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Application of extracellular organic matter from *Micrococcus luteus* to enhance *ex situ* bioremediation of soils polluted with used lubricating oils

ATTILA BODOR

Summary of the Ph.D. thesis

Supervisors:

Dr. Katalin Perei
Assistant Professor

Dr. Gábor Rákhely
Head of Department,
Associate Professor

Doctoral School of Biology

University of Szeged
Faculty of Science and Informatics
Department of Biotechnology

Biological Research Centre
Institute of Biophysics

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INTRODUCTION AND AIMS

Oil pollutions from excessive industrial and agricultural operation, accidental spills or reckless human activities are still among the major environmental concerns due to the fact that petroleum derivatives pose a serious risk to both natural ecosystems and human health. This is especially true for lubricating oils, which consist of a mixture of hydrocarbons with diverse structures and chemical additives, and accumulate various combuston products, polyaromatic compounds and heavy metals over uptime. In aqueous systems or soils (and binding to soil particles), these components can cause long-lasting, difficult-to-handle and persistent contaminations, creating a serious challenge to both the environment and the organisms that live in it. In the immediate vicinity of the pollution, nevertheless, certain microbial species might adapt to the presence of such pollutants or even be able to degrade them; thus, they can play an important role in the biological remediation of the environment.

Under adverse conditions, the activity of microorganisms (including potentially xenobiotic-degrader strains) can be drastically reduced, and cells might enter into a viable but non-culturable (VBNC) state; thuswise, further complicating the biological rehabilitation processes of lubricant-polluted sites. This transition, however, can be reversed and these cells can be re-activated by Resuscitation promoting factors (Rpfs). Moreover, artificial Rpf-supplementation can enhance the biodegradation efficiency of both the indigenous and the inoculated microorganisms for several contaminants. Keeping these in mind, it is not surprising that the number of studies, exploring the environmental potential of Rpf proteins in order to develop more sustainable and environmentally sound remediation technologies, is constantly increasing.

In my Ph.D. thesis, the feasibility of environmental application of the Rpf-containing extracellular organic matter (EOM) from *Micrococcus luteus* was evaluated. To this end, the environmental rehabilitation of a used lubricant oil (ULO)-polluted railway station area was modeled to compare the efficiencies of conventional and EOM-supplemented bioremediation approaches.

METHODS AND TECHNIQUES

The soil samples used in this study were obtained from Szeged, Hungary (Figure S1), from a railway marshaling yard of MÁV Hungarian State Railways (Hungary).

During my experimental work, the main physical and chemical soil characteristics were determined as follows: Soil moisture was quantified gravimetrically, and soil texture was determined according to the Arany yarn number (K_A). Soil pH was measured in the mixture of soil:distilled water=1:2.5, and electric conductivity (EC) was determined in a soil paste completely saturated with water. Saturation percentage (SP) was expressed as the water content of this saturated soil paste. Soil salinity was calculated using the values of EC and SP. The weight loss of the water-saturated soil column was used for the determination of water holding capacity (WHC). Soil organic matter was indicated by the weight loss on ignition (LOI). Total C and total N in soils were analysed using an elemental analyzer equipment, available P (from orthophosphates) was determined by a colorimetric method.

Chemical properties of the fresh lubricant oil and the ULOs extracted from polluted soils were analysed with Fourier transform infrared spectroscopy (FTIR).

For the preparation of EOM from *Micrococcus luteus* IAM 14879, cells were grown on modified lactate minimal medium (LMM), then the culture supernatant was sterile filtered. The protein concentration in EOM, the presence of Rpf protein and its muralytic activity was confirmed by Bradford's method, denaturing gel electrophoresis and zymographic gel (containing the cell wall extract from *M. luteus*), respectively.

The capabilities of the bacterial strains *Rhodococcus qingshengii* KAG C and *R. erythropolis* PR4 for biodegradation of lubricants were tested in liquid minimal media, supplemented with fresh lubricating oil as sole carbon and energy source. At the end of the incubation period, remaining lubricant oils were extracted using liquid/liquid extraction, then quantified gravimetrically.

Bioremediation approaches of the ULO-polluted site were modeled in two scales, using soil micro- and mesocosm systems.

Soil microcosms were constructed to study the effectiveness of various supplementations (i.e. water and minimal medium), as well as, different bacterial inoculum sizes for ULO-polluted soil clean-up. Proportions of 3 g from polluted composite soil (based on dry soil weight) were placed in butyl rubber capped serum vials. Depending on treatment types, sterile water or liquid mineral medium were supplemented to attain 30% soil moisture. For

bioaugmentation treatments, the ULO-polluted soils were combined with bacterial cells to reach approx. 10^7 cells g^{-1} for the small inoculum size and approx. 10^9-10^{10} cells g^{-1} in regard of the increased inoculum size samples. Each vial was incubated in the dark at 28 ^oC for 40 days.

The feasibility of different bioremediation strategies for ULO-polluted soils, including nutrient stimulation and bioaugmentation with or without the addition of EOM, was examined in bench-scale microcosm set-ups. For each microcosm, 10 kg of ULO-polluted composite soil (not autoclaved) was weighed into a plastic pot (volume: 13 L, height: 27 cm, width: 32 cm, depth: 27 cm) and soil moisture was set to 30%. Without nutrient supplementation, NA represented the intrinsic degradative capability of the ULO-polluted soil, and that this mesocosm was considered as control. In every other bioremediation treatment, the C/N/P ratio was adjusted to 500/10/1 using water-soluble sources of inorganic nitrogen, phosphorus, and potassium (NPK). Additionally, both BS+EOM and BAS+EOM were supplemented with 10% Rpf-containing EOM in order to stimulate or reactivate potentially functional degraders in the indigenous microbial community and to enhance the ULO-biodegradation performance of the inoculated strains. Both *R. qingshengii* KAG C and *R. erythropolis* PR4 were introduced into the microcosms to be augmented at inoculation levels of 2×10^7 cells g^{-1} and 2×10^7 cells g^{-1}, respectively. Each mesocosm was incubated at room temperature (20 °C–25 °C) for 60 days.

In each soil systems, TPH concentrations were quantified using a TPH analyzer equipment, following a solid/liquid extraction procedure. Microbial respiration activity was measured using gas chromatography. Plate counting method was used to determine the culturable cell counts of aerobic heterotrophic bacteria (AHB). Catalase (CAT) and dehydrogenase (DH) activities were assayed in accordance with a titrimetric and a colorimetric method, respectively.

16S rDNA amplicon sequencing was carried out in order to reveal the effect of various bioremediation approaches on the microbial community of the ULO-polluted soils. To this end, the total microbial DNA was isolated from each mesocosm using QIAGEN DNeasy® PowerSoil® Kit (QIAGEN, Hilden, Germany). Following the amplification of the V3–V4 regions of the 16S rRNA genes, their sequencing was carried out on the Illumina MiSeq platform (Illumina, San Diego, USA). Data extracted from sequencing were then analyzed with Qiime 2 (Quantitative Insights into Microbial Ecology 2). Qiime 2 was also applied to calculate microbial diversity within a community (alpha diversity) and perform principal coordinate analysis (PCoA) to visualize differences in microbial diversity between samples (beta diversity).

Phytotoxicology tests were conducted at the beginning and end of the 60-day-long bioremediation experiments in order to assess changes in soil conditions following the various treatments. The uncontaminated soil sample was used as a control, and Indian mustard (*Brassica juncea* L. Czern. Var. 'Negro Caballo') was used as a test organism. Relative seed germination and relative root length values of *Brassica* seedlings, grown in the treated soils weighed into Petri dishes, were used to calculate germination indices (IG%) using the following formula: IG%=[(germination %)·(root length %)]/100. The vitality and membrane integrity of the root apical meristem cells of *Brassica* seedlings were determined by means of fluorescence microscopy.

RESULTS

In my research, I constructed soil microcosm experiments in order to model the *ex situ* bioremediation of used lubricant oil (ULO)-polluted soils obtained from a railway marshaling yard. Then, I evaluated the feasibility of EOM from *Micrococcus luteus* for environmental rehabilitation in scaled up systems (soil mesocosms). In the case of all treatments, changes in TPH concentrations, microbial activity, culturable cell counts and microbial community composition were monitored throughout the experiment. Soil rehabilitation experiments were followed by subsequent phytotoxicology tests to assess information about the alterations in fertility.

The main results of my research work are summarized in the following points:

1. Most characteristics (e.g. neutral pH, SOM, carbonates) of the uncontaminated control soil, taken from an area directly adjacent to the chosen ULO-polluted site, were revealed to be favourable for bioremediation. However, its fine clay grains can strongly adsorb water and its salinity can be an inhibiting factor for salt-sensitive species. The C/N ratio the ULO-polluted composite soil was close to an optimal value for biodegradation of lubricant oils, while the available P was really low. Despite the elevated concentration of TPHs, culturable AHB were counted at a similar number both in the contaminated and control soils. Moreover, the FTIR spectrum of spillt ULO showed absorption bands suggesting naturally occuring microbial oil degradation at the site of contamination. Based on the above-mentioned observations, the microbial activity of the studied soil could be accelerated by adequate stimulatory agents (e.g. moisture, NPK, EOM); thus, it could be subjected to bioremediation treatments.
2. Based on the soil microcosm experiments, microbial activity and hydrocarbon biodegradation were enhanced by each treatment (water addition, biostimulation and bioaugmentation) to varying degrees. Biostimulation and bioaugmentation proved to the most effective regarding TPH bioconversion.
3. Regardless of applying a smaller (10^7 CFU g^{-1}) or a larger size (10^9-10^{10} CFU g^{-1}) of inoculum, the cultivable cell counts of AHB significantly decreased with the incubation time in the inoculated soils, possibly due to the transition of introduced strains into a VBNC state induced by environmental stresses (e.g. the declining bioaccessibility of

contaminants, accumulation of toxic intermediates, etc.) or even cell death. TPH bioconversions proved to be affected by the size and type of the inoculum. Applying a smaller inoculum size of *R. qingshengii* KAG C did not improve the biodegradation performance compared to the biostimulation treatment, while the low level of inoculation with *R. erythropolis* PR4 proved to be more effective. By contrast, the usage of a larger size of the *R. qingshengii* KAG C inoculum led to a significantly enhanced ULO biodegradation. Meanwhile, the application of a larger inoculum size did not cause a significantly enhanced performance in ULO bioconversion by *R. erythropolis* PR4 as compared to the use of the smaller size of the inoculum. These findings substantiate that, although the success of bioaugmentation strongly depends on the type and amount of the bacterial strain applied; increasing the number of degrader cells does not necessarily have a positive correlation with increased soil decontamination. Therefore, the application of a smaller inoculum size for bioaugmentation can be more economically justified for the rehabilitation of ULO-polluted soils.

4. Scaling up the bioremediation did not have a remarkable effect on the efficiency of ULO biodegradation, so that, the success of a field-scale remediation can be predicted using the results obtained from the micro- and mesocosm experiments.
5. Rpf protein, produced by *M. luteus* cells growing on my modified LMM, showed muralytic activity. Thus, as a result of replacing several expensive components of LMM, EOM could be used effectively and more economically even in (semi-) field conditions. ULO bioconversion, microbial activity, and culturable cell counts were significantly higher in EOM-treated soils than in the corresponding control soils. Thus, EOM-addition not only stimulated the TPH biodegradation of the indigenous microbiota, but also enhanced biodegradation performance of a bacterial inoculum, comprising previously neither resuscitated nor EOM-treated strains (*R. qingshengii* KAG C and *R. erytropolis* PR4).
6. The supplementation of EOM to the ULO-polluted soils induced the proliferation of unique EOM-responsive hydrocarbonoclastic bacterial genera such as *Pseudomonas*, *Comammonas*, *Stenotrophomonas*, and *Gordonia*, that presumably contributed to the enhanced TPH biodegradation of EOM-treated mesocosms with increased activity.
7. Soil phytotoxicity experiments, performed following bioremediation, demonstrated that, although the germination rate of Indian mustard was inhibited (presumably due to the accumulation of intermediates and by-products from ULO biodegradation), the germinated seedlings became more viable and vital when grown in the remediated soils.

The seemingly contradictory results imply that ecotoxicological responses induced in plants are much more complicated than being characterized by simply the inhibition of germination or root development. Our findings further corroborate that a reduction in TPH and decreased soil toxicity do not necessarily have a direct correlation.

The results of my research demonstrate that the environmental application of EOM can be of paramount importance against the damages caused by contaminants, such as (used) lubricant oils, which are complex in composition and difficult to neutralize with currently available methods.

LIST OF PUBLICATIONS

MTMT identification number: 10053060

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The thesis is based on the following scientific papers

- **Attila Bodor**, Naila Bounedjoum, Gábor Feigl, Ágnes Duzs, Krisztián Laczi, Árpád Szilágyi, Gábor Rákhely, Katalin Perei (2021) Exploitation of extracellular organic matter from *Micrococcus luteus* to enhance *ex situ* bioremediation of soils polluted with used lubricants.
  Journal of Hazardous Materials, 417:125996. **IF: 10.588**

- **Attila Bodor**, Péter Petrovszki, Ágnes Erdeiné Kis, György Erik Vincze, Krisztián Laczi, Naila Bounedjoum, Árpád Szilágyi, Balázs Szalontai, Gábor Feigl, Kornél L. Kovács, Gábor Rákhely, Katalin Perei (2020) Intensification of *ex situ* bioremediation of soils polluted with used lubricant oils: A comparison of biostimulation and bioaugmentation with a special focus on the type and size of the inoculum.
  International Journal of Environmental Research and Public Health, 17(11), 4106. **IF: 3.39**

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  Zöld Újság XI. évfolyam 3. szám (2013)

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- **Attila Bodor**, György Erik Vincze, Péter Petrovszki, Gábor Feigl, Naila Bounedjoum, Krisztián Laczi, Árpád Szilágyi, Gábor Rákhely, Katalin Perei (2020) Extracellular organic matter from *Micrococcus luteus* enhances the bioconversion of used lubricants in polluted soil.

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- György Erik Vincze, **Attila Bodor**, Péter Petrovszki, Tibor Sipos, Naila Bounedjoum, Krisztián Laczi, Balázs Szalontai, Katalin Perei, Gábor Rákhely (2019) Quantitative and qualitative analysis of soil contaminant lubricating oils.
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- Tibor Sipos, **Attila Bodor**, György Erik Vincze, Gábor Feigl, Naila Bounedjoum, Gábor Rákhely, Katalin Perei (2019) Phytotoxicity of remediated soils previously contaminated with ULOs.
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- **Attila Bodor**, Tibor Sipos, György Erik Vincze, Péter Petrovszki, Gábor Feigl, Naila Bounedjoum, Krisztián Laczi, Árpád Szilágyi, Gábor Rákhely, Katalin Perei (2018) Alterations in soil fertility after used lubricating oil bioremediation.
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