Biodegradation of hydrophobic pollutants, isolation and characterization of new solvent-tolerant bacteria

Ph.D. Thesis

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INTRODUCTION

In the last century, the increasing needs of mankind resulted in a rapid industrial development, and unfortunately also caused the accumulation of various pollutants in the environment. The deteriorating conditions, however, gradually inspired the development of an approach that included a conscious strives to protect the nature and also the reduction of environmental impact.

Nowadays, active research is ongoing with the objective to develop environmental friendly technologies and procedures capable of removing the polluting, environmentally harmful substances. In addition to the physical, chemical technologies; biological methods represent an alternative approach, since bioremediation processes - taking the advantages of microbial activity - offer both an efficient, and a cheap and environmentally friendly solution.

Due to their diverse sets of enzymes, a large number of micro-organisms living in the environment are applicable to the highly efficient degradation of different pollutants. One of the most effective specimens can be found among the members of the *Rhodococcus* genus: their extremely varied enzymes and surfactant producing properties enable them to degrade various pollutants - even the ones with hydrophobic properties.

Our country is mostly affected by hydrophobic pollutants: many kinds of crude-oil derived contaminations and food industry wastes (mainly lard and poultry fat).

The primary goal of my research was the development of an environmentally friendly solution for the removal of such pollutants.

Since microorganisms preferring hydrophobic environments can have an important role in bioconversion processes, my further objective was to isolate solvent-tolerant bacteria from diesel and dead oil.

APPLIED METHODS

The diesel oil degrading ability of the *Rhodococcus erythropolis* MK1 strain was examined in microcosm experiments in minimal salt medium and in soil under laboratory conditions. In order to increase the biodegradation efficiency, my samples were supplemented with β- cyclodextrin, and also the cells – when needed - were entrapped in alginate. The efficiency of diesel oil degradation was analyzed using a gas chromatograph mass spectrometer (GC-MS) after a 1:1 ratio extraction of the samples.

The *on site* remediation of diesel oil contaminated soil was performed in 2 m^3 piles, using biostimulation and bioaugmentation. During biostimulation, the piles were treated with minimal salt medium, while during bioaugmentation, the MK1 strain was applied. For the modeling of a fresh contamination, 2800 mg/kg diesel oil was added to some of the piles. The hydrocarbon content of the piles was recorded for 9 weeks with sampling intervals of 3 weeks and analyzed by a GC-MS instrument after a 1:1 rate extraction.

The fat degrading ability of the MK1 strain was compared to the *R. erythropolis* PR4 (NBRC 100887) reference strain. The fatty waste (lard and poultry fat) consumption of the strains were examined in minimal salt medium under laboratory conditions. The samples - when necessary - were supplemented with $NaHCO_3$. The changes in the fat content of the samples were monitored through their methyl esters derivatives by a GC-MS instrument.

The genomic DNA of the MK1 strain was isolated according to phenol-chloroform method. Whole genome sequencing was carried out by Illumina MiSeq next generation platform. The raw sequence data were then assembled with MIRA 4. Annotation of the assembled genome was carried out by the RAST 2.0 annotation server.

Several new microorganisms preferring hydrophobic environment were isolated from diesel oil and dead oil. The diesel oil tolerance of the strains was determined in a two-phase system. The sample consisted of 88.7% diesel oil and 11.3% aqueous solution of mineral salts. The released CO_2 through the active metabolism of the cells was recorded by a gas chromatograph instrument.

RESULTS OF THE THESIS

During my research on hydrophobic pollutants primarily affecting our country, I studied the microbial degradation of diesel oil, lard and poultry fat using the *Rhodococcus erythropolis* MK1 strain.

During my experiments, I also isolated new solvent-tolerant extremophile microorganisms.

I. Findings based on the results obtained from the biodegradation of diesel oil as hydrophobic pollutant

1) The MK1 strain was able to utilize the diesel oil as a sole carbon and energy source.

2) Under laboratory conditions, the MK1 strain degraded nearly 70% of diesel oil in minimal salt medium in a week, but it was found that the addition of cyclodextrin could further increase the bioconversion efficacy by 20%. Cell entrapping did not improve the biodegradation efficiency.

3) Compared to *Alcanivorax borkumensis* – currently considered as one of the most prominent oil-degrading strains - the *Rhodococcus erythropolis* MK1 strain could degrade hydrocarbons significantly more effectively. These degrading capabilities were not restricted only to alkanes, but also the MK1 strain was able to catabolize all types of the hydrocarbons simultaneously.

4) In soil, natural attenuation of the contaminant could be observed, the content of hydrocarbons decreased by almost 40% in the untreated control samples. With the addition of mineral salts (biostimulation) to the samples, the biodegradation efficiency could be increased, the achieved biodegradation yield was nearly 80%. Bioaugmentation did not improve the degradation efficiency.

5) In case of old contamination, stimulating the indigenous microflora with mineral salts proved to be an appropriate treatment. Bioaugmentation did not cause an apparent increase in the efficiency of hydrocarbon degradation in this case.

6) In case of fresh contamination, biostimulation could be an appropriate treatment. However, for faster remediation, bioaugmentation was definitely proved to be a better treatment.

II. Conclusions from the study of degradation of lard- and poultry fat, as a hydrophobic pollutants wastes

1) Besides diesel oil, the MK1 strain could also utilize lard and poultry fat as sole carbon and energy sources.
2) The fat degrading ability of the MK1 strain was compared to the *R. erythropolis* PR4 (NBRC 100887) strain.
3) Fat degradation efficiency was affected by the buffering capacity of the medium and the composition of the fatty acid. In the samples supplemented with $NaHCO_3$, the MK1 strain completely degraded the poultry fat, while the lard biodegradation was 78% in a week.
4) In case of poultry fat, biodegradation was completed in 5 days, while, because of the larger saturated fatty acid content of the lard, more time was required for the biodegradation of this substrate.
5) Although, in case of both substrates, the PR4 strain accomplished a bit faster biodegradation than the MK1 strain, but this bioconversion was effective in a narrower pH range.

III. Conclusions from *Rhodococcus erythropolis* MK1 strain genome analysis

1) Five of the genes in the genome of the MK1 strain code for AlkB enzymes that were presumably involved in the degradation of the alkane components of diesel oil. Another 11 cytochrome P450 proteins were also detected. Potentially 77 genes might have been involved in the degradation of aromatic hydrocarbons.

2) I could identify five genes encoding triacylglycerol lipases and two genes of monoacyl-glycerol lipases from the known lipase enzymes involved in the hydrolysis of fat forming triglycerides. Another 87 ORF were also identified, which might have been involved in the conversion of fatty acids.

IV. Findings and achieved results during the examination of new solvent-tolerant bacteria

1) Eight diesel oil degrading strains were isolated from diesel oil and dead oil.

2) All but one of the isolated strains apparently belonged to the genus *Rhodococcus*. One strain, unlike the previous ones, was Gram-negative and resembled a *Pseudomonas koreensis* strain.
3) The selected strains were extremely diesel oil tolerant; in a two-phase (diesel oil/aqueous) system with 88,7% diesel oil concentration, they were able to engage in active metabolism in the presence of mineral salts.

PUBLICATIONS

Article on which the thesis was based:

1) **Ágnes Kis,** Krisztián Laczi, Szilvia Zsíros, Gábor Rákhely and Katalin Perei: Biodegradation of animal fats and vegetable oils by *Rhodococcus erythropolis* PR4, International Biodeterioration and Biodegradation, (2015) 105: 114-119.
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