acetone and acetic acid as intermediates formed during the biodegradation of DIPE using GC-MS measurements. The detection of 2-propanol and acetic acid is a significant result, as these compounds have not been previously

detected during the biodegradation of DIPE. After the detection of intermediates, I proposed the upper pathway of microbiological DIPE degradation.

10. The DIPE degradation rate of strain CH28 was measured by resting cell experiments. It was in the same order of magnitude as the degradation rate of strains *Rhodococcus ruber* IFP 2001 and *Aquincola tertiaricarbonis* L108. Although strain L108 had the highest DIPE-degradation rate, the strain was not capable of DIPE-mineralisation and acetone accumulated as an end product during biodegradation. In contrast, strain CH28 was excellent at utilizing acetone as the sole source of carbon and energy, therefore carbon dioxide and biomass were produced during mineralization.
11. The DIPE-mineralizing capacity of strain CH28 and the formation of 2-propanol and acetone as intermediates during the degradation process was confirmed by microcosm experiments set up with DIPE-supplemented groundwater samples (75 mg/l and 300 mg/l). Although the strain was able to degrade DIPE at higher concentrations (300 mg/l), and DIPE concentrations of 975 mg/l were still tolerated by the bacterium, biodegradation of DIPE proved to had a higher rate in a system with a lower DIPE concentration (75 mg/l). To the best of our knowledge, this was the first microcosm study ever published on the biodegradation of DIPE.
12. An artificial consortium was formed with strain CH28 and a previously isolated TBA-degrading bacterium, *Hydrogenophaga* sp. strain T4. The consortium was able to efficiently mineralize ETBE. Strain T4 was registered in the National Collection of Agricultural and Industrial Microorganisms with ID: NCAIM B.02575.
13. Using systems set up with ETBE-supplemented groundwater samples I demonstrated that despite the fact that strain CH28 alone cannot mineralize ETBE, a complete biodegradation of ETBE can be achieved with strain CH28 in the polluted site, if the local microbial population is able to degrade TBA − even if it is not able to biodegrade ETBE.
14. The artificial consortium degraded ETBE at a remarkably high rate, it completely mineralized 200 mg/l ETBE in 60 hours. Based on my results, it can be said that the combination of CH28 and T4 strains is capable of significantly more efficient ETBE biodegradation than similar consortia published in the literature so far.
15. Using microcosm experiments assembled with mineral salt medium it was confirmed that although strain CH28 did not grow on MTBE and TAME as the sole carbon and energy sources, it was capable of degrading these two fuel oxygenates in the presence of DIPE. During the biodegradation of MTBE *tert*-butyl alcohol (TBA) accumulated,

while during the biodegradation of TAME *tert*-amyl alcohol (TAA) was formed. To the best of our knowledge, this was the first time when the cometabolic degradation of MTBE and TAME had been reported in the presence of DIPE.

16. In the column experiments, two types of filling materials (1:4 mixture of perlite and peat, clay granules) were identified to which strain CH28 could adhere and grow. In the future this could have a key role e.g. in the planning of a permeable reactive barrier (PRB).
17. The column reactors operated with DIPE as a pollutant ran efficiently for more than four weeks. During the degradation, intermediates (2-propanol, acetone) were detected only near the detection limit (<200 μg/l). This is considered favourable, as it is assumed that these intermediates would not accumulate during an *in situ* remediation process.
18. In the column experiments, I optimized the DM mineral salt medium for strain CH28 (MDM medium).
19. The column reactor operated with ETBE as a pollutant, filled with a 1:4 mixture of perlite and peat, ran efficiently for more than three months with my artificial consortium. The members of the consortium (strains CH28 and T4) were detectable in the effluent of the column throughout the experiment, which confirms that the degradation processes were indeed performed by the microorganisms I inoculated.
20. I did not find any examples of column experiments efficiently operated with either DIPE or ETBE in the literature, so my experiments can be considered unique.
21. In view of my results it can be stated that strain CH28 is a microorganism with a very remarkable bioremediation potential, both for fuel oxygenates and mixed pollutants.

Publications

Hungarian Scientific Bibliography (MTMT) identifier: 10077832
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Articles on which the thesis was based:

1. **Ingrid Zsilinszky**, Balázs Fehér, István Kiss, Attila Komóczi, Péter Gyula, Zsolt Szabó (2021) **Biodegradation of diisopropyl ether, ethyl *tert*-butyl ether, and other fuel oxygenates by *Mycolicibacterium* sp. strain CH28.** *Bioremediation Journal,* DOI:10.1080/10889868.2021.1911924
   IF: 1.724

2. **Ingrid Zsilinszky**, Péter Gyula, Zoltán Bihari, Balázs Fehér, Zsolt Szabó (2019) **Draft genome sequence of *Mycolicibacterium* sp. strain CH28, a potential degrader of diisopropyl ether, isolated from pharmaceutical wastewater.** *Microbiology Resource Announcements 37* DOI: 10.1128/MRA.00682-19

Other publications:

3. Balázs Fehér, **Ingrid Zsilinszky** (2020) **Intelligens kármentesítés.** *inGreen,* 2020. őszi lapszám

Posters:

1. **Ingrid Zsilinszky**, István Kiss, Sándor Mészáros, Balázs Fehér **Laboratory microcosm study of a polluted groundwater.** *Proceedings of the 25th International Symposium on Analytical and Environmental Problems,* October 7-8, 2019, Szeged, Hungary, ISBN 978-963-306-702-4