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BIOSORPTION AND BIOACCUMULATION OF HEAVY METALS

THE USE OF MICROALGAE AND HIGHER AQUATIC PLANTS IN THE ACCUMULATION OF CHROMIUM

Ernazarova G.I.[1], Dzhokebaeva S.A.[1], Orazova S.B.[2], Karpenyuk T.A.[1], Goncharova A.V.[1], Tsurkan Y.S.[2], Kalbaeva A.M.[2], Beisembaeva R.U.[1]
tatyana.karpenyuk@mail.ru
[1]Scientific Research Institute of Biology and Biotechnology Problems, Laboratory of Biochemistry,
[2]Al-Farabi Kazakh National University, Kazakhstan, Almaty.

ABSTRACT

In model experiments the high ability of microalgae (*Anabaena flos-aquae, Anabaenopsis arnoldii*, *Calothrix pariethina, Nostoc linckia*) and higher aquatic plants (*Sparganium stoloniferum Buch-Juncus effusus, How*) to extract the chrome of the aquatic environment in concentrations ranging from 0,05 to 0,2 mg/ml was revealed. Study of interspecific relationships of these cultures shows the largest uptake of chromium ions in cultures with the mutualistic nature of the interaction. Characteristics features of protein contents change in the cells and culture fluids, pigments in microalgae, and the changes of enzymes activity, carbon assimilation, pigments content in higher water plants against a backdrop of increasing concentrations of chromium in medium culture were specified. Using *S. stoloniferum Buch-How* suspension cell culture, it was found that chromium increses cell division, the activity of enzymes, the metabolism of phenolic compounds and nitrogen assimilation. It was shown that during joint cultivation of microalgae and higher aquatic plants extraction of chromium ions from water reaches 95%.

KEY WORDS: microalgae, higher aquatic plants, chromium, accumulation, pigment production

1. INTRODUCTION

Changes in biosphere under the influence of anthropogenic factors become extremely dangerous. Some of the common and very toxic environmental pollutants include heavy metals, for example, chromium, which is widely used in various industrial processes. Despite the treatment activities, the content of compounds in waste water remains quite high. Accumulation of chromium in plant tissues and organs can result to food and cause serious consequences for human health. Migrating on food chain and accumulate in certain levels, chromium and their derivatives are able to dramatically change the metabolic processes in living organisms, causing mutation, reproduction (Rangsayatorn, 2002; Aidor, 1995). So the search of bio-objects capable of sorption and/or accumulation of heavy metals, determine their physiological and biochemical characteristics, their resistance to pollutants is actual problem.

2. MATERIALS AND METHODS

The object of study was algological and axenic culture of microalgae - *Anabaena flos-aquae, Anabaenopsis arnoldii, Calothrix pariethina, Nostoc linckia* and higher aquatic plants *Typha latifolia, Sparganium stoloniferum Buch-Haw, Juncus effusus, Lemna minor*. Microalgae as monocultures and mixtures grew on Fitzgerald medium at 24-hour coverage, 20 days. Potassium bihromat added breeding at concentrations of 0,03-0,6 mM per chrome. Growth rate, calculated as the ratio of dry mass of algae at the beginning and end of the experience, the viability of cultures defined using tripheniltetrazolium chloride (Wong ,1999). Concentration of chrome in medium and biomass of microalgae and higher aquatic plants determined by atomic absorption spectrophotometer AAS-IN, company Carl Zeiss by using flaming atomization. The protein content in cells and culture liquid determined by method (Carpenter, 1992), chlorophyll in microalgae - standard methodology, pigments of higher aquatic plants - double-beam spectrophotometric method (Wellburn, 1994). Cell suspension culture *S. stoloniferum* Buch-Haws received from callus mass, induced from the bar stems, planted on a modified Murasige-Skog medium. Data processed statistically. Figures are arithmetic value of three-five experiments and their standard deviations.

3. RESULTS AND DISCUSSION

In the early stages of experiment the influence of mixed cultivation conditions on the growth of microalgae was studied. To do this, comparing growth monocultures (Figure 1, 1. *A. flos-aquae,* 2. *A. arnoldii*, 3. *C. pariethina,* 4. *N. linckia*) and mixed crops (Figure 1, 5. *A. flos-aquae* x *C. pariethina*, 6. *N. linckia* x *C. pariethina*, 7. *A. flos-aquae* x *N. linckia*, 8. *A. flos-aquae* x *A. arnoldii*). In accordance to Figure 1 after 20 days of cultivation of mono-and mixed cultures in the presence of increasing concentrations of potassium bihromat in the medium *A.*

flos-aquae x *N. linckia* and *A. flos-aquae* x *A. arnoldii* growth was higher than in monocultures. Apparently, there was a mutual stimulation of growth by providing partners with biologically active compounds (positive (allelopathy) interaction between them). In mixed cultures *N. linckia* x *C. pariethina* and *A. flos-aquae* x *C. pariethina* value-growth rate was much lower than in monocultures. Probably between data types have antagonistic relationship (negative allelopathy) that led to the death of cells.

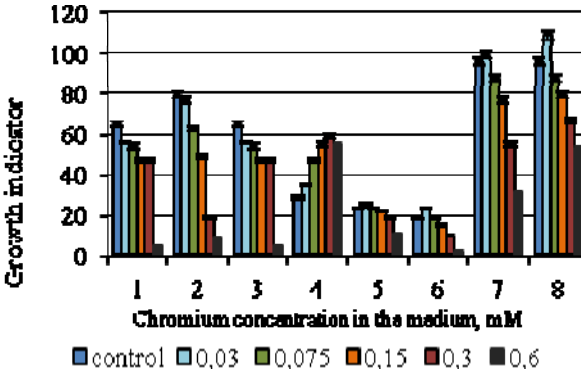

Figure 1**. Growth indicator of mono-and mixed cultures of microalgae in cultivation with various chromium concentration in medium**

The cell viability was defined by vitality coloring using triphenilteterazolium chloride, which characterized the grown and metabolic activity in mono-and mixed cultures. It was found that in mixed cultures in a medium without adding chromium the level of viability of cell cultures partners differed from the monoculture. Thus, in mixed cultures - *A. flos-aquae* x *N. linckia* and *A. flos-aquae* x *A. arnoldi* – the level of viability of both partners cells in two-component mixtures exceed the values of these indicators in monocultures. This confirms that in mixtures *A. flos-aquae* and *N.linckia* and *A. flos-aquae* with *A. arnoldi* between partners developed positive allelopathic relationships. In mixtures with a negative allelopathic interaction (*A. flos-aquae* x *C. pariethina* and *N. linckia* × *C. pariethina*) one partner either die or its viability has been drastically reduced compared to a monoculture. The mixture of *A. flos-aquae* x *C. pariethina,* culture *A. flos-aquae* full has stifled the growth of cell culture of *C. pariethina*. In a mixture of *N. linckia* x *C. pariethina* cell culture *C. pariethina* suppressed growth cell *N. linckia* processes, although the 20 days in their joint cultivation of this culture are not all cells have died. Adding chromium on cultivation medium reflected at the cell viability of all cultures, causing significant declines.

So, when the chromium concentration of 0,6 mM in mixed culture of *A. flos-aquae* x *C. pariethina* did not find a single living cell of *C. pariethina*, while 8% of *A. flos-aquae* cells remained viable in mixed cultures with positive allelopathic, while the chrome was less expressed than in monocultures and mixtures with a negative allelopathic. That is a positive result in increased tolerance allelopathic cells to chromium.

Studying the processes of chromium accumulate by microalgae (Fig. 2) found that biosorbents were the cultures of *A. flos-aquae, N. linckia* and *C. pariethina. A. arnoldii* accumulates chromium in the less amount.

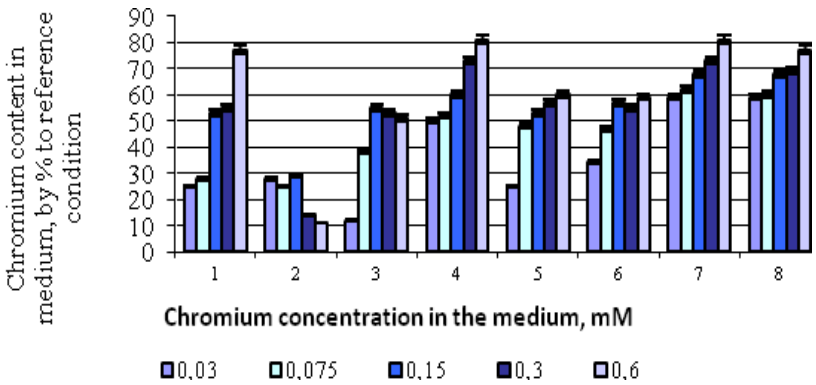

FIGURE 2. CHROMIUM SORPTION BY MICROALGAE

In mixed cultures with the antagonistic nature of the relationships between the intensity of absorption of the chromium was comparable with the intensity of sorption one of the partners of the mixture or was the average of the values retrieved in monocultures of both partners. In mutualistic mixed cultures sorption activity increased in compared to monocultures. In a mixture of *A. flos-aquae* x *N. linckia* and *A. flos aquae* x *A. arnoldii* had linear dependence of chromium extraction from its concentration in the medium. Such intensive sorption capacity is characteristic for only actively metabolize cells.
On figure 3 presented data on the accumulation of chromium by microalgae. A gradual increase of chromium content in cells of monocultures with increased concentrations of this element in the medium was established. The greatest number of intracellular chromium found in the sample with the content of 0,6 mM chromium.

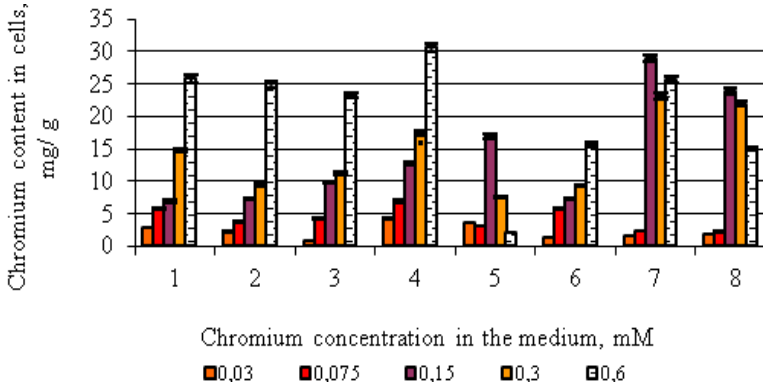

Figure 3. Chromium content in microalgae cells of mono-and mixed cultures

The mutualistic interactions leading to greater growth and cells division of both partners facilitated intensive absorption of chromium from the medium. At 0,05-0,03 mM chromium concentrations, cells absorb small amounts of chromium, but further at increase concentration of chrome in the medium lead to its accumulation in cells. The largest concentration of chrome in the cells *A. flos-aquae* x *N. linckia* discovered under reference concentrations in the medium at 0,15 and 0,6 mM.
A microalgae can perform different functions, for example, the selection of nitrogenous compounds by perform, and protecting cells from toxic action of heavy metals. It was found that increased chromium concentrations of culture medium is increased the excretion of protein from microalgae cell in most cases. In the control options the highest content of investigated monocultures algae protein substances in effluents is defined by *N. Linckia* (8 mg/ml). Increased concentrations of chromium in the medium caused in most cases increased allocation of protein from cells. The sharp increase in the number of proteins in the cultivation of chromium concentration is increased to 0,6 mM were observed for *A. flos-aquae* and *C. pariethina* (7,5-17,5 times to control). However, *N. linckia* and *A. arnoldi* values varied depending on the chromium concentrations in the medium at 0,03 mM protein amount increased, if the concentration of chrome increase up to 0,075 mM protein amount should decrease, and at 0,3 mM again, there was an increase in the protein in liquid culture. Further increase concentration of chromium up to 0,6 mM led to a reduction in extracellular protein from *N. linckia*, but improving – *A. arnoldi*. In mixed cultures with positive allelopathy a significant excess of protein excretion compared to monocultures, if the action is increasing concentrations of chromium. Among the studied compounds far more extracellular protein identified in a mixture of *A. flos - aquae* x *A. arnoldii*. At 0,6 mM chromium concentrations amount of extracellular protein increased at 3,4 times as compared to control.
In according to Figure 4 the content of the intracellular protein in control monocultures varied from 22 to 48% (depending on species). At the increasing chromium concentrations intracellular protein quantity decreased, but not in monoculture. Only *N. linckia* culture at 0,5 mM increased protein content of the cells, if the concentration of chrome 0,6 mM protein content all declined to 10% of monocultures and below.
In mixed cultures with positive allelopathy (*A. flos-aquae* x *A. arnoldii* and *A. flos-aquae* x *N. linckia*) concentration of intracellular protein was 1,5 times higher than in the cells of the same species of monoculture plantations. Under the influence of increasing concentrations of chromium content of protein in these cases has been declining, but did not experience as much as the monocultures. Thus, an increase of chrome concentration led to significant improvements in intra-and extracellular protein content in cells of mixed cultures of microalgae with positive allelopathy. In mixtures of species with negative allelopathy protein in cells and the environment cultivation at high concentrations of chromium are considerably lower.
Toxicity of chromium for microalgae can occur in the form of anion chrome, which is lighter and distributed in a cage and forms a weak complexes with proteins simultaneously inhibiting transport of electrons in electron-transport chain photosystems II (Arjanova, 1978). We have found that chrome has contributed to increase the content of pigments in the cells of most monocultures, especially at relatively low concentrations. Adding

chromium in medium caused by increasing concentrations of chlorophyll content and mixed cultures of most of the content of pigments in mutualistic mixtures. The amount of chlorophyll increased in several times, reaching 19,3 mg/g in the mixed culture of *A. flos-aquae* and *N. linckia* at chromium concentration 0,15 mM and up to16 mg/g-mix *A. flos-aquae* **x** *A. arnoldii* at 0,075 mM of chrome in medium. A further increase of chromium concentration leading to reduced chlorophyll content in cells.
In mutualistic mix culture was also a greater accumulation of phycobillin - additional pigments, performing the function of the light-harvesting complex for cyanoprocaryote. Perhaps, the resistance to the toxic action of mutualistic cultures is connected with high resistance of their pigmented systems.

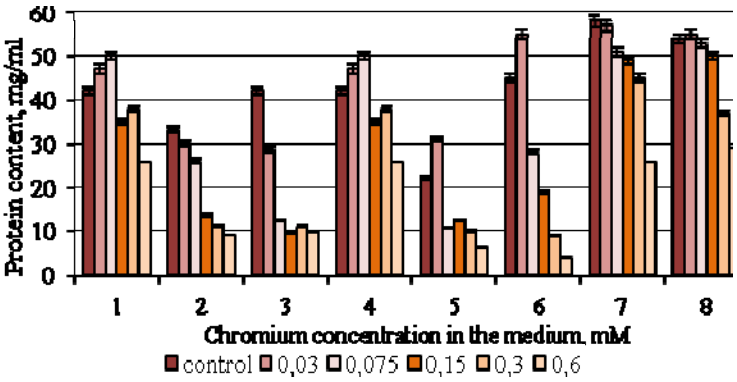

Figure 4**. Protein content in mono-and mixed cultures of microalgae in cultivation with various chromium concentration in medium**

Studying the effects of various concentrations of chromium content of green pigments in the leaves of higher aquatic plants it was found that increased concentrations of chromium in the environment associated with improved amount of chlorophyll *a* and *b* (*Typha latifolia*, *Sparganium stoloniferum Buch-Haw*, *Juncus effusus, Lemna minor*). Higher aquatic plants accumulated a significant amount of chlorophyll *a* and *b*, in the presence of chromium in the medium in concentrations of 0,15-0,6 mM. It was found that increasing concentrations of chromium up to 0,3 mM content of pigments increased in 2 times in comparison with control. Increased concentrations of chromium up to 0,6 mM led to a decline in the number of both pigments and their amounts. However, the content of pigments in the leaves was higher than in the control. So, the sum of both chlorophyll excess 36,5% in compared to control. We can conclude that the species have considerable resistance to high concentrations of chromium in the aquatic environment.
S. stoloniferum culture suspension showed that adding chromium from 0,075mM to 0,6 mM was accompanied by an increase in the number of cells and does not have action to growth processes in cell cultures. At 0,075-0,3 mM chrome there was even accelerating cell proliferation. When added the 0,6 mM of chrome the cell growth was at a level of control. Strengthening growth processes in suspension culture was accompanied by increased activity of polyphenoloxidase, with the maximum enzyme activity characterized cell *S. stoloniferum Buch-Haw*, grown in the presence of 0,6 mM chrome.
Increasing the concentration of chromium affected the activity of nitrate reductase, as indicated by increased nitrate absorption by cells. At 0,6 mM chromium in medium its activity increased in 10 times in compared with control values.
Biosorption activity of higher aquatic plants (*Typha latifolia, Sporganium stoloniferum, Juncus effusus* и *Lemna minor)* and blue-green microalgae (*A. flos-aquae, A. arnoldii, N. linckia, C. pariethina*) in seven days accumulated from 73 to 95% of chromium.

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CONSTRUCTION OF THE PLANT VECTORS WITH HISCUP GENE AND THEIR CLONING INTO FLAX FOR HIGHER HEAVY METAL ACCUMULATION

Jan Fiser[1], Miroslava Vrbova[2], Martina Novakova[1], Martina Mackova[1], Tomas Macek[1]
fiserj@vscht.cz
[1] Institute of Chemical Technology in Prague, Faculty of Food and Biochemical Technology, Department of Biochemistry and Microbiology, Technicka 5, 16628, Prague, CZ
[2] Agritec, Research, Breeding and Services Ltd., Zemedelska 16, 78701, Sumperk, CZ

ABSTRACT

The problems with finding suitable plants for phytoremediation can be solved by preparation of transgenic plants which could express a binding domain for heavy metals. *CUP* gene encodes a yeast protein metallothionein having a high affinity to heavy metals. This gene was fused with histidine tail (*HisCUP*) for another increase of binding capacity. The goal of this work is the preparation of the plant vectors with *HisCUP* gene and their use for the transformation of flax (*Linum usitatissimum* AGT-952). Three vectors with *HisCUP* gene were prepared. First vector was pNOV2819/RbcS/*HisCUP* where *HisCUP* gene was cloned under the control of light-induced promoter of RUBISCO protein. *HisCUP* gene was inserted into second vector pGreen0029/35S/*HisCUP* under the constitutive CaMV 35S promoter and third vector pGreen0029/35S/*HisCUP-GFP* contained the *HisCUP* gene fused with gene for green fluorescent protein. By method of transient expression, the expression of *HisCUP* gene in plant *Nicotiana tabacum* was confirmed at the RNA level for first two vectors and the *HisCUP-GFP* protein was confirmed by fluorescence detection. First two vectors were used for transformation of flax by agrobacterial infection. Regenerating plants with *HisCUP* gene were only obtained using vector pGreen0029/35S/*HisCUP*.

KEY WORDS: phytoremediation, metallothionein, hiscup gene, transgenic plants

1. INTRODUCTION

Natural geological processes and many anthropogenic activities (Dembitsky, 2003) represent dangerous sources of heavy metals contamination. Industrial effluents, atmospheric emissions, fertilizers applications and mining wastes are main sources of heavy metals in the environment (Mudgal et al., 2010).
The conventional remediation methods include soil excavation and washing or immobilization or extraction by physicochemical techniques. These methods are expensive and negative qualitative changes arise after using them. These problems can be solved using *in situ* technology called phytoremediation. Phytoremediation uses plants for accumulation, complexation, volatilization and degradation or transformation of organic and inorganic pollutants (Cherian and Oliveira, 2005). But on the other hand it is long term process (Kumar et al., 1995) and it is difficult to find convenient plant species for this method. For effective phytoremediation, plants have to accumulate high concentration of heavy metals into roots, to transport them into aboveground parts and to produce sufficient amount of biomass (Ghosh and Singh, 2005).
Plants are able to accumulate metals essential for their growth and development. This capability allows plants to accumulate also metals without known biological function such heavy metals (Djingova and Kuleff, 2000). However these metals and essential metals in excess are toxic for plants (Williams et al., 2000). The ability to accumulate heavy metals is in range 0.1 – 100 mg/kg for most plants. Plants with higher storage capacity are so-called hyperaccumulators. Hyperaccumulators have effective transport and detoxification mechanisms which allow accumulate metal in tissue in concentration 50 to 500 times higher compare to the soil concentration (Clemens et al., 2002). Metals are usually stored in vacuoles after binding to compounds decreasing toxicity of metals and enabling their transport (Cherian and Oliveira, 2005).
The disadvantages of hyperaccumulators are mainly slow growth and low biomass production and the ability to accumulate only one specific metal. Hyperaccumulators are also very often bound to the specific habitat (Clemens et al., 2002; Macek et al., 2008). Therefore hyperaccumulators are not suitable plants for phytoremediation. But fast-growing plants with high biomass production, with widely branched and deep root system and easy-harvesting aboveground parts can be modified for phytoremediation by gene manipulation.
One of the most important mechanisms of metals detoxification in plants is their chelatation by ligands rich in thiol group such as glutathion, phytochelatins and metallothioneins. Three traits allow metallothioneins the effective metals detoxification: a) metallothioneins bind metals in polymetalic clusters, b) metallothioneins undergoes facile metal exchange reactions, Zn(II) ions bound by metallothionens are readily displaced by Cd(II), resulting in sequestration of potentially toxic Cd(II) ions, c) the expression of metallothioneins gene in eukaryotes is regulated by metals presence (Jensen et al., 1996). The overexpression of recombinant

metallothioneins increase the resistance to metals, promote heavy metals accumulation and can be considered as promising approach to create plants suitable for phytoremediation.
CUP1 gene is included in heavy metals detoxification in yeast *Saccharomyces cerevisiae* (Fogel and Welch, 1982). This gene was fused with peptide containing histidine tail (6 × His) for additional binding capacity. The best lines of transgenic tobacco containing *HisCUP* gene accumulated about 90 % more cadmium in aboveground parts compare to the wild type plants (Macek et al., 2002).

2. METHODOLOGY AND RESULTS

2.1 THE PREPARATION OF PLANTS VECTORS

Plasmids pNOV2819 (Syngenta) and pGreen0029 were chosen as the plant vectors. Plasmid pNOV2819 has as a selection marker a gene for phosphomannose isomerase. Mannose-6-phosphate, formed in plants on medium with mannose, is toxic for plants because of depletion of inorganic phosphate. Phosphomannose isomerase can transform mannose-6-phosphate to fructose-6-phosphate which is further metabolised in glycolysis (Ondřej et al., 1999). The *HisCUP* gene was amplified from plasmid pTrc*HisCUP* (Truksa et al., 1996) and was inserted into pNOV2819 plant vector under the light-regulated promoter (P-RbcS) used from plasmid ImpactVector. The second plant vector, plasmid pGreen0029, has as selection marker the gene for resistance to kanamycin. Here the *HisCUP* gene was cloned under the control of constitutive promoter of cauliflower mosaic virus (CaMV 35S) used from pSK/35S plasmid. Prepared constructs pNOV2819/RbcS/*HisCUP* and pGreen0029/35S/*HisCUP* were transformed into flax plants by bacterium *Agrobacterium tumefaciens* C58-C1 (pCH32). Third vector based on plasmid pGreen0029 was engineered for easy detection of *HisCUP* gene product by means of fusing this gene with the gene for green fluorescent protein (GFP). *HisCUP* gene was prepared by PCR where plasmid pTrc*HisCUP* was employed as template DNA. *GFP* gene was gained by amplification from pEGFP-C1 plasmid (Clontech). These genes were cloned under the control of 35S promoter into plasmid pGreen0029 (pGreen0029/35S/*HisCUP-GFP*).

2.2 THE VERIFICATION OF *HISCUP* GENE EXPRESSION IN PLANTS

Permanent transformation is long-term process because of long regeneration and selection of transgenic plants. In order to verify gene expression in plants the transient expression method is more suitable. In this method, the suspension of bacteria *A. tumefaciens*, containing vector pNOV2819/RbcS/*HisCUP* or pGreen0029/35S/*HisCUP*, was pressed into leaves of tobacco (*Nicotiana tabacum* var. Wisconsin 38). These were harvested three to five days after the procedure. The total RNA was isolated from plant tissue and rewrote into complementary cDNA by reverse transcriptase and oligodT primer. cDNA was used as template DNA in PCR reaction with specific primers amplifying the *HisCUP* gene. Expression of *HisCUP* gene in tobacco was confirmed for both constructs mentioned above.

2.3 THE CONFIRMATION OF *HISCUP* GENE TRANSLATION

Gold particles were coated by plasmid pGreen0029/35S/*HisCUP-GFP* and shot into tobacco leaves. After one day cultivation, the GFP fluorescence in plant cells was detected. This result confirmed production of fused protein (metallothionein-GFP).

2.4 ISOLATION AND DETECTION OF METALLOTHIONEIN

A. tumefaciens with pNOV2819/RbcS/*HisCUP* or pGreen0029/35S/*HisCUP* was used for metallothionein expression in tobacco leaves by the method of transient expression. Metallothionein was isolated from leaves by affinity chromatography using the specific bound between histidine tail and Ni(II) ions of Ni-NTA. Obtained fractions were separated on Tricine SDS page and afterwards the proteins were transferred on nitrocellulose membrane. Metallothionein was detected imunochemically using commercial mouse antibody specific to histidine tail. The presence of metallothionein in plant tissue wasn't confirmed for prepared vectors above.

2.4 THE TRANSFORMATION OF FLAX (*LINUM USITATISSIMUM* AGT 952)

Bacteria *A. tumefaciens* with plant construct pNOV2819/RbcS/*HisCUP* or pGreen0029/35S/*HisCUP* were used for transformation of flax *Linum usitatissimum* AGT-952. Used flax genotype was prepared by Agritec Ltd as also the transformation procedure protocol. This plant is technical, easily growing crop, climate in the Czech Republic is suitable and therefore it should be possible to use it in the field experiments.

The selection of transgenic plants was performed on media with mannose (in the case of pNOV2819/RbcS/*HisCUP*) or with kanamycin (in the case of pGreen0029/35S/*HisCUP*).Transformed plants created regenerating plants during few weeks. When regenerators achieved a length about 1 cm, they were cut off and transferred on medium where transgenic plants should create the roots. This was achieved only for transformation by *A. tumefaciens* with pGreen0029/35S/*HisCUP*. Genomic DNA was isolated from selected plants and the presence of *HisCUP* gene was successfully confirmed using PCR with primers specific to *HisCUP* gene.

3. Conclusion

The plants with sufficiently rapid growth and biomass production are usually not efficient metal accumulators and vice versa the hyperaccumulators have often unsuitable agrotechnical properties. The growth and biomass production are encoded by many genes but hyperaccumulation is controlled by few key genes (Zhang et al., 2006). Therefore the aim is to prepare transgenic plants with genes providing higher capability of heavy metals accumulation.
Two vectors with *HisCUP* gene encoding for metallothionein were prepared for increasing of heavy metal accumulation in flax (*Linum usitatissimum* AGT-952) and one for confirmation of metallothionein production in plant tissues. Transformation vectors were based on plasmids pNOV2819 (Syngenta) and pGreen0029 which enable two different approaches of transgenic plants selection.
The expression of *HisCUP* gene in plant tobacco was confirmed at RNA level for both, pNOV2819/RbcS/*HisCUP* and pGreen0029/35S/*HisCUP*. Green fluorescence was observed in tobacco leaves cells after bombardment by gold particles coated by plasmid pGreen0029/35S/*HisCUP-GFP*. This suggests that protein is formed in plant tissues.
The plants *Linum usitatissimum* AGT-952, prepared and supplied by Agritec Ltd, were chosen for permanent transformation. Rooting regenerating plants were obtained only in the case of transformation with vector pGreen0029/35S/*HisCUP* and the presence of *HisCUP* transgene in plant genome was confirmed by PCR using primers specific to *HisCUP* gene. Future experiments will further characterize the transgenic plants.

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INFLUENCE OF THE PRESENCE OF COMPLEXING AGENTS ON THE COPPER(II) BIOSORPTION ONTO *POSIDONIA OCEANICA* FROM METAL PLATING WASTEWATER

Marta Izquierdo, Carmen Gabaldón and Paula Marzal
marta.izquierdo-sanchis@uv.es
Departament d'Enginyeria Química, Escola Tècnica Superior d'Enginyeria, Universtitat de Valencia, Avgda. Universitat s/n, 46100 Burjassot, Valencia, Spain

ABSTRACT

Complexing agents (CAs) are widely used in process solutions of the metal plating facilities and wastewater of these facilities usually contains heavy metals and CAs. To investigate the influence of the CAs on the Cu(II) removal onto *Posidonia oceanica*, fixed-bed column experiments were carried out with EDTA and citrate. Experiments were performed at Cu(II) concentrations of 5 and 20 mg Cu(II) dm^{-3} and Cu(II):CA mass ratios of 1:0, 1:0.5: 1:1 and 1:2. Results showed that the presence of CAs strongly modified the development of the copper(II) concentration in the effluent of the operation and thus the Cu(II) removal by *Posidonia oceanica*. In the case of EDTA, metal leak was detected in the column effluent from the beginning of the operation associated to the Cu(II)-EDTA complexes that were not sorbed. In the case of citrate, even with more complexed Cu(II) in the feed solution than in the EDTA experiments, the interference was less important and early metal leaks were not detected. During the operation, however, an effluent constant Cu(II) concentration period was detected, probably associated with Cu(II)-citrate complexes. Total Cu(II) retained was not significantly affected by the presence of CAs. Results highlight the importance of the selection of the CA in the metal plating processes to facilitate wastewater treatment by biosorption.

KEY WORDS: biosorption, chelating agents, citrate, EDTA, heavy metals

1. INTRODUCTION

In facilities of the metal plating sector, organic ligands are found in the composition of different process solutions. EDTA and citric acid are found among the most common CAs of such facilities. EDTA is used in process solutions of autocatalytic copper(II) plating, a key process in printed circuit boards manufacturing as well as for the metallisation of plastics, to extend the process solution lifetime. Citric acid is a common electrolyte in the electropolishing processes. Substituting EDTA by weak and biodegradable CAs is a Best Available Technique (BAT) recommended for this sector when possible (European Comission, 2006); however, high specification work may require the use of EDTA. As a consequence, EDTA and citrate are found in the wastewater of such facilities along with heavy metals.

Chemical speciation of heavy metals in solution is hardly affected by the presence of CAs, and physico-chemical wastewater treatments for heavy metal removal are interfered due to the formation of soluble complexes. Many factors seem to be critical in determining whether heavy metal biosorption onto sorbent materials is enhanced or inhibited by the presence of CAs, such as the nature of the sorbents, the initial metal/CA ratio, the type of CA and metal, the solution pH or the ionic strength. Thus, the study of the effect of the presence of CAs on the biosorption of metals deserves close attention. Literature data show different metal sorption behaviours when CAs are present in the solution. Chu and Hashim (2000) observed an increase in the removal efficiency of copper(II) for two activated carbons when the copper(II):EDTA molar ratio was increased from 1:0 to 1:1 at pH 3; in contrast, a substantial reduction in the copper(II) removal efficiency of the carbons was observed at pH 6 in the presence of any EDTA concentration. In a study of copper(II)-EDTA sorption onto chitosan with variable copper(II):EDTA molar ratios and pHs, Gyliene et al. (2006) concluded that both copper(II) and EDTA from their complex solution were sorbed by chitosan in weakly acidic (pH 3–5) and strongly alkaline solutions (pH>12); and that the increase in the concentration of EDTA diminishes copper(II) sorption. Janos et al. (2006) concluded that the metal uptake by iron humate was affected by the presence of EDTA, salicylate or citrate in solution, but this effect depended strongly on the pH and type of CA. Whereas EDTA suppressed the metal sorption across of the pH range tested (1–6.5), the presence of salicylate increased metal sorption to a certain extent. The presence of citrate only decreased metal sorption at pH values above 4. In our previous works (Gabaldón et al., 2007, 2008), several biosorbents and activated carbons were evaluated for the removal of copper(II) and nickel(II) from wastewater containing EDTA. Biosorbents showed higher removal efficiency of heavy metals than activated carbons, but the presence of EDTA negatively affected the metal uptake of biosorbents, while it increased the removal efficiency of activated carbons. In a study of copper(II) biosorption onto palm shell activated carbon in the presence boric and malonic acids (Issabayeva et al., 2010), a lower

copper(II) biosorption capacity was observed in solutions containing malonic acid that was attributed to the metal complex formation.
The objectives of this work are to evaluate the use of *Posidonia oceanica* as biosorbent for the removal of copper(II) from water containing EDTA and citrate, selected as common CAs used in the metal plating sector. The influence of the presence of these CAs on the biosorption of copper(II) onto *Posidonia oceanica* was investigated from a practical point of view in fixed-bed column experiments, which is the technology used in the industrial operation.

2. Materials and Methods

2.1 Biosorbent

The marine phanerogam *Posidonia oceanica* used in this study is a waste derived from local beach cleaning practices and it is collected by the Denia Compost SA company, whose main activity is managing a composting plant located in Denia (Spain). The raw material was rinsed with tap water to remove impurities until constant conductivity was achieved, and then it was rinsed with distilled water. The biosorbent was then crushed and sieved to 0.5-2 mm particle size, oven dried and stored in a desiccator.

2.2 Fixed-bed column experiments

Column experiments were carried out in Pyrex glass columns of 35.0 cm length and 1.0 cm inner diameter. The biosorbent was packed as an aqueous suspension to avoid air retention in the sorbent bed. The feed solution was pumped with a peristaltic pump in the up-flow mode at ambient temperature. Column effluent samples were collected regularly with an automatic fraction collector. Samples were acidified with concentrated HNO_3 and stored for analysis of copper(II) concentration by FAAS using a Thermo S4 Atomic Absorption Spectrophotometer (Thermo Fisher Scientifics, USA).
Eight column experiments were carried out in the absence and in the presence of citrate and EDTA: five experiments with influent copper(II) concentrations of 5 mg Cu(II) dm^{-3} and copper(II):CA ratios of 1:0, 1:1 and 1:2, and two experiments with 20 mg Cu(II) dm^{-3}, and copper(II):CA ratios of 1:0 and 1:0.5. Sodium chloride 0.01 mol dm^{-3} (NaCl) was used as background electrolyte in order to keep constant the ionic strength. Around one gram of biosorbent was packed in 10 cm bed length. The rest of the column was filled with glass beds. pH in the inlet wastewater was fixed at 6.0. The flow rate was adjusted to 40 cm^3 h^{-1} using a peristaltic pump.

3. Results and discussion

Breakthrough curves (normalized effluent concentration (C/C_0) versus the number of pore volumes of treated water) of the experiments performed with 5 mg Cu(II)dm^{-3} are shown in Figure 1a and 1b for Cu(II):CA ratio of 1:1 and 1:2, respectively, along with the results obtained in the absence of CA (1:0).

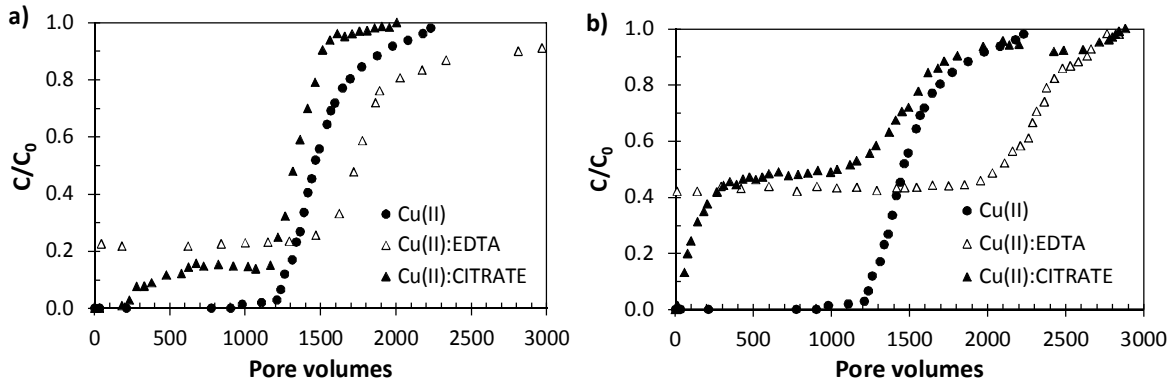

Figure 1. Breakthrough curves of Cu(II) biosorption onto *Posidonia oceanica* with Cu(II) conc. = 5 mg dm^{-3}. a) Cu(II):CA = 1:1 mg mg^{-1}; b) Cu:CA = 1:2 mg mg^{-1} (CA: Complexing Agent).

In the absence of CAs, the breakthrough curve showed a typical S-shape, with an initial period in which copper(II) was not detected in the effluent. The presence of EDTA and citrate greatly interfered the performance of copper(II) removal, though effect of each CA was different.
In the presence of EDTA, a constant copper(II) leak was detected in the effluent at the beginning of each column run, describing a horizontal line in the breakthrough curves at a constant value of C/C_0. Copper(II) concentration

in these initial leaks increased on increasing the EDTA dose, suggesting that the non-sorbed fraction could be related to the copper(II) complexes in the feed solutions. Chemical speciation in the feed solutions of the columns was simulated with Visual MINTEQ. For all the feed solutions, Cu^{2+} was most abundant copper(II) specie. In the presence of EDTA, two copper(II)-EDTA complexes were also present, $CuEDTA^{2-}$ and $CuHEDTA^{-}$, though $CuHEDTA^{-}$ percentage was negligible. For concentration of 5 mg Cu(II) dm^{-3} and copper(II):EDTA mass ratios of 1:1 and 1:2, the percentages of copper(II)-EDTA complexes in the feed solutions of the columns were of 22.05 and 44.10% of the total copper(II) concentration, respectively, which corresponded to concentrations of 1.17 and 2.32 mg Cu(II) dm^{-3}. These values matched up the copper(II) concentration associated to the initial leaks, which were of 1.17 and 2.27 mg dm^{-3}. These results , indicate that the non-sorbed fraction detected at the beginning of the operation corresponded to the copper(II)-EDTA complexes in the feed solutions, so *Posidonia oceanica* did not retained copper(II)-EDTA complexes. Such behaviour was also observed for the copper(II) and nickel(II) biosorption onto grape stalk (Escudero et al., 2008) and for copper(II) biosorption onto spent-grain (Lu and Gibb, 2008). In these works, the authors concluded from column experiments with different metal:EDTA ratios that these biosorbents did not retain the metal-complexed species.

In the presence of high percentages of copper(II)-citrate complexes in the feed, early metal leaks were not detected, indicating than for high concentration of available active sites for copper(II) retention, i.e. at the beginning of the operation, the copper(II) retention is not inhibited by the presence citrate. An important diminution of the breakthrough time (C/C_0 = 0.05) with respect to the results in the absence of citrate was observed. During the operation, however, an effluent constant Cu(II) concentration period was detected at C/C_0 of 0.148 and 0.475 (0.75 and 2.41 mg Cu(II) dm^{-3}) for copper(II):citrate of 1:1 and 1:2, respectively, probably associated with copper(II)-citrate since concentrations increases with the citrate dose. $CuCitrate^{-}$ was revealed as the major complexed specie by Visual MINTEQ simulations, with percentages of 33.44 and 66.56% of the total copper(II) for copper(II):citrate ratios of 1:1 and 1:2 respectively, which corresponded to 1.69 and 3.38 mg Cu(II) dm^{-3}. Concentration in metal leaks are lower than the $CuCitrate^{-}$ concentration in the feed , which corroborates than the interference of citrate in the copper(II) biosorption was weaker than the one of EDTA. This could suggest that the adsorption reaction between copper(II) and *Posidonia oceanica* active sites prevent the formation of $CuCitrate^{-}$ and/or that the anionic specie was retained to some extent. Binding constant between copper(II) and *Posidonia oc*eanica in column operation was obtained from the Langmuir isotherm and resulted in a log(b) = 5.25 [Cu + *Posidonia* ↔ Cu-*Posidonia*] (Izquierdo et al., 2010). Citrate is a weaker chelating agent than EDTA and the formation constant of $CuCitrate^{-}$ is of log(K) = 7.57 [Cu^{2+} + $Citrate^{3-}$ ↔ $CuCitrate^{-}$]. Thus, *Posidonia oceanica* and the ligand may compete for the interaction with copper(II) ions when the concentration of available active sites in the biosorbent surface is high enough to prevent the $CuCitrate^{-}$ formation. Some retention of $CuCitrate^{-}$ may be also be overlapped, though more experiments in batch mode should be performed to elucidate the interaction mechanism in this complex system. Bulgariu et al. (2012) reported a decreased in the efficiency of the Pb(II) sorption onto peat in the presence of citrate in batch mode. A significant suppression of the copper(II) biosorption onto sunflower stem in the presence of citrate and other anionic species was observed by Malik et al. (2005)

Results with 20 mg Cu(II) dm^{-3} confirmed the observations with lower metal concentration (Figure 2). Concentration of copper(II)-EDTA complexes in the feed (2.34 mg Cu(II) dm^{-3}) perfectly matched the measured copper(II) concentration in the initial metal leak (2.39 mg Cu(II) dm^{-3}). In the presence of citrate with a percentage of copper(II)-citrate complexes of 16.78% (3.34 mg Cu(II) dm^{-3}) a constant metal concentration period was not achieved, though a breakthrough curve with two different slopes was observed. This is due to the greater metal concentration provokes the faster saturation of the biosorbent so a lower breakthrough time.

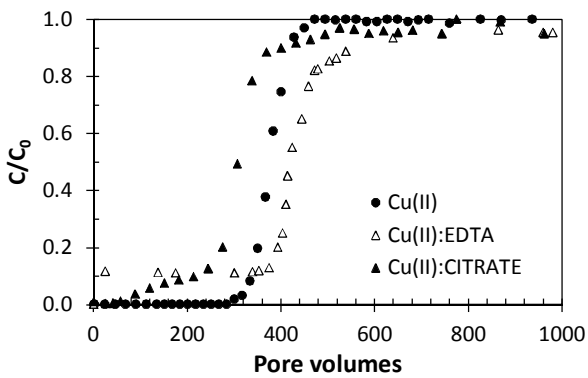

Figure 2. Breakthrough curves of Cu(II) biosorption onto *Posidonia oceanica* with Cu(II) conc. = 20 mg dm^{-3} and Cu:CA = 1:0.5 mg mg^{-1} (CA: Complexing Agent).

From the total mass balance of the system, copper(II) uptake at the saturation point was calculated and results showed that metal uptake varied randomly between 47 and 54 mg g^{-1} for 5 mg Cu(II) dm^{-3}, and between 55 and 58 for 20 mg Cu(II) dm^{-3}, with an average value of 53 mg g^{-1}; thus, the presence of CAs in the wastewater did not greatly affect the total metal sorption capacity of *Posidonia oceanica*.

4. CONCLUSIONS

The main conclusions of the work are:

- The presence of EDTA and citrate adversely affected the copper(II) biosorption onto *Posidonia oceanica* in fixed-bed column operation.
- In the presence of any dose of EDTA, copper(II)-EDTA complexes leaks were detected in the effluent because copper(II)-EDTA complexes were not retained by *Posidonia oceanica*, and its use as a metal wastewater treatment would be strongly limited.
- Interferences of citrate were weaker than of EDTA and for citrate doses lower and equal to 5 mg dm^{-3}, *Posidonia oceanica* may be successfully used as copper(II) biosorbent. For higher citrate concentration, its use should be carefully evaluated.
- Results highlight the importance of the selection of the CA in the metal plating processes to facilitate wastewater treatment by biosorption.

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NEW METAL-TOLERANT BACILLI STRAINS AS A PROSPECTIVE TOOL FOR BIOREMEDIATION

Armine Margaryan
e-mail: arminemargaryan@ysu.am
Yerevan State University, Faculty of Biology, Department of Microbiology, Plants and Microbes Biotechnology, Alex Manoogian 1 str., 0025 Yerevan, Armenia

ABSTRACT

A total of 25 mesophilic and thermophile metal-tolerant bacilli were isolated from soil and silt samples and identified up to species. The morphological, physiological and biochemical properties of metal-tolerant strains were described, as well as 16S rDNA sequence analyses were carried out. Tolerance of Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mo^{2+} and CrO_4^{2-} by isolates was studied and found that all isolates were highly resistant to Mo^{2+}, Cu^{2+} and Ni^{2+}, and sensitive to Cd^{2+} and Zn^{2+}. The ability of the isolates to accumulate mentioned metal ions was studied as well. Strains *B. licheniformis* AD4 and *B. licheniformis* AL1 were selected as highest copper accumulators (70% and 80% from medium respectively).

KEY WORDS: heavy metals, metal-tolerant bacilli, resistance, bioremediation, accumulation of metals.

1. INTRODUCTION

Heavy metal contamination is one of the most important environmental concerns (Xuehui Xie at al., 2010). Some industrial processes such as smelting, mining, metal forging, manufacturing, electroplating etc., result in the release of heavy metals in the environment (Sadin at al., 2011). While some heavy metals are required in trace amounts as nutrients, they become strongly inhibitory for microorganisms at relatively low concentrations (Nies, 1999). Under metal-stress conditions many microorganisms have evolved a variety of adaptation mechanisms, i.e., metal absorption, mineralization, uptake and accumulation, extracellular precipitation, enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell (Nies, 1999; Nies, 2003; Silver at al., 1996). Developed heavy metal resistance mechanisms are mostly plasmid-mediated, but, frequently, related systems localize to chromosomal genes (Abdelatey at al., 2011; Endo at al., 2002; Mark at al., 2000; Nies, 1992; Sadin at al., 2011).

Metal-tolerant microbes are found in all bacterial groups studied, but mostly appear among aerobic and facultative anaerobic chemo-heterotrophic microorganisms of the *Bacillus, Staphylococcus, Escherichia* and *Pseudomonas* genera (Clausen, 2000; Gelmi at al., 1994; Nies, 1999; Rathnayake at al., 2009). It was reported that thermophilic microorganisms in particular, due to their ubiquity, metabolic flexibility and cell wall structure, have the potential to affect the geochemistry of the environment (Cuebas at al., 2011; Hetzer at al., 2006; Sadin at al., 2011; Varnam, 2000).

A good understanding of the heavy metal resistance mechanisms can lead to the development of new approaches for biological environmental clean-up. Heavy metal resistant bacteria could for instance be new resources for the active biosorption of heavy metals and be applied in bioremediation processes (Ahemad at al., 2012; Haq at al., 2000; Lovley at al., 2000; Spain at al., 2003; Tango at al., 2002).

The Republic of Armenia is rich in minerals and ore resources which makes it famous for its mining. Development of mining and smelting industry leads to increasing emissions of large amounts of waste into the environment and this is the primary pollutants of heavy metals resulting in an environmental dispersion of such pollutants.

The aim of this work was isolation and characterization of meso- and thermophile heavy metal resistant bacilli from soil and silt samples with elevated levels of toxic metals collected from different sites on the territory of Armenia (Zangezur Copper and Molybdenum Combine (Kajaran), Zod gold main (Sotk) and Alaverdi Copper Smelter (Alaverdi), Lake Sevan and hot mineral spring Lichk), to select the most resistant strains and characterize for heavy metals accumulation as a potentially useful tool for bioremediation purposes.

2. MATERIALS AND METHODS

2.1 Sampling

Soil and silt samples were collected from five above mentioned locations and were placed into sterile screw cap bottles. Geographical coordinate of studied sites was determined using JPS. Temperature, pH and mineralization

were determined *in situ* using a combined pH/EC/TDS/Temperature electrode (HANNA HI98129/HI98130). The soil and silt samples from different sites were transported immediately after collection to the laboratory for further study. Samples were stored at 4 °C.

2.2 Isolation and enrichment

To isolate aerobic mesophilic and thermophile bacilli 1g of soil/silt samples was suspended in 10 mL of sterile distilled water and vortexed for 1 min. Supernatants were transferred to a glass tube with a screw cup and pasteurized at 80 °C for 10 min in a water bath. Then 0.5 mL aliquots of appropriate dilution were placed on enrichment agar medium in plates and incubated overnight at 37ºC and 55 ºC. Enrichment medium had following composition: yeast extract 0,5 %, peptone 0,5 %, NaCl 0,5 %, glucose 0,1%, agar 2%. The different concentrations (from 1-10 mg/l) of heavy metal salts ($CuSO_4x5H_2O$, $NiCI_2x6H_2O$, $ZnSO_4x7H_2O$, $CdCl_2xH_2O$, $CoCl_2x6H_2O$, K_2CrO_4, and $Na_2MoO_4x2H_2O$) were supplied into the medium after sterilization (autoclaving at 121 ºC for 15 min) and cooling. All colonies obtained on plates were picked and purified by streaking onto same medium at least three times. The subcultures were considered pure after microscopic observation - one type of bacterium per culture.

2.3 Identification of bacilli

2.3.1. Study of Colonial and Cellular Morphology:
Isolated colonies of purified strains grown on solidified agar plates were observed and data was recorded regarding the form (circular, flamentous and irregular); elevation (flat, convex, and umbonate); margin (entire, undulate, erose and filamentous); and optical feature (opaque, translucent,and transparent) of the colonies (Pelczar and Reid, 1958).
Cellular morphology and motility were determined by light microscope (Olimpus CH-2, oil immersion, 100 X). Shape of the cell (bacilli and cocco-bacilli) and arrangement of cells (scattered, bunches and chain) along with the Gram-reaction was observed (Netrusov at al., 2005). For revealing of endospores the cells were stained according to the Peshkov's method (Netrusov at al., 2005).

2.3.2 Physiology and Biochemical Properties:
The temperature range for growth was determined after incubation of isolates at 5 to 75 ºC with 10 ºC intervals. The pH dependence of growth was tested at pH range from 5 to 10. The range of NaCl concentrations for growth was determined by adding from 0 to 10% NaCl to the incubation medium. Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Anaerobic growth of the isolates was tested by growth in solid anaerobic stab cultures covered by paraffin layer. Reduction of nitrate to nitrite, Voges-Proskauer reaction, formation of dihydroxyacetone was determined according to (Gordon et al., 1973). The utilization of citrate and propionate was determined using the Simmon's medium (Netrusov at al., 2005). The casein and starch hydrolyses were tested by streak plate technique (Bergey's Manual of Systematic Bacteriology, 1986; Netrusov at al., 2005). The gelatin liquefaction was determined by inoculating the bacilli into gelatin nutrient medium (gelatin 10g; distilled water 100ml) and incubation at 37ºC for 24 hours (Bergey's Manual of Systematic Bacteriology, 1986; Netrusov at al., 2005). The lipolytic activity was determined by the hydrolysis of Tweens 20 (polyoxyethylene sorbitanmonolaurate), 40 (polyoxyethylene sorbitanmonopalmitate), 60 (polyoxyethylene sorbitanmonostearate), 80 (polyoxyethylene sorbitanmonooleate) (Bergey's Manual of Systematic Bacteriology, 1986; Netrusov at al., 2005). The basal medium with inverted Durham tubes was used to test acid and gas production from D-(+)-glucose, L-(+)-arabinose, D-(+)-xylose and D-(-)-mannitol.

2.3.3 Nucleic acid extraction and polymerase chain reaction (PCR):
Extraction of genomic DNA from bacilli isolates was carried out according to (William S. and Helene Feil, A. Copeland, Bacterial genomic DNA isolation using CTAB). Extracted DNA was used as templates for amplification 16S rRNA gene sequenses by PCR. The "universal" oligonucleotide primers 16SF (5'-GAGTTTGATCCTGGCTCAG-3') and 16SR (5'-GAAAGGAGGTGATCCAGCC-3') were used for amplification of bacterial 16S rRNA genes.
PCR mixtures used for amplification of sequences contained 1 µl DNA, 10 µl 10 iProof Buffer, 1 µl 10 mM dNTP mix, 2,5 µl primer (final concentration 100nM), 0,5 µl iProof *Taq* DNA polymerase, and sterile water to a final volume of 50 µl. PCR amplification was done with the following program: 96ºC for 3 min; 30 cycles of denaturation at 96ºC for 30 s, annealing at 55ºC for 30ºC, and extension at 72ºC for 2,30 min; and a single final extension at 72ºC for 10 min.

2.3.4 Sequencing and phylogenetic analysis:

PCR products were purified with GenEluteTM PCR Clean-up Kit. Sequencing was performed on ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). Homology searches from the nucleic acid sequences were performed using the Blast algorithm (Altschul et al. 1990) at the NCBI (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov). All sequences were compared with other sequences in the GenBank database to identify closest relatives. Phylogenetic tree was made by DNASTAR and ClastalX2 computer programs.

2.4 Determination of minimal inhibition concentration (MIC) of heavy metals

MIC of each metal was determined by plating cultures on solid basal medium with different concentrations of separate metal ions Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mo^{2+} and CrO_4^{2-} (from 1 to 1000 mg/l). Plates were incubated at 37ºC for mesophiles and 55ºC for thermotolerant strains during 24-72 hours (Anyanwu at al., 2011; Affan at al., 2009).

2.5 Heavy metal bioaccumulation by isolates

Bacterial cultures were grown in 25ml of growth medium (containing mix of heavy metals salts in concentration of 2,5 mg/l for each metal) in 250ml Erlenmayer flasks by shaking (150 rpm) at 55 ºC for 24 hours. Bacterial cells were harvested by centrifugation at 6000 rpm for 25 min at 4ºC. Pellet was washed twice with 0,9 % NaCl solution, and then centrifuged at 6000 rpm for 25 minutes. Pellet was disrupted by enzymatic digestion in lyses buffer (50 mM Tris-HCl, pH 8,0, 100 mM EDTA, 10mg/l lysozyme) at room temperature for 3 h (Sadin at al., 2012). Digested cell aliquots were conserved with double distilled 1N nitric acid. The concentrations of heavy metals were measured by Elan 9000 ICP mass-spectrometer with indium internal standard and argon plasma (Lenore at al., 1998).

3. RESULTS AND DISCUSSION

3.1 Isolates and identification

A total of 25 metal-tolerant aerobic chemoorganotrophic bacilli strains were isolated from soil and silt samples (Table 1).

Table 1: Isolated strains and some characteristics of sampling sites.

Location	Geographical coordinates	pH	Mineralization (mg/l)	Isolated strains
Zangezur Copper and Molybdenum Combine (Kajaran)*	39°09′04″N 46°09′36″E	6,55	70	AK1, AK2, AK3, AK4, AK5, AK6, AK7, AK8, AK9, AK10
River Debed: Alaverdy Copper Smelter	40°57′26.1″N 44°37′57.1″E	8,56	204,2	AD1, AD2, AD3, AD4, AD5, AD6, AD7
Lichk	39°03′22″N 46°10′27″E	9,15	700	AL1, AL2
Gold Sotk Mine**	40°11’904“N 45°52’431“E	8,33	545,47	AG1, AG2, AG3, AG4
Sevan Lake	40°18′38″ N 45°20′57″ E	8,5-9,0	-	AS1, AS2

(-) - *Not determined*; (*) - *The mine of Kajaran contains 87% of copper and 8% of molybdenum*; (**) - *Content of metal in the ore is estimated to be at least 4 to 4.5 gram in one ton.*

The results of the morphological and biochemical identification experiments are shown in Table 2. All studied strains were Gram-positive, motile rod with oval endospors. Optimal temperature of growth for strains AK1-AK7 was 35-40°C, for the rest isolates - 50-55°C. The pH range for growth was 6-9 with the optimum at 7. The optimal NaCl concentration for AK1, AK2, AK4, AK8, AK9 and AK10 strains was 2-3 %, for the rest strains was 5-6%. The catalase and oxidizing tests were positive for all strains. They used citrate, assimilated D-glucose, L-arabinose and D-mannitol by forming acids, but did not produce gas from glucose. The strains 1A and 1C were positive according to Voges-Proskauer test. The isolates hydrolyzed gelatin, casein, starch, Tween 20 and Tween 40.

Table 2: Some phenotyping characteristics of isolates.

Characteristics	AG1	AG2	AG3	AG4	AD1	AD2	AD3	AD4	AD5	AD6	AD7	AK1	AK2	AK3	AK4	AK5	AK6	AK7	AK8	AK9	AK10	AL1	AL2	AS1	AS2
Cell size > 10µm	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Voges-Proskauer test	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+
pH in V-P broth	> 7	> 7	< 6	< 6	> 7	> 7	> 7	> 7	> 7	> 7	> 7	> 7	> 7	> 7	> 7	< 6	> 7	< 6	< 6	< 6	> 7	< 6	< 6	< 6	< 6
Acid from																									
D-Glucose	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
D-Manitol	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	+	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+
Nitrate reduction to nitrite	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-
Formation of Dihydroxyacetone	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+	+	+

Following to the criteria of Bergey's Manual of Determinative Bacteriology [1, 11] the isolates were identified as *Bacillus subtilis* (AK7, AK8, AK9, AK10); B. *licheniformis* (AL1, AL2, AS1, AS2, AG3, AG4); *B. megaterium* (AK1, AK2); *B. circulans* (AK3, AK4, AK6), *B. pumilus* (AK5) and *Geobacillus stearothermophilus* (AG1, AG2, AD1, AD2, AD3, AD4, AD5, AD6, AD7).
The identification of strains AG1, AG2, AG3, AD1, AD2, AL1, AS1 and AS2 was confirmed by 16S rDNA sequence analysis. The 16S rDNA sequence of strain AG1 and AD1 were almost identical to the 16S rDNA sequence of *Brevibacillus thermoruber* – 97%. 16S rDNA sequence analysis of AG2 and AD2 strains were nearly identical to the 16S rDNA sequence of *Geobacillus thermodenitrificans* – 98%, and AG3, AL1, AS1 and AS2 strains – *B. licheniformis* (99%), that confirmed identification by phenotype characters. Based on the molecular analysis data, a phylogenetic tree was constructed by comparing nucleotide sequences with available 16S rDNA sequences (Fig. 1).

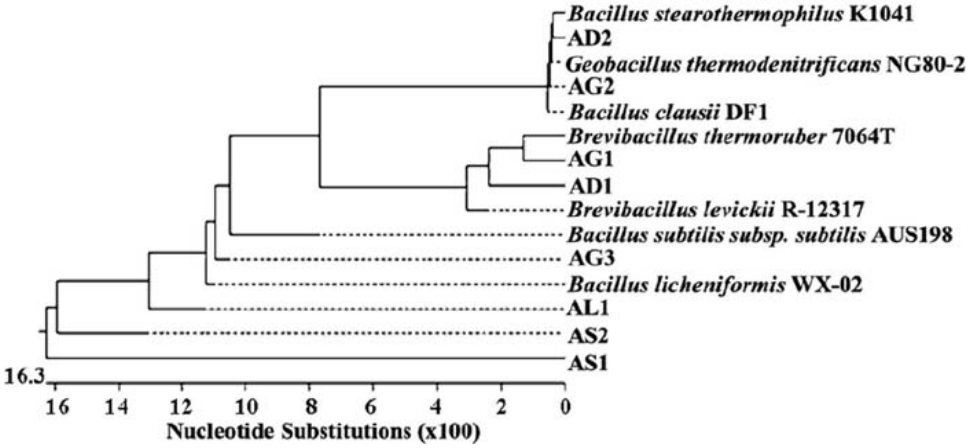

Figure 1. Phylogenetic tree of isolates based on 16S rDNA comparison.

3.2 Metal tolerance and bioaccumulation

The isolated metal-tolerant bacilli showed high resistance to Mo^{2+} (700mg/L), as well as to Cu^{2+} and Ni^{2+} for some strains (up to 300 mg/L). The results are shown in the table 3. The strains were sensitive to Cd^{2+} and Zn^{2+} ions.
The high resistance of strains to Mo^{2+} and Cu^{2+} ions correlates with the high concentration of mentioned metals in the sampling environments.

Table 3: MIC of Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mo^{2+} and CrO_4^{2-} ions (mg/L) for strains.

Metals/ Strains	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Co^{2+}	Mo^{2+}	CrO_4^{2-}
AG1	250	150	30	10	150	700	45
AG2	250	150	30	10	150	700	45
AG3	250	150	60	50	200	700	300
AG4	250	150	60	50	200	700	300
AD1	250	150	30	10	150	700	45
AD2	250	150	30	10	150	700	45
AD3	250	150	50	50	150	700	45
AD4	250	150	50	50	50	700	45
AD5	250	220	50	50	50	700	45
AD6	250	220	50	50	50	700	45
AD7	250	300	50	50	50	700	45
AL1	250	150	60	50	200	700	300
AL2	250	150	60	50	200	700	300

Metals/ Strains	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Co^{2+}	Mo^{2+}	CrO_4^{2-}
AK1	250	300	50	10	200	700	350
AK2	250	220	50	10	200	700	350
AK3	250	150	50	50	150	700	300
AK4	250	150	50	50	150	700	300
AK5	250	150	50	50	150	700	300
AK6	200	40	50	10	50	700	20
AK7	200	35	50	10	50	700	25
AK8	200	35	25	10	50	700	20
AK9	200	10	15	5	50	700	20
AK10	200	20	30	10	50	700	20
AS1	250	150	60	50	200	700	300
AS2	250	150	60	50	200	700	300

We chose three of the most resistant *B. licheniformis* AD4, *B. licheniformis* AL1 and *B. licheniformis* AL2 strains and studied their ability to accumulate heavy metals. Results exhibited the high accumulation of Cu^{2+} by *B. licheniformis* AD4 and *B. licheniformis* AL1 strains (70% and 80% from medium respectively). Results were presented in the Fig. 2.

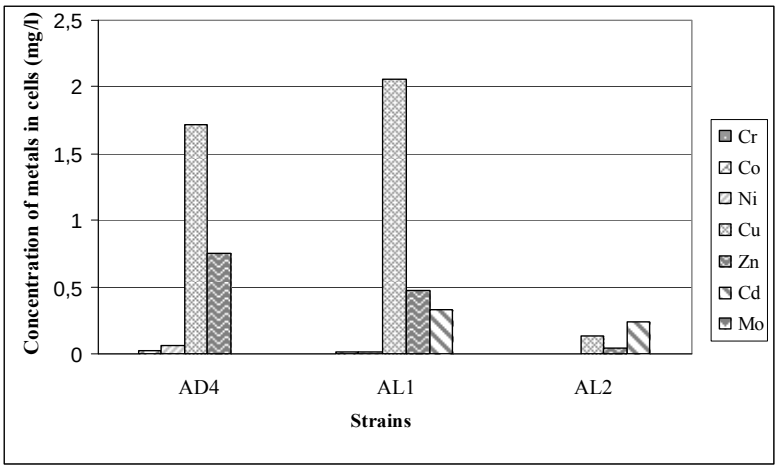

Figure 2. Heavy metal ions accumulation by strains.

B. licheniformis AL2 strain accumulated mostly Cd^{2+}. Mo^{2+}, Co^{2+}, Ni^{2+} and CrO_4^{2-} almost were not accumulated. Ability of strains to accumulate the metals is characterized as follows: for *B. licheniformis* AD4 - Cu^{2+}> Zn^{2+}> Ni^{2+}, for *B. licheniformis* AL1 - Cu^{2+}> Zn^{2+}> Cd^{2+}, and for *B. licheniformis* AL2 - Cd^{2+}> Cu^{2+}> Zn^{2+}.
According to the primary results *B. licheniformis* AD4 and *B. licheniformis* AL1 strains were selected as highest copper bioaccumulators.
All isolated strains are maintained at the Department of Department of Microbiology, Plants and Microbes Biotechnology of YSU and will be used in the further research to study their potential in bioremediation and biotechnology.

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ISOLATION AND CHARACTERIZATION OF MANGANESE-OXIDIZING BACTERIA (MOB) FROM A FORMER URANIUM MINING DISTRICT, CONTAMINATED WITH HEAVY METALS

Sathish Mayanna[1], Franziska Schäffner[1], Matthias Gube[2], Dirk Merten[1], Erika Kothe[2] and Georg Büchel[1]
sathish.mayanna@uni-jena.de
[1] Institute of Geosciences, Friedrich Schiller-University Jena, Burgweg 11, 07749, Jena, Germany.
[2] Institute of Microbiology, Friedrich Schiller-University Jena, Neugasse 25, 07743, Jena, Germany.

ABSTRACT

Heavy metal contamination in uranium mining sites is a common problem. The former uranium mining district in Eastern Thuringia, Germany, which was mined for four decades, was one of the most intensively mined and polluted sites. During mining the rocks consisting of low grade of uranium ores were leached leading to contamination with heavy metals forming secondary mineral enriched (SME) layers. The appearance of SME layers with manganese (Mn) and iron (Fe) oxides in the mining site provides a research platform on bioremediation and microbe-mineral interaction. This research focuses on the isolation and characterization of the manganese-oxidizing bacteria (MOB) from SME layers. Total digestion of the SME layer show high metal content of 18 mg/g, 55 mg/g and 73 mg/g soil of Mn, Al and Fe respectively. In contrast, the total carbon content of the SME layer is very low (0.28 g/kg soil) and pH is around 4.5. Culture dependent techniques revealed that total colony forming units (CFU) on modified K-medium from the SME layer is up to 10^7/g soil. The initial full sequencing results of the isolates represent four different manganese oxidizers which belong to the genus *Arthrobacter*, *Sphingomonas*, *Bacillus* and *Brevibacillus*. The manganese oxidizers were tested using leukoberbelin blue, which reduces Mn oxides turning colonies to dark blue if manganese oxidation activity is present. Mn enrichment was further confirmed using LA-ICP-MS. Further perspectives will be to elucidate the microbial influence on the sorption of heavy metals to biogenic/synthetic Mn oxides.

KEY WORDS: isolation, manganese oxidizing bacteria, uranium, biogenic manganese oxides

1. INTRODUCTION

Heavy metal contamination through uranium mining activities causes huge losses of flora and fauna in the polluted ecosystems. Habitats that have developed as a consequence of mining activities are often characterized as extreme environmental conditions due to low pH, sparse nutrients, high metal content and intense salt load (Haferburg et al., 2007). Uranium mining was performed in the former German Democratic republic (GDR) from 1946 to 1990. At the site studied, heap leaching was performed by application of acid to low grade ore, leaching not only uranium, but also leaching of other heavy metals. Recent investigations (Carlsson et al., 2005) had discovered several local epigenetic zones that consist of strata-bound 10 - 20 cm thick black to dark reddish layers consisting of iron and manganese oxides and hydroxides.
Manganese represents the 12th most abundant element in the Earth's crust, and constitutes, after iron (Fe) the most prevalent transition metal. It is a micronutrient in biological system and is an important electron acceptor for anaerobic respiration of different prokaryotes. The natural cycle of manganese actually is controlled by prokaryotes (Falamin et al., 2006). Bacteria can catalyze the oxidation of manganese (Beijerinck 1913), which has led to interest in the role of such organisms in the environmental manganese cycle. Manganese oxides are among the strongest oxidants in the environment and are able to degrade or oxidize different organic and inorganic compounds, including humic and fulvic acids, aromatic hydrocarbons, Cr(III), Co(II), or hydrogen sulfide (Huang 1991, Jenne 1968, Post 1999). In addition, Mn oxides have high sorptive capacities and therefore sorb a wide range of ions, controlling the distributions and bioavailability of many toxic as well as essential elements.
The present research focusses on the process of manganese oxide formation and its influence on the immobilization of heavy metals in the former leaching heap (Gessenhalde) of the uranium mining district in Eastern Thuringia, Germany. Since the site is characterized by low pH and high heavy metal content, we hypothesize that microbes might play a significant role dominating the formation of Mn oxides. To this end, we investigated bacterial Mn(II) oxidation processes, their mechanisms, and products. This knowledge is prerequisite to harnessing these natural processes for biogeotechnological applications such as *in situ* bioremediation of contaminated soils or ground waters (Bargar et al., 2005).

2. Materials and methods

2.1 Sampling site Gessenhalde

The former Gessenhalde was located in the uranium mining district, Ronneburg, which was built up in the former loam pits of the village Gessen that had to give way to the leaching heap (Fig. 1). After uranium leaching was stopped in 1989, the dump material including up to 10 m of excavated underground material was filled into the former open pit Lichtenberg. Then, the basement area was recontoured using a heterogeneous allochthonic soil as filling material. In 2004, the test site Gessenwiese was created, covering 2500 m^2 in the north-west of the former leaching area. Since 2004, most part of the Gessenhalde is used as a platform for applied and fundamental research activities dealing with phytoextraction, mobilization, and microbial alterations of minerals.

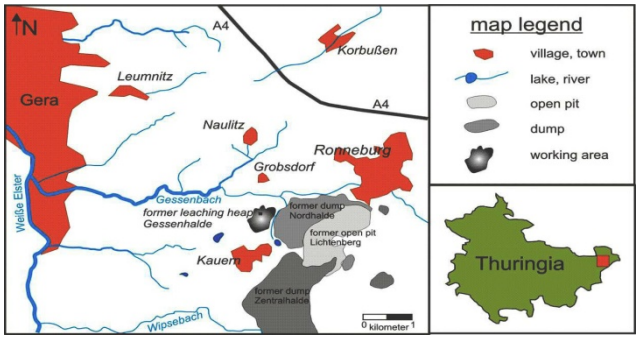

Figure 1. Map depicts the former leaching heaps, waste dumps and open pit in the former uranium mining district, Ronneburg, Germany (Grawunder et al., 2009).

Sampling locations in the Gessenhalde were first screened for secondary mineral enriched (SME) layers with Fe and Mn oxides/hydroxides. The screening was performed through 1 m deep bore holes and the SME layers were visually identified. The identified sites were selected for detailed soil characterization and soil samples were taken for geochemical, mineralogical and microbiological investigations.

2.2 Total carbon and total metal content

For total carbon, about 100 to 500 mg (n=3) of the ground sample were filled with WO_3 in little tin shuttles and measured with a CHNOS vario EL elemental analyzer (Elementar). For total digestion, 200 mg ground samples were digested with a pressure digestion system (DAS, PicoTrace) for the total metal content, which was analyzed with ICP-OES (725 ES, Varian) and ICP-MS (XSeries II, Thermo Fisher Scientific).

2.3 Identification of bacteria

Soil samples were collected in Falcon tubes from the manganese enriched layer and within two to three hours after sampling, the samples were transferred at 4 ^{o}C to the laboratory and processed. 5 g of soil was shaken for 1 hr with 45 ml of 0.7 % NaCl in a 50 ml Falcon tube. Subsequently, a dilution series up to 10^{-5} were spread on modified K-media with Mn sources at different concentrations.
The colonies were tested for Mn oxidation with leucoberbelin blue (LBB) and positive (dark blue color) colonies were streak plated to isolate get pure cultures. Colony PCR was carried out using universal primers (27F and 1427R) to amplify the 16S rRNA for purposes of identification. The PCR products were sequenced (GATC, Germany) and a phylogenetic analysis were performed using MAFFT 6 for alignment and Treefinder Oct 2010 for phylogenetic reconstruction.

3. Results and Discussion

3.1 Low carbon with high heavy metal content

Results of total carbon analysis show very low (up to 1.06 g/kg C contents) at the site, with even lower (up to 0.28 g/kg C) in the SME layers at a depth of 87 cm (Fig. 2A). Since the pH of the soil is 4.5 the only sources of carbon are of biological materials like roots. However, contribution from roots can be excluded at a depth of 87 cm as roots of on-site plants cannot reach the SME layer.

The SME layer at a depth of 75 to 95 cm is characterized by high metal contents (Fig. 2B). Previous studies (Carlsson et al., 2005) indicated the shales and sulphide minerals as the sources of heavy metals.

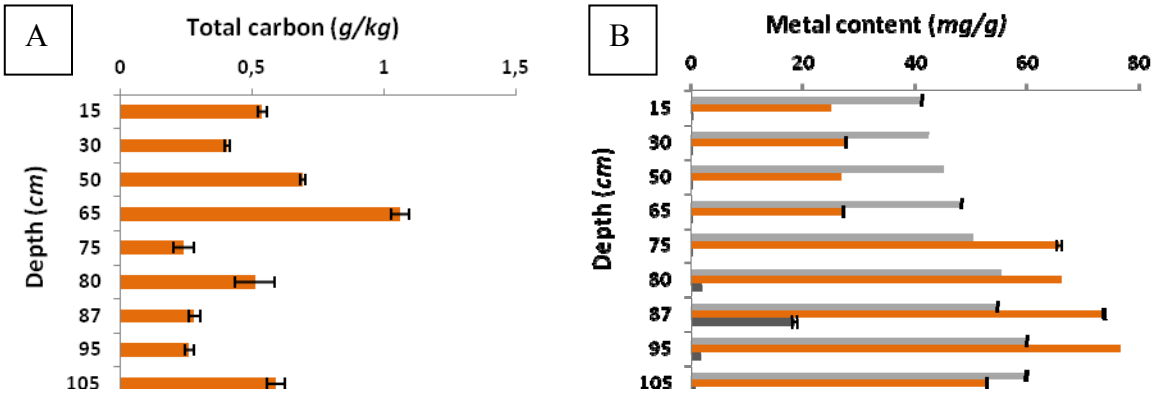

Figure 2. A) The bar graph represents the total carbon (g/kg) with respect to depth of soil profile. B) The multiple bar graphs show the total metal content (mg/g) with respect to depth of soil profile. Orange, light gray and dark gray bar represents Iron (Fe), Aluminum (Al) and Manganese (Mn) content respectively.

3.2 Isolation of MOB and 16S rRNA gene sequencing

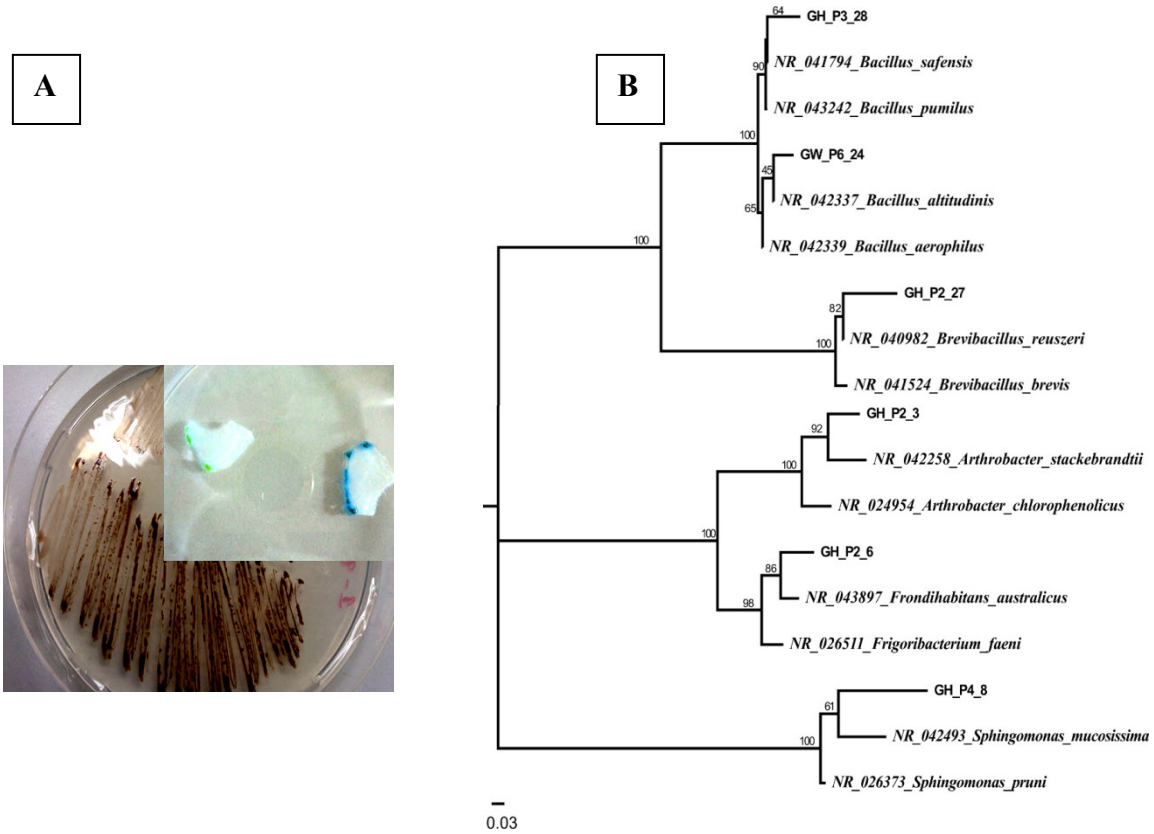

Figure 3. A) Shows the colonies of *Bacillus safensis* on the agar plate, the dark blue color on the sides of filter paper positively indicate Mn oxides with LBB reagent. B) Maximum-likelihood (ML) trees showing the phylogenetic relationships of Mn(II)-oxidizing bacteria isolated using 16S rRNA gene analysis. Isolates from this study are shown in bold and the related strains are shown in italics. ML trees were constructed with Treefinder Oct2010 using the generalized time reversible (GTR) nucleotide substitution model with 1,000 LR-ELW branch support replicates. Scale bar, 0.03 substitutions per nucleotide site.

Culture dependent techniques show higher colony forming units (CFU's), with up to 10^7 per g soil compared with previous results (Burkhardt et al., 2009) carried out on a nearby site. We could isolate four different strains of bacteria which gave positive result (dark blue color colonies) up on reacting with LBB reagent (Fig. 3B) and manganese content on the colonies was confirmed with LA-ICP-MS (results not shown here).
Initial result of comparative 16S rRNA gene sequences of isolates revealed that our strains belong to three bacterial phyla, Firmicutes, Actinobacteria and Alphaproteobacteria. The Firmicutes isolates form two clusters which are affiliated to genera *Bacillus* and *Brevibacillus*. In contrast, our Actinobacteria strains are affiliated to the genus *Arthrobacter*. The last group of isolates belonging Alphaproteobacteria associated with the genus *Sphingomonas*. All these isolated strains are able to oxidize manganese, and our results are in accordance with others findings, that these groups are manganese oxidizers (Bargar et al., 2005, Palmer et al., 1986).
In conclusion, despite the extreme energy limitation, acidic pH and high metal content in the former Gessenhalde, we isolated strains which are able to survive in these harsh environmental conditions while oxidize manganese. These might subsequently be utilized to immobilize toxic heavy metals in the former uranium mining districts and other acid mine drainage (AMD) sites. Further perspective, will be to carry out sorption studies of heavy metals on biogenic (Mn oxide produced from isolated strains) and synthetic (chemical oxidation) Mn oxides.

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CADMIUM AND ZINC SORPTION FROM SINGLE AND BINARY SOLUTIONS BY STRAW BIOCHAR: THE ROLE OF FUNCTIONAL GROUPS

Lucia Remenárová[1], Martin Pipíška[1], Miroslav Horník[1], Marián Rozložník[1], Jozef Augustín[1], Gerhard Soja[2]
remenarova@ucm.sk
[1]University of SS. Cyril and Methodius in Trnava, Faculty of Natural Sciences, Department of Ecochemistry and Radioecology, J. Herdu 2, Trnava, SK-917 01 Slovak Republic
[2]AIT Austrian Institute of Technology GmbH, Health & Environment Department, 2444 Seibersdorf, Austria

ABSTRACT

Biochar produced by pyrolysis of a straw biomass was investigated as a potential sorbent for the removal of cadmium and zinc from single and binary solutions using radiotracer technique. The suitability of Langmuir model for description of Zn and Cd sorption in single systems was determined by modified Akaike´s information criteria. The maximum sorption capacity Q_{max} at pH 6.0 calculated from Langmuir isotherm was 192.7 µmol Cd^{2+}/g and 147.5 µmol Zn^{2+}/g. Competitive Langmuir model well described data in Cd-Zn binary system obtained. Maximum sorption capacities of biochar in binary system Cd-Zn were Q_{max} 204.8 µmol Cd^{2+}/g and 151.6 µmol Zn^{2+}/g. The surface characteristics and mechanisms of Cd and Zn biosorption by biochar were examined by SEM-EDX analysis, potentiometric titration and chemical blocking of functional groups. Potentiometric titration of biochar revealed carboxyl, phosphoryl, hydroxyl and amino group in concentrations 0.069, 0.011, 0.240 and 0.910 mmol/g, respectively. To determine their participation in the sorption process, functional groups were chemically blocked and modified biochar was further used in sorption experiments. Results revealed the dominant role of carboxyl, hydroxyl and amino groups in Cd and Zn ions binding.

KEY WORDS: biochar, sorption, ^{65}Zn, ^{109}Cd, SEM-EDX, multicomponent systems, functional groups

1. INTRODUCTION

In recent years, much attention has become focused on biochar due to its proven in solving of the most environmental problems today. Biochar presents the carbonaceous product of pyrolyzing biomass without an activation (Chun et al., 2004). Due to the wide availability of its raw materials, biochar may be produced from direct burning of precursors, which makes the production of biochar much less expensive, as compared to activated carbon (Qiu et al., 2009). Biochar has a relatively structured carbon matrix with high degree of porosity and extensive surface area, suggesting that it may act as a sorbent which is similar in some aspects to activated carbon and thereby play an important role in controlling contaminants in the environment (Chen et al., 2011). Many papers deal with using of biochar derived from various biological wastes for wastewater treatment. Most of them devote removal of toxic organic compounds such as pentachlorphenol, dyes, phenanthrene etc. However, only limited information about the mechanism and the role of active sites in sorption process is available (Xu et al., 2011; Qiu et al., 2009). The objective of this study was to investigate the sorption capacity of straw based biochar for Cd^{2+} and Zn^{2+} cations from single and binary solutions. The characterization, quantification and involvement of functional groups present on the biochar surface in metal binding were studied.

2. MATERIALS AND METHODS

2.1 Biosorbent preparation

Biochar produced by pyrolysis of straw was provided by AIT – Austrian Institute of Technology, Seibersdorf, Austria. Before experiments, the biochar was washed twice in deionised water (Simplicity 185, Millipore, USA), oven-dried for 72 h at 90°C. After drying biochar was ground to various particle sizes, from which particle size <125 µm was used in biosorption experiments.

2.2 Biosorption experiments in single and binary systems

The metal sorption capacity of sorbent was determined by suspending of straw biochar (SB) (2.5 g/L, d.w.) in metal solutions (pH 6.0) containing $CdCl_2$ or $ZnCl_2$ in concentration range 100-4000 µM spiked with $^{109}CdCl_2$ or $^{65}ZnCl_2$ and exposing for 4h at 20 °C on a reciprocal shaker (40 rpm). At the end of the experiments biosorbent was filtered out, washed in deionised water and radioactivity of both SB and liquid phase was

measured. Biosorption in binary-metal system Cd-Zn was carried out at the same operating conditions in solutions containing both $CdCl_2$ and $ZnCl_2$ in different initial molar ratios 2:1, 1:1, 1:2, spiked with $^{109}CdCl_2$ or $^{65}ZnCl_2$.

2.3 Radiometric analysis

The gamma spectrometric assembly using the well type scintillation detector 54BP54/2-X, NaI(Tl) (Scionix, the Netherlands) and the data processing software Scintivision 32 (ORTEC, USA) were used for ^{109}Cd and ^{65}Zn determination in SB and supernatant fluids at the energy of γ- photons: ^{109}Cd – 88.04 keV and ^{65}Zn – 1115.52 keV. Standardized $^{109}CdCl_2$ solution (3.857 MBq/mL, $CdCl_2$ 50 mg/L in 3 g/L HCl) and $^{65}ZnCl_2$ solution (0.8767 MBq/ml; 50 mg $ZnCl_2$/L in 3 g/L HCl) were obtained from the Czech Institute of Metrology, Prague (Czech Republic).

2.3 Sorbent characterization and the role functional groups

The surface structure analysis and EDX microanalysis of SB before and after Cd^{2+} and Zn^{2+} sorption from single systems were performed by scanning electron microscope VEGA 2 SEM (TESCAN Inc., Czech Republic) coupled with an EDX, QUANTAX QX2 detector (RONTEC, Germany) for electron dispersive X-ray analysis. Potentiometric titration was carried out according to the modified procedure described by Zhang et al. (2010).
Carboxyl, hydroxyl and amino groups were modified as follows: *Esterification of carboxylic groups* of SB was performed in accordance with the method of Gardea-Torresdey *et al*. (1990), which includes reaction with methanol. *Formylation of hydroxyl groups* of SB was carried out in reaction with formaldehyde according to procedure of Chen and Yang (2006). *Methylation of amino groups* of SB was realized in accordance with the method of Fu and Virararghavan (2002).
To identify the functional groups present on biochar FTIR analysis (Nicolet NEXUS 470 spectrometer, Thermo Scientific, USA) of control sample and metal loaded biochar was performed. Samples were mixed with KBr at a ratio 1:100 for making pellets. The FTIR spectra were obtained within the range of 400-4000 cm^{-1}.

3. Results and discussion

3.1 Sorption equilibrium in single and binary systems

Due to the fact that equilibrium modeling offers useful information about studied sorption system, biosorption equilibrium in single systems were analyzed using two well known adsorption isotherms – Langmuir and Freundlich. The suitability of the isotherm to describe the sorption process is often determined only on the marginal differences beteween coefficients of determination (R^2). In our work the adequacy of models for mathematical description of Cd and Zn biosorption was also compared using modified Akaike´s information criterion AIC_c (Burnham and Anderson, 2002). The AIC_c is able to answer the question which isotherm is better for description of metal sorption – the model with lower AIC_c value is considered most likely to be correct. This approach was successfully used in our previous papers (Remenárová *et al.*, 2012; Pipíška *et al.*, 2010). From Table 1 it is evident that there is more than 99 % (Cd) and 87 % (Zn) probability that the Langmuir model is correct. The maximum sorption capacity Q_{max} of SB for Cd^{2+} calculated from Langmuir isotherm was 193 ± 10 μmol/g. The lower value of Q_{max} (148 ± 3 μmol/g) was observed in the case of Zn^{2+} sorption. Despite the fact that higher affinity constant *b* (0.021 L/μmol) of Langmuir isotherm and also higher affinity constant *K* (39.8 L/g) of Freundlich isotherm suggest that biochar has higher affinity for Zn^{2+} ions, the binding capacity of SB was higher for Cd^{2+} ions, reflected by higher Q_{max} value. However, it should be realized that although the isotherms present useful tool for comparing of equilibrium on a quantitative basis (Q_{max}, *b*, *K*), they offer no insight into the mechanism of Cd and Zn biosorption.

Table 1: The parameters of Langmuir and Freundlich isotherm for Cd^{2+} and Zn^{2+} ions sorption by straw biochar (2.5 g/L, d.w.) from single system at pH 6.0 and 20 °C.

Metal	Langmuir				Freundlich			
	Q_{max} [μmol/g]	*b* [L/μmol]	R^2	AIC_C	*K* [L/g]	*1/n*	R^2	AIC_C
Cd^{2+}	193 ± 10	0.006 ± 0.001	0.977	0.9998	23.6 ± 7.9	0.26 ± 0.05	0.949	0.0002
Zn^{2+}	148 ± 3	0.021 ± 0.003	0.992	0.8782	39.8 ± 19.3	0.17 ± 0.07	0.767	0.1218

Sorption in binary Cd-Zn system is rather complicated, because of possible interactions among the metal ions. Romera et al. (2008) mentioned that the most appropriate form to describe sorption equilibrium in binary

systems is to adjust the experimental data to a mathematical model from which number of parameters can be obtained for quantitative interpretation of sorption equilibrium uptake. Following the results of AIC_c from single systems, for description of sorption equilibrium from binary system Cd-Zn, competitive model derived from Langmuir isotherm was used (Marešová et al., 2011). 3-D sorption isotherm surfaces of Cd-Zn binary system predicted by the competitive Langmuir model are shown in Fig. 1. High values of R^2 (0.963 for Zn) and (0.902 for Cd) indicate that competitive Langmuir model well described equilibrium data in binary system. We also found the existence of competition among metal ions for binding sites on SB surface. The addition of co-ion caused the decrease of sorption of primary ion. Increasing concentration of zinc caused significant decrease of Cd^{2+} sorption from 150 to 76 µmol/g. On the contrary, the increasing concentration of cadmium caused less pronounced decrease of Zn^{2+} sorption from 131 to 97 µmol/g. When Cd^{2+} and Zn^{2+} ions are present in equimolar ratio 1:1 maximum uptake was 109 µmol/g for cadmium and 127 µmol/g for zinc. Analysis of competitive Langmuir isotherm parameters reflected higher affinity of SB for zinc (b_{Zn} = 0.011 L/µmol) ions compared to cadmium ions (b_{Cd} = 0.004 L/µmol).

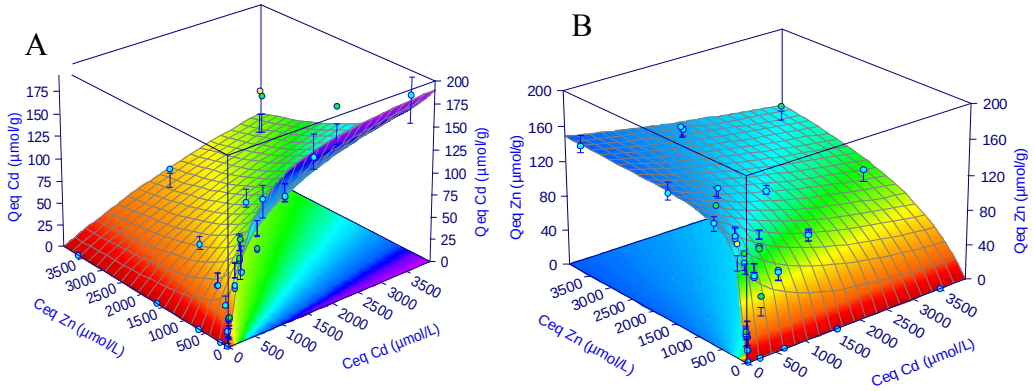

Figure 1. 3D **sorption isotherm surfaces of Cd^{2+} and Zn^{2+} binary system:** (A) Cd^{2+} and (B) Zn^{2+} sorption (pH 6.0, 20° C). The surfaces are predicted by the competitive Langmuir model, bars represent 95 % confidence interval.

3.2 The role of functional groups in Cd^{2+} and Zn^{2+} biosorption

The surface morphology of straw biochar was studied using SEM connected with EDX. The SEM images show that biochar (Fig. 2A) has an irregular amorphous surface with a porous structure. However, as was described by Liu et al. (2010), char surface are greatly depended on pyrolysis temperature. The SEM was also performed to observe biochar surface after sorption of Cd^{2+} and Zn^{2+} ions. After comparing of sorbent images before and after sorption we can concluded that there are no morphological changes on biochar surface after metal sorption (Fig. 2). The presence of cadmium and zinc on biochar is clearly evident from a uniform distribution of metals on biochar surface. For the purpose of having knowledge about composition of SB, the elemental analysis was done with use of EDX analysis. The EDX of unloaded SB (data not shown) showed the presence of various elements with no peaks for cadmium and zinc, while the EDX spectra after sorption of Cd^{2+} and Zn^{2+} confirmed the presence of Zn and Cd on biochar. Lu et al. (2012) postulated similar results using SEM-EDX analysis to study relative distribution of Pb^{2+} onto sludge-derived biochar. The distribution of Cd and Zn on the surface of biochar particles was confirmed by element mapping (Fig. 2B,C).

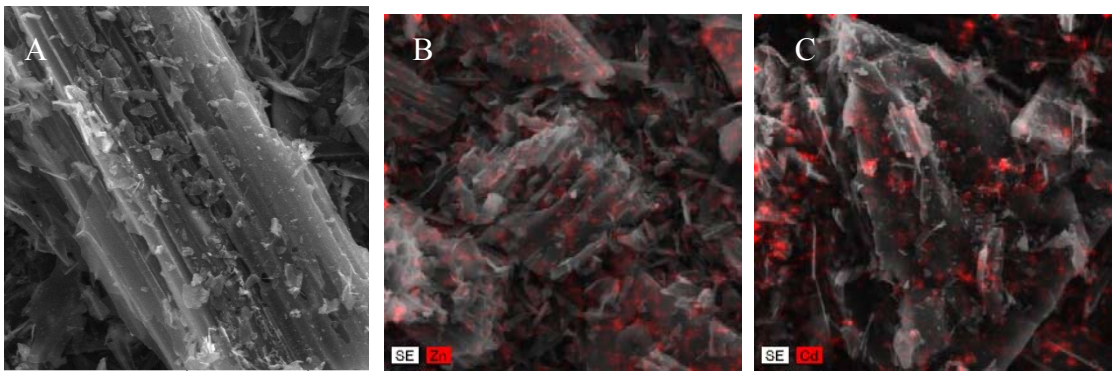

Figure 2. SEM-EDX analysis and element mapping of the straw biochar: (A) before; (B) after Zn^{2+} (4000 µM $ZnCl_2$) sorption; and (C) after Cd^{2+} (4000 µM $CdCl_2$) sorption from single system.

From the view of metal cations biosorption is necessary to know the proton binding active zone on biosorbent surface. To identify the types and the amount of functional groups present on the SB surface, potentiometric titration of SB was performed. For calculation of pK_a values (data not shown) of functional groups and corresponding site concentrations c_{An} from titration curve behavior the modeling software ProtoFit using the non-electrostatic model (NEM) was used. It was found that a four-site model provided a good fit of titration data and the results revealed carboxyl, phosphoryl, hydroxyl and amino groups in concentrations 0.069, 0.011, 0.240 and 0.910 mmol/g, respectively. It can be expected, that in biosorption of Cd^{2+} and Zn^{2+} ions above mentioned groups will play a significant role. To determine which of functional groups are involved in sorption process, FTIR analysis before and after Cd^{2+} and Zn^{2+} sorption was carried out. FTIR spectra of SB after metal sorption showed that the peaks of carboxyl and hydroxyl groups expected at 1655 and 3676 cm^{-1} had shifted to 1657 and 3696 in case of SB after cadmium sorption, respectively, to 1665 and 3682 after zinc sorption.
To quantify the participation of functional groups determined by potentiometric titration and FTIR analysis, chemically modified SB was used in sorption experiments. Blocking of –OH, –COOH and $–NH_2$ on SB caused the significant decrease of cadmium uptake from 134 to 41.6, 16.0 and 15.2 µmol/g, and also the decrease of zinc uptake from 142 to 47.7, 31.1 and 31.2 µmol/g. This suggests the dominant role of all examined functional groups in sorption process beside the role of other functional groups or mechanism. As was described by Lu et al. (2012), sorption of metal cations by sludge-derived biochar (SDBC) involved mechanism that can be divided in few groups: (1) metal exchange with K^+ and Na^+, which can be mainly attributed to the electrostatic outer-sphere complexation; (2) exchange with Ca^{2+} and Mg^{2+}, the majority of which can be attributed to co-precipitation and inner-sphere complexation with complexed humic matter and mineral oxides of SDBC; (3) surface complexation with free –COOH functional groups; (4) surface complexation with free –OH functional groups, and (5) the others, including inner-sphere complexation with free hydroxyl groups or mineral oxides and other surface precipitation.
We can conclude that the functional groups present in SB play a significant role in Cd and Zn sorption by straw biochar. SB represents effective alternative to activated carbon in metal removal from single and multicomponent systems.

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MODELING ADSORPTION OF CADMIUM BY PLANT GROWTH PROMOTING RHIZOBACTERIA AT DIFFEREN PH SOLUTIONS

Marzieh Safari[1], Ali Sorooshzadeh[1], Ahmad Asgharzadeh[2] and Saeed Saadat[2]
m.safari@modares.ac.ir
1 Department of Agronomy, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.
[2] Soil Microbiology Research Department of Soil and Water Research Institute (SWRI), Karaj, Iran.

ABSTRACT

The biosorption behaviors of two Plant Growth Promoting Rhizobacteria (*Pseudomonas fluorescence* and *P. putida*) for cadmium at different pH (5.5, 6, 6.5, 7 and 7.5) and different cadmium concentration (5, 15, 25 and 35 mg/l) by factorial experiment arrangement in randomized complete design with 3 replications were surveyed. Analysis of variance showed that metal removal significantly affected by all treatments. Means comparison between two species of bacteria was showed that *Pseudomonas florescence* is superior species for removing cadmium from all cadmium concentration. The maximum cadmium absorption by *Pseudomonas florescence* was in 5 mg/l of cadmium concentration at pH=6 (79.13%), while *Pseudomonas putida* at pH=7 (75.93%). Equilibrium adsorption data well fitted with Langmuir and Freundlich adsorption models. Langmuir and Freundlich models results showed that P. fluorescence at low Cd equilibrium concentration, uptake higher cadmium in pH 6, but with increasing Cd equilibrium concentration, higher uptake was absorved in pH 7. P. putida had higher uptake values at low and high level of Cd equilibrium concentration in both isotherm models in pH 7 and 7.5 respectively. The comparison of two isotherm parameters (Qm and a) further confirmed that, Pseudomonas fluorescence was stronger species in binding cadmium ions (52.6 mg/g and 7.7 respectively).

KEY WORDS: biosorption, *Pseudomonas,* Langmuir isotherm, Freundlich isotherm

1. INTRODUCTION

The low mobility of phosphorus in agricultural soils leads to excessive usage of phosphate fertilizers by farmers that could result in cadmium pollution in these soils (as cadmium is one component of these fertilizers); cadmium is an important heavy metal environmental contaminant that causes damage even at very low concentrations. Several physical–chemical treatments are used for remediation of heavy metal-contaminated sites; however, they are not suitable. Currently, microbes (especially bacteria) are often used for bioremediation, consistent with developing goals to reduce heavy metals pollution for sustainable agriculture. *Pseudomonas* is an abundant and cost effective bacterium that could solubilize phosphate fertilizer; in addition to supplying phosphate for plants, this genus of bacteria could be a good candidate for the adsorption of cadmium from these fertilizers and is resistant to cadmium stress (Rani et al. 2010). Biosorption isotherm data can explain the distribution of adsorbate (heavy metal) molecules between liquid (metal salt solution) and solid (biomass) phases when the adsorption process reaches an equilibrium state (Munir et al. 2010). Using adsorption modeling to assess the capacity of bacteria for metal adsorption under certain experimental conditions, such as different metal concentrations and pH levels, could allow for the prediction of metal adsorption under other conditions.

2. MATERIALS AND METHODS

2.1 Preparation of microorganism and metal solution

In this work, we used two strains of *Pseudomonas*: *Pseudomonas fluorescence*str. P169 and P. putida str. P108. Bacterial species were prepared at the Soil Biology Center of the Soil and Water Research Institute. Cadmium stock solution (1000 mg/l) was prepared by dissolving 2.2 g of cadmium sulfate salt ($CdSO_4.8/3\ H_2O$) (Sigma-Aldrich) into 0.02 M maleate buffer that was prepared using deionized water. The pH was adjusted to pH levels of 5.5, 6, 6.5, 7 and 7.5 using maleate buffer. Different cadmium concentrations (5, 15, 25 and 35 mg/l) were created by diluting the stock solution.

2.2 Adsorption experiment

First, 20 ml of each metal solution concentration treatment was prepared in 100 ml Erlenmeyer flasks; 10 ml of each bacterial suspension was then added to the flasks. Solutions were shaken at 135 rpm at room temperature (25±2 ^{o}C) for one hour and were then centrifuged (10000 rpm) at 10°C for 10 min. After the bacterial bodies were separated, the cadmium concentrations of the supernatants were analyzed using atomic adsorption

spectroscopy (Perkin-Elmer). Metal removal by both bacterial species was determined according to the following formula:

$$R = \left(\frac{C0 - Ce}{C0}\right) \times 100$$

Where R is the percentage of metal absorbed, C_0 is the initial concentration of cadmium ions (mg/l) and C_e is the equilibrium (final) concentration of cadmium ions (mg/l).

2.3 Isoterm models

The metal sorption efficiency of biosorbents was calculated using the following equation:

$$Q = (V(C0 - Ce)/X$$

where Q is the metal uptake (mg metal per g biosorbent), V is the metal liquid volume (ml), C_0 is the initial concentration of the metal in the solution (mg/l), C_e is the equilibrium concentration of the metal in the solution (mg/l) and X is the dry weight of the added biosorbent (mg).
Sorption isotherm models (Langmuir and Freundlich) were chosen for comparison with equilibrium sorption data (Ziagova et al. 2007; Vásquez et al. 2007).
Langmuir model (theoretical model):

$$Q = \frac{(Qmax \times b \times Ce)}{(1 + b \times Ce)}$$

Where Qmax is the maximum metal uptake under the given conditions and b is a constant related to the affinity between the biosorbent and metal.
Freundlich model (empirical model):

$$Q = k \times Ce^{1/n}$$

Where k and 1/n are Freundlich constants that are correlated to the maximum adsorption capacity and adsorption intensity, respectively.

3. Results and discussion

3.1 Effect of pH on metal uptake

The maximum adsorption of cadmium was observed in the 5 mg/l cadmium concentration treatment at pH values 6 and 7 for *Pseudomonas florescence* and *P. putida,* respectively; in contrast, the minimum adsorption was observed in the 35 mg/l cadmium concentration treatment at pH values 7 and 7.5 for *Pseudomonas florescence* and *P. putida,* respectively (table 1). In addition, we found that *Pseudomonas florescence* exhibited greater cadmium adsorption than *P. putida*.

The results showed that the amount of cadmium absorbed by *P. putida* in the different cadmium concentration treatments increased with increasing pH; the optimum pH was 7.0 for this species, with approximately 76 % removal capacity. However, in *P. florescence*, absorbance increased with increasing pH until pH=6, greatly decreased at pH= 6.5 and then increased again at higher pH levels (table 1). Our results were consistent with the results from other studies, where the highest Cu removal by a *Pseudomonas* sp. occurred at pH 6 (Andreazza et al. 2010). Other results demonstrated that the optimum pH values for maximum cadmium adsorption by *Pseudomonas* spp. were pH 6 (Rani et al. 2010) and pH 7 (Ziagova et al. 2007), with 86.66% and 60% of cadmium absorbed, respectively. It has been suggested that greater cadmium adsorption with increasing pH is closely related to an increase in the number of negative surface charges.

3.2 Effect of cadmium concentration

As shown in table 1, the percentage of cadmium removed decreased as the initial cadmium concentration was increased. The results show that the highest percentage of cadmium adsorption for the two species studied was observed at the 5 mg/l cadmium concentration level (table 1). This result is consistent with findings reported by other researchers (Zeng et al. 2009).

3.3 Langmuir and Freundlich models

High correlation coefficient values (R^2) of Langmuir and Freundlich isotherm models indicated that both models have high accuracy when fit to the experimental data (table 2). The results of the Langmuir and Freundlich models showed that at low Cd equilibrium concentrations, *P. fluorescence* uptake of cadmium was greater at pH 6, but with increasing Cd equilibrium concentration, higher uptake was observed at pH 7. In both isotherm models, *P. putida* had higher uptake values at pH 7 and 7.5 in low and high levels of Cd equilibrium

concentration, respectively; As shown in table 2, Comparison among the numeric quantities of the Freundlich constant (k) confirmed the optimum pH for maximum adsorption capacity for each bacterium. The comparison of two isotherm parameters (Qm and k) further confirmed that *Pseudomonas fluorescence* has a greater capacity to bind cadmium ions than *P. putida* (table 2). *P. fluorescence* had a higher removal percentage (R) (79.13%) than *P. putida* (75.93%) (table 1). According to the Langmuir and Freundlich isotherms, the maximum binding capacities of Cd(II) in the highest equilibrium concentration were 33.42 and 36.38 mg/g for *Pseudomonas fluorescence* and 33.77 and 32.79 mg/g for *P. putida* at pH 7 and 7.5, respectively. Equilibrium adsorption data was well described by both the Langmuir and Freundlich adsorption models; however, as shown by the R^2 values in table 2, the Freundlich model fit the experimental data much better than did the Langmuir model. The Q_m quantity obtained by the two bacterial species in this experiment is consistent with the results of other studies (Vullo et al. 2008; Fang et al. 2010). The comparison of the two isotherm parameters further confirmed that, *P.florescence* has the greater capacity to bind cadmium ions.

Table 1 Equilibrium adsorbed quantities, maximum adsorption and removal percentage of Cd (II) ion in different cadmium concentration for two bacteria species at different acidity level

	C_0 (mg/g)	Pseudomonas fluorescence R (%)	Pseudomonas putida R (%)
pH= 5.5	5	76.133 b	69.333 e
	15	63.623 fgh	49.377 nop
	25	54.587 kl	39.307 st
	35	49.227 onp	35.817 u
pH= 6	5	79.133 a	71.267 de
	15	65.600 f	50.557 mno
	25	59.187 ij	40.347 rs
	35	52.483 lm	37.323 tu
pH= 6.5	5	71.200 de	72.133 cd
	15	57.223 jk	51.177 mn
	25	49.880 mnop	42.280 qr
	35	44.477 q	37.907 stu
pH= 7	5	74.267 bc	75.933 b
	15	63.823 fg	60.513 i
	25	54.720 kl	51.133 mn
	35	61.143 hi	47.773 p
pH= 7.5	5	74.333 bc	74.333 bc
	15	65.337 f	61.157 ghi
	25	56.213 k	50.307 mnop
	35	51.570 mn	47.963 op

Table 2. Sorption isotherm coefficients of Langmuir and Freundlich models

Langmuir isotherm					Freundlich isotherm				
		Qm	k	R^2			a	n	R^2
Pseudomonas fluorescence	pH=5.5	40	0.19	0.996	*Pseudomonas fluorescence*	pH=5.5	7.02	0.56	0.997
	pH=6	41.6	0.22	0.992		pH=6	7.7	0.561	0.998
	pH=6.5	37.03	0.16	0.994		pH=6.5	5.8	0.57	0.999
	pH=7	52.6	0.12	0.987		pH=7	6.1	0.68	0.968
	pH=7.5	47.6	0.14	0.998		pH=7.5	6.5	0.61	0.993
Pseudomonas putida	pH=5.5	25.6	0.24	0.986	*Pseudomonas putida*	pH=5.5	5.6	0.47	0.998
	pH=6	26.3	0.25	0.983		pH=6	5.9	0.46	0.996
	pH=6.5	27.02	0.26	0.982		pH=6.5	6.1	0.46	0.999
	pH=7	35.7	0.22	0.990		pH=7	6.8	0.53	0.998
	pH=7.5	38.4	0.18	0.994		pH=7.5	6.5	0.55	0.995

Therefore, applying these *Pseudomonas* strains in soil with cadmium contamination could be a promising way to reduce cadmium uptake by plants. Therefore, plants that grow in sites inoculated with *Pseudomonas* species may be devoid of heavy metals. In addition, the growth of the plants may be promoted by these bacteria.

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RISK ELEMENTS UPTAKE BY LESS COMMON TYPES OF VEGETABLES

Milan Sehnal, Jana Tremlová, Jiřina Száková, Jana Najmanová, Pavel Tlustoš
tremlova@af.czu.cz
Department of Agroenvironmental Chemistry and Plant Nutrition, Czech University of Life Sciences, CZ-165 21 Prague 6, Czech Republic

ABSTRACT

The risk element (As, Cd, Pb, Zn) uptake by less frequented vegetable species was tested in contaminated soils. *Brassica oleracea* L. var. Acephala – savoy cabbage, *Brassica napus L.* var. Napobrassica – swede turnip, and *Anthriscus cerefolium* L., - chervil, were compared and lettuce (*Lactuca sativa* L.) representing the common vegetable species was included for comparison. Influences of vegetable species and soil properties were taken into account. Data analysis did not show any influence of soil types that were used for this pot experiment, but showed influence of plant species. Total contents of As in edible parts of the plants ranged from 1.86 mg kg $^{-1}$ (*L. sativa*) to 33.6 mg kg $^{-1}$ (*A. cerefolium*), for Cd from 1.01 mg kg $^{-1}$ (*B. napus*) to 36.8 mg kg $^{-1}$ (*A. cerefolium*), for Pb from 0.78 mg kg $^{-1}$ (*B. napus*) to 130 mg kg $^{-1}$ (*A. cerefolium*), for Zn from 57.4 mg.kg $^{-1}$ (*B. napus*) to 269 mg kg $^{-1}$ (*A. cerefolium*), respectively. In most of cases, the element contents exceeded the maximum permissible levels for edible vegetables given by the EU Commission Regulation 1881/2006. Therefore, the choice of vegetable species for cultivation in risk element contaminated areas must be carried out with good knowledge of soil contamination levels.

KEY WORDS: risk element, vegetable, uptake, ICP OES, pot experiment

1. INTRODUCTION

Vegetable is part of a healthy balanced diet of the human (WHO, 2005; SZU, 2007) and according to their recommendations should be represented in the diet several times a day. The protective effect of micro-nutrients, fiber and other nutrients contained in vegetables is indisputable, however, problems may occur if the vegetables contain some anti- nutritional compounds or substances (Reddy and Pierson, 1994). Anti-nutritional substances include risk elements such as arsenic (As), copper (Cu), cadmium (Cd), lead (Pb), mercury (Hg), zinc (Zn), etc. These elements are received by roots or/and plant surfaces from soil solution or/and from the atmospheric fallout, respectively (Tremlová, 2011). The uptake ability for each plant species varies depending on its metabolism (Wang et al., 2003). Many studies deal with uptake and accumulation of risk elements in traditionally and commonly grown crop plants (Zheng et al., 2007, Wang et al., 2004), but for own consumption there are often grown also lesser known and lesser common types of vegetable such as savoy cabbage (*Brassica oleracea* L. var. Acephala), swede turnip (*Brassica napus L.* var. Napobrassica), chervil (*Anthriscus cerefolium* L.), etc. If these are grown on sites highly contaminated by risk elements in the soil or/and air, then it could mean a potential and significant source of uptake into the human body *via* diet. Some previous studies (Turkdogan et al., 2002) shown that elevated concentrations of trace elements such as copper, cobalt (Co), cadmium, lead, zinc, manganese (Mn), nickel (Ni) in the diet can cause the occurrence of some cancers, especially those invading the digestive tract. We can predict that the increased levels of some risk elements are carcinogenic for human gastrointestinal system. The main task of this study is to estimate and assess the risk of growing lesser known types of vegetable for own consumption on the contaminated sites.

2. MATERIALS AND METHODS

In pot experiment were grown 3 lesser common types of vegetable namely savoy cabbage (*B. oleracea convar.* L. var. Acephala), chervil (*A. cerefolium* L.) and swede turnip (*B. napus* L. var. Napobrassica) for comparison was grown lettuce (*Lactuca sativa* L.). This well-known and in pot experiment frequently used plant seems like an ideal reference plant for this study. All plants were grown in soils - Kutná Hora and Příbram, respectively. Both this soils derive from anthropogenically highly contaminated areas. After harvesting the plants were dried at 70 °C, homogenized and prepared for following analyses.
The „pseudo-total“ concentrations of elements in the soils were determined in the digests obtained by the following decomposition procedure: Aliquots (~ 0.5 g) of air-dried soil samples were decomposed in a digestion vessel with 10 ml of *Aqua Regia* (*i.e.* nitric and hydrochloric acid mixture in ratio 1+3). The mixture was heated in an Ethos 1 (MLS GmbH, Germany) microwave assisted wet digestion system for 33 minutes at 210 °C. After cooling, the digest was quantitatively transferred into a 25 ml glass tube, topped up by deionized water, and kept

at laboratory temperature until measurement. A certified reference material RM 7001 Light Sandy Soil was applied for the quality assurance of analytical data. Plant samples were decomposed by using the dry ashing procedure as follows: An aliquot (~ 1 g) of the dried and powdered aboveground biomass or roots were weighed to 1 mg, placed into a borosilicate glass test-tube and decomposed in a mixture of oxidizing gases (O_2 + O_3 + NO_x) at 400 °C for 10 hours in a Dry Mode Mineralizer Apion (Tessek, Czech Republic). The ash was dissolved in 20 mL of 1.5 % HNO_3 (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until the analysis (Miholová et al. 1993). The concentrations of elements in soil and plant digests were determined by optical emission spectroscopy with inductively coupled plasma (ICP – OES) with axial plasma configuration (Varian VistaPro, Varian, Australia), equipped with an autosampler SPS – 5. For the determination of low arsenic concentrations in soil extracts and plant digests, hydride generation atomic absorption spectrometry (VARIAN AA280Z, Varian, Australia), equipped with a continuous hydride generator VGA – 77. Data analyses were done by SAS 9.2 software (SAS Institute Inc., USA).

3. Results and discussion

According to the public notice (Anonymous, 1994) the element concentrations in soils cannot exceed a maximum of 30 mg kg^{-1} As, 1.0 mg kg^{-1} Cd, 140 mg kg^{-1} Pb and 200 mg kg^{-1} Zn. The total arsenic and cadmium concentrations reached the legislation threshold limit in both investigated soils. Elevated concentrations of zinc and lead were determined in the soil from the Příbram location. The uptake comparison of risk elements into plants was carried out according to the soil in which plants were grown and the type of plant as well. We used two soils with different physico-chemical properties (see Table 1). The both derived from highly contaminated areas. Another variable was the type of plant. We focused on the dependence of total contents of risk elements in edible tissues in four chosen plants (see Table 2). The total contents of As in the plants tissues ranged from 1.86 mg kg^{-1} (lettuce) to 33.61 mg kg^{-1} (chervil), for Cd from 1.01 mg kg^{-1} (swede turnip) to 36.80 mg kg^{-1} (chervil) for Pb of 0.78 mg kg^{-1} (swede turnip) to 130.42 mg kg^{-1} (chervil), Zn from 57.36 mg kg^{-1} (swede turnip) to 269.27 mg kg^{-1} (chervil).

Table 1: Physico-chemical soil properties

soil	As	Cd	Pb	Zn	Ca*	Mg*	K*	P*	KVK	C_{ox}	pH
KH	473	13.8	67.8	156	2296	76.7	62.2	69.5	148	4.0	7.4
P	36.0	5.7	822	267	7246	513	758	50.5	123	1.9	4.5

KH – Kutná Hora, P – Příbram, concentrations of As – KVK are given in mg kg^{-1}, C_{ox} in %
*available element contents determined according to Mehlich III soil extraction procedure (Mehlich, 1984)

Table 2: Total contents in edible part of plants

soil	plant	As	Cd	Pb	Zn
KH	lettuce	4.48±1.46	3.30±0.99	1.16±0.18	98.7±10.9
	chervil	33.6±6.3	3.12±0.51	2.32±0.55	144±9
	swede turnip	6.70±1.28	1.01±0.22	0.78±0.10	57.4±7.8
	savoy cabbage	2.44±0.70	2.01±0.91	1.43±0.34	146±30
P	lettuce	1.86±0.54	33.5±9.2	13.6±4.3	177±65
	chervil	11.9±3.4	36.8±6.2	130±12	269±19
	swede turnip	4.93±2.67	11.6±4.9	71.6±11.3	216±86
	savoy cabbage	2.44±0.53	12.4±0.9	15.6±3.2	185±6

KH – Kutná Hora, P – Příbram, concentrations and standard deviations are given in mg kg^{-1}

Effect of soil and different physico-chemical properties was not clearly demonstrated. Although some earlier studies (Jedynak et al., 2008; Kabat-Pendias et Pendias, 2001) show that total elements contents, pH value and organic carbon in soil play a role in plant uptakes. In our case, however, the dominant factor determining the elements uptake of plants was the content of these elements in soils. The plant element contents reflected the elevated element contents in soils but the element translocation within the plants was affected by individual elements as documented by an example of savoy cabbage in the Figure 1. Regardless of the soil, higher accumulations of Cd and Zn in leaves in contrast to dominant accumulation of As and Pb in roots were confirmed. However, higher percentage of individual elements translocated to aboveground biomass was observed in plants growing in the soilwith higher content of these elements.

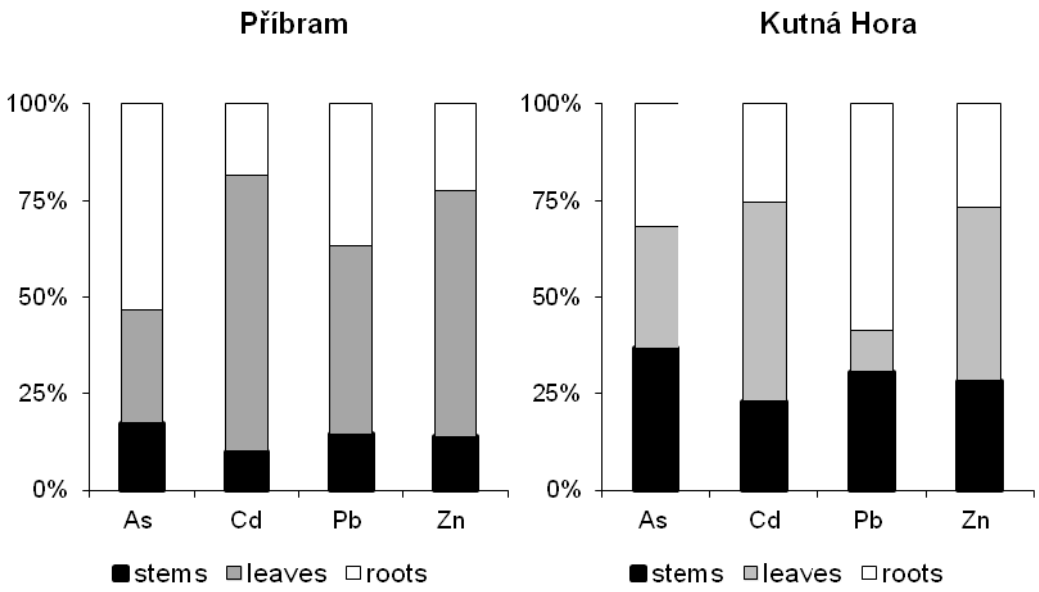

Figure 1: Element distribution within the savoy cabbage (*B. oleracea* L. convar. Acephala) plants.

Effect of plant species was clearly demonstrated. The similar results are shown in the papers of Chen et al. (2004) and Li et al. (2006) that conducted analogous study on ten different crop plants. The capability of risk elements accumulation, especially of Cd, Pb and Zn, is significant particularly for chervil. On the other hand it seems that the minimum accumulation capacity has a swede turnip. In this paper we compare the edible parts of plants that might be physiologically different. According to Tlustoš et al. (2002) different plants can accumulate different amount of these elements in various plant tissues depending on plant species metabolism. The ability to uptake and to accumulate risk elements in four chosen crop plants can be generalized as follows, in decreasing As content in the plants *A. cerefolium* > *B. napus* > *B. oleracea* convar. Acephela ~ *L. sativa*, for Cd as follows *A. cerefolium* > *L. sativa* > *B. napus* ~ *B. oleracea* convar. Acephela, for Pb *A. cerefolium* > *B. napus* > *B. oleracea* convar. Acephela ~ *L. sativa*, for Zn *A. cerefolium* > *B. oleracea* convar. Acephela > *B. napus* ~ *L. sativa,* respectively. The ability uptake for single elements declined in the order Cd > Zn > Pb > As. This trend is confirmed by study of Sipter et al. (2008), which also dealt with risk elements uptake into vegetable grown on contaminated sites. The total content calculated on a fresh mass in most cases exceeded the EU Commission Regulation No. 1881/2006, therefore plant selecting and plant growing should carry out with good knowledge of risk elements levels in the soil.

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THE EFFECTIVITY OF VARIOUS EXTRACTION AGENTS TO RELEASE MERCURY FROM ANTHROPOGENICALLY CONTAMINATED SOILS

Adéla Šípková[1], Jiřina Száková[1], Pavel Coufalík[2], Pavel Tlustoš[1]
sipkovaa@af.czu.cz
[1] Department of Agroenvironmental Chemistry and Plant Nutrition, Czech University of Life Sciences, Kamýcká 129, 165 21 Prague 6, Czech Republic
[2] Department of Chemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic

ABSTRACT

The potential bioavailability of the Hg from the soil might by characterized by variety of chemical processes, differing in the extraction agent, its concentration, the sample weight or the time of extraction. In this study, a comparative analysis of several extraction methods, commonly used for obtaining the mobile phase of the mercury from anthropogenically contaminated soils, was carried out. The aim was to estimate the rate of mercury sorption by soil, especially by its organic matter. Concentrated HNO_3, 0.01 M $Na_2S_2O_3$, 0.05 M EDTA and 0.11 M CH_3COOH were used as extraction agents. The inductively coupled plasma mass spectroscopy (ICP-MS) was used to estimate the mobile phase of the mercury within each extract and the Advanced Mercury Analyzer (AMA-254) for the determination of total Hg, respectively. The results showed that even strong acid HNO_3 is unable to release the mercury tightly bound to the soil matrix. This particular method with microwave digestion is commonly used for the estimation of this type of anthropogenic pollution. Conversely, the lowest mercury yield was obtained using the acetic acid. In all experiments, the concentrations were below 0.15 % of the total Hg content, which is a proportion generally defined as biologically available to plants.

KEY WORDS: mercury, contaminated soil, single extraction, inductively coupled plasma mass spectroscopy, Advanced Mercury Analyzer AMA-254

1. INTRODUCTION

Soil is a heterogenous system containing colloid inorganic and organic matter in the form of small particles, water, and various gases. The natural origin of mercury depends particularly on the character of bedrock (Závadská et al., 1999). The Hg concentration in uncontaminated soil does not usually exceed 200 $\mu g.kg^{-1}$ (Adriano, 2001). If the concentration is higher, the source of pollution is the point of interest. The anthropogenic sources may result from the proximity of the mining industry or metallurgy, agriculture, sewage sludge treatment or incinerators, and also landfills (Lasat, 2002). In the Czech Republic, the permissible content of the harmful substances in the soil are determined by the directive of Ministry of the Environment no. 13/1994. The maximum acceptable Hg content is 800 $\mu g.kg^{-1}$; however, the concentration of mercury in anthropogenically contaminated soils can be substantially higher. For instance, according to Kacálková et al., 2009 the mercury content near the former waste incineration plant in Hradec Kralove, Czech Republic, was found to be up to 12 $\mu g.g^{-1}$.
Mercury can be released from soil by different extraction procedures. These procedures enable the determination of particular species present, the varying amounts of Hg bound to the soil, and also the bioavailability or toxicity. Numerous of these procedures are described in the literature. The least tightly bound water-soluble fraction of mercury is obtained by the simple extraction using deionised water (Séguin et al., 2004, Rodrigues et al., 2010). It represents mercury present in pore water in soil. This form of mercury is usually not in the form of the water-soluble ionic species but as a species bound to organic matter; nevertheless, not directly on carbon (Biester and Scholz, 1997). Biologically available mercury is further released into the soil solution. The extraction with CH_3COOH is commonly used to simulate the approximate composition of this solution. Beside this most common approach other extraction agents, such as $CaCl_2$ solution, might be used (Novozamsky et al., 1993). The extraction solutions based on the chelating agents such as EDTA or DTPA represent another more efficient possibility. These extraction agents simulate well the interaction of plant´s roots with the dissolved organic acids, which further leads to the dissolution of some forms of particular elements, which are not originaly present in the soil solution (Cibulka, 1991).
In the soil, mercury can be bound very tightly to the sulfur forming the insoluble HgS. The portion of this mercury bound to sulfide may be up to 60 % of the total Hg content (Boszke et al., 2008). This phase of mercury can be obtained either by aqua regia extraction in a microwave oven (Fernándes-Martínez and Rucandio, 2003), or using the saturated Na_2S solution from the residue remaining in the second stage (Revis et al, 2003). Extraction with $Na_2S_2O_3$ is used for speciation analysis, because it is causing no Hg transformations. Issaro et al. (2010) studied the effect of the concentration of sodium thiosulphate on the extraction efficiency. The $Na_2S_2O_3$ is also used for increasing the Hg availability for fytoextraction methods (Moreno et al., 2005).

The proportion of Hg, which is not firmly bound in the silicate matrix of the soil, is often obtained by using HNO_3 as an extraction agent (Reis et al., 2010). The mercury concentration in these extracts enables to estimate the amount of Hg from anthropogenic sources. It might also be used in sequential extractions to obtain elemental mercury (Bloom et al., 2003, Coufalík et al., 2011). In this work, four various extraction agents were applied to assess Hg mobility in anthropogenically contaminated soils.

2. Materials and Methods

2.1 Samples

Ten soil samples were collected from the former waste incineration plant in the suburb of Hradec Kralove, Czech Republic. The hazardous waste containing: oil, agricultural waste, preservatives, waste from chemical processes and percolation, degreasing waste containing solvents, waste containing metals, halogens, sulphur, dyes, fertilizers, pesticides and others were burnt there between the years 1993 and 2002. This hazardous waste was stored without protection during the running period of the incinerator (Kacálková et al., 2009).

2.2 Total and extractable portions of mercury

Total contents of mercury were determined, without chemical pretreatment of the samples, by thermal decomposition atomic absorption spectroscopy (AAS) with gold amalgamation (LECO model AMA-254), a rapid total mercury determination method.
Four extraction reagents HNO_3, $Na_2S_2O_3$, EDTA, and CH_3COOH were used to determine the mobile and mobilizable phases of Hg. For determination of potentially mobilizable mercury portions, 0.25 g of each sample was decomposed in 5 ml of HNO_3. The reaction mixture was digested at 280°C during 75 min by using microwave heating in MLS ultraCLAVE IV system (Milestone, Germany) and then milli-q water was added to a final volume of 50 ml.
The mild extraction procedures were performed as follows: i) Sodium thiosulfate extraction proceeded overnight in 10 ml of 0.01 M solution, which was added to 1 g of the sample. ii) 0.05 M EDTA was adjusted with NaOH to pH 7. Subsequently, 1 g of soil was added to 10 ml of extraction solution and shaken for 1 h. iii) 0.5 g of sample was added to 10 ml of 0.11 M solution of CH_3COOH and shaken overnight. Subsequently, all the the samples were centrifuged for 10 minutes at 3000 rpm, filtered and the extract was acidified by the mixture of acids (HNO_3 : HCl = 4 : 1). Mercury content in all extracts was measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., USA).

3. Results and discussion

The measurements of the total Hg content indicate that in all ten samples from the vicinity of the former waste incineration plant in Hradec Králové there are places where the concentration of mercury is relatively low; however, sites with above limits concentrations are present as well. In the most polluted sample the amount of mercury reaches almost 29 $mg.kg^{-1}$, which exceeds the required limit 36 times. Kacálková et al. (2009) report the same variability of the mercury content present in the same area showing the concentrations ranging from 0.15 to 12 $mg.kg^{-1}$.
The extraction yields by individual extraction agents are shown in Table I representing the total amount of mercury and also the its rate to total Hg content. The nitric acid proved to be the most efficient extraction agent. Concentrations of Hg range between 48 % and 56 % of total content in 8 of 10 samples using nitric acid. In the case of the most contaminated sample the concentration was approximately 70 %. In the last sample, where the total content exceeded the limit 13 times, the extraction yield was using nitric acid was almost 96 %. The mercury concentration obtained by this extraction method should correspond to the rate of anthropogenic contamination.
Using $Na_2S_2O_3$ as an extraction agent the mercury yield was also highest in two last samples. The average yield of these particular samples attains approximately 20 %. In other samples, the content of mercury ranged from 1.2 % to 3.4 %. Thus, it might be inferred that in places with higher anthropogenic contamination, the presence of mercury species bound to sulfur is significantly higher than in less contaminated samples. In the case of the most contaminated site the rate of extractable Hg using $Na_2S_2O_3$ corresponds with the results reported by Issaro et al. (2010). They showed that the extraction yield of $Na_2S_2O_3$ usually reaches 50 ± 5% of mercury obtained by nitric acid. Moreover, they showed that the rate of extractable Hg by sodium thisulphate is decreasing with decreasing total Hg content. On the other hand, Subiréz-Munoz et al. (2011) obtained by this type of extraction only approximately 20 % of the total Hg content.

Table 1: Extractable contents of mercury after individual extraction (μg.kg^{-1}).

Sample	total	HNO_3	$Na_2S_2O_3$	EDTA	CH_3COOH
1	1070	578	14.3	6.92	0.493
2	236	132	7.04	1.99	0.339
3	415	201	7.82	5.21	0.373
4	419	234	8.03	8.80	0.480
5	550	273	10.4	6.27	0.450
6	2050	1132	52.1	9.37	0.727
7	396	223	13.5	2.95	0.436
8	580	300	6.95	2.94	0.784
9	28800	20108	5877	481	11.3
10	10500	10040	2094	64.7	8.64

Further, using chelating agent EDTA, the values of extractable Hg ranged between 0.46 % and 2.1 % of the total Hg content, in all experimental samples. These results correspond to those reported by Subiréz-Munoz et al. (2011) who obtained less than 2 % of the total Hg using the same 0.01 M EDTA solution. This small variability suggests that the amount of mercury bound to organic matter, which might serve as a source for plant uptake, is similar both in more and less anthropogenically contaminated places. Coinciding results were also obtained by extraction with a solution of CH_3COOH, which simulates natural conditions of soil solution. The yields of CH_3COOH confirmed no significant differences between more and less contaminated samples. However, contrastingly to the EDTA, the average mercury yield was below 0.15 % of the total Hg.
The obtained results are also shown in Fig. 1. The mercury concentrations of individual samples are plotted in log-normal scale due to its comparability, because the differences between the various extraction agents are in order of several orders. The graph illustrates the differences between the Hg concentration of the majority of samples and the two most contaminated sites using nitric acid and sodium thiosulphate. While in the case of extractions with EDTA and CH_3COOH, the differences between samples from various sites are not reaching up to these vales.

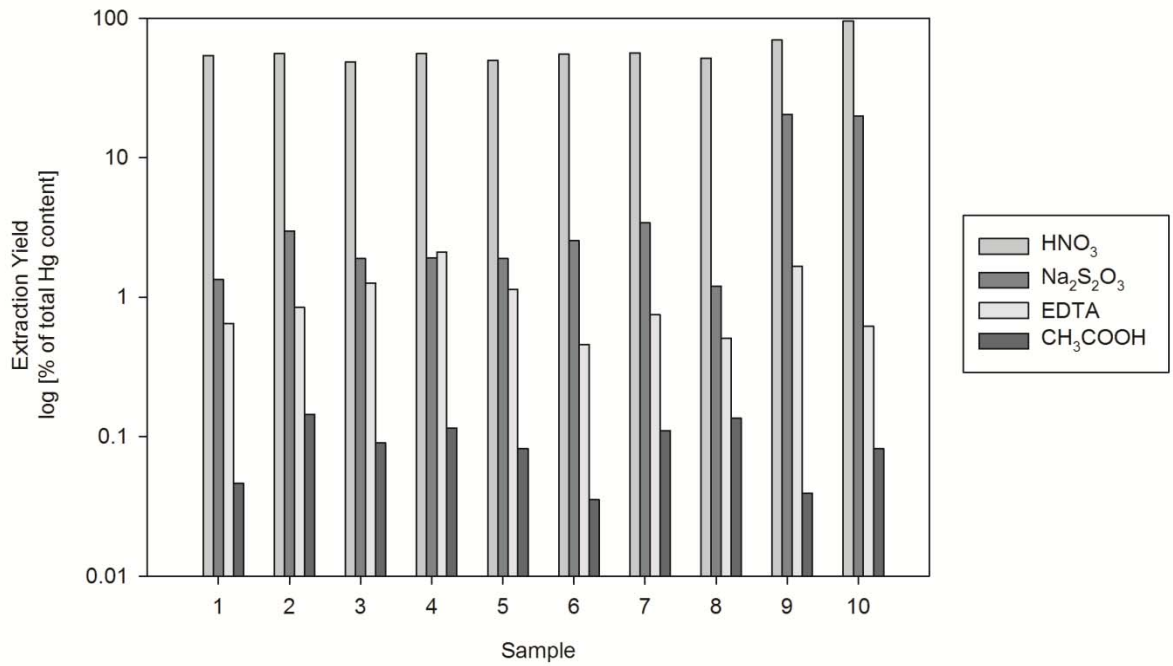

Figure 1: Extraction yield using individual extraction agents.

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PHYTOREMEDIATION AND RHIZOREMEDIATION

IMPROVING THE PHYTOREMEDIATION POTENTIAL OF PLANTS USING ENDOPHYTIC BACTERIA: CULTIVABLE BACTERIAL COMMUNITIES ASSOCIATED WITH TWO MONOCOTYLEDONOUS PLANTS USED FOR WASTE TREATMENT

Guiomar Garcia-Cabellos Dunne*, Elaine Keogh*, Clare Menton, Dina Brazil, David Ryan, Kieran J. Germaine and David N. Dowling
David.Dowling@itcarlow.ie
Institute of Technology Carlow, Department of Science & Health, Kilkenny Road , Carlow, Ireland

* Both authors contributed equally to this work

ABSTRACT

This study investigated the culturable-aerobic bacterial diversity associated with two plants species (*Miscanthus giganteus* and *Iris pseudacorus*) that were being used in waste treatment systems. *Miscanthus* and *Iris* bacterial isolates (163) were isolated and identified through 16S rDNA sequencing. The majority of these isolates were gamma-Proteobacteria. *In Miscanthus, Pseudomonas* and *Acinetobacter* species dominated the isolate collection while in *Iris, Microbacterium, Enterobacter, Pseudomonas* and *Rahnella* were dominant. Many of the isolates were found to possess resistance to heavy metals and could degrade organic xenobiotics. These isolates may prove to be useful inoculants for improving plant biomass and phytoremediation efficiency within *Miscanthus* and *Iris* plants.

KEY WORDS: *Miscanthus, Iris*, bacteria, endophytes, phytoremediation

1. INTRODUCTION

The application of wastewaters or bio-solids to renewable energy crops may provide a practical solution to tertiary wastewater treatment and provide an economically viable biomass crop for the renewable energy industry. However, the presence of toxic chemicals such as heavy metals and volatile organic compounds (VOCs) within waste streams can have a negative effect on plant biomass production. Biomass production and phytoremediation capacity can be increased though the use of plant associated bacteria. Many rhizospheric and endophytic bacteria possess plant growth promotion traits, are resistant to heavy metals and are capable of degrading a range of organic pollutants (Doty et al., 2009; Aravind et al., 2010). There are many reports on the use of such bacteria to enhance biomass production and increase phytoremediation ability of various different plants (Andria et al., 2009; Mastretta et al., 2009; Sun et al., 2010). *Iris pseudacorus* (or flag Iris) is a popular plant of choice in constructed wetlands. It is one of the fastest growing plants used in wetland systems, producing more biomass, inducing higher microbial activity and greater contaminant removal ability than other wetland plants (Camacho et al., 2007). *Miscanthus giganteus* is a vigorous perennial grass that reproduces vegetatively by rhizomes and has potential as a bio-energy crop and as a phytoremediation plant (Arduini et al., 2006; Zub and Brancourt-Hulmel, 2010). Davis et al., (2010) modelled greenhouse gas (GHG) emissions from agricultural production of a number of bioenergy crops and found that Miscanthus gave the greatest GHG reduction. The aims of this work was to gain a better understanding of the culturable bacterial communities associated with *Iris* and *Miscanthus* plants used for waste treatment, with the objective of isolating strains that could be used to enhance plant growth and phytoremediation potential.

2. MATERIALS AND METHODS

2.1 Source of plants

Miscanthus (*Miscanthus sinensis* x *giganteus)* plants were collected from the Teagasc site in Carlow, Ireland, from plots treated with brewers waste. Iris (*Iris pseudacorus*) plants were collected from a constructed wetland system located in Kilmeaden, Co. Waterford, Ireland, used to treat wastewaters from a dairy factory.

2.2 Isolation and identification of endophytes

Bacteria from the leaves, stems, rhizomes and rhizosphere were isolated as described previously (Germaine et al., 2009). The bacterial isolates were sent to MIDI laboratories (USA) for identification based on partial (500bp) 16S rDNA sequencing and comparison to sequences in the 16S rDNA MicroSeqTM Database. Sequences were

deposited in the National Centre for Biotechnology Information (NCBI) Genbank under accession numbers GU726490-GU726589 (*Iris* isolates), and HM102445- HM102499 (*Miscanthus* isolates). Phylogenetic trees of the 16S rDNA sequences were constructed using the CLC DNA Workbench software programme (Software Labs, UK) by first performing a multiple sequence alignment and then creating a tree using the UPGMA algorithm with 1000 bootstraps.

2.3 Characterisation of endophytes

Bacterial isolates were tested for their tolerance to various concentrations of heavy metals. The metals tested were copper, zinc and cadmium. The bacterial strains were streaked onto nutrient agar plates containing 2, 3 or 4 mmol l-1 of the heavy metal and incubated at 30°C for 72 hr. To test whether the isolates could utilize toluene, naphthalene or biphenyl as a sole carbon and energy source, they were streaked onto minimal media agar plates (Abraham et al., 2002) with these as the sole carbon source. The plates were sealed using parafilm, incubated at 30°C for 72 hr.

3. Results and discussion

3.1 Phylogenetic relationship of endophytes and their distrubution *in planta*

Endophyte isolates (63) were identified from *Miscanthus* plants (28 from inside the leaf tissue, 14 from inside the stems and 21 from inside the rhizome) using 16S rDNA gene sequencing and ten different genera were identified. The identifications are indicated in Figure 1B which shows their phylogenetic relationship. *Pseudomonas, Acinetobacter, Enterobacter* and *Pantoea* were the most dominant genera at 56%, 21%, 6% and 6% respectively. *Pantoea* were found in both the leaf and stem tissue and Enterobacter were found in both the stem and rhizome tissue. Each of the other seven genera were exclusive to one compartment of the plant (Figure 2 A). In total, ~96% of the leaf endophytes isolated were from phylum Proteobacteria (class gammaproteobacteria) and the remaining 4% were Firmicutes. A total of 100 isolates were isolated from *Iris pseudacorus* plants (6 from the phyllosphere, 22 from inside the leaf tissue, 52 from inside the rhizome and 20 from the rhizosphere) and seventeen different genera were identified in total. Figure 1B shows the phylogenetic relationship among these isolates. *Enterobacter, Microbacterium, Rahnella and Pseudomonas* were the most dominant genera isolated. *Bacillus, Microbacterium, Pseudomonas* and *Stenotrophomonas* were common genera to all three compartments of *Iris* (rhizosphere, rhizome and leaf) (Figure 2B), whereas *Ochrobactrum* was unique to the rhizosphere.
The diversity of the culturable endophytes isolated from both plants was biased towards gammaproteobacteria (95% of *Miscanthus* isolates and 69% of Iris isolates). Similar results were shown by Porteous-Moore et al., (2006) where 60% of the culturable endophytes isolated from willow and poplar were found to be from the gammaproteobacteria. Li et al., (2010) created a 16S rDNA library from DNA extracted from the roots of the wetland plant *Phragmites australis*. 79% of the clones in this library were Proteobacteria and included all five classes. Only three of their genera (*Pseudomonas, Enterobacter* and *Janthinobacterium)* were common to those identified in this study from *Miscanthus* and *Iris*.

3. 2 Characterisation of endophytic isolates

The bacterial isolates from both Miscanthus and Iris were characterised with respect to their heavy metal resistance. For the *Miscanthus* isolates, 17% of leaf endophytes, 29% of stem endophytes and 20% of rhizome endophytes had a Minimum Inhibitory Concentration (MIC) value of 4mM copper. None of the *Miscanthus* isolates were resistant to cadmium even at the lowest concentration tested. When the *Miscanthus* endophytes were tested for zinc resistance 10% of rhizome, 7% of stem and 0% of leaf endophytes were resistant to 4mM zinc. Bacterial isolates from *Iris* displayed a similar pattern of resistance to heavy metals, although the number of heavy metal resistant isolates was higher in each of the plant compartments. 85% of the isolates were resistant to 4mM copper, 49% to 4mM zinc and only 5% of the isolates were resistant to 4mM cadmium.
There are numerous reports detailing the isolation of endophytic bacteria that are resistant to a range of heavy metals (Idris et al., 2004; Mastretta et al., 2009). Sun et al., (2010) found that inoculation of *Brassica napus* with Cu resistant endophytes significantly increased plant biomass and the levels of copper (63 to 125%)

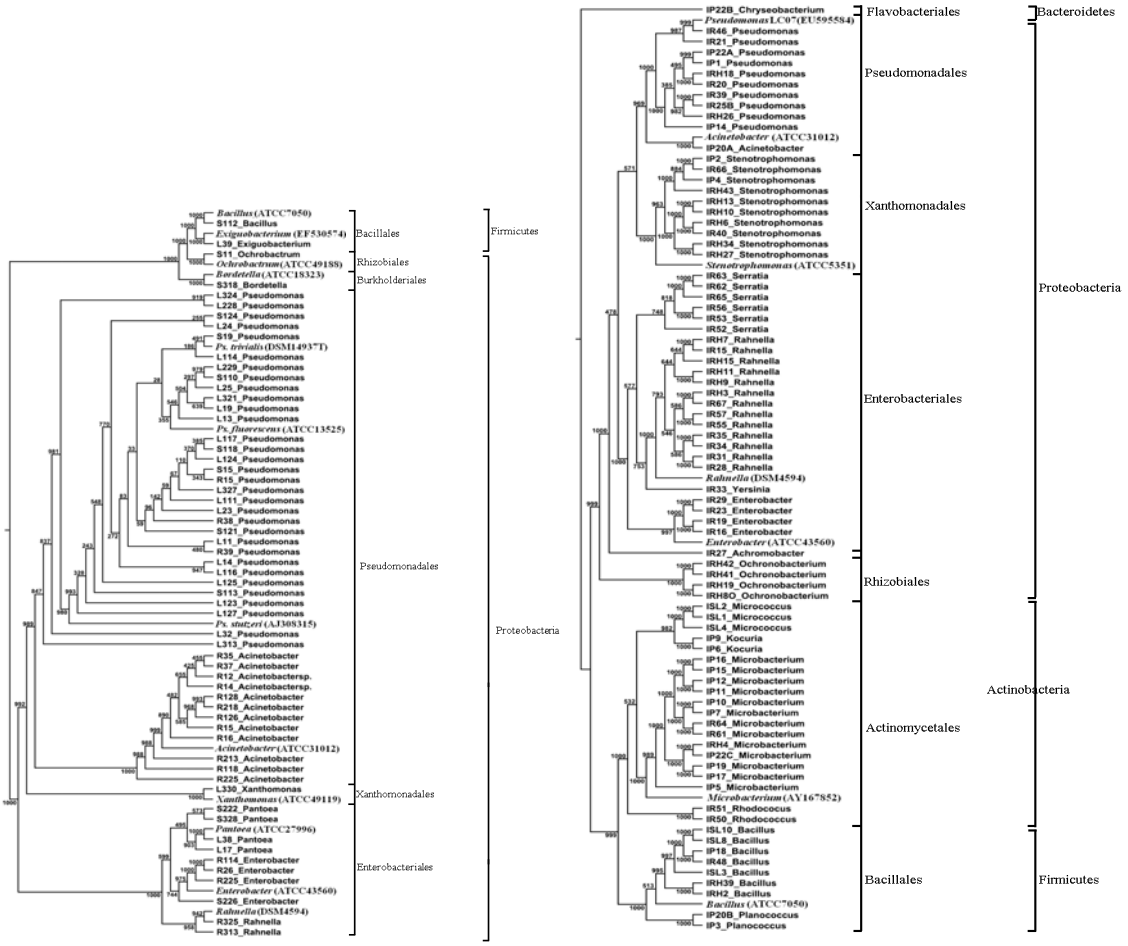

Figure 1. Phylogenetic relationship among the bacterial isolates from (A) *Miscanthus* and (B) *Iris*

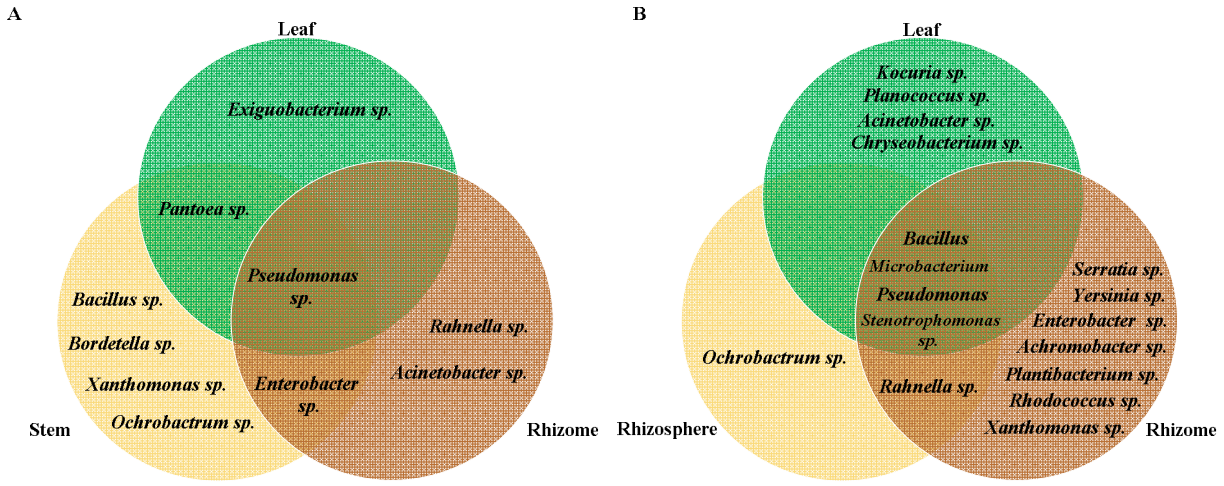

Figure 2. Distribution of bacterial taxa between the compartments of (A) *Miscanthus* and (B) *Iris* within the shoots compared to the un-inoculated controls. In the current study a high prevalence of resistance to

copper and zinc was observed in the endophytic isolates from both *Miscanthus* and *Iris* plants. These strains may prove useful for enhancing heavy metal removal from wastewater and sludges using *Miscanthus* or *Iris* plants.

The bacterial isolates from both *Miscanthus* and *Iris* were also tested for their ability to utilise organic xenobiotics as a sole carbon and energy source. Of the isolates from *Miscanthus* 5% could utilise naphthalene, 5 % toluene and 5 % biphenyl as a sole carbon source. Four strains (L39, S323, R315 and R318) were found to utilise all three organic compounds. 5% of the isolates from *Iris* plants could utilise naphthalene, and 4% could utilise toluene as a sole carbon source. None of these isolates could use biphenyl. Many endophytic bacteria with inherent organic xenobiotic degrading abilities (including oil, diesels, BTEX, PAHs, aliphatic hydrocarbons and nitroaromatics) have been isolated (Phillips et al., 2008). The intimate nature of the plant-endophyte relationship, provides a unique opportunity to utilise endophytic bacteria to complement the metabolic potential of their host plant thus improving phytoremediation (Dowling and Doty, 2009). Seven bacterial isolates from *Iris* and 38 from *Miscanthus* could utilize naphthalene, toluene or biphenyl as a sole carbon source and therefore may have potential for improving the phytoremediation of these xenobiotics. They may also have the potential to improve biomass production in *Miscanthus*, and may further the possibility of combining both bioenergy crop production with waste water or sludge treatment.

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SCREENING OF PLANTS – PHYTOREMEDIATORS RESISTANT TO OIL POLLUTION

Togzhan D. Mukasheva, Lyudmila V. Ignatova, Ramza Zh. Berzhanova, Raihan K. Sydykbekova, Anel A. Omirbekova, Dinara Dautova
Togzhan.Mukasheva@kaznu.kz
Al-Farabi Kazakh National University, Faculty of Biology and Biotechnology, 71 Al-Farabi street, 050038, Almaty, Kazakhstan

ABSTRACT

Selection of plants – phytoremediators resistant to various concentrations of oil was carried out among 12 species of cereals, legumes, cruciferous and grass mixture consisting of red fescue, ryegrass and Kentucky blue grass. It was established that oil at a concentration of 2 to 5% in most cases only slightly affected the germination energy and germination rate of plant seeds. Among the Poaceae family seed germination in the presence of oil has decreased from 8% to 20%. The plants of the Brassicaceae family showed a slight decrease in germination within 3-12%. The most sensitive plants were among Legume family. Mixture showed high germination energy and germination of seeds.Oil in low concentrations exerted no inhibitory effect on the length of underground and aboveground parts of most plants. With increasing concentration of oil, a decrease in the length of the underground and above-ground parts in cruciferous plants was by 50%. Resistance showed barley, rape and alfalfa and grass mixture, regardless of the concentration of oil. Thus, the identified plants that can be used as phytoremediators at the final stages of the remediation of contaminated soils.

KEY WORDS: phytoremediators, resistance, oil, energy of germination, rate of germination

1. INTRODUCTION

The problem of environmental pollution by oil and oil products is actual for Kazakhstan as oil production and processing is one of the major industries in the country. As a result of oil production, refining, oil transportation, accidents at oil pipelines, entering of oil hydrocarbons in natural objects take place. For cleaning of contaminated surfaces a variety of natural technologies, including phytoremedition, as biological method of remediation of contaminated soils using plants and rhizosphere microorganisms can be used (Glick et al, 2003). Today, there are numerous experimental data that demonstrate a number of protective mechanisms that plants have, by which they resist the toxic action of foreign substances. They include excretion; conjugation of toxic compounds with intracellular compounds, degradation of toxicants to standard cellular metabolites and carbon dioxide. The importance of phytoremediation increases in final stages of soil purification, when the rate of degradation is slowing down, the number of heterocyclic compounds and aromatic structures is increasing. The main advantages of phytoremediation are the possibility of large areas reclaiming, relatively low cost compared with other technologies, high efficiency and no negative impact on the environment.
Oil pollution is toxic to plants at relatively low concentrations. Survival of plants in contaminated soil contributes to the rhizosphere microbial community due to its catalytic activity and phytotoxicity reduction of pollutant in the process of degradation. Use of plants suitable for the remediation described in many papers. For determination of characteristics of plants growth involved in the reduction of oil content in the soil, the studies used different groups of plants – grasses, woody plants, monocotyledonous and dicotyledonous plants and plants of different familes: Fabaceae (Ogbo, 2009), Poaceae (Kireeva et al, 2006), Brassicaceae (Dominguez-Rosado, 2004).

2. MATERIALS AND METHODS

2.1 Plants

The plants used were: Fabaceae – pea (*Pisum sativum* L.), red clover (*Trifolium pretense* L.), alfalfa (*Medicago sativa*), beans (*Phaseolus vulgaris*); Poaceae – corn (*Zea mays* L.), (*Agrostis stolonifera* L.), wheat (*Triticum aestivum*), barley (*Hordeum sativum.*); Brassicaceae – mustard (*Sinapis alba*), rape (*Brassica napus biennis*), radish (*Raphanus sativus*); grass mixture (red fescue – 75%, ryegrass – 20% and Kentucky blue grass – 10%).

2.2 Assessing the impact of oil on seed germination and the length of overground and underground parts of seedling

The experiment was conducted under laboratory conditions in petri dishes. Pre-seeds were sterilized 10% solution of sodium hypochloride for 30 min, and then washed several times with distilled water. Petri dish covered with filter paper and introduced as a control either distilled water or an aqueous emulsion of oil, the rate of final concentrations of oil - 2, 3, 5% (Amakiri, Onofeghara, 1984). In each dish 10 seeds of each plant were laid out. The seeds were germinated at 23 ° C. The duration of was 7 days. To assess plant resistance to oil following parameters were counted: germination energy, germination rate, length of overground and underground parts of the seedling.
Germination energy that characterizes the speed of germination is a percentage of germinated seeds at the time of the most energy intensive growth of seed germination and was calculated as the percentage of seeds germinated in half of the time required to calculate the germination rate of seeds (the period was 3.5 days). Germination rate was determined on 7day of seed germination (Hartmann, 2002).

3. Results and discussion

3.1 Germination energy of seeds in the presence of oil

The use of plants as bioassays allows to determine the effect of pollutant on plant growth and the level of phytoremediation process (Varun et al, 2011). One of the indicators in the evaluation of phytotoxicity of the soil for plants is the energy of germination. In assessing the impact of oil on the germination energy of plants tested, the little effect of oil at a concentration of 2% was showed . At a concentration of 3 and 5%, the reduction in seed germination energy was observed. The high values of germination energy of seeds were showen by mustard, barley, alfalfa, radish and grass mixture. So, mustard, alfaalfa and barley sprouted over 50% of the seeds compared to the control, the radish and grass mixture of more than 36% regardless of the oil concentration.

Table 1. Germination energy of seeds in the presence of oil

Plants	Control	Concentration of oil,		
		2	3	5
	Germination energy, %			
Pea	60	42,5	32,5	15
Red clover	55,5	30	7,5	5
Alfaalfa	80	75,5	67,5	55,5
Bean	60	47,5	20	7,5
Corn	47,5	22,5	8,5	2,5
Wheat	52,5	32,5	20	5
Bent	40	15	7,5	5
Barley	70	67,5	52,5	47,5
Mustard	90	75	57,5	50
Rape	60	57,5	37,5	22,5
Radish	90	67,5	50	37,5
Grass mixture	62,5	47,5	50	37,5

The lowest energy of germination was observed for clover, maize and bent; the figure was within the range of 2, 5 to 7, 5%.

3.2 Effect of different concentrations of oil on the germination rate of seeds

Along with the energy of germination, plant resistance to the effects of pollutant can be judged by indicators of germination rate of seeds. Increase or decrease of the number of germinated seeds is a parameter that affects whether the oil affect on the morphological characteristics of plant growth (Varun, 2011). Good germination rates were also found in mustard, alfalafa, barley and grass mixture. During oil concentration increasing a significant reduction in the number of germinated seeds was not observed, decreasing was not observed either. These data demonstrate the stability of these plants to the action of oil.

Table 2: Indicators of germination rate of seeds under the oil influence

Plants	Control	Concentration of oil %		
		2	3	5
	Germination rate, %			
Pea	90	60	50	32,5
Red clover	70	55	22,5	15
Alfaalfa	90	90	85	77,5
Bean	87,5	72,5	32,5	12,5
Corn	60	37,5	20	5
Wheat	87,5	60	30,5	20
Bent	72,5	27,5	10	5
Barley	90	87,5	72,5	67,5
Mustard	100	87,5	77,5	75
Rape	100	82,5	55,5	37,5
Radish	100	85	70	42,5
Grass mixture	85	80	72,5	62,5

Oil inhibited the growth of some seeds of plants, both at 2 and 5%. For example, the bent grass seeds showed low germination rate of seeds, percentage of germinated seeds at concentration of oil was only 2-5% from 27,5 to 5%. This shows the inhibitory effect of crude oil. The same can be said about maize. Different characteristics of germination rate which depended on the concentration of oil were observed.

3.3 Influence of oil concentration on the length of overground and underground parts of plant

After measuring of over- and underground parts of plants, it was shown that the concentration of 2% of the affected oil had no inhibitory effect on the growth of all plants, except of bent grass. At the concentration of 5%, oil ecreased the length f overground and underground parts of most of plants. High rates of shoot and root length of ssedlings have been observed in pea, alfalfa and rape regardless of the concentration. At seven day seedlings of these plants well-developed root system was awarded. So, pea and rape's length of the underground part was equal to approximately 70mm.

Table 3: Characteristics of overground and underground parts of plants measurement under the effect of different concentrations of oil

Plants	Length of overground part, mm				Length of underground part, mm			
	Control	Oil, %			Control	Oil, %		
		2	3	5		2	3	5
Pea	65,8±4,6	60,7±4,2	64,5±4,3	62,7±4,2	72,5±4,5	71,7±4,5	65,8±4,5	64,3±5,6
Red clover	8,5±0,5	8,1±0,4	6,4±0,3	5,9±0,3	12,5±1,1	11,7±1,1	6,5±0,4	4,3±0,2
Alfaalfa	19,6±1,2	19,4±1,2	18,7±1,2	18,3±1,2	15,6±1,1	13,8±0,9	12,2±1,1	11,6±1,0
Bean	34,7±1,5	34,1±1,4	27,5±2,22	19,1±1,8	30,5±2,8	30,1±2,8	21,%±1	13,7±1,2
Maize	32,2±3,1	30,7±2,9	24,5±2,4	20,7±1,9	55,3±5,2	55,5±4,9	49,3±4,2	48,1±3,5
Wheat	22,7±2,0	17,5±1,4	17,2±1,5	11,4±0,8	17,5±1,5	14,3±1,1	14,1±1,1	8,4±0,6
Bent	18,3±1,7	17,6±1,4	13,5±1,1	12,1±1,0	12,5±1,0	10,7±0,9	10,2±0,6	8,6±0,6
Barley	14,6±1,2	14,1±1,2	9,3±0,6	8,1±0,6	13,7±1,1	13,2±1,0	11,4±0,7	10,3±0,8
Mustard	21,4±1,9	20,3±1,8	19,9±1,7	15,6±1,3	28,6±2,5	28,3±2,1	24,7±2,1	21,5±1,8
Rape	47,4±4,6	43,5±4,1	41,7±3,8	47,5±4,3	67,7±5,9	65,4±5,7	66,8±5,9	57,8±4,7
Radish	26,5±2,5	24,1±2,2	23,7±2,1	19,6±1,6	87,2±8,0	77,6±7,2	73,2±6,8	72,1±6,9
Grass mixture	16,3±1,4	15,6±1,3	11,2±0,8	8,7±0,6	14,5±1,1	13,7±0,7	12,9±0,9	11,3±0,7

During the screening of selected plants-phytoremediators, species most resistant to petroleum hydrocarbons, pertaining to different families were selected. They are mustard, alfalfa, rape, pea, grass mixture and barley. They have shown resistance to the oil concentration from 2 to 5%. The highest rates of the length of

undergroundparts of plants in the presence of oil are typical for pea, alfaalfa, rape, mustard and grass mixture.It is likely that these plants can be used to improve the treatment of contaminated soils and soil remediation in the latter stages

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THE EFFECT OF PLANTS AND NATURAL COMPOUNDS ON BACTERIAL POPULATION IN THE CONTAMINATED SOIL

Petra Prouzová, Eva Hoskovcová, Lucie Musilová, Michal Strejček, Ondřej Uhlík, Kateřina Demnerová a Martina Macková
Dept. of Biochemistry and Microbiology, ICT Prague, Technicka 3, Prague 6, Czech Republic, 166 28
petra.prouzova@vscht.cz

ABSTRACT

Phytoremediation and rhizoremediation, the use of plants and their associated microorganisms to remove or degrade contaminants, are promising technologies for the remediation of soil contaminants such as PCB, PAH etc. Many plant-derived chemicals stimulate microorganisms to biodegrade xenobiotics or serve as inducers for bioremediation processes in the contaminated soil.
This study investigates the impact of plants and added natural compounds – caffeic acid, naringin and their mixture as possible PCB degradation inducers on indigenous microbial communities and activity of degrading bacteria. As a model plants tobacco (*Nicotiana tabacum*) and horseradish (*Armoracia rusticana*) were used. After 4 months cultivation soil bacteria were isolated from each soil sample and total amount of bacteria on PCA media and amount of bacteria which are able to grow on minimal media with biphenyl (MM) as carbon source were determined and identified by MALDI-TOF MS. The results showed that plants and caffeic acid and naringin not only affect the growth of bacterial populations, but also their diversity. The horseradish rhizosphere, especially with mixture of caffeic acid and naringin, contains greater variety of cultivable bacteria species than the rhizosphere of tobacco. The total DNA was isolated from soil samples and 16S rRNA gene analysis was performed. DNA-based pyrosequencing was used to characterize the structure of bacterial communities in used contaminated soil. Stable isotope probing (SIP) was used to determine the active members of benzoic acid metabolizing soil bacterial communities.
The changes of PCB concentration in contaminated soil after cultivation were investigated using GC analysis. The positive effect of plants could be seen in all parallels. The PCB decrease was approximately 20 mg/kg for tobacco and 30 mg/kg in the case of horseradish.

KEY WORDS: Secondary plant metabolites, plants, bacteria, PCB degradation

1. INTRODUCTION

Plant roots exude an enormous range of potentially valuable small molecular weight compounds into the rhizosphere. Some of the most complex chemical, physical, and biological interactions experienced by terrestrial plants are those that occur between roots and their surrounding environment of soil (i.e., the rhizosphere) (Bais et al, 2006). Plant root exudates also affect the level of contamination found in soil and ground water from various environmental pollutants. This rhizoremediation results from root exudate mediated stimulation of bacterial growth and survival, resulting in more efficient degradation of environmental pollutants (Kuiper et al, 2004).

2. MATERIAL AND METHODS

2.1 Soil, plants and secondary plant metabolites

Long-term PCB contaminated soil from Lhenice dumpsite (South Bohemia) was used for pot experiments. Tobacco (*Nicotiana tabacum*) and horseradish (*Armoracia rusticana*) were pregrown in noncontaminated soil and after cleaning their root zone where planted into standard pots with 450g of contaminated soil. Secondary plant metabolites (caffeic acid, naringin and mixture of caffeic acid and naringin) were added repeatedly in concentration– 8 $mg \cdot ml^{-1}$ or 4 $mg \cdot ml^{-1}$ of each compound in the mixture respectively.

2.1 Isolation and identification of soil bacteria

10 g samples of bulk soil and rhizospheric soil were extracted with 90 ml of 0,1% pyrophosphate. Samples were shaken in 250 ml flasks for 2 hours then aliquots of the extract were diluted with saline solution and spread on Petri dishes containing Plate count agar (PCA, Oxoid) for estimation of the total number of bacteria and minimal medium (MM) with biphenyl as the sole carbon source for identification of PCB degraders. Total counts of grown bacteria were estimated after 24 and 48 hours, respectively.

2.2 PCB content in soil

Gas chromatography with ECD detection was used for evaluation of decrease of PCB content in soil after 4 months cultivation. Indicator congeners were measured: PCB 28 — 2,4,4-trichlorobiphenyl, PCB 52 — 2,2‘,5,5‘-tetrachlorobiphenyl, PCB 101 — 2, 2‘, 4,5,5‘-pentachlorobiphenyl, PCB 118 — 2,3‘,4,4‘,5-pentachlorobiphenyl, PCB 138 — 2,2‘,3,4,4‘,5-hexachlorobiphenyl, PCB 153 — 2,2‘,4,4‘,5,5‘-hexachlorobiphenyl a PCB 180 — 2,2‘,3,4,4‘,5,5‘-heptachlorobiphenyl (US EPA methods 8089/8081) (Macková *et al*., 2009).

2.3 DNA extraction

DNA was extracted with a PowerMax soil DNA isolation kit (MoBio Laboratories Inc., United States) using the standard protocol. After the final elution the DNA was concentrated by adding 0.2 ml 5 M NaCl and 10.4 ml ethanol, incubated overnight at -20°C and transferred gradually into 2 ml microtubes with 20 µg glycogen (Roche, Germany), which were centrifuged after each addition in order to obtain a single pellet. The pellet was then dissolved in 20 µl of water.

2.4 Metagenomic analysis

The metagenomic analysis were performed according to Uhlik et al (in press)

3. RESULTS AND DISCUSSION

The aim of this work was to study the impact of secondary plant metabolites on microbial diversity and PCB degradation in the rhizosphere of horseradish (*Armoracia rusticana*) and tobacco (*Nicotiana tabacum*) cultivated in PCB contaminated soil.

3.1 Determination of total amount of bacteria

Soil bacteria were isolated from each soil sample and total amount of bacteria on PCA media and amount of bacteria which are able to growth on minimal media with biphenyl (MM) as carbon source were determinate (Tab. 1, Tab. 2).

Table 1: Microbial counts from pot experiments in non-vegetated soil and soil vegetated with tobacco

	PCA		**MM**	
	CFUx10^4/g soil		CFUx10^5/g soil	
Control Soil	5,6	1,34	6,34	1,2
Tobacco	6	2,3	8,8	3,9
Tobacco + Caffeic acid	20,4	6,6	12,4	3,4
Tobacco + Naringin	50,4	18,3	49,0	8,4
Tobacco + Mix	27,8	9,3	44,3	8,3

Table 1: Microbial counts from pot experiments in non-vegetated soil and soil vegetated with horseradish

	PCA		**MM**	
	CFUx10^4/g soil		CFUx10^5/g soil	
Control Soil	5,6	1,34	6,34	1,2
Horseradish	37,7	5,3	33,7	5,3
Horseradish + Caffeic acid	17,3	4,1	8,7	2,3
Horseradish + Naringin	23,7	7,2	10,4	4,1
Horseradish + Mix	32,1	3,3	38,1	13,2

PCA - Plate count agar
MM – Minimal medium
CFU – Colony forming units

In the case of tobacco the positive effect of secondary plant metabolites on total amount of microorganism and microorganism which are able to use biphenyl as a sole carbon source was observed, above all the addition of narigin. The presence of plant without secondary plant metabolites had no effect on amount of bacteria. Unlike

tobacco, for horseradish the significant effect of plant presence was found. Mackova et al confirmed similar results in their long-term phyto/rhizoremediation experiments. Tobacco, blacknightshade and alfalfa were used as model plants and the benefial effect of plants on the number of microorganisms found in the rhizosphere area was showed. McGuinnes and Dowling (2009) showed that 10-100x more microorganisms were found in the rhizosphere soil than in the nonvegetated soil.

3.2 PCB content in soil

The decrease of PCB content in contaminated soil was measured after 4 months cultivations with tobacco and horseradish.
The positive effect of the presence of plants (tobacco and horseradish) was found out. The decrease was higher in the case of samples vegetated by horseradish (around 30 mg/kg) than samples vegetated by tobacco (around 20 mg/kg). Results for all four parallels were similar in the case of horseradish. Tobacco with caffeic acid and tobacco itself got the best results for this plant. The positive effect of plants on degradation abilities was discussed in van Aken (2010) paper.

3.4 Metagenomic analysis
For confirmation of differences in bacterial communities the metagenomic analysis was used. The dominant phylum found in all samples was Proteobacteria - Alpha and Gammaproteobacteria (Fig. 2)

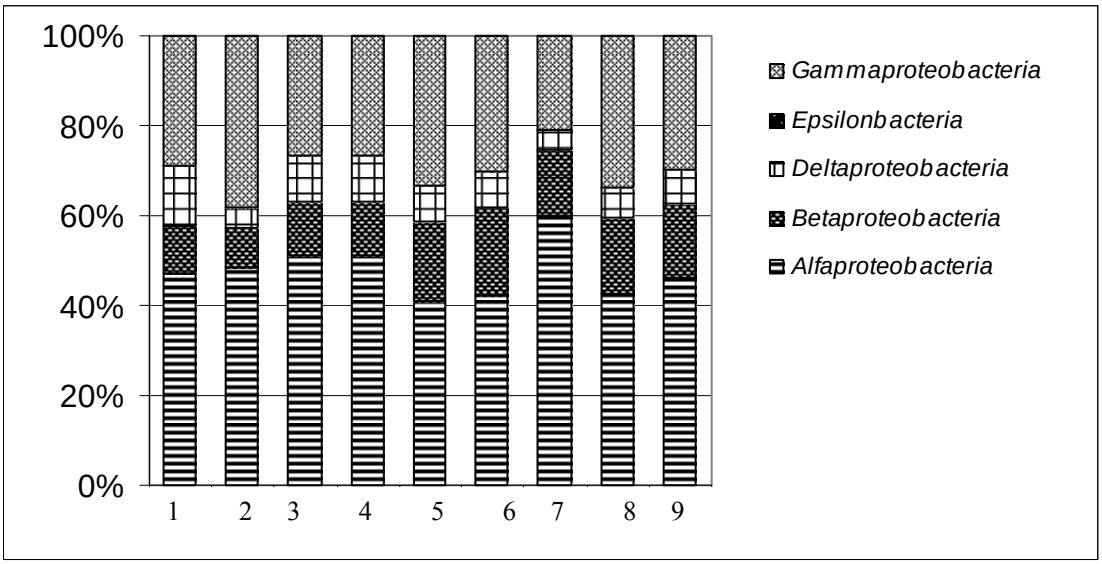

Figure 2: Microbial diversity (Class level)

Control soil, 2. Horseradish, 3. Horseradish+caffeic acid, 4. Horseradish+naringin, 5. Horseradish +mix, 6. Tobacco, 7. Tobacco+caffeic acid, 8. Tobacco+naringin, 9. Tobacco+mix

Same results were obtained in study of Nogales et al (2001). They found the most dominant phylum Proteobacteria also and beta, alpha and gamma subdivisions between the most abundant classes in this phylum.

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1 2 3 4 5 6 7 8 9

PLANT-BACTERIA COOPERATION ON METABOLISATION OF CHLOROBENZOIC ACIDS IN SOIL

Blanka Vrchotová, Tomáš Macek, Kateřina Demnerová and Martina Macková
vrchotob@vscht.cz
Institute of Chemical Technology, Prague, Faculty of Food and Biochemical Technology, Department of Biochemistry and Microbiology, Technicka 3, 166 28 Prague, Czech Republic

ABSTRACT

Chlorobenzoic acids, like other xenobiotics present in the environment, can be detoxified by biological systems, plants or microorganisms or by the cooperation of both. In this work we tested the metabolic potential of plants and bacteria in soil contaminated by a mixture of chlorobenzoic acids, and compared this potential with metabolisation activity of plants or bacterial strains alone. Plants of black nightshade (*Solanum nigrum*) and strain A18 (*Pseudomonas pseudoalcaligenes*) or UH82 (*Arthrobacte*r sp.) were used for testing. After six months of the experiment, the amount of bacteria, concentration of individual chlorobenzoic acids in plant biomass and in soil were analyzed.
In the plant biomass all chlorobenzoic acids initially present in the soil were determined in trace amounts. Bacterial counts were comparable in all tested cases. In the soil seven from eleven added chlorobenzoic acids were below detection limit at the end of experiment. Detected were 2,3,5-tri; 2,3-di; 2,6-di a 2,4,6-trichlorobenzoic acids in ascending order. The positive effect of bacterial strains addition on chlorobenzoic acids removal from soil was not unequivocally demonstrated. On the other hand, the presence of black nightshade plants had positive effect on chlorobenzoic acids disappearance from the contaminated soil.

KEY WORDS: chlorobenzoic acid, plant, cooperation, microorganism, metabolisation

1. INTRODUCTION

Chlorobenzoic acids are known degradation products formed during degradation of polychlorinated biphenyls. Microbial degradation of polychlorinated biphenyls resulted in formation of mixture of chlorobenzoic acids with different number and position of chlorine atoms on aromatic ring (Adebusoye et al 2008). Thereby formed mixture of chlorobenzoic acids can slow down or completely inhibit the degradation of the initial compound (Deavers et al 2010). For this reason, it is necessary to determine the degradation of not only initial compound but also intermediates or products formed during the degradation pathway. Examples of such compounds are chlorobenzoic acids whose metabolization by plants and microorganisms is determined here.

2. MATERIALS AND METHODS

2.1 Metabolization experiment

For testing cooperation of plant and bacteria on chlorobenzoic acid metabolization in contaminated soil black nightshade (*Solanum nigrum*), strains A18 (*Pseudomonas pseudoalcaligenes*) or UH82 (*Arthrobacte*r sp.) were chosen because of their metabolic activity proved in previous tests. Soil was before experiment comtaminated by mixture of chlorobenzoic acids (2-; 3-; 4-; 2,3-di; 2,4-di; 2,5-di; 26-di; 3,4-di; 3,5-di; 2,3,5-tri and 2,4,6-trichlorobenzoic acid) to the concentration of 5 mg/l from individual chlorobenzoic acid. To the soil with plant of black nightshade, defined amount of microbial cells were added. After six months the amount of bacterial cells, mass of the plant biomass and the concentration of chlorobenzoic acid in the soil and in the different parts of plant biomass were measured. For comparison of degradation activity also soil just with plants or microorgansms was similarly prepared.. As a control of metabolization soil without addition of microorganisms or cultivation of plants was used.

2.2 Measurement of chlorobenzoic acids concentration

Soil or plant biomass was before measurement of chlorobenzoic acids concentration dried on air, homogenised and extracted. Soil samples were extracted by water with H_2SO_4 addition to pH 2 and biomass by methanol. Concentration of chlorobenzoic acids was measured on HPLC with DAD detector (HP 1100, USA). The column Kinetex C18 100A (150 mm x 2,1 mm x 2,6 μm) (Phenomenex, USA) was used. Isocratic elution with 45% of methanol with 55% of buffer pH 3,15 and flow 0.14 ml/min was used. Detection of chlorobenzoic acids was in λ = 205 nm.

3. RESULTS AND DISCUSSION

3.1 Bacterial counts after 6 months

At the end of experiment counts of bacterial cells were determined on plate count agar (PCA) and on minimal medium with benzoic acid (MM) for determination of potencialy chlorobenzoic acids degradation mikrooganisms (Fig. 1.).
In all tested cases counts of microorganisms were comparable. In cases when plants were present, amounts of microorganisms were higher in root zone in comparison with other parts of soil. Just in non contaminated soil higher amounts of microorganisms were detrmined in upper part of soil.

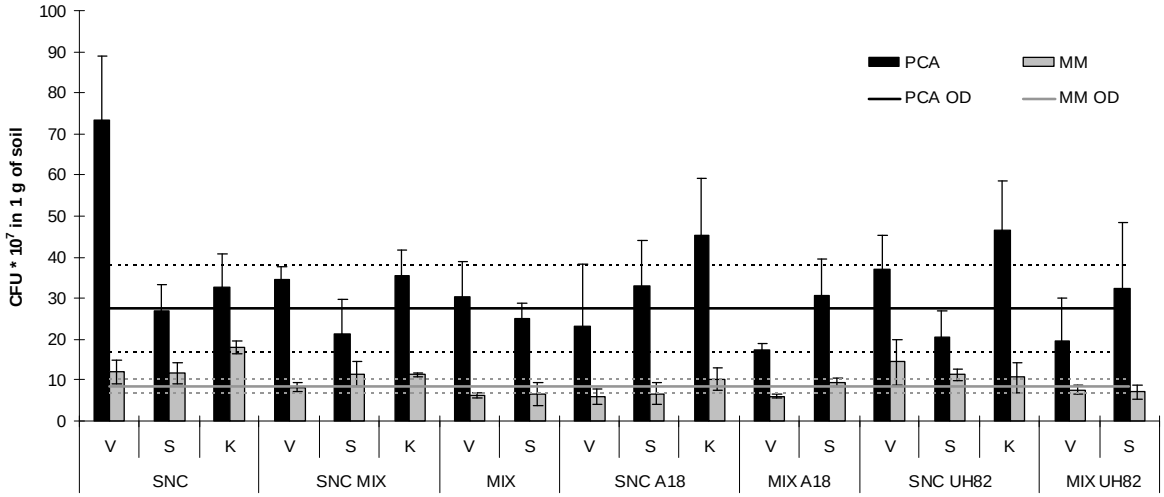

Figure 1. Microbial counts in chlorobenzoic acids contaminated soil after 6 months experiment. PCA – Plate count agar; MM - minimal medium with benzoic acid; SNC – black nightshade (Solanum nigrum); MIX – mixture of chlorobenzoic acids; A18 - Pseudomonas pseudoalcaligenes; UH82 - Arthrobacter sp.; V – upper part of soil; S – lower part of soil; K – soil from root zone.

3.2 Concentration of chlorobenzoic acids in soil

Next to amounts of microorganisms concentration of chlorobenzoic acids in soil after six months was determined (Fig. 2.).
At the end of the experiment just four chlorobenzoic acids were detected in soil. Other seven from eleven used for soil contamination at the beginning of the experiment were under detection limit. Detected were 2,3-di; 2,6-di; 2,3,5-tri and 2,4,6-trichlorobenzoic acid. The highest residual concentration was 2,4,6-trichlorobenzoic acid followed by 2,6-dichlorobenzoic acid and 2,3-dichlorobenzoic acid and the lowest concentration from detected acids was measured for 2,3,5-trichlorobenzoic acid.
From these data is possible to conclude that in our experiment chlorobenzoic acids were in soil metabolised according position and number of chlorine atoms on aromatic ring. Chlorobenzoic acids with chlorine atoms in *ortho*- position were metabolised with the lowest efficiency followed by *meta*- position. This is the reason why in case of 2,6-dichlorobenzoic acid and 2,4,6-trichlorobenzoic acid were determined the highest residual concentrations. Both acids contain two chlorine atoms in *ortho*- position.
Presence of black nightshade plant leads to higher efficiency of chlorobenzoic acids metabolization. Residual concentration of chlorobenzoic acids were in vegetated soil lower than in non vegetated soil. Again like in case of microbial content positive effect of root zone on chlorobenzoic acids disappearance was proved.

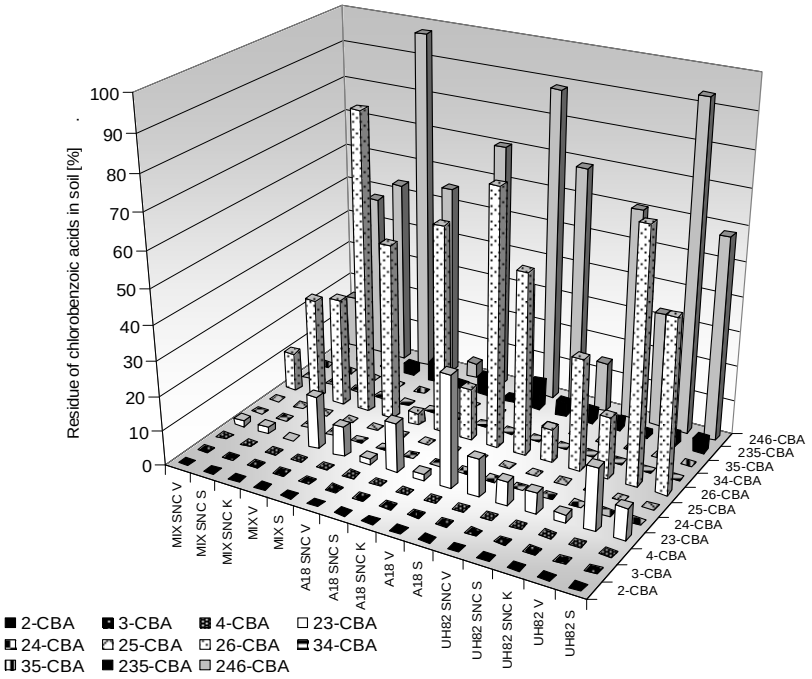

Figure 2. **Microbial counts in chlorobenzoic acids contaminated soil after 6 months experiment.** PCA – Plate count agar; MM - minimal medium with benzoic acid; SNC – black nightshade (*Solanum nigrum*); MIX – mixture of chlorobenzoic acids; A18 - *Pseudomonas pseudoalcaligenes*; UH82 - *Arthrobacter* sp.; V – upper part of soil; S – lower part of soil; K – soil from root zone.

3.3 Concentration of chlorobenzoic acids in black nightshade biomass

Also concentration of chlorobenzoic acids in different parts of plant biomass was determined (Fig. 3.). Like in soil the highest concentration was determined for 2,4,6-trichlorobenzoic acid. But others chlorobenzoic acids were determined just in trace amounts.
The determination of chlorobenzoic acids concentration in plant biomass is only approximate, because extracts from plant biomass contained many interfering substances.
In plants vegetated in soil with contamination of chlorobenzoic acids it was observed later flowering and the morphology of the flowers was different then in plants vegetated in non contaminated soil.

4. CONCLUSIONS

Experiment proved that chlorobenzoic acids were relatively efficiently metabolized in the soil. During the six months were removed seven from eleven added chlorobenzoic acids and the remaining four were in most cases significantly reduced. Also according to the results, loss of this 4 chlorobenzoic acid in soil vegetated by plants was greater than in soil without the presence of plants. The positive effect of bacteria addition on the results was very little and was not unequivocally demonstrated. It can be assumed that the loss of chlorobenzoic acids in soil is rather caused by microorganisms present in soil even present before contamination.
Based on the results obtained by measuring of the chlorobenzoic acids concentrations in plant biomass, it is clear that there is no significant accumulation of the tested chlorobenzoic acids. The highest concentrations were found in flowers, fruits and stalks in other parts of plants were found only traces of chlorobenzoic acids. It is possible, that increased concentrations of chlorobenzoic acids in flowers, could be the cause of the later flowering of the plants grown in soil with the addition of chlorobenzoic acids, and poor development of flowers of these plants.

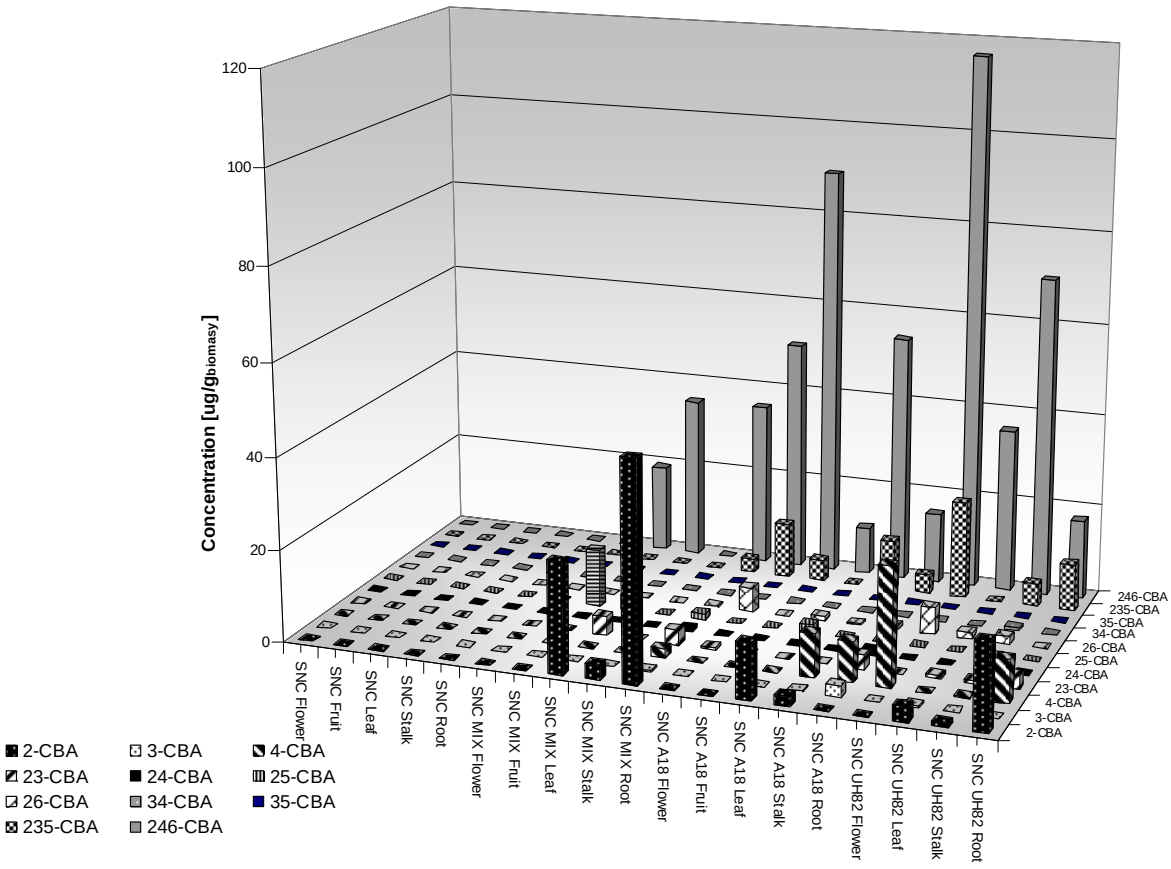

Figure 3. Concentration of chlorobenzoic acids in different parts of black nightshade plants after six months of experiment. CBA - chlorobenzoic acid; SNC – black nightshade (Solanum nigrum); MIX – mixture of chlorobenzoic acids; A18 - Pseudomonas pseudoalcaligenes; UH82 - Arthrobacter sp.

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BIODEGRADATION AND TOXICITY OF POPs

DEGRADATION OF PHENOL AND P-NITROPHENOL BY THE WHITE-ROT POLYPORE TRAMETES VERSICOLOR

Maira Carabajal[1], René Ullrich[2], Laura Levin[1], Martin Kluge[2] and Martin Hofrichter[2]
mcarabajal@conicet.gov.ar
[1]University of Buenos Aires, Faculty of Exact and Natural Sciences, Department of Biodiversity and Experimental Biology, Intendente Güiraldes 2160 - Ciudad Universitaria - C1428EGA, Argentina.
[2] International Graduate School of Zittau, Unit of Environmental Biotechnology, Markt 23, 02763 Zittau, Germany

ABSTRACT

The aim of this study was to evaluate the potential of *Trametes versicolor* (BAFC 2344) to degrade phenol and p-nitrophenol (NP). Due to their widespread industrial usage and toxicity, these compounds cause serious environmental problems. In vivo-degradation experiments were carried out in immobilized culture for phenol and non-immobilized for NP and evaluated by HPLC. *T. versicolor* was precultivated on natural sponge (*Luffa cilindrica*) in tomato juice medium and was then transferred to 15 mM phenol solution. Degradation was performed in static culture over a period of 23 days. Phenol was added three times, when its concentration decreased by 60-70%. Degradation rates ranging between 3.6 $mM.d^{-1}$ and 1.3 $mM.d^{-1}$ could be achieved. Along with phenol addition laccase activities were sharply decreasing but were increasing again. Non-immobilized fungal cultures with 0.5 mM NP in glucose-asparagine medium were also able to degrade the pollutant. NP concentration was reduced by 92% within 25 days. The most abundant extracellular enzyme activity was laccase (1,400 U l^{-1}). These results point out that *T. versicolor* is able to tolerate and degrade phenol and NP, considering NP as usually resistant to fungal degradation. Immobilization on natural sponge combines a high rate of phenol degradation and reutilization in several removal cycles.

KEY WORDS: nitrophenol, phenol, *Trametes versicolor*, degradation

1. INTRODUCTION

Phenols and nitrophenols are known as hazardous pollutants. The main sources of nitrophenols are industrial manufacturing and processing. They are used mainly to produce dyes, paint coloring, rubber chemicals drugs, fungicides. Nitroaromatic compounds constitute a major class of widely distributed environmental contaminants (Ye et al., 2004; Teramoto et al., 2004). Phenols compounds are also recognized as environmentally hazardous and the pollution is associated with pulp mills, coal mines, refineries, wood preservation plants and various chemical industries (Nair et al., 2008).
Trametes versicolor is a white rot fungus with a well known ability to degrade a variety of toxic industrial pollutants. This strain is capable of completely degrading polymers of phenolic origin, including lignin. The successful bioremediation of a phenolic wastewater by this strain was found to be dependent on fungal growth, enzyme production and some inducers (Ryan et al., 2007; Yemendzhiev et al., 2008).
Most of the published investigations on the degradation of phenol have been done using bacterial strains and some yeast. The use of mycelial fungal strains is a relatively untouched area (Yemendzhiev et al., 2008). The aim of this study was to investigate the potential of *Trametes versicolor* (BAFC 2344) to degrade phenol and p-nitrophenol.

2. MATERIALS AND METHODS

2.1 Culture conditions

The degradation study of NP was carried out under submerged fermentation in glucose-asparagine medium (GA) (Levin and Forchiassin, 2001) NP 0.25 mM was added after autoclaving. 250 ml Erlenmeyer flasks containing 80 ml of the medium were inoculated with mycelium grown on MEA plates at 25 ◦C for 10-12 days. Stock cultures were maintained on 2% malt extract agar (MEA) at 4 ◦C in the dark. The content of an agar plate was homogenized in 80 ml of sterile water and the mycelial suspension used to inoculate liquid cultures (5%, v/v). Controls consisted of *T. versicolor* grown in GA medium without NP. The cultures were harvested at 25th day of incubation. Samples (1 ml of the culture liquid) were taken every 2–3 days. Each sample was centrifuged (10,000 × *g* for 8 min) at 4 °C. The supernatant was used to quantify NP degradation.
To evaluate phenol degradation, immobilized cultures of *T. versicolor* on natural sponge (*Luffa cilindrica*) were grown in a complex liquid medium (TJM) which consisted of eco-tomato juice (Albi & Co., Germany) and distilled water (50:50 v/v). To stimulate enzyme production, $MnCl_2$ and $CuSO_4.5H_2O$ were added before

autoclaving (final concentration 250 µM). 500-ml flasks containing 200 ml of TJM and 5 pieces of sponge (each 150 x 250 mm) were incubated in a rotary shaker at 100 rpm and 24 ◦C. The cultures were harvested at 19th day of incubation and the immobilized mycelium was then transferred to 15 mM phenol solution. Degradation was performed under static conditions over a period of 23 days at 24 °C. Phenol was added three times, when its concentration in the liquid medium decreased by 60-70%. Heat-inactivated cultures were used as controls.

2.2 Extracellular enzyme assays and NP and Phenol estimation

Laccase activity was determined in the liquid medium by following the oxidation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM citrate/phosphate buffer (pH 4.5 , 25ºC) at 420 nm (ε 420, 36,000 M^{-1} cm^{-1}) (Bourbonnais et al., 1995). The activity of Mn-peroxidase (MnP) was measured at 270 nm by following the formation of Mn^{3+}-malonate-complexes (Wariishi et al., 1992). The enzymatic activity was expressed as International units (U) defined as the amount of enzyme required to produce 1 µmol product min^{-1} and expressed as UL^{-1}. NP and phenol concentration were quantified by HPLC. All chemicals used were obtained from Sigma–Aldrich (Weinheim, Germany) and Merck (Darmstadt, Germany).

2.3 Statistical analysis

Experiments were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using analysis of variance (ANOVA) followed by Tukey's Test using Infostat program. Differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1 *P*-nitrophenol degradation

T. versicolor (BAFC 2344) cultivated with 0.5 mM *p*-nitrophenol in glucose-asparagine medium (GA) not only was able to grow but also degrade the pollutant. NP concentration was reduced by 0.04 mM (92%) within 23 days (Fig. 2). In Czapek medium with 2-nitrophenol, 3-nitrophenol and 4-nitrophenol as single carbon and energy sources, different strains of *T. Versicolor* has also demostrated this ability of removing the pollutants (Yemendzhiev et al., 2011), however only around 30% of 4-nitrophenol (initial concentration 0.5 g/l) was degraded after 10 days. Ligninolytic enzymes (laccase, MnP and lignin-peroxidase) are believed to be responsible for degradation or even mineralization of nitroaromatic compounds (Tripathi et al., 2011). The *ligninolytic enzymes* of white rot fungi are non-stereoselective and catalyze the degradation of pollutants by using a *non-specific* free radical mechanism (Pointing, 2001). In our study the main ligninolytic enzyme activity was laccase (1,400 U l^{-1}). Its activity increased when nitrophenol concentration decreased. This suggests that laccase activity could be responsible for NP degradation. In control cultures laccase activity was higher and relatively constant after the 7th day (it increased from 790 U l^{-1} to a maximun of 1,780 U l^{-1}in the 16th day) (Fig. 1). Lower titres of MnP activity were measured. The enzymatic production showed an oscillant patron, is some cases MnP activity could not be detected in the culture supernatants (data not shown).

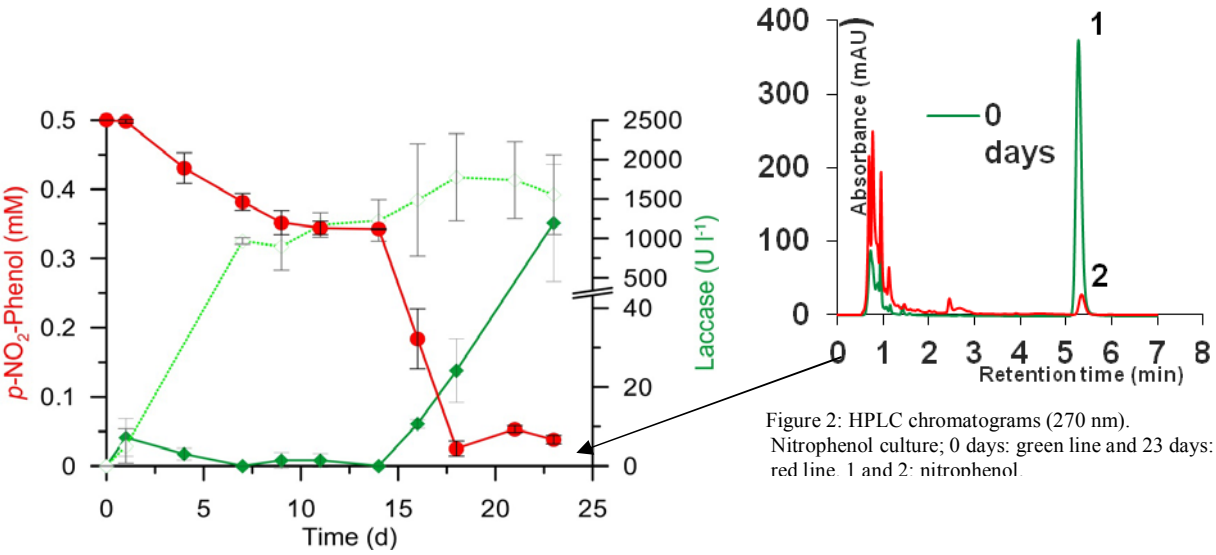

Figure 2: HPLC chromatograms (270 nm). Nitrophenol culture; 0 days: green line and 23 days: red line. 1 and 2: nitrophenol.

Figure 1: *P*-nitrophenol concentration: red filled circles, Laccase activity: green filled diamonds, Laccase activity in the control without *p*-nitrophenol: green open diamonds

3.2 Phenol degradation

T. versicolor immobilized in natural sponge could remove 71, 62, 74 and 73% of phenol after 3, 10, 16 and 21 days respectively (degradation rates: 3.6 $mM.d^{-1}$ and 1.3 $mM.d^{-1}$) (Fig. 3 and 4). Ligninolytic enzymes of white rot fungi such as *T. versicolor* proved to be involved in phenol degradation. Several *T. versicolor* strains produced laccase, MnP and LiP (Bourbonnais et al., 1995; Dodson et al.,1987; Paice et al., 1993). Along with phenol addition to *T. versicolor* BAFC 2344, laccase was the only extracellular enzyme detected. This activity sharply decreased with the addition of the pollutant but increased again in correlation with phenol degradation. MnP or LiP were not detected during the degradacion process. In white rot fungi like *Pleurotus spp.*, *Phanerochaete chrysosporium*, *Phanerochaete laevis* and *T. versicolor* laccase activity increased following the addition of certain phenolic compounds (Tripathi et al., 2011). One of the attributed functions of laccases is to detoxify aromatic compounds by promoting polymerization. This was further verified by the fact that laccase production increased in coincidence with the formation of dark precipitates resulting from the polymerization of the phenolic substrates (Thurston, 1994). These precipitates were observed since the 3rd day of cultivation. Phenol concentration in heat-inactivated controls remained constant (data not shown).

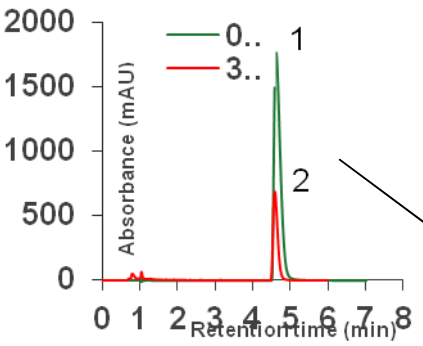

Figure 4. HPLC chromatograms (270 nm). Phenol culture; 0 days: green line and 23 days: red line. 1 and 2: phenol

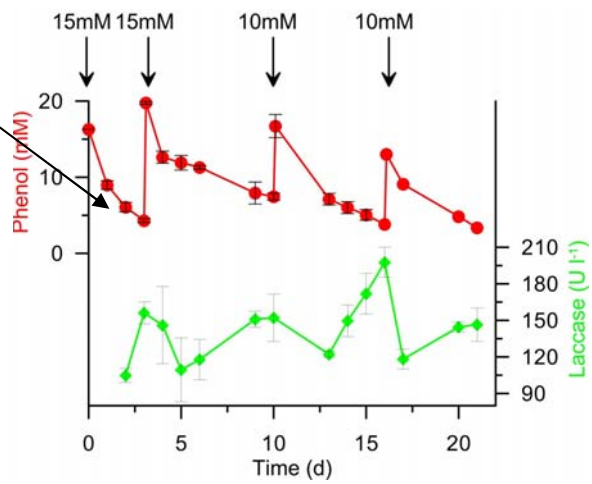

Figure 3. In vivo degradation of phenol by Trametes versicolor. Phenol concentration: red filled circles, Laccase activity: green filled diamonds.

These results pointed out that *T. versicolor* (BAFC 2344) is able to tolerate and degrade phenol and NP, considering NP as usually resistant to fungal degradation (Teramoto et al., 2004). Immobilization on natural sponge combines a high rate of phenol degradation and reutilization in several removal cycles.

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AEROBIC COMETABOLIC BIOREMEDIATION OF AN AQUIFER POLLUTED BY CHLORINATED SOLVENTS: PRELIMINARY INVESTIGATION OF THE FEASIBILITY OF A PACKED BED REACTOR *ON-SITE* PROCESS

Ciavarelli R.[1], Bucchi G.[1], Doria F.[1], Salviulo R.[1], Zanaroli G.[2], Fraraccio S.[2], Frascari D.[1], Pinelli D.[1] and Fava F.[2]
dario.frascari@unibo.it
[1]University of Bologna, Department of Chemical, Mining and Environmental Engineering, Via Terracini 28, 40131 Bologna, Italy.
[2]University of Bologna, Department of Civil, Environmental and Materials Engineering, Via Terracini 28, 40131 Bologna, Italy.

ABSTRACT

This work focuses on the enrichment of a trichloroethylene (TCE) and 1,1,2,2-tetrachloroethane (TeCA) co-metabolizing aerobic microbial consortium from a contaminated site in Northern Italy, for the development of an *on-site* bioremediation treatment. The goals were: (i) to select the best growth substrate and the best-performing consortium in terms of contaminant degradation capability; (ii) to perform a preliminary suspended-cell kinetic test; (iii) to perform a preliminary evaluation of the feasibility of the co-metabolic treatment of the tested groundwater in a packed-bed reactor operating at the site's temperature (15°C). The best degradation results were obtained with a butane-growing consortium. At 15° C, the selected culture resulted in short lag-times for the onset of butane utilization (< 1 day) and TCE and TeCA degradation (< 1 - 8 days). Once immobilized on 4 different porous carriers, the selected consortium was able to grow and form a stable biofilm. The TCE and TeCA degradation rates resulted similar for the 4 carriers tested. Further experiments are in progress to estimate the kinetic parameters of the co-metabolic processes and to understand which carrier leads to the best performances in a continuous-flow packed-bed reactor process.

KEY WORDS: aerobic co-metabolism, chlorinated solvents, biofilm, bioremediation.

1. INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs) are widespread subsurface contaminants and several of them are known or suspected cancer-causing agents (US EPA, 2000). CAH-contaminated sites are usually treated by means of physical–chemical methods, which are often limited to contaminant transfer to a different matrix. On the other hand, numerous studies showed that aerobic co-metabolism can lead to the complete and rapid dechlorination of a wide range of chlorinated solvents (McCarty et al., 1998; Frascari et al., 2005; Semprini et al., 2009). Packed bed reactors (PBRs) represent an interesting solution for biodegradation processes, in particular in the presence of recalcitrant and toxic compounds (Karamanev et al., 1991). Indeed attached cells presents specific advantages over suspended cells, such as higher biomass concentrations, higher cell retention times, the elimination of the biomass settling step, a partial protection of cells against toxic substances and lower inhibition due to high substrate concentrations.
This work focuses on the enrichment of an aerobic trichloroethylene (TCE) and 1,1,2,2-tetrachloroethane (TeCA) co-metabolizing microbial consortium from the indigenous biomass of a contaminated site in Northern Italy. The goals of this study were: (i) to select the best growth substrate and the best-performing consortium in terms of contaminant degradation capability; (ii) to perform a preliminary suspended-cell kinetic test; (iii) to perform a preliminary evaluation of the feasibility of the co-metabolic treatment of the tested groundwater in a packed-bed reactor operating at the site's temperature (15°C).

2. MATERIALS AND METHODS

2.1 Experimental scheme

The tests were conducted in 119-mL glass vials, closed with Teflon-lined rubber septa, containing 60 mL of site or synthetic groundwater. The bioreactors were agitated at 125 rpm and 30°C, unless otherwise indicated. Macronutrients (KNO_3, KH_2PO_4, K_2HPO_4) were periodically added. The first part of the work, aimed at selecting the best performing culture, was articulated in 2 steps. In the 1st step, a 25-vial set was obtained by exposing groundwater sampled from 5 wells of the contaminated-site (indicated as 1, 2, 3, 4 or 5) to 5 candidate growth substrates: methane (M), propane (PR), butane (B), pentane (PE) and phenol (F). The tested growth substrates were always injected at an aqueous concentration of 2 mg L^{-1}. The vials were initially enriched with

substrates and oxygen. After 4 pulses of growth substrate they were spiked with TCE and TeCA at increasing concentrations (1-7 mg L^{-1}) to test their co-metabolizing capacity. TCE and TeCA were re-added when their concentration decreased down to 1% of the initial value. In the 2nd step, kinetic tests were conducted with the best 8 microcosms selected in step 1, as described in the "Results and Discusson".
In the second part of the work, the best-performing microcosm (B4) was tested (i) as immobilized biomass (at 30°C) and (ii) at 15°C (average aquifer temperature), in preliminary suspended-cell tests. The goal was to verify the feasibility of the co-metabolic treatment of the tested groundwater in a packed-bed reactor operating at the site's temperature. Further tests aimed at evaluating the performances of the selected consortium in immobilized form at 15°C are currently in progress. In the attached-cell tests, consortium B4 was re-inoculated in 4 vials with fresh synthetic water (10% v/v of inoculum) and 50 mL of porous carriers (Biomax®, Biopearl®, Biomech®, Cerambios®). In order to reduce the suspended biomass to a negligible level, before each butane re-spike the carriers were washed in physiological solution and re-supplied in 60 mL of sterilized synthetic water.
For each experimental condition, negative control vials sterilized with NaN_3 were setup. The latter resulted in negligible depletion rates in comparison with those obtained in the viable-cell tests (data not shown).
Metagenomic DNA was extracted from suspended and attached-cell grown cultures with UltraClean Soil DNA kit (MoBio Laboratories) after enzymatic (Protease K 100 µg mL^{-1}, Lysozime 160 µg mL^{-1} and Mutanolysin 30 U mL^{-1}) and mechanical cell lysis. The enriched bacterial consortia were characterized via PCR-DGGE analysis followed by bands sequencing and phylogenetic analysis as described by Zanaroli et al. (2010).

2.2 Estimation of model parameters

Considering the low substrate and contaminant concentrations tested in this work, the experimental data of substrate utilization and TCE or TeCA co-metabolism were interpreted by means of a first-order model: $r_i = k_i \cdot C_i \cdot X$, where r_i is the initial degradation rate (mg $L^{-1}d^{-1}$), k_i the first order kinetic constant (L $g_{protein}^{-1}$ d^{-1}), C_i the liquid phase concentration (mg L^{-1}) and X_i the cell concentration ($g_{protein}$ L^{-1}). In each test, the lag-time for the onset of degradation of each contaminant and - for each pulse - the initial degradation rate of each compound was calculated as described previously (Frascari et al., 2007 and 2008). Total amounts in the vial and liquid-phase concentrations of each compound were calculated on the basis of the following gas-water partition coefficients (dimensionless, 30°C): methane and propane 31, pentane 60, butane 44, phenol $1.36 \cdot 10^{-5}$, TCE 0.49, TeCA 0.02 (Sander, 1999). The first order kinetic constants (k_S, k_{TCE}, k_{TeCA}) were obtained as $r_i / (C_i \cdot X)$.

2.3 Analysis and chemicals

Methane, propane, butane, pentane, phenol, TCE, TeCA (Aldrich, Gillingham, UK) and oxygen were measured in the vial headspace with a Hewlett Packard 6890 GC equipped with a flame ionization detector and an electron capture detector. The details relative to the chromatographic methods are described by Frascari et al. (2005). Suspended biomass concentration was measured as proteins, using the Lowry method (Frascari et al., 2005). The quantification of attached cells were performed as described by Cappelletti et al. (2012).

3. Results and discussion

3.1 Substrate and consortium selection at 30°C

Substrate utilization was observed in all the microcosms. The substrate lag time was considerably short in the methane-, butane- and pentane-consuming consortia (4-5 days), while a longer period was required for the onset of propane and phenol consumption (15-20 days). The average TCE lag time was shorter in the butane- and methane-consuming tests (11 and 14 days, respectively) than in the pentane- and propane-fed microcosms (18 and 25 days). TeCA depletion started after an average of 23 days in the butane- and pentane-consuming consortia, while a longer period was necessary with methane (34 days) and propane (35 days). The longest TCE lag time was observed in the phenol-consuming microcosms (40 days), which did not show any TeCA depletion ability.
A sub-set of 8 microcosms was selected for a kinetic test aimed at identifying the best CAH-degrading consortia. The selection was made on the basis of the following parameters: (i) substrate lag-time, (ii) TCE lag-time, (iii) TeCA lag-time, (iv) TCE and TeCA degradation rates. The selected consortia were: methane-consuming M1 and M4, propane-consuming PR2 and PR4, butane-consuming B4 and B5, and pentane consuming PE1 and PE4. No phenol-consuming consortia were selected. The kinetic test consisted of a pulse of only substrate (2 mg L^{-1}), followed by a pulse of only TCE (1 mg L^{-1}), by a 2nd substrate pulse (2 mg L^{-1}) and by a pulse of only TeCA (1 mg L^{-1}).
The biomass concentrations at the initial time of the kinetic test are reported in Figure 1. The estimates of the 1st order constants k_S, k_{TCE} and k_{TeCA} obtained from the kinetic test are presented in Figures 1 and 2. The highest k_S

was obtained for the methane-growing consortia (750-1700 L $g_{protein}^{-1}$ d^{-1}). In the propane- and pentane-consuming consortia the estimated k_S ranged between 210 and 310 L $g_{protein}^{-1}$ d^{-1}, whereas the values obtained with butane were even lower (110-170 L $g_{protein}^{-1}$ d^{-1}). From the standpoint of a full-scale PBR, a low k_S is preferable, as it implies a slower substrate consumption and therefore a better distribution of biomass growth along the bioreactor. The estimated k_{TCE} were at least one order of magnitude lower. Interestingly, the highest k_{TCE} (42-96 L $g_{protein}^{-1}$ d^{-1} with butane, 56 L $g_{protein}^{-1}$ d^{-1} with propane-fed PR4) were obtained with the consortia characterized by the lowest k_S. The k_{TCE} evaluated in PR2, M1, M4, PE1 and PE4 were one order of magnitude lower (0.6-2.7 L $g_{protein}^{-1}$ d^{-1}). Furthermore, the k_{TeCA} of all the 8 consortia were of the same order of magnitude (0.4-1.2 L$g_{protein}^{-1}d^{-1}$), about 10-fold lower than the highest k_{TCE} values and approximately 100-fold lower than the average k_S values.

Butane-consuming consortium B4 was selected as the most promising one for a process of aerobic co-metabolic treatment of the studied groundwater in a PBR, on the basis of its short lag-time for the onset of substrate, TCE and TeCA degradation, its low k_S and its high k_{TCE}. k_{TeCA} was not included in the criteria of consortium selection, considering that the 8 consortia showed similar TeCA degradation abilities, and that TeCA can be rapidly converted into TCE in a preliminary abiotic treatment step characterized by a high pH (Joens et al., 1995). Consortium B4 mainly consisted of Bacteroidetes and Firmicutes (data not shown). Phylotypes related to CAH-cometabolising bacteria, such as *Methylosinus trichosporium* and *Mycobacterium chubuense*, were also detected although they represented, according to relative band intensities, a minor fraction of the bacterial community enriched.

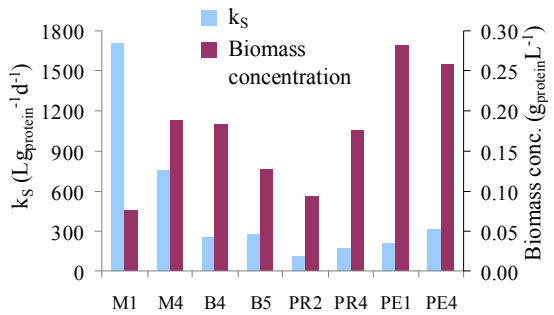

Figure 1. Biomass concentration and k_S (L $g_{protein}^{-1}$ d^{-1}) of the 8 selected microcosms.

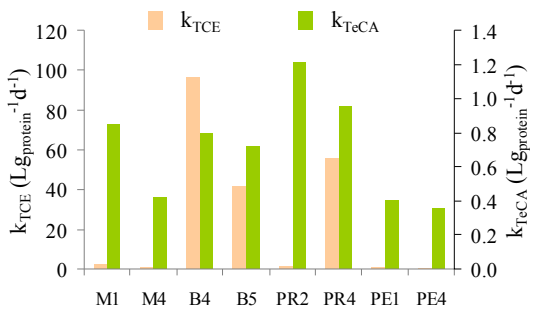

Figure 2. k_{TCE} and k_{TeCA} (L $g_{protein}^{-1}$ d^{-1}) of the 8 selected microcosms.

3.2 Attached cell test

In further tests, the ability of consortium B4 to form biofilms and to co-metabolize TCE in attached form was tested. B4 was able to grow and form a stable biofilm on 4 different ceramic or sintered glass supports. The butane degradation rate ranged between 285 and 453 mg L^{-1} d^{-1} for Biomax®, Biomech® and Biopearl®, whereas the value obtained with Cerambios® was sensibly lower (39 mg L^{-1} d^{-1}). The TCE degradation rates ranged between 0.19 mg L^{-1} d^{-1} (Biopearl®) and 0.29 mg L^{-1} d^{-1} (Biomech®). While the butane k_S resulted comparable among the four carriers (Figure 3), Cerambios® allowed B4 to reach a significantly higher k_{TCE}, despite the small differences observed for the TCE volumetric degradation rates. This result can be explained by the differences in attached biomass concentration (data not shown). Interestingly, no remarkable differences were detected in the composition of B4 biofilms grown of the 4 supports, that differed markedly from the consortium grown under suspended cell conditions and were mainly composed by phylotypes distantly related to known CAH-cometabolising *Cupriavidus necator* strains. Further tests in packed-bed columns are in progress, to investigate the process under continuous flow conditions.

3.3 Substrate and consortium selection at 15°C

In order to assess the feasibility of a full scale PBR process at the aquifer average temperature (15°C), a second set of enrichment cultures from the 5 groundwater samples was set up at 15°C, and a further batch bioreactor was inoculated (10% v/v) with consortium B4 (enriched at 30°C) and maintained at 15°C. Every microcosm showed the ability to consume butane as the main carbon source and to degrade TCE and TeCA via co-metabolic pathways. The B4-inoculated test showed considerably short lag-times for the onset of butane consumption (< 1 day) and for the degradation of TCE (< 1 day) and TeCA (< 8 days). The average lag-times for the onset of butane consumption and TCE and TeCA degradation were higher than those observed in the test at 30°C (8, 30 and 30 days, respectively). A kinetic study similar to that performed at 30°C is in progress, in order to estimate the first-order constants at 15°C.

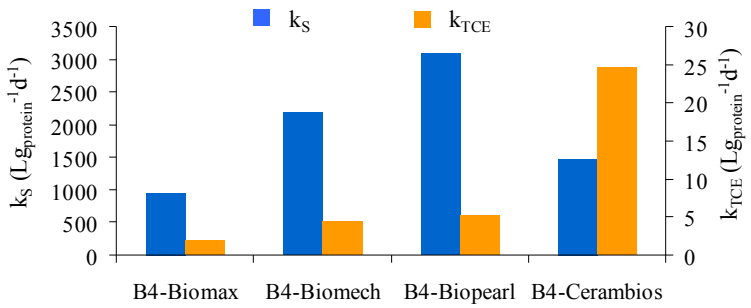

Figure 3. k_S and k_{TCE} obtained in attached cells test conducted with consortium B4.

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BIOFILM MEDIATED OIL SANDS PROCESS WATER DETOXIFICATION

Marc Demeter[1,2,3], Iain George[1,2], Joe Lemire[1,2], Howard Ceri[1,2,3] and Raymond Turner[1,2,3]
turnerr@ucalgary.ca
[1]Department of Biological Sciences, University of Calgary, Calgary AB, Canada
[2]Biofilm Research Group, University of Calgary, Calgary AB, Canada
[3]Institute for Sustainable Energy, Environment, and Economy, University of Calgary, Calgary AB, Canada

ABSTRACT

Alberta oil sands process water (OSPW) is collected and stored in tailings ponds. This tailings pond water (TPW) contains naphthenic acids (NAs), which are responsible for its acute, aquatic toxicity. TPW toxicity can be mitigated by aerobic microbial degradation of the NAs by microorganisms found naturally within the TPW, but is an extremely long and slow process. *Ex*-situ bioremediation of TPW presents a much more efficient method to detoxify TPW. The microorganisms chosen, and their method of growth have consequences on the success of a bioremediation effort. The aim of this research was to evaluate biofilm-mediated bioremediation of model NAs, specifically comparing mixed species biofilms to single species biofilms. The Calgary Biofilm Device (CBD) was used to grow both mixed and single species biofilms from an OSPW/TPW inoculum. Gas Chromatography reveals that multispecies biofilms are capable of degrading simple model NAs, and single species biofilms are not. These results are thought to be a result of a cooperation/division of labor amongst the many bacterial species found within the multispecies biofilm, allowing the heterogeneous and diverse biofilms to metabolize the NAs.

KEY WORDS: oil sands, tailings ponds, biofilms, bioremediation, naphthenic acids

1. INTRODUCTION

The process used to release bitumen (heavy oil) from the sand and clay of the Canadian Alberta oil sands results in the formation of large quantities of a waste product known as tailings, which contains oil sands process water (OSPW) (Clemente et al. 2004). OSPWs collected in tailings ponds, are highly toxic to both aquatic organisms and mammals. The principle component of this toxicity is a vast group of alkyl-substituted acyclic and cyclo-aliphatic carboxylic acids known as Naphthenic Acids (NAs) (Lo et al. 2006; Kannel & Gan 2012). Bioremediation via aerobic microbial degradation of the NAs is a promising proposed solution to reduce the toxicity of the OSPW prior to environmental release (Del Rio et al. 2006). One major obstacle to bioremediation is the complexity and variability of the NA fraction within OSPWs; the more recalcitrant NAs are often left intact (Smith et al. 2008).

Planktonic cultures of known NA degraders are not a viable bioremediation solution because the complete degradation of complex and variable NAs, likely requires the coordination of a diverse and spatially close group of bacteria resembling the diversity found within the TPW, along with other such properties like metal resistance (Stach & Burns 2002). Such a group is commonly found in nature, and is known as a biofilm; a multicellular aggregate of surface attached bacterial cells bound in an exopolysaccharide matrix (J. William Costerton et al. 1995). Biofilm mediated bioremediation is more proficient as biofilms have a better chance of adaptation and survival attributed to their protection within the matrix. Degradation of recalcitrant toxins is often a synergistic or division of labor effort, made possible by the high microbial diversity within a multispecies biofilm (Singh et al. 2006; Jefferson 2004). Thus, biofilm reactors have become a focus within the field of bioremediation. We hypothesize that the difficulties to bioremediation exposed by the recalcitrant NA fraction may be overcome by the use of selected enriched multispecies biofilms. The goal of this research was to first establish that multispecies biofilms could be grown from a TPW inoculant using the CBD, then to examine their ability to degrade two model NAs. The NA degradation capabilities were compared to those of single species biofilms, grown using TPW bacterial isolates.

2. MATERIALS AND METHODS

2.1 Multispecies biofilm culturing

All biofilms used in this study were grown using the CBD. The CBD is a two-part reaction vessel consisting of a standard 96-well microtiter plate with a specialized lid with 96 identical pegs, whereby each peg is suspended

within a single well of the microtiter plate. Media in the wells is inoculated, and identical biofilms form and adhere to the polystyrene pegs (H Ceri et al. 1999; Harrison et al. 2010).
For multispecies biofilm growth, the method used was very similar to that of previous work from our group (Golby et al. 2012). In short, each well of the 96-well microtiter plate received 75 µL of OSPW from a northern Alberta oil sands tailings pond as the bacterial inoculant, and 75 µL of media. The media used was a modified Bushnell-Haas (BH) minimal salts media (Wyndham & J W Costerton 1981). Some biofilms were grown on BH media supplemented with 1 g/L glucose (BH-G) as a complementary stimulatory carbon source. Positive control biofilms were grown on the rich media, trypticase soy broth (TSB) (Difco). For the biofilms grown in the presence of NAs, the model NAs, cyclohexane carboxylic acid (CHCA) and cyclohexane acetic acid (CHAA) (Sigma-Aldrich), were mixed at a 50:50 ratio (mixture referred to as 2χNA) and incorporated into the BH media (as salt naphthenates) at a final NA concentration of 100 mg/L.
Once inoculated the CBD was incubated on a gyrorotator shaker at 25 °C and 125 rpm. Biofilms were replenished once with 150 µL of fresh media 2 days after the initial inoculation. This provides emergent biofilms with fresh resources, but more importantly rids the reaction vessel of NAs contained within the TPW inoculant that may interfere with later NA measurements. Total incubation time lasted up to 14 days. Un-inoculated BH-glucose media with 2χNA served as a negative control.

2.2 Single species biofilm culturing

TPW single species colony isolates were obtained by spread plating 100 µL of OSPW on Bushnell-Haas glucose 2χNA agar plates incubated at 25°C for approximately 21 days. Three individual colony morphologies were identified, and subsequently isolated. These 3 isolates are referred to as G1, G2, and G3.
The methods used for growing single species biofilms are identical to those used for multispecies biofilms with the exception that the bacterial inoculant consisted of 75 µL of a 1.0 McFarland standard of an individual isolate. Single species biofilm growth media used was consistent with the media used to obtain these isolates, BH-G 2χNA.

2.3 Confocal Laser Scanning Microscopy (CLSM)

Biofilms were visualized by microscopy (Harrison et al. 2006) using the fluorescent nucleic acid stain Syto® 61 (Invitrogen, OR, USA), a Leica DM IRE2 with a 64x water immersion objective, and a Texas Red filter. Images were captured using Leica Confocal Software (LCS, Leica Microsystems), and processed in 3D using Imaris x64 Image Processing Software (Bitplane Scientific Software, South Windsor, CT, USA).

2.4 Gas Chromatography (GC)

Using dichloromethane (DCM) as an organic solvent, re-acidified NAs were extracted from the growth media using a method similar to Quesnel *et al* (Quesnel et al. 2011). Prior to analysis by gas chromatography (GC), NAs were derivatized into trimethylsilylates using 150 µL of BSA for multispecies biofilms, and 150 µL of BSTFA for single species biofilms. Upon derivatization mixed species biofilm NA levels were analyzed using GC coupled to a mass spectrometer (GC-MS). Single species biofilm NA levels were analyzed using GC coupled to a flame ionization detector (GC-FID). Day zero (initial NA) levels were determined by extracting NAs from BH-G 100 mg/L 2χNA media. NAs were also extracted from un-inoculated media to serve as abiotic controls.

3. Results and discussion

3.1 TPW biofilm growth

Multispecies biofilms may be grown on the CBD in the presence of NAs using TPW as an inoculum source, and grew under every condition tested. Thick and confluent biofilms grew on TSB (Figure 1A), which was expected, as TSB is an extremely rich medium. This condition existed as a positive control confirming the presence of viable bacteria in the TPW. Thin and sparse biofilms grew on BH minimal media alone, which suggests that there is a minimal amount of carbon available in the TPW. Sparse biofilms also grew when BH media was supplemented with 2χNA. When 1 g/L glucose was added to BH media, biofilm growth was more populous than just BH alone, but less populous than the TSB biofilms. Lastly, biofilms grew on BH-G media supplemented with 2χNA, and were roughly as populous as the BH-G biofilms (Figure 1B). For NA degradation experiments, all biofilms will be grown using BH-G 2χNA media to ensure that a thick and confluent biofilm is obtained for use in the experiments.
Single species biofilms using the isolates G1, G2, and G3 as inoculants, were also capable of growing as

biofilms on BH-G 2χNA media. These biofilms were all comparable in structure and size to each other, and to the BH-G 2χNA multispecies biofilms.

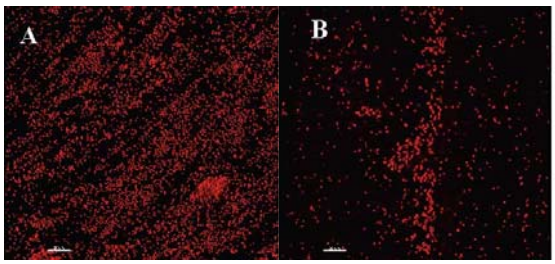

Figure 1: Confocal laser scanning microscopy images of tailings pond water biofilms. Images are 3D renderings of the biofilms created using Imaris software. Biofilms were grown aerobically (25 °C, 125 rpm) for 14 days using a tailings pond water inoculant. Growth media used included TSB (A), and BH-G 2χNA (B). Media was not replenished.

3.2 Model NA degradation

3.2.1 TPW Multispecies Biofilms

Figure 2, which displays the 2χNA levels in the CBD over a 10-day period, clearly demonstrates that model NA levels drop significantly over this time course. Although the observed loss in 2χNAs cannot be unequivocally attributed to metabolic degradation of CHAA and CHCA, this strongly supports our hypothesis that selected enriched multispecies biofilms are capable of degrading NAs. Our hypothesis was based upon other studies that have previously suggested that biofilm-mediated bioremediation of xenobiotic substrates (such as NAs) is more proficient than bioremediation with planktonic bacteria, as multispecies biofilms are better able to survive harsh conditions, and are extremely adaptable allowing the biofilm community as a whole to work together to metabolize substrates. This community interaction is often described as a heterogeneous division of labor effort, whereby different organisms within the biofilm contribute to the overall degradation/metabolism of the substrate at different steps within the degradation process (Singh et al. 2006; Stach & Burns 2002; Jefferson 2004). Furthermore, the model NAs tested (CHAA and CHCA) are relatively simple, and literature suggests therefore that these NAs are likely capable of being degraded by microorganisms via β-oxidation (Clemente et al. 2004; Kannel & Gan 2012; Quagraine et al. 2005). It is also interesting to note that 2χNA levels do not appear to drop until day 4 (Figure 2). One possible explanation for this may be that until day 4 the multispecies TPW biofilms are growing using the glucose as a carbon source, and once this resource is exhausted they start to metabolize the NAs.

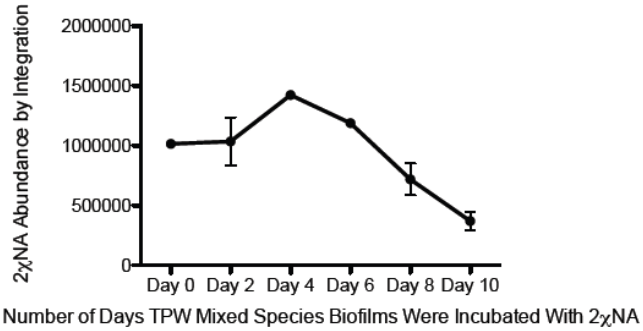

Figure 2: Abundance of NAs over a 10-day time course. Tailings ponds water multispecies biofilms were grown on the Calgary Biofilm Device (CBD) in the presence of 100 mg/L 2χNA (50:50 mix of CHCA ad CHAA) using Bushnell-Haas glucose (BH-G) media. Incubation occurred at 25 °C and 125 rpm. Media (with 100 mg/L 2χNAs) was replenished/supplied at Day 0, which represents starting NA levels. Biofilms were monitored for NA levels 2 days after inoculation. 2χNA abundance is measured by low resolution GC-MS (n=2).

3.2.2 TPW Single Species Biofilms

Only a few single species isolates were cultured from the community. As hypothesized, none of the single colony isolate biofilms were capable of degrading the model NAs tested. After 12 days of incubation, none of these biofilms had 2χNA levels significantly below that of initial NA levels (Figure 3). It is possible that these biofilms simply lack the community or village necessary to successfully metabolize CHCA or CHAA.

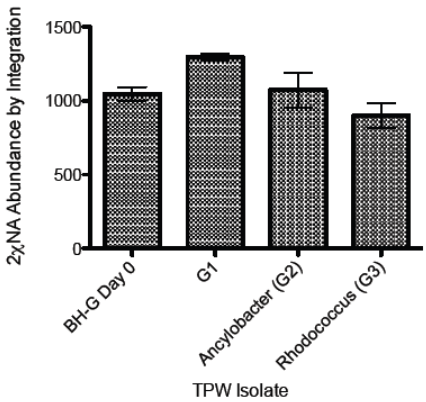

Figure 3: Abundance of 2χNA after 12 days of incubation with single species TPW biofilms. Biofilms were grown aerobically, using TPW single colony isolates as an inoculum source in the CBD, with Bushnell-Haas glucose and 100 mg/L 2χNA as the growth media. Incubation occurred at 25°C and 125 rpm on a rotary shaker for 14 total days (media and NAs replenished at day 2). 2χNA abundance is measured by GC-FID (n=2).

In conclusion both mixed and single species biofilms may be grown on the CBD using TPW as an inoculum source in the presence of NAs. Mixed species TPW biofilms are likely able to degrade the model NAs CHCA and CHAA, whereas single species TPW biofilms are not. The observed loss of NAs incubated with multispecies TPW biofilms is likely due to a cooperative/division of labor relationship between the organisms within the heterogeneous biofilm working as a village to metabolize the model NAs.

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SEDIMENT-FREE ENRICHMENTS ANAEROBICALLY DECHLORINATING AROCLOR 1260

Vlasta Dudková and Kateřina Demnerová
dudkovav@vscht.cz
Institute of Chemical Technology, Prague; Faculty of Food and Biochemical Technology; Department of Biochemistry and Microbiology; Technická 3, 166 28 Prague 6; phone: (00420) 220 443 021 Czech Republic

ABSTRACT

Polychlorinated biphenyls (PCBs) are ubiquitous manmade contaminants that pose a continuing risk to the health of humans and ecosystems. Bacteria that live in PCB-contaminated sediments have adapted to dechlorinate and detoxify of PCBs, but very little is known about these organisms. Learning how to effectively stimulate and grow these dechlorinators offers the best chance for developing safe, effective, and economic *in situ* remediation techniques in the future.
By a series of sequential transfers we developed enriched sediment-free consortia with stable dechlorinating activity against Aroclor 1260 from sediment of the river Strážský Sewer (Slovakia). Nona-, octa-, and heptachlorobiphenyls were dechlorinated to tri-, tetra-, penta- and hexachlorobiphenyls when pyruvate or lactate was added as a carbon source. The chlorophenyl rings targeted were: 23456-, 2346-, 2345-, 234- and 245-. Our consortia removed flanked *meta*- and *para*- chlorines. Under laboratory conditions, we examined the influence of known haloprimers: 44'-dibromobiphenyl or 26-dibromobiphenyl on the rate of dechlorination and the lag period. Furthermore, we explored the onset addition of 2-bromethanesulphonate, molybdate and selected antibiotics (ampicillin, kanamycin and vankomycin) on PCB dechlorination.
We enriched completely new dechlorinating enrichments which do not correspond to any of the known microbial PCB dechlorination processes or their combination. The key role in our isolated enrichments played sulphate-reducing bacteria.

KEY WORDS: PCB, Aroclor 1260, dechlorination, bioremediation

1. INTRODUCTION

Polychlorinated biphenyls known also under abbreviation PCBs are ubiquitous compounds contaminating the environment. PCBs are chemicaly stable, non-flamable and ironically, these properties made them hazardous for the environment. Luckily, it was foud that they could be transformed by several kinds of organism, one group of them are the anaerobic bacteria. Anaerobic bacteria are able to transform higher chlorinated PCBs into lower chlorinated PCBs The natural occurrence of anaerobic dechlorination was confirmed all over the world.
Our intentions were to confirm natural attenuation of PCBs in the long-term contaminated site, Strážský sewer (Slovak), by series of sequential transfers, to develop sediment-free microbial enrichment dechlorinating Aroclor 1260 and to identify dechlorination pattern and match it with known dechlorination patterns.

2. MATERIALS AND METHODS

To develop anaerobically dechlorinating enrichment in the sediment-free composition we used approach of Donna Bedard (2006). We used fine silica as a support for halogenated biphenyls and serial transfer of a small volume when dechlorination sufficiently progressed. For detailed description of the experiment see Dudková (2012).

3. RESULTS AND DISCUSSION

3.1 Characteristics of long term contaminated site

The original sediment used for our studies came from a long-term PCB contaminated site. The PCB composition on site was altered. We detected high levels of di-chlorinated and trichlorinated PCBs. On the figure 1 it is shown percentual distribution of *ortho*-, *meta*- and *para*- chlorines in commercial PCB mixtuers that were produced nearby and the composition of the sediment. The numbers in the chart represent the total amout of particular chlorine. We can see anormously high concentration of *ortho*- chlorines which we could presume was caused due to partial dechlorination of the present PCBs. As hallmarks supporting our presumption we detect high level of 26- and 22'-CB which were present in 35 mole %. The homologue distribution also supports our hypothesis about on site dechlorination.

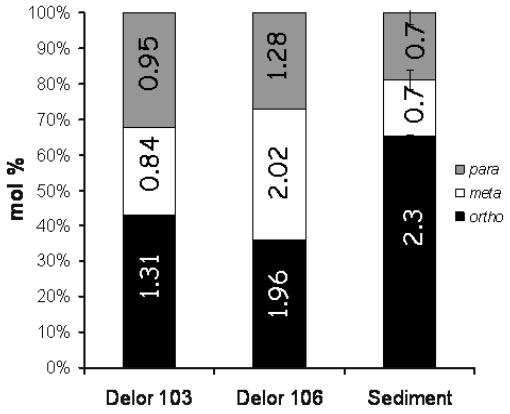

Figure 1. Comparison of chlorine distribution in Strážské Sewer sediment and in Delors 103 and 106.

3.2 Primary sediment slurry culture experiments

Because halopriming is useful stimulating technique used for dechlorination enhancement, we used two different brominated biphenyls 26 and 44' BB. 26 BB should stimulate dechlorination pattern N – *meta*- dechlorination and 44' BB should stimulate dechlorination pattern H – the *para* dechlorination. The original sediment slurry samples were prepared by resuspension of the contaminated sediment into the low-sulfur mineral medium and were amended by the selected primers.
At the figure 2, the mole distribution of hexa-nona chlorinated PCBs is listed. The PCBs which undergo dechlorination were the same for sediment slurry treated with haloprimers as well as without them. We did not observe the enhancement of dechlorination in case when we used the haloprimers.

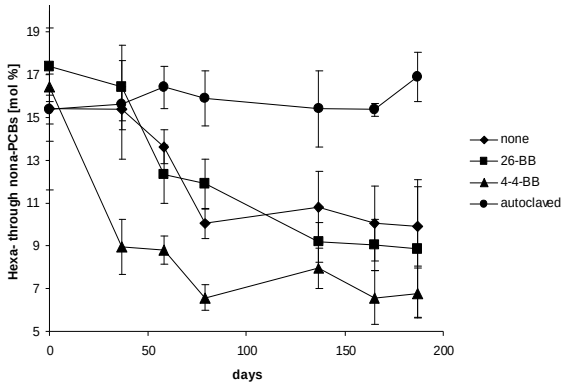

Figure 2. Changes in the proportions of hexa- through nona-chlorinated biphenyls in the primary sediment slurry samples during cultivation.

3.3 First serial transfer

We used Aroclor 1260 as a PCB source. Cultures were prepared by transfering of 10 vol. % sediment slurry samples into fresh low-sulfur mineral medium. As a potential carbon source, we used pyruvate, lactate or acetate amended by hydrogen. The addition of haloprimers was sustained. Flanked *meta*- and *para*- chlorines were dechlorinated and we did not detect any *ortho*- dechlorination.

3.4 Follow-up serial transfer

During sequential transfers, we decided not to add haloprimers anymore into the cultivation medium. This decision was made due to (1) hinderance of PCBs dechlorination in case when haloprimers were present and (2) because of no effect on enhancement of dechlorination. Lag time, the time before dechloriantion occurred, had variable character in particular sequential transfers. The lag time was almost none in primary cultures, approximately two-three weeks in the first transfer, 150 days in the second transfer and almost year in the third serial transfer, and no more than week in the fifth transfer.

General characteristics all of the mentioned batch cultures: The overal dechlorination activity expressed as molar homologue distribution over time is shown in figure 3. We can clearly see the decreasing concentration of hepta and octa chlorinated biphenyls. At the beginning of the dechlorination, hexachlorinated congeners are disappearing and later different are formed from hepta and octa chlorinated congeners. Penta and tetra chlorinated congeners are mainly formed.

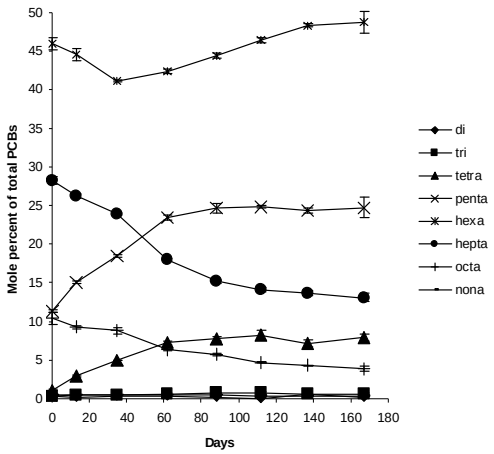

Figure 3. Changes in the PCB homolog distribution during dechlorination in sediment-free microbial enrichments using lactate as a carbon source
Data are the average of triplicate samples. Standard deviations are plotted and in many cases are smaller than the data symbols.

Closer inspection of congeners which were being dechlorinated revealed that only flanked chlorines from 23456-, 2346- and 2345- phenyl rings were dechlorinated. The other phenyl rings (234- and 245-) were also dechlorinated, but we were not able to show accurately which of the chlorines were removed.
We determined that effective carbon sources were pyruvate and lactate, and that dechlorination activity of our cultures was different from what we observed in the sediment. The attacked rings were 23456-, 2346-, 2345-, 234- and 245-phenyl and we observed sequential dechlorination.
The final experiment should tell as something more about microbes which were responsible for dechlorination and the syntrophic culture. We used two antibiotics, ampicillin and vacomycin, acting on gram positive bacteria, we used the molybdate as an inhibitor of sulphate reducing bacteria, we used 2-bromoethane sulphonate as an inhibitor of methanogenic bacteria and heat as detection of sporeforming bacteria. Our data showed more consistent results for cultures fed with lactate than with pyruvate. They also showed that gram positive bacteria did not play significant role in the dechlorination. The inhibitor of methanogenesis did not cause any harm either. But the heat completely hindered dechlorination suggesting that either syntrophic or dechlorinating or both

microbial population did not belong between sporeformers. From the experiment with molybdate was important, that sulfate reducing bacteria played key role in our dechlorinating cultures.

4. Conclusion

We believe that natural attenuation of PCB on site is highly likely. We develop stable sediment-free anaerobic culture dechlorinating Aroclor 1260 and using lactate as a possible carbon source. Our isolated culture is only minoritely responsible for the PCB distribution in the original sediment.
We were not able to match dechlorination pattern to any of known dechloriantion patterns. The culture removed flanked *meta*- and *para*- chlorines and that sulphate reducing bacteria play a key role in dechloriantion and that gram positive bacteria do not have any significant role.

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TOXICITY OF BENZONITRILE HERBICIDES AND THEIR BACTERIAL DEGRADATION PRODUCTS

Petra Lovecka, Marketa Thimova, Pavlina Janu, Petra Grznarova, Martina Mackova, Katerina Demnerova
loveckap@vscht.cz
Department of Biochemistry and Microbiology, ICT Prague, Technicka 3, 166 28 Prague 6, Czech Republic

ABSTRACT

Pesticides are xenobiotic compounds continually introduced into the environment to selectively eliminate pests. This work is focused to determination of toxicity and genotoxicity of the worldwide commonly used benzonitrile herbicides dichlobenil, chloroxynil, bromoxynil and ioxynil and their bacterial metabolites - amides and acides. Various systems for the testing of ecotoxicity were used to estimate the toxicity of intermediates of bacterial metabolism in comparison with initial benzonitrile herbicides Sea luminescent bacteria *Vibrio fischeri* was chosen as the prokaryotic model systems for investigation of the acute ecotoxicity. The eukaryotic model systems were represented by the seeds of *Lactuca sativa*, var. *capitata*. We tested also the cytotoxicity effect of herbicides on human hepatocellular carcinoma HepG2. The cytotoxic effect of various concentrations was measured with Real-Time Cell Analyzer RTCA DP. Genotoxicity was determinated using the Ames test with bacteria *Salmonella typhimurium* his- TA98 and TA100.
The test with the luminescent bacteria *Vibrio fischeri* proved high toxicity of the herbicides, but the toxicity of metabolites did not exhibit so high values. For the eukaryotic model, represented by the seeds of *Lactuca sativa*, var. *capitata*, the toxicity of each single insecticide was comparable. The results showed cytotoxicity for mammalian cells only of bromoxynil, chloroxynil and ioxynil.

KEY WORDS: benzonitrile herbucide, toxicity, cytotoxicity, genotoxicity, HepG2, *Vibrio fischeri*

1. INTRODUCTION

Halogenated benzonitrile analogues comprise a widely used group of herbicides. Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) as a selective agent for the management of broad-leaved weeds in cereal crops has been applied to large areas. It will be probably used even more intensively in future as a substitute of atrazine, which becomes banned in an increasing number of countries (Holtze et al. 2008). Ioxynil (3,5-diiodo-4-hydroxybenzonitrile) has a similar usage as bromoxynil. On the other hand, dichlobenil (2,6-dichlorobenzonitrile) was used for the treatment of smaller areas such as private gardens, orchards, plant nurseries or paths. Contamination of groundwater with its common metabolite 2,6-dichlorobenzamide persists after the application of this herbicide was abolished in some countries like Denmark (Holtze et al. 2006). The formation of this recalcitrant metabolite raised concerns as it is more mobile and hence more prone to spreading in the environment than the parent compound. Moreover, its biological effects have not yet been fully recognized (Holtze et al. 2006). Though it has been shown that a slow mineralization of the benzonitrile herbicides occurs in soils (for a review see Holtze et al. 2008), little is known on the organisms participating in this process. As a result, it has been frequently found in groundwater. Soil degradation studies also detected amides as the major products of bromoxynil and ioxynil. These metabolites are also more soluble than the original compounds, and presumably possess a medium mobility in soils. Their toxicity and environmental dissipation remain largely unknown.

2. MATERIALS AND METHODS

2.1 Chemicals

Benzonitrile, 3,5-dichloro-4-hydroxybenzonitrile (chloroxynil), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil), 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) and 2,6-dichlorobenzonitrile (dichlobenil) – and the authentic standards of their biotransformations – benzamide, benzoic acid, 3,5-dichloro-4-hydroxybenzoic acid,

3,5-dibromo-4-hydroxybenzoic acid, 3,5-diiodo-4-hydroxybenzoic acid, 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid - were purchased from standard commercial sources (Sigma Aldrich, Alfa Aesar) and were of analytical grade purity. Authentic standards of 3,5-dichloro-4-hydroxybenzamide, 3,5-dibromo-4-hydroxybenzamide, 3,5-diiodo-4-hydroxybenzamide were prepared from the corresponding nitriles by incubation with concentrated sulfuric acid at room temperature as described by Nielsen et al. (2007). Identity of the reaction products was confirmed by NMR and MS analysis.

2.2 Toxicity test using bacteria *Vibrio fischeri*

Lyophilized cells of *Vibrio fischeri* were resuscitated in 2 % NaCl at 15 °C. The cell suspensions were incubated with the examined compounds for 15 min at 15 °C and luminescence was determined using a Lumac Biocounter 1500 at the beginning of incubation (about 6000 relative light units) and at its end. The EC50 value was defined as the compound concentration at which luminescence was inhibited by 50 %, and was calculated from four measurements carried out for each of four concentrations of the examined compounds.

2.3 Toxicity test using *Lactuca sativa*

Germinating seeds of Lactuca sativa were incubated with 0.5 mM of the examined compounds at 22°C in dark and the root length was determined after 4 days. The inhibition coefficient I was defined as $I = Lc- L_T/ Lc *100$, where Lc is the average root length in seeds germinating in reference solution (cm), and L_T is average root length in seeds germinating in test solutions (cm)..A statistically significant difference between the average root length of seeds germinating in reference solution and test solutions was determined using the ANOVA test with a significance level of 95 %.

2.4 Measurement of genotoxicity using Ames test

The Ames test has a range of specific modifications and enables detection of a wide variety of mutagens. The detection system using *Salmonella typhimurium* his- differs in mutations within histidine operon. (Ames, 1975)

2.5 Measurement of cytotoxicity using system RTCA DP

An in vitro cytotoxicity evaluation of materials was carried out using an RTCA DP system (Roche Inc.). The system measures electrical impedance across interdigitated microelectrodes integrated on the bottom of tissue culture E-plates. The impedance measurement provides quantitative information of cell number and viability. Dynamic cell proliferation of cells was monitored in 90-min intervals from the time of treatment until the end of the experiment (48 h). Cell Index values were analyzed by RTCA software (Roche Inc.) to get I_{48} (%) (Oztűrk, 2011).

3. RESULTS

3.1 Acute toxicity of benzonitrile herbicides and their biodegradation products

Table 1. Determination of acute toxicity of chloroxynil, bromoxynil, ioxynil, dichlobenil and standards of their biodegradation products using the luminescent bacterium *Vibrio fischeri*

Compound	EC_{50} ± SD (μM)
2,6-dichlorobenzonitrile (dichlobenil)	505± 29
3,5-dichloro-4-hydroxybenzonitrile (chloroxynil)	5± 2
3,5-dibromo-4-hydroxybenzonitrile (bromoxynil)	14± 3
3,5-diiodo-4-hydroxybenzonitrile (ioxynil)	8± 2
3,5-dichloro-4-hydroxybenzoic acid	54±11
3,5-dibromo-4-hydroxybenzoic acid	42±2
3,5-diiodo-4-hydroxybenzoic acid	6 ±3
2,6-dichlorobenzoic acid	54±11
2,6-dichlorobenzamide	1773±53
3,5-dichloro-4-hydroxybenzamide	14±1
3,5-dibromo-4-hydroxybenzamide	8,±1
3,5-diiodo-4-hydroxybenzamide	5±3

EC_{50} = compound concentration causing 50 % inhibition of luminescence
SD = standard deviation

In chemoluminescence inhibition tests (Table 1), bromoxynil was found to be considerably more toxic than the corresponding carboxylic acid. The toxicities of ioxynil and its acid were similar and the highest of all the compounds. Dichlobenil and its corresponding amide exhibited a much lower toxicity than all the hydroxylated compounds. The toxicity of 2,6-dichlorobenzoic acid, however, was comparable to that of 3,5-dibromo-4-hydroxybenzoic acid. In luminescence inhibition tests (Table 1) 3,5-dichloro-4-hydroxybenzamide has larger inhibitory effect on the bacteria *Vibrio fischeri* than other the biodegradation products 3,5-dibromo-4-hydroxybenzamide, 3,5-diiodo-4-hydroxybenzamide.

Table 2. Determination of the effect of chloroxynil, bromoxynil, ioxynil, dichlobenil and standards of their biodegradation products (0.5 mM each) on root growth of germinating seeds of *Lactuca sativa*

Compound	Inhibition (%)
2,6-dichlorobenzonitrile (dichlobenil)	100
3,5-dichloro-4-hydroxybenzonitrile (chloroxynil)	100
3,5-dibromo-4-hydroxybenzonitrile (bromoxynil)	98
3,5- diiodo -4-hydroxybenzonitrile (ioxynil)	100
3,5-dichloro-4-hydroxybenzoic acid	52
3,5-dibromo-4-hydroxybenzoic acid	42
3,5-diiodo-4-hydroxybenzoic acid	67
2,6-dichlorobenzoic acid	60
2,6-dichlorobenzamide	93
3,5-dichloro-4-hydroxybenzamide,	53
3,5-dibromo-4-hydroxybenzamide,	50
3,5-diiodo-4-hydroxybenzamide	87

In toxicity assays using *Lactuca sativa* seeds (Table 2) 3,5-dibromo-4-hydroxybenzamide, 3,5-dichloro-4-hydroxybenzamide, have 50 % inhibitory effect on the plants. Toxicity of 3,5-diiodo-4-hydroxybenzamide has larger inhibitory effect than all compounds 87 %. In toxicity assays using *Lactuca sativa* seeds (Table 2), all of the benzonitrile analogues tested at 0.5 mM caused complete inhibition of root growth. At these concentrations, all the biodegradation products exerted less inhibitory effect on the plants than the parent compounds. However, at concentrations of 0.5 mM, 3,5-dichloro-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzoic acid exhibited a similar inhibitory activity as the corresponding nitriles.

3.2 Cytotoxicity of benzonitrile herbicides and their biodegradation products

The RTCA showed the cytotoxicity of benzonitrile herbicides and their metabolic products on HepG2 cells (Table 3).

Table 3. Determination of the inhibition effect I_{48} (%) of chloroxynil, bromoxynil, ioxynil, dichlobenil and standards of their biodegradation products on HepG2 cells

Compound	Inhibition effect I_{48} (%) for 100 mg/l
2,6-dichlorobenzonitrile (dichlobenil)	15
3,5-dichloro-4-hydroxybenzonitrile (chloroxynil)	99
3,5-dibromo-4-hydroxybenzonitrile (bromoxynil)	92
3,5- diiodo -4-hydroxybenzonitrile (ioxynil)	100
3,5-dichloro-4-hydroxybenzoic acid	56
3,5-dibromo-4-hydroxybenzoic acid	20
3,5-diiodo-4-hydroxybenzoic acid	19
2,6-dichlorobenzoic acid	12
2,6-dichlorobenzamide	13
3,5-dichloro-4-hydroxybenzamide,	13
3,5-dibromo-4-hydroxybenzamide,	12
3,5-diiodo-4-hydroxybenzamide	16

The results showed that the herbicides bromoxynil, chloroxynil and ioxynil as very cytotoxic. The other tested substances (dichlobenil and microbial metabolites) showed a moderate inhibition of cell growth.

Genotoxicity of benzonitrile herbicides and their biodegradation products
Genotoxic effect wasn´t proved in any case of benzonitrile herbicides and their biodegradation products.

4. Conclusions

Degradation experiments with benzonitrile herbicides identified as the principal products of biodegradation by soil microorganisms acids and amides of these substances (Holtze et al, 2007a; Holtze et al, 2007b; Holtze et al, 2008). Another follow-up revealed that the amide of dichlobenil BAM is poorly degradable in envirlonment and it became a serious groundwater contaminant. All the biodegradation products exerted less inhibitory effect on the plants than the parent compounds (Vesela, 2010). In chemiluminescence inhibition tests bromoxynil was found to be considerably more toxic than the corresponding carboxylic acid. The toxicities of ioxynil and its acid were similar and the highest of all tested compounds. Dichlobenil and its corresponding amide exhibited a much lower toxicity than all the hydroxylated compounds. On the other hand, the toxicity of 2,6-dichlorobenzoic acid was comparable to that of 3,5-dibromo-4-hydroxybenzoic acid. 3,5-dibromo-4-hydroxybenzamide, 3,5-diiodo-4-hydroxybenzamide have simile toxicity, 3,5-dichloro-4-hydroxybenzamide has less toxicity than others. In toxicity assays using *Lactuca sativa* seeds all the biodegradation products (2,6-dichlorobenzamide, 3,5-dibromo-4-hydroxybenzamide, 3,5-diiodo-4-hydroxybenzamide) exerted less inhibitory effect on the plants than the parent compounds, but higher than acids. Only toxicity of 3,5-dichloro-4-hydroxybenzamide has less inhibitory effect then all compounds. The herbicides bromoxynil, chloroxynil and ioxynil as very cytotoxic for HepG2 cells and all of substances are not genotoxic by Ames test.
The toxicological studies with benzonitrile herbicides bromoxynil, dichlobenil, ioxynil and their bacterial metabolic products however showed very low risk of these substances for human and other living organisms (U.S.EPA, 1998a; U.S.EPA, 1998b; EC, 2004a; EC, 2004b; Björklund et al., 2011a).

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COMPARATIVE MINERALISATION OF PYRENE IN A SPIKED AND AGED SOIL BY A PYRENE-DEGRADING BACTERIUM ISOLATED FROM A PAHS-CONTAMINATED SOIL AND EFFECT OF THE PRESENCE OF CYCLODEXTRIN

M.A. Sánchez-Trujillo, J. Villaverde, L. Láiz, E. Morillo.
jvillaverde@irnase.csic.es
Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC) Apartado 1052, Sevilla, SPAIN

ABSTRACT

A pyrene-degrading bacterial strain isolated from an aged PAHs-contaminated soil and identified as Achromobacter sp. was used to screen for its ability to mineralise pyrene. When the strain was exposed to ^{14}C-pyrene and 0.5 mg of nonradiolabeled pyrene as sole carbon source, Achromobacter sp. mineralised (degraded to CO_2) 55.5% of the pyrene at the end of the study. To investigate how degradation might be optimized in a pyrene spiked and aged soil, pyrene mineralisation by the indigenous microbial community was monitored over 140 days, and compared with mineralization in the presence of: i) a hydroxyporpil-β-cyclodextrin (HPBCD) solution as amendment; ii) Achromobacter sp. addition; iii) a combination of HPBCD and Achromobacter sp. The ability of indigenous microflora to mineralise ^{14}C-pyrene was appreciable (30.5%). Addition of HPBCD resulted in an important reduction of the lag phase from 79 to 54 days, but with no increase of the total extent of mineralization (31.0%). On the other hand, the addition of Achromobacter sp. alone resulted also in a drastic reduction of lag phase, from 79 to 45 days, however the total extent of mineralization of ^{14}C-pyrene was slightly lower (25.7%) than in non amended soil. After the addition of HPBCD and Achromobacter sp. in combination the total extent of mineralization reached was slightly lower than in non amended soil (23.5%), but however there was also an important reduction in lag phase duration, from 79 to 46 days, related with the addition of Achromobacter sp.

KEY WORDS: pyrene, mineralisation, biostimulation, cyclodextrin, contaminated soil.

1. INTRODUCTION

Pyrene, together with other polycyclic aromatic hydrocarbons (PAHs) resulting of both natural and anthropogenic production, are ubiquitous pollutants. These compounds persist in the environment, due to their hydrophobicity, deposited in soils and sediments (Ravindra et al., 2008). Some of these PAHs are considered to be possible or probable human carcinogens, and their distribution in the environment causes hazard to human beings (Seo et al., 2009). The US Environmental Protection Agency has monitored PAHs as priority pollutants in ecosystems since the 1970s. PAHs released into the environment could be removed through many processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation and adsorption. However, the principal process for successful removal and elimination of PAHs from the environment is the microbial transformation and degradation (Zhao et al., 2009). Nowadays, in order to obtain the elimination of these compounds in natural environments, considerable efforts have been focused on the isolation of microorganisms able to degrade them. Bioremediation based on certain species of microorganisms is a cheap and effective way to decontaminate PAHs-contaminated soils. PAHs with high molecular weight were less significant to biodegradation due to their low water solubility and strong adsorption onto the soil particle surface than PAHs with low molecular weight and aliphatic hydrocarbons (Kanaly and Harayama, 2000).

The persistence of hydrocarbons in soils is thought to be due to the absence of a bioavailable or biodegradable fraction (Semple et al., 2003). There are two controlling factors which determine the biodegradation: the rate of transfer of the contaminant to an organism in the soil, and the rate of uptake and metabolism of the hydrophobic organic compounds (HOC). To enhance biodegradation of organic contaminants in soil, it would be desirable to increase the bioavailable fraction within the total HOC burden within the soil (Semple et al., 2007). This may be enhanced by the addition of chemicals, such as cyclodextrins, to soils. Such amendments as bioavailability enhancers have been shown to enhance the rate and extent of the biodegradation of organic contaminants in soil (Allan et al., 2007).

Cyclodextrins (CDs) are a group of macrocyclic molecules derived from starch that have a torus-shaped cyclical structure comprised of α-1,4-linked glucose units, with a hydrophilic exterior and a hydrophobic central cavity (Shieh and Hedges, 1996). Hydroxypropyl-β-cyclodextrin (HPBCD) is a modified β-CD with increased hydroxyl functional groups on the exterior of the torus to improve its water solubility (Ko and Lebeault, 1999). This

structure can incorporate hydrocarbons, and increase their HOC solubility (Stockes et al., 2005), and, consequently, its biodegradability (Wang et al., 1998). Furthermore, column experiments identified that cyclodextrins have the ability to solubilise anthracene, pyrene and 2,4,4-trichlorobiphenyl in soils (Brusseau et al., 1994). This transfer of the contaminant from the soil-solid phase into soil solution is an important way of improving the biodegradable fraction of HOC in soils.
The aims of this study were: i) to screen for the ability of a bacterial strain, Achromobacter sp., isolated from an aged PAHs contaminated soil, to mineralise pyrene; ii) to investigate if degradation might be optimized in a pyrene spiked and aged soil by the presence of: 1. hydroxyporpil-β-cyclodextrin (HPBCD) as amendment (biostimulation), 2. Achromobacter sp. and 3. a combination of HPBCD and Achromobacter sp.

2. MATERIALS AND METHODS

2.1 Materials

Analytical standard (98%) pyrene and radiolabelled [4,5,9,10-^{14}C] pyrene (specific activity of 58.7 mCi mmol^{-1} and radiochemical purity >98%) were purchased from Sigma-Aldrich Chemical Co (Germany). The cyclodextrin (CD) employed was hydroxypropil-β-cyclodextrin (HPBCD), purchased from Cyclolab, Budapest, Hungary and with a chemical purity >97%.
A pyrene-degrading bacterial strain E2BCU-2008-S8.3, previously isolated from an aged PAHs-contaminated soil from the North of Spain, and identified as Achromobacter sp. by 16S rDNA gene sequence analysis technique was used. The strain was cultured in Tryptic Soy Broth (TSB) medium diluted 20 times.
The investigated soil was located at the experimental farm 'La Hampa' in Coria (Seville, Spain), presenting a clay texture and classified as Entic Pelloxerert (Morillo et al., 2004). Soil was sampled from the top 0-20 cm layer, air dried for 24 h and crashed to pass through a 2-mm aperture sieve, in order to remove stones, plant material and facilitate mixing, before its use. The characteristics of this soil are: pH 8.0; $CaCO_3$ 24.1%; organic matter 1.76%; sand 2.7%; stil 31.5%; and clay 65.9%.

2.2 Methods

2.2.1 Pyrene mineralisation assay in a culture medium inoculated by Achromobacter sp.

Mineralisation of ^{14}C-labelled pyrene assays were carried out in respirometers: modified 250 mL Erlenmeyers containing 100 mL of TSB medium diluted 20 times (TSB 0.05x) were used. Methanol stock solution containing ^{14}C-labelled and unlabelled pyrene was added to the TSB (0.05x) to obtain a final concentration of 5 mg L^{-1} and a radiactivity of approximately 1660 Bq per flask. After the addition of 1 mL of Achromobacter sp. inoculum containing 3 x 10^{9} CFU mL^{-1} the flasks were closed with Teflon-lined stoppers, and incubated at 20±1ºC. Production of $^{14}CO_2$ was measured as radioactivity appearing in the alkali trap of the biometer flasks. The trap contained 1 mL of 0.5 M NaOH. Periodically, the solution was removed from the trap and replaced with fresh alkali. The NaOH solution was mixed with 5 mL of liquid scintillation cocktail (Ready safe from PerkinElmer, Inc., USA) and the mixture kept in darkness for about 8 h for dissipation of chemiluminescence.
Controls were prepared at the same form and under the same conditions but in absence of Achromobacter sp. inoculum.

2.2.2. Pyrene mineralisation assay in a spiked and aged soil. Effect of Achromobacter sp. inoculation and/or HPBCD addition.

Mineralisation of ^{14}C-labelled pyrene assays were carried out in respirometers into which 10 g of the selected soil, were spiked with a methanol stock solution containing ^{14}C-labelled and unlabelled pyrene to obtain a final concentration of 50 mg kg^{-1} and a radiactivity of 1660 Bq per flask. The respirometers were closed and kept in dark for 90 days; after this period of aging, 100 mL of TSB (0.05x) culture medium per falsk were added and they were kept in a platform shaker for the time necessary to reach the soil-solution equilibrium. Then the respirometers were subjected to one of this treatments: addition of a HPBCD solution, with a concentration corresponding to 20 times the millimoles of the pyrene previously added in the soil, to enhance the PAH bioavailability and increase its mineralisation rate; adittion of 1 mL of Achromobacter sp. inoculum; and combination of HPBCD addition and Achromobacter sp. inoculation. Controls were prepared at the same form

and under the same conditions in absence of HPBCD solution and/or Achromobacter sp. inoculum. Afterwards, flasks were closed with Teflon-lined stoppers, and incubated at 20±1ºC during 140 days. Production of $^{14}CO_2$ was measured as described before.

3. Results and discussion

3.1 Pyrene mineralisation by Achromobacter sp in TSB (0.05x) culture medium.

Figure 1 shows pyerene mineralisation curve by Achromobacter sp. It is a standard 3-stage mineralisation curve. Fisrtly, there was a lag phase where the inoculum added adapted to the freshly amended pyrene and myneralisation was minimal (below 5%). Following this, there was an increase in the rate of mineralisation, as displayed by an "exponential" phase; after which catabolic activity reached a *plateau.* Achromobacter sp. in TSB (0.05x) culture medium was able to mineralise pyrene. Extensive mineralisation (55.5%) was observed after an initial 42 days lag phase; the maximun rate of mineralisation obtained was 1% day^{-1} and the time necessary to mineralise 50% if the initial pyrene amount added to the experiment were 128 days.

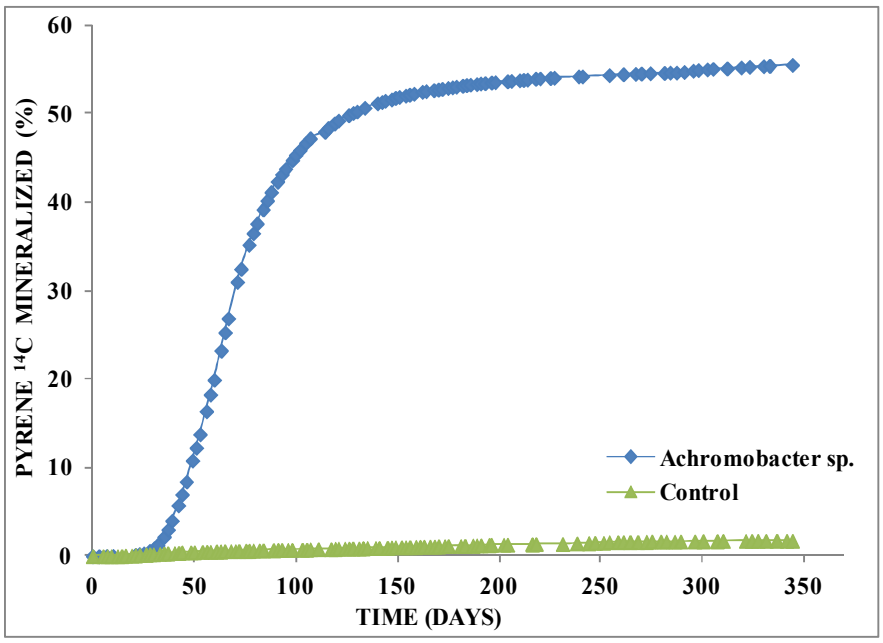

Figure 1. Mineralisation by Achromobacter sp of ^{14}C-pyrene in TSB (0.05x) culture medium supplemented with pyrene (5 mg L^{-1}).

The reached pyrene mineralisation percentage confirms that Achromobacter sp., isolated from a PAHs and aged contaminated soil, is a specific pyrene-degrader strain. Similar results were obtained by Tiwari et al. (2010), who isolated a bacterial strain, identified as Achromobacter sp., from oil refinery sludge. In that case, it was revealed that the strain was able to degrade 80% of hydrocarbon. They concluded that the strain Achromobacter sp. should be consider as a promising microorganism to be used for planning biorremediation strategies of contaminated sites.

3.2 Mineralisation experiments in spiked and aged pyrene-soil in presence of HPBCD and /or Achromobacter sp.

Figure 2 shows the pyrene mineralisation curves obtained after 140 days by natural soil atenuation (not inoculated) and in the presence of HPBCD solution and/or Achromobacter sp. Inoculum.

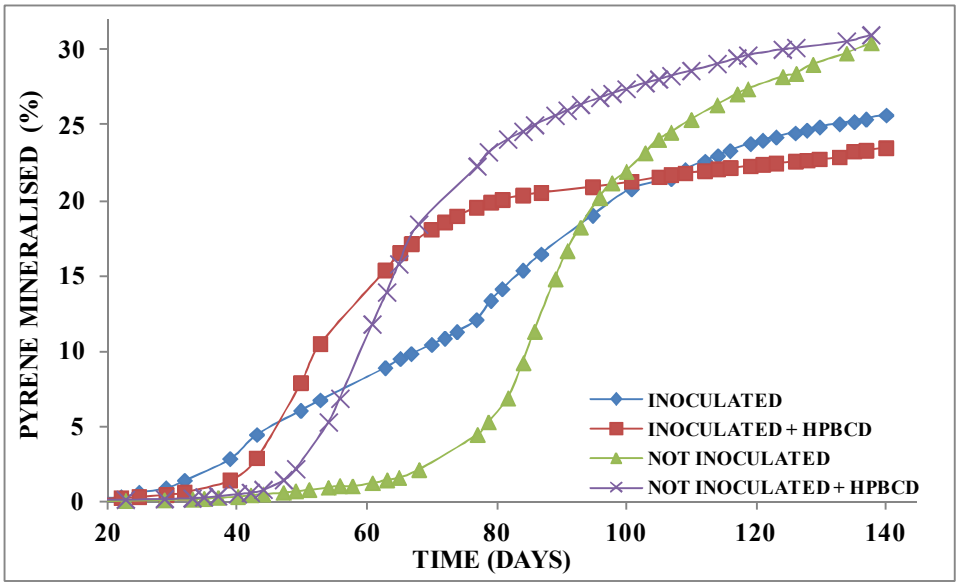

Figure 2. Pyrene mineralisation in spiked and aged soil by natural soil atenuation, and in the presence of HPBCD solution and/or Achromobacter sp. inoculum.

The ability of indigenous microflora to mineralise ^{14}C-pyrene was appreciable (30.5%). Addition of HPBCD resulted in an important reduction of lag phase duration, from 79 to 54 days (Table 1), but with no increase of the total extent of mineralization (31.0%). The high reduction of lag phase is related with the fact that HPBCD improves the solubility of pyrene and as a consequence the pyrene bioavailable fraction is ready to be degraded as soon as HPBCD is added.

Table 1. Effect of HPBCD addition and/or Achromobacter sp. inoculation on natural soil attenuation.

	Lag phase (days)	Overall extent of ^{14}C mineralisation (%)
NOT INOCULATED	79	30.5
NOT INOCULATED + HPBCD	54	31.0
INOCULATED	45	25.7
INOCULATED + HPBCD	46	23.5

The addition of Achromobacter sp. alone resulted also in a drastic reduction of lag phase, from 79 to 45 days (Table 1), similar to the lag phase obtained in pyrene mineralization in TSB medium with Acromobacter sp. However, the total extent of mineralization of ^{14}C-pyrene was slightly lower (25.7%) than in non amended soil. This result indicates that the introduced bacteria limit the activity of the indigenous microorganisms in the studied soil.

After the addition of HPBCD and Achromobacter sp. in combination the total extent of mineralization reached was slightly lower than in non amended soil (23.5%), this result suggests again that the presence of Achromobacter sp. interferes with the activity of the indigenous microorganisms in the soil; there was also an important reduction in lag phase duration, from 79 to 46 days, related with the addition of Achromobacter sp.

In conclusion and based on these results, this study shows that the indigenous microbial communities in a soil spiked with pyrene and aged for 90 days have the potential to actively and extensively mineralize target PAH. The use of HPBCD solution at a very low concentration of only 20 times the pyrene equimolar concentration in soil will act as an bioavailability enhancer, accelerating the pass of the pyrene desorbing fraction from the soil particle surface to the soil solution , and improving the microorganism accessibility to the PAH, provoking a reduction of lag phase duration with no interferences in the activity of the indigenous microorganisms.

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DEGRADATION OF HCH ISOMERS, DDT AND THEIR DEGRADATION INTERMEDIATES IN HIGHLY CONTAMINATED SOIL

Ljuba Zidkova[1], Costel Bumbac[2], Cristiana Cosma[2]
zidkova@dekonta.cz
[1]Dekonta, a.s., Dretovice 109, 27342 Stehelceves, Czech Republic
[2]The National Research and Development Institute for Industrial Ecology,
90-92 Panduri street, 050663 Bucharest, Romania

ABSTRACT

This work was focused on chemical reduction of HCH isomers (α-ε), DDT and their degradation intermediates using zero valent iron (steel grit G200, Kovobrasiv Mnisek s.r.o, Czech Republic) in comparison to a Daramend ISCR technology (in situ chemical reduction; Adventus Americas, Inc.). The technology was developed for persistent organic compound degradation and consist in application of micro zero valent iron with an organic substrate under sequential anaerobic/aerobic conditions.
In the framework of these experiments highly contaminated soil with both contaminants (HCH and DDT) was used. During 3.5 months HCH removal efficiency (α-ε HCH isomers) 63% and DDX removal efficiency (DDT and its degradation intermediates) 70% were achieved when the Daramend technology was used. The HCH and DDX removal efficiency of the same soil sample was lower, 32% and 58% respectively, when only zero valent iron (steel grit G200) was used.
Results of two control variants were compared to above mentioned efficiencies. Degradation of HCH was observed even in the control of the Daramend experiment (60%) where sequential anaerobic/aerobic cycles were tested. The concentration of HCH in the other experiment (steel grit, only anaerobic condition) did not change. However, degradation of DDX was observed in both controls. The results show that the most important technological condition for an effective HCH degradation is the application of sequential anaerobic/aerobic cycles.

KEY WORDS: zero valent iron, in situ chemical reduction, chlorinated pesticides, HCH, DDT

1. INTRODUCTION

Hexachlorocyclohexane was produced industrially in large quantities for its insecticidal effect and used for removing human and animal parasites and for a protection of forests, agricultural crops and other vegetation in the Czech Republic, former Czechoslovakia, and in Romania. The insecticidal effect has only gamma isomer of HCH (γ-HCH, Lindane). Lindane was separated from the mixture of HCH isomers using fractional distillation. Waste isomers were usually stored at place with often inefficient safety precautions. Lindane was usually used together with DDT, after restriction of DDT was further used for protection of seed grain. Lindane was restricted in 1989. HCH isomers are relatively stable compounds with higher solubility in water in comparison to other POPs (DDT, Aldrin, Heptachlor) therefore has also higher mobility in atmosphere and hydrospere.
DDT was widely used in former Czechoslovakia and Romania approximately from 1944. During its production DDD was generated as well. Further, DDT is transferred to DDE in the environment. DDT was restricted in 1974. DDT and its degradation intermediates are relatively stable non-polar compounds with low water solubility but they also accumulate in lipid parts of organisms and absorb on solid particles of soil.
This work was focused on decontamination of soil polluted by with HCH isomers (403 g/kg), DDT (4 mg/kg) and their degradation intermediates. The chemical reduction was selected for those experiments with highly contaminated soil and two different ZVI (zero valent iron) forms were tested. Steel grit G200 (particles 0.18-0.065 mm), Kovobrasiv Mnisek s.r.o, Czech Republic, and microscale iron as a component of a Daramend preparation.

2. MATERIALS AND METHODS

The Daramend technology consists in application of the Daramend preparation (40 % ZVI, 60 % organic substrate) and sequential anaerobic (longer) and aerobic (shorter) phases. In this work one cycle was comprised from 10 days of the anaerobic phase followed by 4 days of the aerobic phase. The soil was homogenized and placed to glass reaction bottles (1 kg of contaminated soil, 2 l bottles), soil was watered to dry mass around 50 % and 1% w/w of Daramend was added. Next 2% w/w of Daramend was added after 50 days to accelerate the

degradation process. At the end of the anaerobic phase ORP and pH were measured, reaction bottles were open and the soil was regularly stirred during subsequent aerobic phase. At the end of the aerobic phase samples for chemical analyses were taken.

In case of the experiment with steel grit addition 250 ml reaction bottles with 100 g of contaminated soil were used. The whole bottle was subsequently analysed including the composition of a gas phase. 5 % w/w of steel grit was used and water was added to achieve around 50 % of dry mass. Further, resazurin (1 mg/kg) was added for visual check of anaerobic conditions. The aerobic phase was not included in the ZVI experiment.

3. Results and discussion

During 3.5 months HCH removal efficiency 63 % and 32 % in D (Daramend) and ZVI (steel grit) variants were achieved, respectively (Figure 1). Both isomers α and β were degraded more efficiently than γ, δ and ε isomers. The composition of degradation isomers was similar in both variants, the highest concentration (634 and 758 mg/kg for ZVI and D experiment, respectively) was observed for chlorobenzene, then benzene and dichlorobenzenes were detected in both variants whereas concentration of trichlorobenzenes and tetrachlorobenzenes was slightly decreased in both variants, again. 70% of DDX (DDT and DDD) was degraded in D variant, 58% in ZVI variant. The generation of DDE was not proved (<0.5 mg/kg). The anaerobic conditions were monitored by ORP values which decreased to final -405 mV in variant D (Figure 3) and were proven by disappearance of pink colour of resazurin in variant ZVI. Further, generation of H_2 (92.4%) and CH_4 (1.4%) was observed in the ZVI variant (Figure 3) whereas in the ZVI control decrease of O_2 and slight increase of CO_2 was found.

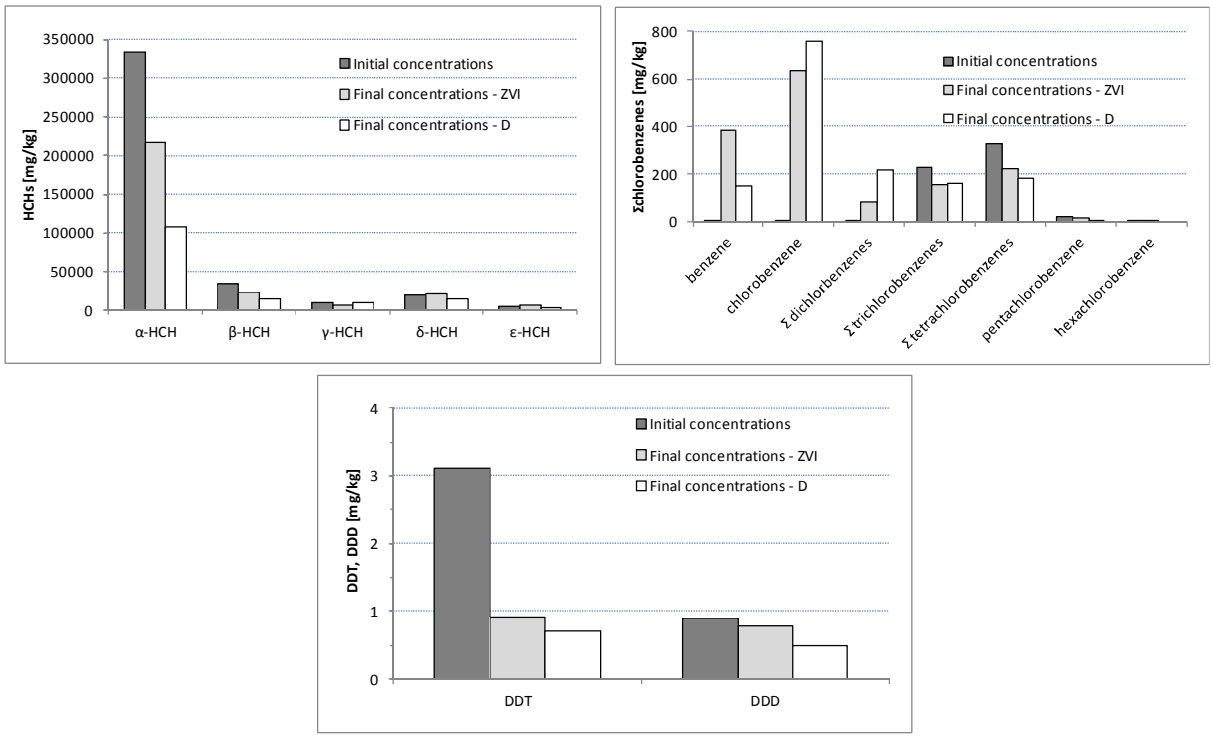

Figure 1. The initial and the final concentrations of HCH isomers (HCHs), DDT and their degradation intermediates – chlorobenzenes and DDD in ZVI (steel grit) and D (Daramend) variants after 3.5 months. DDE was not detected (<0,5 mg/kg).

The respective control variant of D and ZVI experiments (without Daramend or steel grit addition) revealed the key role of sequential anaerobic and aerobic phases. The HCH removal efficiency in the control of the D experiment was 60 %; in the control of the ZVI experiment no decrease of HCHs was observed (Figure 2). Therefore inclusion of the aerobic phase had a significant positive effect on the HCH degradation. 68% and 58 % of DDX were degraded in control variants of D and ZVI experiments, respectively. The system of sequential anaerobic/aerobic phases had no significant influence on DDT (and DDD) degradation, DDE was not detected. The difference of controls was observed also in the composition of chlorobenzenes. No generation of benzene, cholorobenzene or dichlorbenzenes was found, however the decrease of trichlorobenzenes concentration (both

experiments) and tetrachlorobenzenes (D experiment) was observed. ORP values in the control of D experiment ranged from 411 to 218 mV (Figure 3).

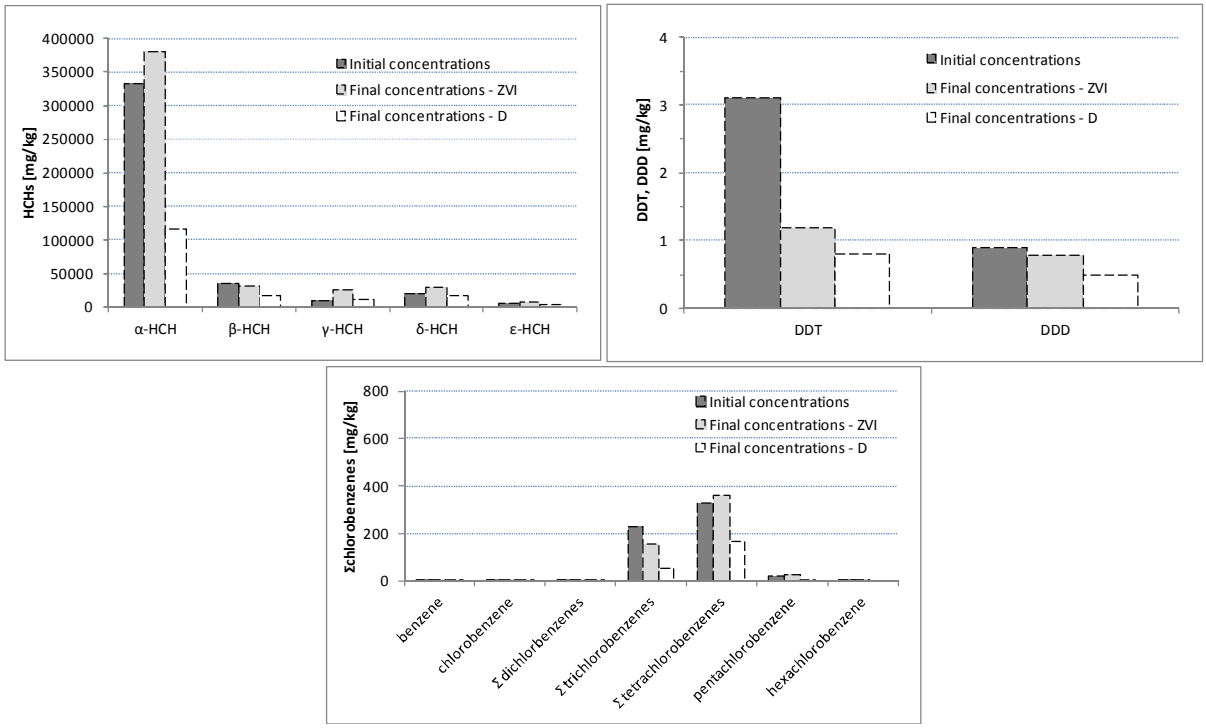

Figure 2. The initial and the final concentrations of HCH isomers (HCHs), DDT and their degradation intermediates (chlorobenzenes and DDD) after 3.5 months in respective (ZVI (steel grit) and D (Daramend)) **control variants**. DDE was not detected (<0,5 mg/kg).

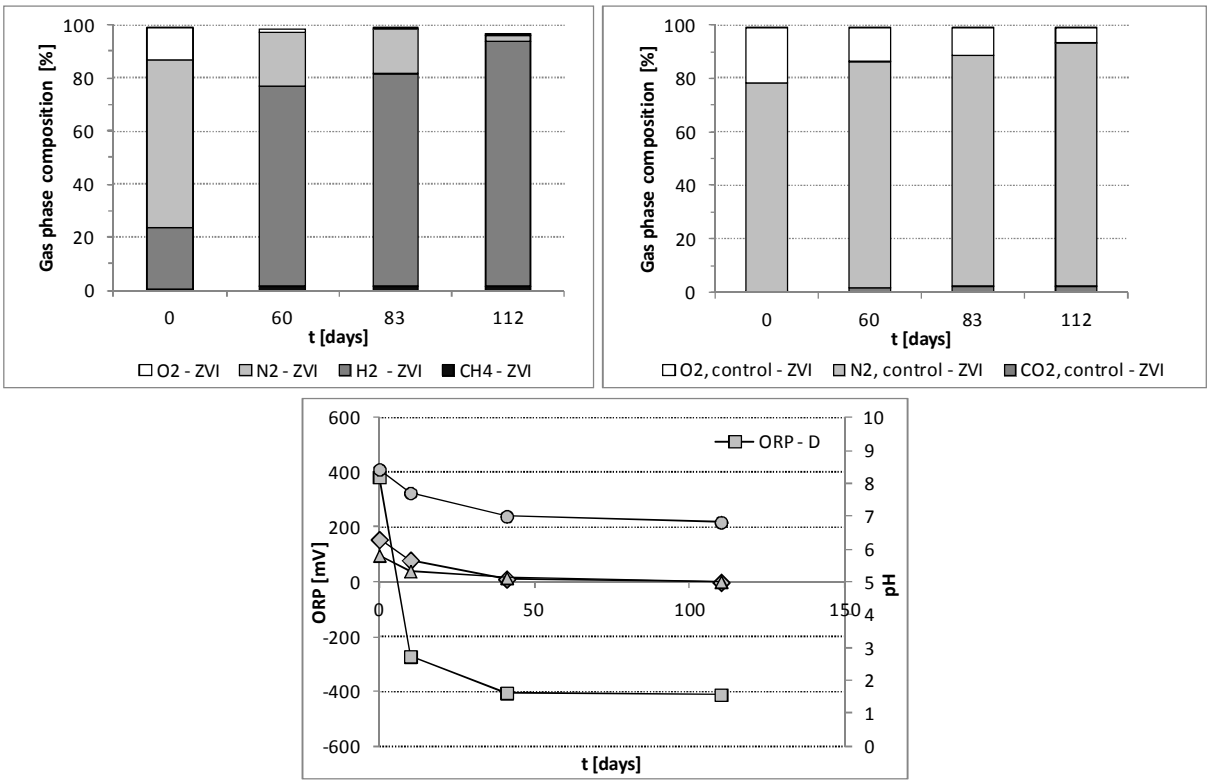

Figure 3. The chenges in gas phase composition in ZVI (steel grit) experiment, variant with steel grit and control, and changes in pH and ORP values in D (Daramend) experiment, variant with Daramend addition and control.

4. CONCLUSION

The degradation under sequential anaerobic/aerobic phases was evaluated as more efficient technology for simultaneous HCH and DDT degradation. Under these conditions removal of 63% of HCHs and 70 % of DDX (DDT and DDD) was achieved in 3.5 months. No decrease of HCH was observed in the control of ZVI experiment whereas 60% of HCHs were degraded in the control of Daramend experiment. However, the decrease was not caused by the presence of the Daramend preparation (the comparison with the control). Therefore sequential anaerobic/aerobic cycles had a significant influence on the efficiency of the HCH degradation and were the key technological step.
Very low concentrations of degradation intermediates were found in both experiments. The highest final concentration belonged to chlorobenzene in Daramend variant (758 mg/kg).
HCHs and DDX were degraded in both variant with zero valent iron addition (no matter independently on the ZVI form). Results from this test indicate that extending the treatment period could result in a more efficient treatment.

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USE OF GENETICALLY MODIFIED ORGANISMS IN BIOREMEDIATION

SIMBIOTIC LEGHEMOGLOBIN GENE TARGETED TO TOBACCO GENOME AFFECTS PLANT GROWTH AND ENERGY STATUS DIRING CADMIUM TREATMENT

Dmitryukova[1] M.Yu., Fedyaev[1] V.V., Vershinina[2] Z.R., Postrigan[2] B.N., Baimiev[2] A.Kh
zilyaver@mail.ru
[1] Bashkir State University, Department of Biology, 450074, Bashkortostan, Ufa, Validy Str. 32, Russia
[2] Institute of biochemistry and genetics of Ufa science centre RAS, Department of Molecular Biology and Plant Physiology, 450054, Bashkortostan, Ufa, Prospekt Oktyabrya 71, Russia

ABSTRACT

Transgenic tobacco (*Nicotiana tabacum* L., cv. Samsun) plants bearing the soybean (*Glycine max* (L.) Merr.) leghemoglobin A gene under the control of 35S CaMV promoter were produced. The effects of this gene expression on tobacco growth and respiration, MDA content, and activities of catalase and guaicol peroxidase were investigated. The growth rate of transformed plant was reduced, respiratory losses were increased, and lipid peroxidation was substantially suppressed. In plants expressing the leghemoglobin A gene, the weakening of cadmium negative effects on growth parameters and plant oxidative status were observed.

KEY WORDS: leghemoglobin, cadmium growth, respiration, MDA

1. INTRODUCTION

Leghemoglobin (legoglobin, Lb) is a myoglobin-like hemoprotein transferring oxygen and it has a very high oxygen binding affinity (Jokipii-Lukkari et al., 2009). Lb is synthesized in the nodules of symbiotic legumes during rhizobia-legume symbiosis. Its function is to maintain low oxygen concentration sufficient for bacteroids' respiration and safe for nitrogenase, which is sensitive to oxygen (Vinogradov and Moens, 2008). Lb was shown to enhance oxygen uptake by plant mitochondria (Suganuma et al., 1987), and is capable of interaction with reactive species of oxygen (ROS) and nitrogen, thus manifesting anti- or proxidant properties (Kosmachevskaya and Topunov 2009), i.e., participating in plant protection against oxidative and nitrosative stress (Badouin et al., 2006).
Since the main function of most nonsymbiotic Hb is to protect plants against a variety of adverse factors, it is important to study the effect of symbiotic Hb on the metabolism of plants under stressful conditions. As a source of nonspecific oxidative stress may serve heavy metals, including cadmium, which does not participate in the normal plant metabolism and is toxic even at low concentrations (Hatata et al., 2008).
The purpose of this work was studying growth, energy metabolism, intensity of lipid peroxidation, and activities of antioxidant enzymes in tobacco plants under normal conditions and under oxidative stress induced by toxic concentrations of cadmium salts.

2. MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

Bacteria *Escherichia coli* strain XL1-Blue and *Agrobacterium tumefaciens* strain AGL0 was used. For plant transformation a binary vector pCambia 1301 was used.

2.2 Manipulations with DNA

Genomic tobacco DNA was obtained by the salt extraction method (Aljanabi and Martinez, 1997). Total RNA was isolated using TRIzol (Invitrogen, United States). Total RNA was isolated using trizol (Invitrogen" (USA). RT-PCR was performed with MuLV-reverse transcriptase ("Fermentas", Lithuania). Plasmid DNA was isolated by alkaline lysis of bacterial colonies. Ligation was performed using T4 DNA ligase (Silex, Russia) as described in manual. Quality and quantity of the obtained preparations were determined by analytic electrophoresis on 1% agarose gel. Agarose gel electrophoresis was carried out in the SubCell GT WIDE MINI (Bio-Rad, United States) as described in Sambrook, 1989. PCR was performed in thermocycler (DNA Technology, Russia). The soybean Lb A gene was amplified using forward TATGGTTGCTTTCACTGAGA and reverse GATACTAATTATGCCTTCTT primers. Primers were selected using Lasergene programs (DNASTAR,United States). Electroporation of *A. tumefaciens* competent cells was performed using a Micropulser electroporator (Bio Rad).

2.3 Producing transgenic plants

Transgenic tobacco plants were obtained by agrobacterial transformation of leaf explants (Horsh et al., 1985). Histochemical analysis was assayed as described (Jefferson, 1987) with some modification (Kosugi et al., 1990).

2.4 Studying the morphophysiological characteristics of transgenic plants

In experiments transgenic tobacco plants of the first generation (T1) were taken. As a control wild type tobacco plants were used. Seeds obtained from transgenic tobacco plants were germinated on medium with antibiotic (hygromycin) and then were transferred into commercial soil. The plants were maintained in a greenhouse with a 16-h photoperiod and the temperature about a 26^0C. for approximately 4 weeks. Then plants were transferred to water culture containing Hogland –Arnon salts (pH 7) and, after 3 day preculture, mineral nutrient solution was supplemented with cadmium acetate up to the final concentration of 2.5 mg/l.
Growth estimation was carried out in 14 days after transferring. Changes in plant dry weight (ΔW), total respiration, lipid peroxidation intense and catalase and guayacol peroxidase activity were measured. Total respiration (ΣR) was determined by the monometric method in the Warburg apparatus and expressed in µl of O_2/day. Gross photosynthesis (Pg) was calculated as a sum of dark respiration (ΣR) and increasing of dry biomass per a day. Respiration costs for adaptation was calculated, using relative growth rate (RGR) and the ratio of dark respiration to gross photosynthesis.
The inhibitory analysis was conducted on 4 week old plants. To inhibit cytochrome oxidase 2 mM NaN3 was used. Cyanide resistant oxidase was inhibited with 15 mM salicylhydroxamic acid. The solutions of inhibitors were prepared in 0.1 M K phosphate buffer (pH 4.5). MDA content was determined by the thiobarbituric acid (TBA) as described by Heath and Packer (1968). Catalase activity was assayed by the method based on the capability of hydrogen peroxide to produce stable colored complex with molybdenum salts. Peroxidase activity was assayed spectrophotometrically in reaction with guaiacol
All measurements were performed at least in ten replications. Data statistic processing was performed using the Microsoft Excel 2007 software.

3. Results and discussion

To confirm plant transgenecity and transgene expression activity, the seeds obtained from regenerated T0 shoots were germinated on medium containing hygromycin. The segregation ratio in T1 generation after the trait of resistance to the antibiotic made up 3 : 1, which indicates the presence of a single copy of vector T-DNA insertion into the plant genome. PCR with DNA from tobacco seedlings resistant to hygromycin demonstrated the presence of the soybean Lb A insertion (Fig.1). Also, the synthesis of Lb A mRNA was confirmed by RT-PCR on RNA isolated from the leaves of transgenic tobacco.

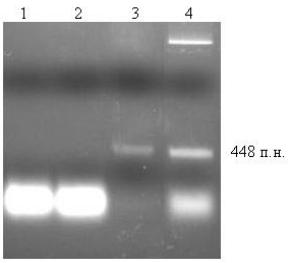

Figure 1. RT-PCR in transgenic tobacco plants. Lane 1-2 wild type tobacco; lanes 3 transgenic tobacco; lane 4 molecular weight marker.

Expression of Lb A gene led to growth inhibition of transgenic tobacco plants (Fig. 2). In transformed plants the RGR was by 23% lower than in wild type plants. Our results agree with studies demonstrating growth retardation in potato plants with soybean Lb A gene inserted into their plastids. Medium supplement with cadmium reduced RGR in transgenic plants by 2.6 times and in wild type plants – by 1.8 times, i.e., toxic cadmium concentrations more substantially reduced growth of wild type plants.

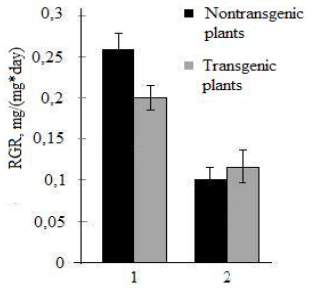

Figure 2. Relative growth ratio. Measuring took during 14 days after transferring to cadmium containing medium.

Dark respiration as a main source of energy plays has an important role in the regulation of metabolism; and is a key indicator of oxidative processes in the cell (Atkin et al., 2007). The ratio of total dark respiration (Σ*R*) to gross photosynthesis (*P*g) characterizing plant energy balance was calculated based on the measuring of the respiration rate and growth parameters. We demonstrated that in transgenic plants the ratio was higher by 15% than in wild type plants. After cadmium treatment, this ratio increased in transgenic plants only by 5%, whereas in wild type plants it increased more significantly (by 23%) (Fig. 3).

Previously it has been shown that the Σ*R*/*P*g ratio increases under unfavorable conditions. The degree of increase in the Σ*R*/*P*g ratio depends on plant tolerance to stress factors: the more tolerant organism the less changes in the Σ*R*/*P*g ratio (Rachmankulova et al., 2003).

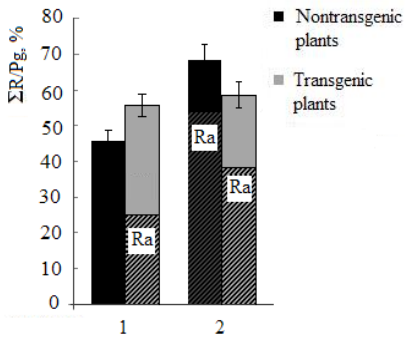

Figure 3. The ratio of total dark respiration (Σ*R*) to gross photosynthesis (*P*g).1-control, 2-after 14 days on cadmium acetate (2.5 mg/l). Ra –adaptation component of total respiration.

The increasing in the total dark respiration under stress is strongly associated with an increasing in so-called adaptation component of total respiration (*Ra*), not related to the growth and raising under adverse conditions. The more increases this component, the less resistance of plants to this stress (Usmanov et al., 2001). Calculation of the respiratory cost of adaptation showed that the value of *R*a in transformed plants was 26%. After cadmium salts supplement, the costs of adaptation in transgenic plants were 39%, that is lower than in wild type plants (54%). Thus, our data shows that transformed plants are characterized by better balance and stability of energy transforming processes.

Respiration is a multicomponent process including several biochemical pathways differing in energetic efficiency.To understand reasons underlying the increasing in the respiratory cost in transgenic plants under influence of heavy metal, we performed the inhibitory analysis of the pathways of dark respiration. In transgenic plants, cyanide resistant respiration was higher by 15% and cytochrome respiration was reduced slightly (by 10%) (Fig. 4).

After cadmium addition to nutrient medium, the rate of cyanide resistant and residual respiration increased, especially in wild type plants. The rate of the alternative respiration pathway slightly increased in transgenic plants and in wild type plants.

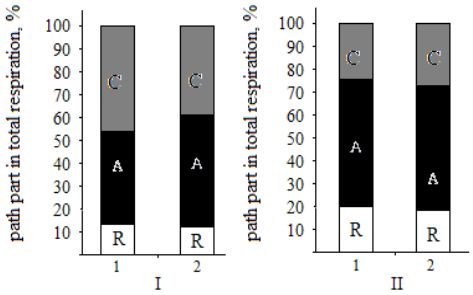

Figure 4. The ratio of respiration paths. I – control, II- after 14 day after cadmium supplement. C – cytochrome respiration, A – alternative (cyanide resistant) respiration, R – residual respiration

One of the early nonspecific plant responses to stressors is an active ROS generation (Juszczuk, Rychter, 2003). Lipid peroxiadation activity in plants was determined by the content of MDA. In transgenic plants, it was lower by 30% than in wild type plants. Reduced MDA content in transgenic plants was evidently related to AO activation. The alternative cyanide resistant pathway of respiration is known to prevent generation of superoxide radical in the electron transporting chain and accumulation of hydrogen peroxide in mitochondria. Under the influence of cadmium, the content of MDA increased in both genotypes; however, this increasing was less in transformed plants (by 12%) than in wild type plants (by 24%) (Fig. 5). Thus, transgenic plants were characterized by more favorable antioxidant status.

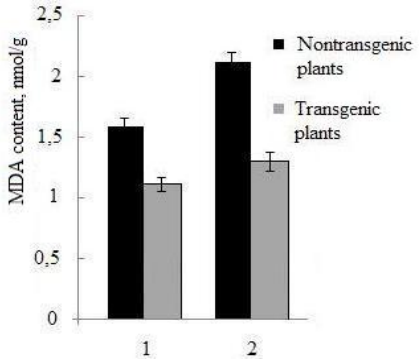

Figure 5. Effect of *lba* on MDA content. 1- one month old plants, 2- after 14 days on cadmium containing medium.

Lb autooxidation or its interaction with ROS leads to the loss of Lb oxygen carrier properties. Therefore, to maintain Lb in its active form, the presence of peroxidases declining the content of peroxides in the cell is required. Thus, enzymes involved in oxidative stress protection can participate in Lb maintenance in the active state. To study the effect of Lb on activities of antioxidant enzymes in the presence of heavy metal, activities of guaiacol peroxidase and catalase were assayed. Activity of guaiacol peroxidase in transgenic plants was by 19% lower than in wild- type plants. Cadmium addition to medium activated guaiacol peroxidase substantially in wild type plants and insignificantly in transgenic plants (Fig. 6). Catalase activity was not statistically different in transgenic and wild type plants. Cadmium treatment activated catalase by 20% in transgenic plants and by 60% in wild type plants. Thus, we demonstrated that transgenic plants bearing the soybean Lb A gene were more resistant to unfavorable action of heavy metal.

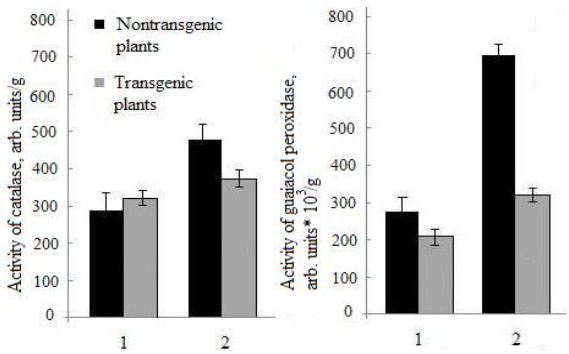

Figure 6. Effect of lba on catalase and guaiacol peroxidase activity. 1- control, 2 – after 14 days of cadmium treatment.

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PREPARATION OF TRANSGENIC PLANTS FOR MORE EFFECTIVE RHIZOREMEDIATION OF PCBs

Veronika Kurzawova, Martina Novakova, Martina Chovancova, Tomas Macek, Martina Mackova
veronika.kurzawova@vscht.cz
Institute of Chemical Technology Prague, Faculty of Food and Biochemical Technology Department of Biochemistry and Microbiology, Technicka 3, 166 28 Prague 6, Czech Republic

ABSTRACT

Polychlorinated biphenyls (PCBs) are organic compounds widely spread in the environment. Plants, microorganisms and their consortia can be used as an effective tool for the decontamination of PCB-polluted areas. Some bacteria degrade PCBs anaerobically, others by aerobic pathway determined by biphenyl operon. Expression of genes of biphenyl operon is inducible and it was found that several plant secondary metabolites including flavonoids (e.g. quercetin) and terpenes can serve as efficient inducers. Investigation of plants or construction of transgenic ones producing high amounts of plant secondary metabolites inducing bacterial biphenyl operon can be one of the approaches for rhizoremediation enhancement.
The aim of this study is to prepare transgenic *Nicotiana tabacum* plants overexpressing genes for flavonoid-3'-hydroxylase (*AtF3'H*), the enzyme converting indigenous kaempferol to quercetin. The ability of quercetin to induce the expression of *bphA* gene, the first gene of the biphenyl operon, was verified and consequently the *AtF3'H* gene was cloned to the pQE30 vector to obtain the enzyme with histidine tail (6 x His) after expression in bacteria. Following the activity assay of expressed enzyme, the *AtF3'H* gene with His-Tag will be cloned to the plant vector under the control of the root specific promoter targeting the expression of *AtF3'H* into roots of transgenic plants. This should lead to a higher production of quercetin in plants and consequently to releasing of this compound to the rhizosphere where this flavonoid should stimulate PCB degradation by rhizosphere bacteria.

KEY WORDS: phytoremediation, quercetin, flavonoid-3'-hydroxylase, PCBs

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are xenobiotic organic compounds which were used widely throughout the 20th century. There are 209 possible congeners of PCBs differing in position and number of chlorines located on the biphenyl core (Abraham et al 2002). More chlorines on the biphenyl core increase chemical stability and decrease water-solubility. Due to these properties, higher-chlorinated PCBs are more recalcitrant to biodegradation (Van Aken et al 2010). These PCBs are metabolized by anaerobic bacterial pathway (reductive dechlorination) resulting in products degraded by aerobic pathway determined in the biphenyl operon (*bph* operon) occurring in genome of some aerobic bacteria (Fig. 1) (Furukawa and Fujihara 2008).

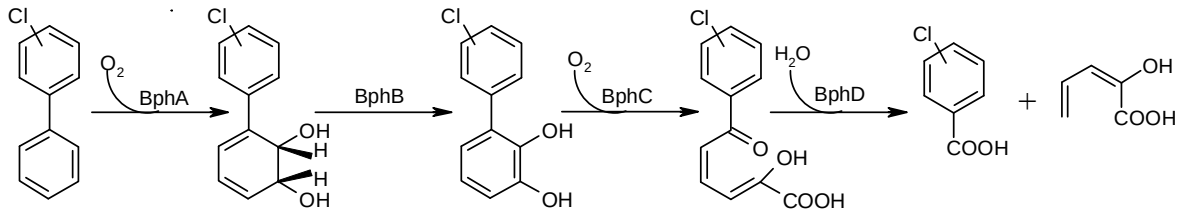

Figure 1. Bacterial PCBs degradation pathway (BphA: multienzyme complex - biphenyl-2,3-dioxygenase, BphB: *cis* -2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase, BphC: 2,3-dihydroxybiphenyl 1,2-dioxygenase, BphD: 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate hydrolase) (Furukawa and Fujihara 2008).

Biodegradation of PCBs by rhizospheric bacteria is affected also by vegetation of contaminated soil. Mutual interactions of these two groups of organisms may result in increased biodegradation of PCBs. Bacteria can be beneficial for plants which is shown already in the early studies focused on the biodegradation of herbicides and pesticides. Consequently these experiments suggested the ability of rhizospheric bacteria to degrade herbicides and pesticides serves as a plants protection against these compounds (Kuiper et al 2004). Meanwhile plant roots improve the aeration of soil which positively stimulates processes of aerobic bacteria and simultaneously plants release products of primary and secondary metabolism to the rhizosphere where these compounds positively

affect bacterial growth and activity (Aprill and Sims 1990). Plant exudates in addition to small-molecular weight metabolites as amino acids, sugars, enzymes, etc. (Bais et al 2006) consist of organic compounds with biphenyl-like structure (flavonoids, phenolic compounds) potentially inducing the bacterial *bph* operon (Hegde and Fletcher 1996). However, the native and well studied inducer of *bph* operon is biphenyl. Soil with biphenyl enhances the PCBs removal (Focht and Brunner 1985), but this compound is toxic and therefore its addition to contaminated soil is not appropriate for the use in bioremediation *in situ* (Tandlich et al 2001). To avoid biphenyl usage, some secondary plant metabolites were studied for their ability of *bph* operon induction. For example naringenin and coumarin were found as compounds not only supporting the growth of PCBs degraders but inducing PCBs degradation as well (Donnelly et al 1994). Concluded, plant secondary metabolites may serve as inducers instead of the biphenyl. This brings the question which plant secondary metabolite could be the best alternative compound to support rhizospheric microbes in rhizoremediation of PCBs.
The aim of this study is to prepare transgenic *Nicotiana tabacum* plants with enhanced production of the plant secondary metabolite quercetin in roots. Targeting quercetin to roots should lead to its releasing to the rhizosphere where this compound may serve as an inducer of *bph* operon and consequently can positively stimulate the PCBs biodegradation by rhizosphere microorganisms.

2. Materials and methods

2.1 Quercetin as an inducer of bacterial *bph* operon

The first step of this experiment was to study the ability of quercetin to induce the expression of the biphenyl operon. Bacteria degrading PCBs, namely *Pseudomonas* spp. and *Achromobacter* sp., were cultivated with quercetin as a source of carbon and energy and the growth curve was measured. Bacterial RNA was isolated at the exponential phase of growth and the *bphA* gene (fig. 1) was amplified and detected after reverse transcription of RNA.

2.2 Cloning and expression of plant *AtF3'H* gene

Consequently the gene encoding for flavonoid 3'-hydroxylase isolated from *Arabidopsis thaliana* (*AtF3'H*) kindly provided by professor Weisshaar from the University of Bielefeld (Schoenbohm et al 2000) was cloned into the commercial pQE30 vector. This vector contains a hexahistidine tail which serves as a tag for immunochemical detection and isolation of tagged protein by affinity chromatography (Ni-NTA column). For the protein isolation it was necessary to localise the *AtF3'H* gene expression. After transformation of bacteria *Escherichia coli* SG13009 by vector pQE30-*AtF3'H* we proceeded to the expression of AtF3'H protein which was performed at 37 °C while shaking at 200 RPM. *AtF3'H* gene expression was induced by adding IPTG to the final concentration of 0.4 mM. Fractions during the expression were collected and analysed by SDS PAGE and by immunochemical detection (Western Blot + commercial mouse antibody against histidine tail).

2.3 Fusion of *AtF3'H* gene with β-Glu promoter

Expression of *AtF3'H* gene under control of the root specific promoter should lead to the overexpression of the gene of our interest in roots. *AtF3'H* gene with fused β-Glu promoter will be subcloned to the vector for suitable plant transformation.
Plants will be transformed by agrobacterial infection. First transient expression assay will be performed to verify the efficiency of expression followed by stable transformation of *Nicotiana tabacum* plants.

3. Results and discussion

The ability of quercetin to induce expression of biphenyl operon genes in selected bacteria was verified at the RNA level. Consequently the plant gene for flavonoid-3'-hydroxylase was cloned to the bacterial expression vector containing a hexahistidine tail. Bacteria *Escherichia coli* SG13009 were transformed by prepared vector pQE30-*AtF3'H* and the expressed protein was localised in the inclusion bodies. We are currently performing steps which will lead to the preparation of plant vector bearing *AtF3'H* gene under the control of root specific promoter of β-glucosidase from maize (Gu et al 2006).

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GM PLANTS EXPRESSING BACTERIAL DIOXYGENASE AND YEAST METALLOTHIONEIN GENES CAN ENHANCE PHYTOREMEDIATION LEVEL

Martina Novakova, Ladislava Trbolova, Jitka Viktorova, Eliska Neumannova, Petra Lovecka, Jan Fiser, Martina Mackova and Tomas Macek
suram@vscht.cz
Institute of Chemical Technology, Department of Biochemistry and Microbiology, Technická 5, Prague, 166 28, Czech Republic.

ABSTRACT

The aim of this work is to construct and study GM plants with increased capability to degrade organic pollutants such as polychlorinated biphenyls (PCBs) and also to accumulate heavy metals.
We have prepared GM plants of *Nicotiana tabacum* containing bacterial *bphC* gene. *BphC* gene codes for 2,3-dihydroxybiphenyl 1,2-dioxygenase cleaving the aromatic ring of dihydroxybiphenyl and was cloned in fusion with the gene for β-glucuronidase (G), luciferase (L) or with a histidine tail (H). The presence of transgenic DNA and its expression into protein was already determined in parental, first filial and second filial generation of transgenic plants. The ability to remove the toxic substrate 2,3-dihydroxybiphenyl from media was studied with selected transgenic lines. Transgenic line H3 showed 95 % higher decrease of the substrate content in medium than nontransgenic plants. Further the toxic effect of Delor 103 (PCB mix) and selected congeners of PCBs on transgenic tobacco lines was studied, where transgenic plants grew better than nontransgenic on Delor 103. To increase the phytoremediation abilities also for heavy metals accumulation, we have inserted yeast *CUP* gene coding for heavy metals binding protein metallothionein. This gene was cloned in the fusion with histidine tail into the *bphC* transgenic plants. Properties of prepared transgenic plants will be studied more, nevertheless they sound to be promising in phytoremediation technologies.

KEY WORDS: phytoremediation, PCB, heavy metals, transgenic plants

1. INTRODUCTION

Because of the wide spread contamination of the environment several approaches to decontaminate polluted sites have been used. Since the abilities of pollutant remediation of several living organisms are known, the science focused on characterization and further development of bioremediation techniques. Biological remediation is also cheaper, in comparison to the physical-chemical methods biological one does not destroy the environment and is generally acceptable technology today. At the beginning of the nineties scientists focused their interest on research of the plant ability to decontaminate pollutants from the environment. This technology is called phytoremediation and can be defined as the use of green plants for transfer, accumulation and removal of pollutants from the environment, or at least reduction of their spreading (Cunningham et al., 1995; Macek et al., 2000). In an optimal case, this approach might lead to the mineralization of organic compounds, with the main aim of preventing the migration of pollutants to a site of actual danger, to human health.
With the aim to improve the phytoremediation abilities of selected plants (fast growing, forming high amount of biomass) research is oriented to preparation of genetically modified plants with the help of genetic engineering (Macek et al., 2008; Novakova et al., 2010). The target is the formation of plants combining a high ability to accumulate, detoxify, or degrade xenobiotics and pollutants, with resistance toward the toxic compounds present and with suitable agrotechnical characteristics.
We have already prepared the genetically modified plants with increased abilities to degrade organic pollutants (aromates, toluene) (Novakova et al., 2009). These transgenic plants contain bacterial *bphC* gene in their genome. *BphC* gene encodes for enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase that catalyses opening of aromatic ring of polychlorinated biphenyls (PCBs) (Fig.1) (Sylvestre et al., 2009). This gene was prepared in three variants: in a fusion with gene for β-glucuronidase, luciferase and with histidine tail. The transgenic lines containing *bphC/LUC* genes were named L1, L2, L3, L4 and L5; the transgenic lines containing *bphC/GUS* genes were named G1, G2 and G3; the transgenic lines containing *bphC/His* genes were named H1, H2, H3 and H4. Formed transgenic plants should degrade PCBs from contaminated soil and thus help to maintain uncontaminated and clean environment.

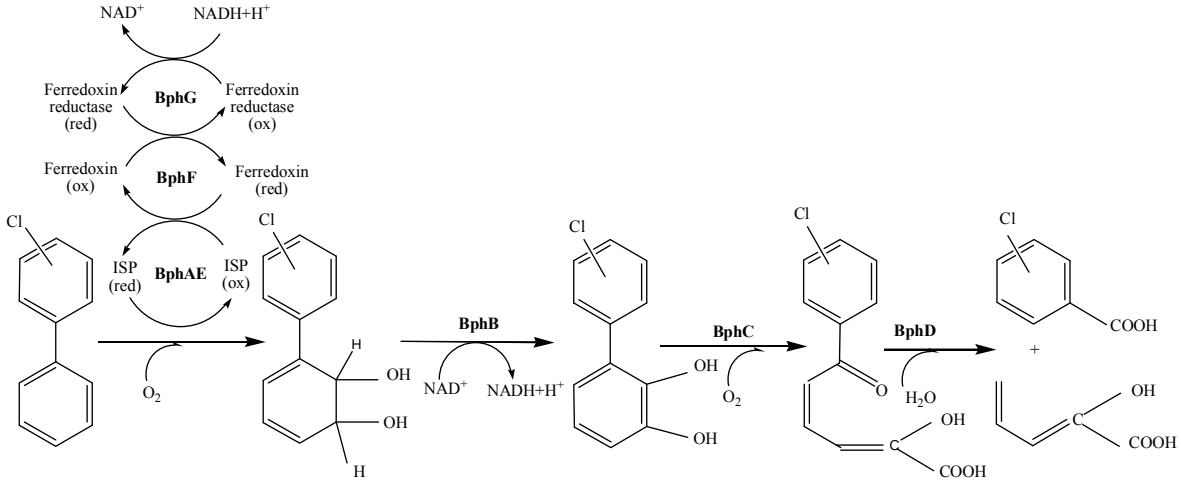

Figure 1. Bacterial metabolic pathway of PCBs (Sylvestre et al., 2009). BphAE – biphenyl-2,3-dioxygenase (*bphAE*), BphF – ferredoxin (*bphF*), BphG – ferredoxin reductase (*bphG*), BphB – biphenyldihydrodiol dehydrogenase (*bphB*), BphC – 2,3-dihydroxybiphenyl 1,2-dioxygenase (*bphC*), BphD – hydrolase (*bphD*).

There is usually not only one pollutant in real contaminated sites, but there are present several organic and inorganic pollutants. Some transgenic plants have been already prepared containing bacterial, yeast or human genes to accumulate e.g. heavy metals and to be resistant to toxic concentration of these inorganic pollutants. Such gene has been also yeast *CUP1* cloned in the fusion with histidine tail into tobacco plants (Macek et al., 2002). *CUP* gene encodes for metallothionein, a peptide having the ability to bind heavy metal ions. Prepared transgenic plants showed increased accumulation of Cd(II) in the plant tissues (Macek et al., 2002). The aim of this work was therefore not only to characterize *bphC* transgenic plants, but also to increase the phytoremediation abilities of existing *bphC* transgenic plants towards the heavy metals pollution by cloning of *HisCUP* (Fiser et al., 2011) gene into their genome.

2. MATERIALS AND METHODS

2.1 Confirmation of the presence of the *bphC* gene in transgenic plants

The lines of the parental (T0), first (T1) and second (T2) filial generation of *Nicotiana tabacum* cv. Wisconsin 38 containing bacterial *bphC* gene in fusion with different markers were used (Novakova et al., 2009): *bphC*/*GUS* – G1, G2, G3 transgenic lines containing *bphC* in fusion with *GUS* gene encoding β-glucuronidase; *bphC*/*LUC* – L1, L2, L3, L4, L5 transgenic lines containing *bphC* in fusion with *LUC* gene encoding luciferase; *bphC*/His – H1, H2, H3, H4 transgenic lines containing *bphC* gene in fusion with histidine tail.
Presence of *bphC/LUC*, *bphC/GUS* and *bphC/His* transgenes was confirmed after the isolation of plant DNA (DNeasy Plant Mini Kit, Qiagen, USA) by PCR with specific primers bphC1/F, bphC2/R (Novakova et al., 2009) (Generi Biotech, Hradec Kralove, Czech Republic). Amplified fragments of *bphC* gene were 880 bp long. These experiments were performed in parental (T0), first filial generation (T1) and also second filial generation (T2) of transgenic plants. Also histochemical studies were performed to prove the expression of the protein BphC/LUC and BphC/GUS (Novakova et al., 2009). The expression of BphC/His protein was studied using SDS PAGE and Western blot with immunochemical detection (Anti-His antibody, Invitrogen).

2.2 Growing of transgenic plants on toxic medium

Toxic effect of Delor 103 (PCB mix) and selected congeners of PCBs on transgenic tobacco lines was studied. Seeds of H1, H3, G1 a L3 lines were planted on MS medium (Murashige and Skoog, 1962) with Delor 103 (20 mg/l, 150 mg/l, 250 mg/l, 350 mg/l) and grown for 14 days. Further tested PCB congeners were: PCB 3 (4-chlorobiphenyl), PCB 8 (2,4´-dichlorobiphenyl), PCB 28 (2,4,4´-trichlorobiphenyl) (0 mg/l, 50 mg/l, 100 mg/l). Measured parameters were length of root, length of stem and weight of biomass. The results were evaluated as the tolerance index (Hannink et al., 2001). The index for e.g. plant height was calculated as $I = ((\text{plant hight}_{\text{on tested concentration}})/(\text{plant hight}_{\text{control concentration – 0 mg/l}})) * 100$.

2.3 Comet assay

The genotoxic effect of Delor 103 (PCB mix) in the hydroponic medium, in the gel and in the real contaminated soil (Lhenice soil, Czech Republic, contamination with PCBs) were performed according to Gichner et al. (2007).

2.4 Preparation of double transgenic plants (the supertransgenics)

The yeast metallothionein gene *CUP* has been chosen to clone into the genome of *bphC* transgenic plants. Preparation of the vector containing *His/CUP* gene is described at Fiser et al. (2011). The agrobacterial transformation using *A. tumefaciens* C58-C1 (pCH32) was performed to obtain the transgenic plants.

3. RESULTS AND DISCUSSION

3.1 Confirmation of the presence of the *bphC* gene in plants

To proof the presence of desired transgenes in the transgenic plant lines the plant DNA was isolated and transgenes were detected in the transgenic lines using PCR. The transgenes were detected in all prepared lines of parental, first filial and second filial generation.
The histochemical assay with T0 plants containing *bphC/LUC* genes determined the expression of BphC/LUC protein in the lines L1, L2, L3, not in the lines L4 and L5 (Novakova et al., 2009). The T1 generation of L1, L2 and L3 plants was expressing the BphC/LUC protein as well. The histochemical assay with T0 and T1 plants containing *bphC/GUS* genes determined the expression of BphC/GUS protein in all studied lines (G1, G2, G3). The presence of BphC/His protein in transgenic lines containing *bphC/His* gene was characterized using Western blot with immunochemical detection. Transgenic lines H1, H2, H3 and H4 of T0 generation showed the expression of desired protein, on the other hand when seeding the seeds for obtaining plants of T1 generation, H2 and H4 plants were dying in the MS medium and therefore were excluded from further experiments. This fact could be caused by transgene insertion into the key site of plant genome as has been already described (Primrose et al., 2001; Slater et al., 2008). The presence of BphC/His protein in T1 generation was proved in H1 and H3 plants.

3.2 Growing of transgenic plants on toxic medium

Tolerance indexes of selected transgenic lines grown in medium with Delor 103 were higher than those of wild type plants. These experiments showed better viability of transgenic plants in the PCB presence in comparison with non-transgenic plants. Furter testing showed also higher decrease of the substrate content (2,3-DHB) in medium than nontransgenic plants in the case of H1, H3, L1, G1 and G3 lines (fig. 2).

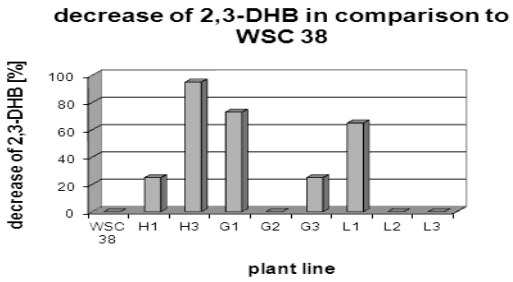

Figure 2: Decrease of 2,3-dihydroxybiphenyl (2,3-DHB) in medium in comparison to wild type plants (WSC 38) evaluated in %.

When comparing the tolerance indexes of transgenic lines and wild type plants grown in media with selected PCB congeners, there has been found no significant difference.

3.3 Comet assay

Comet assay didn't show any genotoxic effect of studied contaminants, neither with Delor 103 nor with real contaminated soil. These results were contrary to results shown by Gichner et al. (2007) cultivating *Nicotiana tabacum* cv. xanthi in real contaminated soil and achieving higher genotoxicity of the PCBs from the soil.

3.4 Preparation of double transgenic plants (the supertransgenics)

For further enhancement of phytoremediation abilities also to heavy metals we have cloned *His/CUP* gene encoding for yeast metallothionein into plant genome of *bphC* transgenic plants.

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STRATEGIES FOR SUCCESSFUL RHIZOREMEDIATION OF PCBs USING ENGINEERED PLANTS AND MICROBES

Michel Sylvestre
Michel.Sylvestre@iaf.inrs.ca
Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, Laval, Quebec
Canada H7V1B7

ABSTRACT

The fate of polychlorinated biphenyls (PCBs) in soil is driven by a combination of interacting biological processes. Several investigations have brought evidence that the rhizosphere provides a remarkable ecological niche to enhance the PCB degradation process by rhizobacteria. The bacterial oxidative enzymes involved in PCB degradation have been investigated extensively. Furthermore, recent studies suggest that approaches involving processes based on plant-microbe association are very promising to remediate PCB-contaminated sites. In this review emphasis will be placed on the current state of knowledge regarding the strategies that are proposed to engineer the enzymes of the PCB-degrading bacterial oxidative pathway and to design PCB-degrading plant-microbe systems to remediate PCB-contaminated soil.

KEY WORDS: PCB, rhizoremediation, flavonoids, biphenyl dioxygenase

1. INTRODUCTION

Although polychlorinated biphenyls (PCBs) are now out of production worldwide, they still persist in the environment. Because of the high cost of land filling or incineration, biological degradation of PCBs has received increasing attention over the years. Bacteria can co-metabolize PCBs oxidatively through the biphenyl catabolic pathway. This pathway has been thoroughly investigated. However, bioremediation strategies based solely on the use of PCB-degrading bacteria are not viable because of the low bioavailability of PCBs and because efficient effectors other than biphenyl are required to induce the catabolic enzymes. To overcome these difficulties, processes based on plant-microbe interactions have been proposed. Plants can help overcome these difficulties. In this review we will present an overview of the current literature about the PCB-degrading enzymes and about the various ways through which plants may interact with bacteria to promote PCB degradation. We will also discuss how we may apply this knowledge for designing efficient approaches to remediate PCBs in soil.

2. ENGINEERING BACTERIAL ENZYMES TO DEGRADE PCBS

The first step of the upper biphenyl/chlorobiphenyl pathway is catalyzed by the biphenyl 2,3-dioxygenase (BPDO). The enzyme introduces one molecule of oxygen on two vicinal *ortho-meta* carbons of the aromatic ring. The metabolite, *cis*-2,3-dihydro-2,3-dihydroxybiphenyl is re-aromatized by the 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (2,3-DDHBD). The catechol metabolite is then cleaved by the 2,3-dihydroxybiphenyl-1,2-dioxygenase (2,3-DHBD) to generate 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), which is hydrolyzed to produce benzoic and pentanoic acids. The limitations of each enzymatic steps of the pathway have recently been reviewed (Sylvestre and Toussaint 2011).
The BPDO is an interesting enzyme. This Rieske-type dioxygenase (RO) metabolizes many biphenyl analogs including heterocyclic aromatics such as dibenzofuran and flavonoids. Understanding how BPDO catalytic pocket interacts with its substrates to bind them and orient their reactive carbons toward the protein reactive atoms will help design novel biocatalysts useful in biotechnological processes for the destruction of persistent pollutants or biocatalytic processes for green production of chemicals. BPDO comprises three components. The catalytic component, which is a RO protein (BphAE), is a hetero hexamer made up of three α (BphA) and three β subunits (BphE). The other two components are the ferredoxin (BphF) and the ferredoxin reductase (BphG) which are involved in electron transfer from NADH to BphAE.
Burkholderia xenovorans LB400 BphAE ($BphAE_{LB400}$) has been thoroughly investigated because this organism is considered as one of the best PCB degrader of natural occurrence (Kumar et al 2011). Using directed-evolution approaches, we evolved $BphAE_{LB400}$ and obtained $BphAE_{p4}$ (Barriault and Sylvestre 2004) and $BphAE_{RR41}$ (Mohammadi and Sylvestre 2005), two variants that metabolize a much broader range of substrates than the parent enzyme. Structural analyses showed that Thr335Ala substitution, common to both variants relieves intramolecular constraints on the Val320Gly321Gln322 segment lining the catalytic pocket allowing for significant movement of this segment during substrate binding, thus increasing the space available to

accommodate larger substrates (Kumar et al 2011). In addition, we found that the combined Asn338Gln and Leu409Phe substitutions of $BphAE_{RR41}$ alters a substrate-induced mechanism required to retune the alignment of protein atoms involved in the chemical steps of the reaction (Mohammadi et al 2011). This mechanism speeds up the electron transport process during the catalytic reaction. As a result, the enzyme was able to catalyze the oxygenation of dibenzofuran and chlorodibenzofurans (Mohammadi et al 2011) as well as of PCBs (Viger 2012) more efficiently than the parent enzyme. $BphAE_{RR41}$ was able to metabolize 17 of the 18 tested di- to penta-chlorinated PCBs, most of which, including the toxic 3,3',4,4'-tetrachlorobiphenyl are congeners that the parent $BphAE_{LB400}$ was unable to metabolize. Therefore, these mutant enzymes are regarded as promising tools to be transferred into a PCB-degrading soil bacteria to broaden their PCB substrates range.
The second enzyme of the pathway, the 2,3-DDHBD can oxidize a very wide range of dihydrodiol substrates, including 3,4-dihydro-3,4-dihydroxybiphenyl and metabolites produced from the dioxygenation of aromatic and heteroaromatic compounds such as naphthalene (Barriault et al 1999), dibenzofuran (Mohammadi and Sylvestre 2005) and flavonoids (Misawa et al 2002, Pham et al 2012, Toussaint et al 2011). Recently, crystal structure analysis revealed that the substrate binding loop of the 2,3-DDHBD is highly mobile and conformational changes are induced during ligand-binding where the disorganized loop becomes organized forming a well defined cavity to accommodate a wide variety of substrates which explains its versatility (Dhindwal et al 2011).
The 2,3-DHBD is less versatile than the 2,3-DDHBD and is unable to cleave the *meta-para* hydroxylated metabolites. Fortunately, the homologous enzyme 1,2-dihydroxynaphthalene dioxygenase (Dox G) of *Pseudomonas* sp. C18 was found to catalyze the ring cleavage of these *meta-para*-hydroxylated metabolites more efficiently than the 2,3-DHBD. DoxG variants exhibiting higher ability to cleave 3,4-dihydroxybiphenyl metabolites were obtained by directed evolution (Fortin et al 2005), showing the feasibility of engineering extradiol dioxygenases to expand the range of PCB substrates metabolized by the biphenyl pathway.
HOPDAs bearing chlorine atoms on the phenyl ring are in general good substrates for the HOPDA hydrolase. However 3- and 4-chloroHOPDA produced from chlorobiphenyls that bear chlorine on both rings, are poor substrates. Nevertheless, the fact that HOPDA hydrolases obtained from various bacteria respond differently to chloroHOPDAs (Seah et al 2001) makes it possible to engineer enzymes with features that allow a more efficient degradation of congeners bearing chlorine atoms on both rings.

3. EXPLOITING PLANTS TO PROMOTE PCB DEGRADATION BY RHIZOSPHERE BACTERIA

Phytoremediation is a promising technology that has a lot of potential in alleviating contaminated soils from PCBs. It is generally accepted that plants can metabolize xenobiotics, including PCBs, through a three-phase process. However, it has been demonstrated that the first phase of the degradation of PCBs in plants may result in the formation and accumulation of hydroxylated PCBs, which are more toxic than the parent compounds (Rezek et al 2008). Furthermore, because plants are autotrophic organisms, they do not have the capacity to completely transform PCBs, mainly due to a lack of catabolic enzymes required to do so, which results in slow and/or partial transformation of such compounds. In addition, the efficiency of a plant to metabolize PCBs is highly dependent on both the plant species and PCB congeners (Rezek et al 2008, Sylvestre et al 2009).
Alternatively, harnessing the potential of plants and their root-associated microorganisms in order to facilitate the remediation of PCBs in contaminated soils (Mackova et al 2007, Mackova et al 2009) has shown a lot of potential in the past decades. Plant secondary metabolites released in root exudates may act as signal molecules in the rhizosphere (Shaw et al 2006, Singer 2006). Many investigations have shown that flavonoids and terpenes that share chemical similarity with biphenyl can act as co-metabolites or pathway inducers to stimulate the bacterial biphenyl degradation pathway (for a review see (Singer 2006)). Direct evidences that root exudates can promote PCB degradation in soil were obtained by Narasimhan et al (2003) who showed that PCB removal by *Pseudomonas putida* PML2 which is a phenylpropanoid-utilizing and PCB-degrading rhizobacteria was significantly lower in the rhizosphere of *Arabidopsis* mutant exuding less flavonoids than in the rhizosphere of the wild-type strain. More recently, Toussaint et al (2011) have shown that although *Rhodococcus erythropolis* U23A, a PCB-degrader rhizobacterium was unable to grow on flavonoids, its biphenyl catabolic pathway was induced by *Arabidopsis* root exudates and they identified flavanone as being an active exudates component. Pham et al (2012) have shown that some bacteria such as *Pandoraea pnomenusa* B356 are better fitted than others to metabolize flavonoids. Hence the biphenyl dioxygenase from strain B356 is significantly better fitted to metabolize flavone, isoflavone and flavanone than the biphenyl dioxygenase of the well characterized *B. xenovorans* LB400. Unlike $BphAE_{LB400}$, the kinetic parameters of $BphAE_{B356}$ toward these flavonoids were in the same range as for biphenyl. In addition, remarkably, the biphenyl catabolic pathway of strain B356 was strongly induced by isoflavone whereas none of the three flavonoids induced the catabolic pathway of strain LB400. Together, these data provide evidences supporting the hypothesis whereby the biphenyl catabolic pathways may have evolved in bacteria to serve ecological functions, perhaps related to the metabolism of plants secondary metabolites in soil.

Although the perspective of using rhizoremediation as a tool to remove persistent contaminants from the soil is quite appealing, most of the well characterized PCB-degrading bacteria are not endowed with the genetic background to colonize plants roots and, in addition, they cannot fully degrade complex PCBs. However, it is possible to engineer rhizobacterial strains to express PCB-degrading genes efficiently in the rhizosphere (Villacieros et al 2005). Therefore the use of transgenic plants and/or bacteria may offer yet another potential approach to remediate PCB-contaminated soils (Macek et al 2008, Sylvestre et al 2009). By introducing one or several genes from organisms that can degrade xenobiotic pollutants to candidate plants or bacteria, it may be possible to design an efficient plant-microbe system to metabolize PCBs efficiently in soil.
Since accumulation of toxic metabolites produced by plants P-450 systems and release of *trans*-diols may hamper efficient PCB removal by combined plant-rhizobacterial systems, it has been suggested that transgenic plants producing bacterial PCB-degrading enzymes can overcome these difficulties and be advantageously used for PCB-rhizoremediation processes (Novakova et al 2009, Sylvestre et al 2009). Analyses of tobacco plants transiently expressing *B. xenovorans* LB400 genes encoding the BPDO components or transformed with them (Sylvestre et al 2009) have shown that each component can be produced individually as active protein in plants. Furthermore, active BphAE and BphG were co-purified from *Nicotiana benthamiana* leaves agroinfiltrated with pGreen-*bphA*+*bphE* + pGreen-*bphG*. However, the simultaneous expression of all four BPDO genes in transgenic tobacco is hampered by genetic or physiological reasons.
Unlike BPDO, 2,3-DHBD consists of a single homo-octamer component. Therefore, a single gene is required to produce active enzyme. Macek et al (2008) have recently discussed an approach to overcome the inability of plants to cleave dihydroxybiphenyls by cloning 2,3-DHBD into plants. Gene *bphC* from *P. pnomenusa* B-356 was successfully cloned in *Nicotiana tabacum* (Novakova et al 2009). Interestingly, plants expressing 2,3-DHBD were more resistant to PCBs than non-transgenic ones (Macek et al 2005, Novakova et al 2009). This feature might be attributed to the fact that 2,3-DHBD can remove 2,3-dihydroxy-chlorobiphenyls derived from PCBs (Mackova et al 2007) which are potentially toxic to plants. Although the toxicity of 2,3-dihydroxy-chlorobiphenyls to plants has never been examined directly, Camara et al (2004) have shown they are toxic to bacterial cells, Novakova et al (2009) have shown they are toxic to tobacco plants and Lovecka et al (2004) have shown that monohydroxylated PCB metabolites are toxic to plants depending on number and position of the chlorine atoms. Furthermore, the toxicity of catechol to plants has been clearly demonstrated by Liao et al (2006). These are among the many observations supporting the use of transgenic plants producing 2,3-DHBD for rhizoremediation of PCB-contaminated sites since these plants are likely to be more resistant to the PCB metabolites produced by plants and their associated rhizobacteria than the non-transgenic parents.
Together the recent investigations about the bacterial PCB-degrading enzymes and the possible exploitation of plant-microbe interactions to promote the PCB degradation in soil are very encouraging. These investigations allow us to draw the conclusions that engineering rhizobacterial strains to enhance their PCB degrading abilities is very well possible. Furthermore engineering plants to metabolize the PCB metabolites produced from rhizobacteria is also a promising and feasible approach to facilitate the rhizoremediation of PCBs. Finally, the recent development with regard to the identity of the plant secondary metabolites able to induce the bacterial PCB-degrading abilities imply that the efficiency of the process will depend on the choice of appropriate bacterial strains responding to the specific plant secondary metabolites produced by the plants to which they are associated.

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PREPARATION OF SUPER-TRANSGENIC PLANTS EXPRESSING BOTH DIOXYGENASE AND METALLOTHIONEIN GENES

Viktorová Jitka, Neumannová Eliška, Fišer Jan, Bečvářová Zuzana, Nováková Martina, Macek Tomáš and Macková Martina
jitka.viktorova@vscht.cz
Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague 6, Czech Republic

ABSTRACT

The aim of this work is the preparation of transgenic plant resistant to both organic and inorganic pollutants. First transgenic plants containing bacterial *bphC* gene were prepared. The *bphC* gene is coding for 2,3-dihydroxybiphenyl-1,2-dioxygenase, cleaving the biphenyl ring to form a product which could be further metabolized. The presence of *bphC* gene in first and second filial generation of transgenic plants was detected on the DNA and RNA level using PCR or RT-PCR methods. Transgenic plants contain the *bphC* gene in fusion with genes for detection markers *GUS* (gene for β-glucuronidase) and *LUC* (gene for luciferase) and also *bphC* gene with histidine tail. The expression of the *bphC*/*GUS* and the *bphC*/*LUC* genes in plants was tested by histochemical assay proving the expression of detection markers. Also the expression of the *bphC/His* gene was verified after purification of the enzyme by affinity chromatography followed by Western blot and immunochemical assay. The expression level differed among transgenic lines.
Based on results of degradation experiment with 2,3-dihydroxybiphenyl, the transgenic lines G1, H1 and L1 were transformed by plasmid carrying yeast metallothionein gene *CUP* in fusion with histidine tail. The metallothioneins are known for their ability to bind heavy metals, histidine tail is an additional binding domain enhancing the effect. The transformation of transgenic plants was mediated by *Agrobacterium tumefaciens*. Transgenic plants containing both *bphC* and *His/CUP* genes were tested and the transcription of genes was confirmed using transient expression as well as permanent transformation.

KEY WORDS: transgenic plants, BphC, metallothionein, phytoremediation

1. INTRODUCTION

The use of plants to clean up contaminated soil and water provide a cheap, natural and aesthetic method for bioremediation (Macek et al., 2008). Genetically modified plants can serve as an efficient alternative for remediation of diverse dangerous pollutants from the environment (Macek et al., 2000).
Various authors (Frančová et al., 2004) have also demonstrated that mono- and dihydroxybiphenyls, formed during the degradation of PCBs by plants, can serve as substrates for the bacterial enzyme dihydroxybiphenyl dioxygenase (BphC). Thus, the inability of plants to cleave the aromatic structure of dihydroxybiphenyl can be overcome by the preparation of transgenic plants bearing bacterial genes known to cleave such compounds.
This is the reason why Novakova et al. (2009) chose gene *bphC* from the bacterial pathway of *Pandoraea pnomenusa* B-356, which encodes the enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC). The gene was cloned into the plants of *Nicotiana tabacum.* BphC catalyzes the conversion of 2,3-dihydroxybiphenyl to 6-oxo-6-phenyl-hexa-2,4-dienoic acid (figure 1). Thus this gene can serve to improve the biodegradation ability of plants.

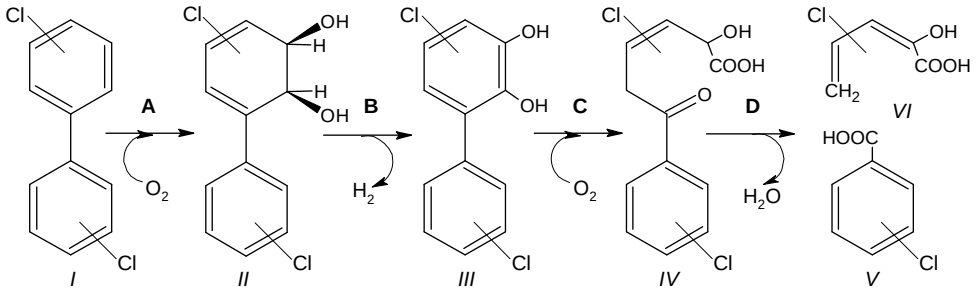

Figure 1. Bacterial degradation pathway of PCBs (Novakova et al., 2009). A, biphenyl-2,3-dioxygenase; B, biphenyldihydrodiol dehydrogenase; C, 2,3-dihydroxybiphenyl 1,2-dioxygenase; D, hydrolase; I, chlorobiphenyl; II, 2,3,-dihydro-2,3-dihydroxychlorobiphenyl; III, 2,3-dihydroxychlorobiphenyl; IV, 2-hydroxychloro-6-oxo-6-phenylhexa-2,4-dienoic acid; V, chlorobenzoic acid; VI, 2-hydroxychloro- 2,4-pentadienoic acid.

Heavy metals are another group of high risk environmental contaminants. Heavy metals have the inhibitory influence on plant growth and affect a lot of the metabolic paths. The overproduction of metallothionein peptides can be one of the possible ways to enhance the plant resistance and also how to improve the plant ability to accumulate heavy metals. Metallothioneins are known for high affinity to heavy metals. There is a diversity of metal-binding metallothioneins with the potential to perform distinct roles in the metabolism of different metal ions. This is the reason why Fišer et al. (2011) chose gene *CUP* from the yeast *Saccharomyces cerevisiae*, which encodes for the metallothionein, and cloned the gene into the pGreen0029 vector.

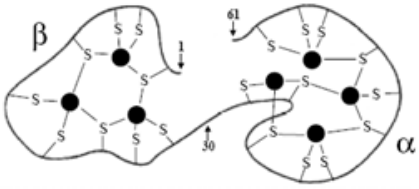

Figure 2. An example of binding of metal ions (spots) to metallothionein consisting of two domains.

The aim of this work is to prepare genetically modified plants of *Nicotiana tabacum* containing genetic elements allowing remediation of environment contaminated by both inorganic and organic pollutants together. In order to increase metal accumulation ability, the gene *CUP1* was fused with gene for an additional metal binding domain. In this case a polyhistidine tail was the peptide of choice yielding the construct *His/CUP*.

2. MATERIALS AND METHODS

2.1 Plant lines

The lines of the first and second filial generation of *Nicotiana tabacum* cv. Wisconsin 38 containing bacterial *bphC* gene in fusion with different markers were used (Novakova et al., 2009):
bphC/GUS – G1, G2, G3 transgenic lines containing *bphC* in fusion with *GUS* gene encoding β-glucuronidase,
bphC/LUC – L1, L2, L3, L4, L5 transgenic lines containing *bphC* in fusion with *LUC* gene encoding luciferase,
bphC/His – H1, H2, H3, H4 transgenic lines containing *bphC* gene in fusion with histidine tail.
Transgenic plants were resistant to hygromycin. When seeding the seeds, concentration of hygromycin in medium was 100 mg l^{-1}, when growing plants, concentration of hygromycin was 15 mg l^{-1}.

2.2 Treatment of transgenic seeds

Seeds of *N. tabacum* were surface-sterilized by immersion for 20 minutes in a solution of 14% Chloramin B. After being rinsed thoroughly with 3% Chloramin B and then with distilled water, seeds were germinated on MS (Murashige and Skoog, 1962) medium containing 100 mg l^{-1} of hygromycin.

2.3 Detection of transgene in plants on the DNA and RNA level

The presence of transgene on the DNA level was confirmed after the isolation and purification of plant DNA (DNeasy Plant Mini Kit, Qiagen, USA). Transgene was amplified by PCR using the combination of specific primers (Generi Biotech, Hradec Kralove, Czech Republic). These primers amplified the 880 bp fragment using the PCR program: 94 °C – 3 min, 35 cycles of 94 °C – 0.5 min, 55 °C – 0.5 min, 72 °C – 1.5 min in case of *bphC* gene and 324 bp fragment during amplification of *CUP* gene. The reactions were terminated at 72 °C for 7 min. The presence of the transgene on the RNA level was detected after isolation of plant RNA (RNeasy Plant Mini Kit, Qiagen, USA) and RT-PCR (Omniscript RT Kit, Qiagen) using the same primers.

2.4 Study of toxicity of $CdCl_2$ on prepared transgenic plants

Sterile transgenic plants containing both *bphC* and *His/CUP* gene were cut into the pieces and cultivated on MS (Murashige and Skoog) medium containing cadmium (three duplicates containing 16.2 mg.l^{-1} $CdCl_2$ for each transgenic line as well as for negative control – non transgenic tobacco). Seedlings were cultivated for 6 weeks at 26 ºC. After this period the height of plant, length of root and weight of biomass was measured.

3. Results and discussion

The already transgenic plants carrying *bphC* gene in fusion with sequence for histidine tail (H1), β-glucuronidase (G1) and luciferase (L1) were used for the transformation of the plants by genus *Agrobacterium*. These lines were chosen based on previous results of decrease of 2,3-dihydroxybiphenyl from solution and decrease of different congeners of polychlorinated biphenyls in contaminated soil in presence of each tested line (figure 3).

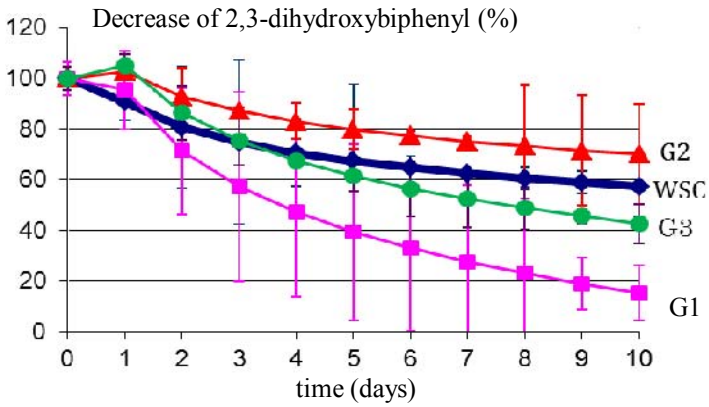

Figure 3. The decrease of toxic substrate content in water solution in presence of line G1, G2 or G3 as well as in presence of non transgenic tobacco (WSC). Initially, the 100 ml flask contained half gram of 14 days old seedlings peer 40 ml of distilled water containing 40 mg l^{-1} of 2,3-dihydroxybiphenyl.

The *bphC* gene presence was verified first at the DNA level using PCR method. Then the transcription was checked up by RT-PCR method. Finally the translation was verified using methods SDS PAGE and Western blot with following immunochemical detection (gene *bphC/His*), histochemical detection of glucuronidase enzyme (gene *bphC/GUS* gene) and histochemical detection of luciferase enzyme (gene *bphC/LUC*).
Prior to the permanent transformation the *His/CUP* gene expression was at first checked in transgenic plant cells by the transient expression method using agrobacterial infiltration. Then the permanent transformation of the plant *Nicotiana tabacum* followed using prepared *Agrobacterium*. In total 7 lines of transgenic plants *Nicotiana tabacum* carrying *bphC* and *His/CUP* genes were prepared: 3 lines carrying *bphC/GUS* and *His/CUP* genes labeled according to detection markers of both genes as G1H1, G1H2, G1H3 (figure 4) and 4 lines carrying *bphC/LUC* and *His/CUP* genes labeled as L1H1, L1H2, L1H3 and L1H4.

Figure 4. The prepared transgenic plants containing both *bphC* and *CUP* gene form roots in presence of selection antibiotics.

In these transgenic plants the presence of the *CUP* gene was proved at the level of DNA and RNA using methods of PCR and RT-PCR. The growth of newly prepared transgenic plants should not be affected by heavy metals, therefore in case of lines G1H1, G1H2 and G1H3 the influence of cadmium has been evaluated. The conclusion was that the G1H3 line regenerates and creates roots on the MS medium with concentration 16.2 mg $CdCl_2.l^{-1}$ (figure 5).

Figure 5. Study of physiological effect of medium containing cadmium on transgenic (G1H2) and non transgenic (WSC) plant.

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DIVERSITY OF MICROORGANISMS INVOLVED IN BIOREMEDIATION PROCESSES & OMICS

MULTI-COMPONENT COMPOSITION FOR EX SITU BIOREMEDIATION EX SITU OF SOILS AND OIL SLUDGE

Lyudmila V. Ignatova, Togzhan D. Mukasheva, Ramza Zh. Berzhanova, Raihan K. Sydykbekova, Tatyana A. Karpenyuk, Alla V. Goncharova, Anel A. Omirbekova
Lyudmila.Ignatova@kaznu.kz
Al-Farabi Kazakh National University, Faculty of Biology and Biotechnology, 71 Al-Farabi street, 050038, Almaty, Kazakhstan

ABSTRACT

The most effective way to ex situ bioremediation of soil and sludge, with a high content of various fractions of petroleum is the use of complex products. To date, the bottleneck in the use of drugs is its composition.
In our works, it was previously shown that in soils with high content of hydrocarbons there are predominant species of the *Rhodococcus, Mycobacterium, Pseudomonas, Bacillus, Candida, Trichosporon* and *Aureobasidium* genera.
Field experiment on bioremediation of soils and sludges was conducted at the landfill. Before making preparations, such agricultural activities like irrigation and tillage were conducted. The maximum of hydrocarbon products activity came on the 10th day after the start of the experiment, the content of hydrocarbons in the soils decreased to 364.3 g per kg of soil, after 2.5 months the residual amount was 121.0 g / kg soil. More than 58.1% of hydrocarbons were subjected to destruction. In the sludge of more than, their residual amount was 57.4 g per kg of soil. Used drugs in one field season give combine effect of ex situ bioremediation to 87.5 % with a high content of hydrocarbons in the treated sites.

KEY WORDS: bioremediation, yeast, soil, oil sludge, oil, petroleum products, destructive activity

1. INTRODUCTION

Slow the paces of environmental pollution require the development of environmentally sound and economically feasible methods aimed at intensifying the processes of degradation of oil (Margesin R., Schinner F., 2001). The most effective way to ex situ bioremediation of soil and sludge, with a high content of various fractions of petroleum find the use of complex products. To date, the bottleneck of developing such technology is the use of drugs, consisting of hydrocarbon-oxidizing microorganisms with desired characteristics: the ability to oxidize a wide range of polycyclic aromatic hydrocarbons, dispose of high concentrations of oil and oil products, and synthesize surfactants and other (Phillips TM at al, 2000). Their scope is different from cleaning oil sludge and soil, surface waters to the deep cleaning of waters and sediments, as well as cleaning of oil tanks. To improve the bioavailability of hydrocarbon pollutants using surface-active agents (surfactants, surfactants), which contribute to the desorption of petroleum hydrocarbons, thereby facilitating their assimilation by microbial cells. Aureobasidium fungi have the ability to produce various biologically active compounds, including biosurfactant (Crescenzi V., 1995).
We have previously shown that in soils with high content of hydrocarbons total number of microorganisms is very low, were predominant among bacteria of the genera Rhodococcus, Mycobacterium, Pseudomonas, Bacillus, and yeasts and of Candida, Trichosporon, and Aureobasidium. In order to develop complex products microorganisms isolated from soil contaminated with hydrocarbons above 200 g / kg soil were used.

2. MATERIALS AND METHODS

2.1 Microorganisms

In this work we used Mycobacterium thermoresistible 119-3GM, Rhodococcus egui 51KS, Pseudomonas cepacia AC 122, Bacillus lentus 109KS, Candida nitratiovorans B1, Candida chilensis B2, Trichosporon cutaneum R20SO2 isolated from soil with a high content of hydrocarbons; Aureobasidium pullulans P7, isolated from ordinary chernozem Almaty field. Cultures maintained by periodic reseeding in MPA medium, wort agar and KAA, as well as under a layer of vaseline oil at temperature +4 ° C (Mukasheva e t al, 2004).
The composition of the nutrient medium for growing strains-destructors: Wednesday Evans - (NH4) 2SO4 - 1 g / L, K2HPO4 - 1 g / l, MgSO4 × 7H2O - 0,3 g / l, SaSl2 - 0.1 g / L, FeSO4 × 7H2O - 0,02 g / l (Evans GG and Herbert D. et al, 1965).

2.2 The study of oil-oxidizing microbial activity

Quantitative evaluation of the ability of hydrocarbon was carried out in liquid mineral medium according to the residual amount of oil and oil by weight at 10 days of cultivation (Lurie YY and Rybnikova AI, 1974).
Fuel-oil activity was studied by "nitrocellulose filters." On the surface of agar medium made from the appropriate seeding cultivation, filter impregnated with heavy oil was covered, pressed it with a spatula and smooth. Sown in this way Petri dishes were incubated in a thermostat at 280S. Fuel-oil activity of the research cultures of microorganisms was evaluated by the intensity of illumination zones, which are formed by the consumption of fuel oil in the area of microbial colonies.
Qualitative changes in crude oil were studied by infrared spectroscopy. IR spectra were recorded on a spectrophotometer IR-200 firms «Thermo Electron» in v = 4000-500 cm -1 in the two-beam mode. The thickness of 0.4-0.6 mm cuvette. Intermediates of degradation of polycyclic aromatic hydrocarbons extracted from the culture fluid with ethyl ether. Separation of metabolites extracted hydrocarbons were carried out on thin-layer chromatography plates «Sorbfil» 100 x 150 cm, followed by detection in the ultraviolet. The solvents used: chloroform, ethyl acetate, acetic acid (Kiyohara H. et al, 1982).

2.3 Testing the activity of the composition of the oil-oxidizing microorganisms in model studies and field testing of the studied composition

Studies performed in model experiments for 30 days on soil and sludge. The samples were moistened to 60% of full capacity and made of composition at the rate of 109 cells per 1 g of soil. Samples were placed in desiccators for 30 days. The number of hydrocarbons was determined gravimetrically. The experiments were performed within 1 month of the contaminated soils, landfill Uralsk. After every 15 days, have made drug, conducted tillage, residual hydrocarbons checked gravimetrically. The drug is introduced into the amount of 10 to 15 g; nitroammophos - 80 - 100 g per 1 kg of soil or sludge. Re-introduction of a biological product was performed after 15 days.

3. Results and discussion

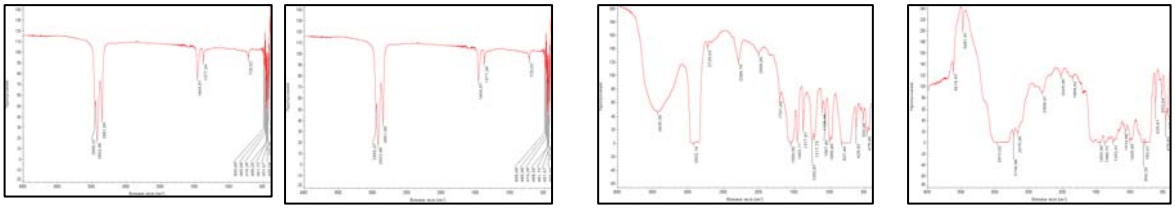
3.1 Characteristics of the strains of biopreparation

One of the main requirements for the introduced microorganisms for bioremediation of contaminated ecosystems is high activity in hydrocarbon-heavy levels of pollution. Strains of Mycobacterium thermoresistible 119-3GM, Rhodococcus egui 51KS, Pseudomonas cepacia AC 122, Bacillus lentus 109KS, Candida nitratiovorans B1, Candida chilensis B2, Trichosporon cutaneum R20SO2, Aureobasidium pullulans P7 characterized by the ability to dispose of high concentrations of oil and aromatic, light and heavy fractions of different types of oil, have the ability to synthesize metabolites with the surface active and emulsifying properties by culturing them on the hydrophobic and hydrophilic substrates (Table 1). Thus, the consumption of oil tengizkoy researched cultures ranged from 28.9 to 70.6%, diesel fuel - by 24.9% to 68.8%.
In fuel oil resin-asphaltene compounds are several times higher than in crude oil, so oil refers to petroleum trudnodegradiruemym. Accordingly, the first utilization of fuel oil, as in bacteria and yeasts were determined qualitatively using nitrocellulose filters. By the appearance of zones of enlightenment found that all strains were able to dispose of oil. At the rate of appearance of zones of enlightenment and their value noted that most hydrocarbon activity, strains of Mycobacterium thermoresistible had a 119-3GM and Rhodococcus egui 51KS. The results of these studies coincide with those obtained for the disposal of fuel oil by the gravimetric method.

Analysis of the IR specters of culture fluids of microorganisms grown on crude oil and fuel oil was carried out in all strains. It was established that under the action of microorganisms their qualitative composition has changed. Namely, in the region 1400-1600 cm-1 a broad and intense band at 1550 cm-1, which can be attributed to the stretching vibration of C = O ketones of different structure, which indicates that oxidation of the oil is prescribed. The oxidation of Tengiz oil accumulates esters, alcohols and carboxylic acids and after oxidation of fuel oil different products were found: ethers, esters, alcohols, ketones, carboxylic acids and aromatic compounds (Figure 1).

Table 1. Characteristics of strains used to create drug

Strains	Consumption,%				E24 on diesel fuel,%
	Tengiz oil	Kumkol oil	Diesel fuel	Fuel oil	
Mycobacterium thermoresistible 119- 3GM	70,6±2,2	54,5±1,8	68,5±2,1	48,1±0,9	58,1
Rhodococcus equi 51KS	69,3±2,3	55,3±3,1	67,3±2,1	44,3±1,4	60,2
Bacillus lentus 109KS	65,9±2,4	52,2±2,4	62,7±1,8	41,2±1,5	54,1
Pseudomonas cepacia 122AC	68,5±2,1	53,3±2,3	65,9±2,4	45,4±1,6	52,1
Candida nitratiovorans B1	32,2±0,3	25,4±0,3	36,7±2,2	12,9±0,6	60,1
Candida chilensis B2	28,9±0,1	23,9±1,3	24,9±1,2	12,9±0,6	54,1
Trichosporon cutaneum P20CO2	31,8±0,2	26,1±1,0	36,9±1,5	13,5±0,7	76,2
Aureobasidium pullulans P7	39,8±0,2	25,1±1,0	38,4±1,5	12,3±0,7	78,4



1 (control) 2 (control) 3 4

Figure 1. **IR spectral analysis of petroleum hydrocarbons after growth of strain *Mycobacterium thermoresistible* 119 - 3GM**
Note: 1, 3 - Tengiz oil, 2,3-oil.

In the thin-layer chromatography plates for bacteria *Mycobacterium thermoresistible* 119-3GM, *Rhodococcus egui* 51KS, *Pseudomonas cepacia* AC 122, *Bacillus lentus* 109KS and yeast *Candida nitratiovorans* B1, *Candida chilensis* B2 and *Thichosporon cutaneum* R20SO2 oxidation products of naphthalene, anthracene, and phenanthrene accumulated in the culture liquid after 10 days of incubation were identified. It was shown that the intermediate products of metabolism of polycyclic aromatic hydrocarbons are salicylic acid, phthalic acid and catechol.

3.2 The study of the destructive activity of a multicomponent composition in model experiments

Tested composition consisting of eight cultures: bacterial cultures *Mycobacterium thermoresistible* 119-3GM, *Rhodococcus egui* 51KS, *Pseudomonas cepacia* AC 122, *Bacillus lentus* 109KS, and yeast *Candida nitratiovorans* B1, *Candida chilensis* B2, *Thichosporon cutaneum* R20SO2 and *Aureobasidium pullulans* P7. Biomass used for thickening filings.
The composition of the oil-oxidizing microorganisms have made at the rate of 500 mg per 100 g of contaminated soil. Model studies were carried out on soil and sludge. In the initial degree of contamination of soils by hydrocarbons was 230.9 g / kg soil. After 20 days the amount of oil in the control without introducing microorganisms decreased to only 219.1 g / kg soil. In advanced options when you make biological product oil content at 20 days decreased to 38.37 g / kg, the degree of utilization of petroleum hydrocarbons was 83.1% (table 2).

Table 2. Model studies to verify the destructive activity of biopreparation

Objects	Quantity of hydrocarbons in g / kg of dry soil		
	Day 0	After 20 days	The extent of oil consumption in the soil,%
Soil	230,9±3,6	38,37±2,2	83,1
Oil sludge	119,5 ±2,15	23,02±1,9	80,8

3.3 Field testing of multi-component compositions of soil and sludge Field experiment on bioremediation of soil and sludge at the landfill was held in Uralsk. The initial oil content in soil was 750.7 g / kg and sludge - 137.2 g / kg. Oil sludge is a product of long-term storage of petroleum and petroleum products with predominance in it of heavy hydrocarbon fractions. Before making preparations conducted agricultural activities - irrigation and tillage. The maximum activity of the preparation of hydrocarbon occurred on the 10th day after the start of the experiment. After 10 days the hydrocarbon content in soil decreased to 364.3 g / kg of soil, waste subjected to 51.4% of the oil. After 2.5 months after the introduction of biologic residual, oil was 121.0 g / kg soil, and the percentage of recycled oil was 86.3%. In the sludge was subjected to destruction of more than 87.5% of hydrocarbons, their residual amount was 16.5 g / kg. High rates of decomposition of oil contamination were in the beginning of the experiment.

Table 3. Field tests on the composition of soil and sludge

Objects	Quantity of hydrocarbons in g / kg dry soil		
	Day 0	After 3 months	The extent of oil consumption in the soil,%
Soil	750,7±2,6	121,1±1,6	86,3
Oil sludge	137,2 ±3,5	16,8±1,6	87,5

It was found that multi-component drugs are urgently needed to handle the ex situ extracted the surface layer of contaminated soil and treatment of chronically polluted ecosystems where pollutant levels exceed the allowable dose. Studied composition of field season, gives the total effect of ex situ bioremediation to 87.5% with a high content of hydrocarbons in the treated sites. Prior to bioremediation work is necessary to identify the level and nature of contamination, depth of contamination, to determine the flow rate of the drug. Tillage biological product should be carried out twice a season.

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EVALUATION OF BIOREMEDIATED ACTIVITY OF MICROORGANISMS ISOLATED FROM ACTIVE SILT

Karpenyuk T.A. [1], Goncharova A.V. [1], Tsurkan Y.S. [2], Kalbaeva A.M. [2], Mukasheva T.D. [1], Ignatova L.V[2].
Alla.Goncharova@kaznu.kz; goncharova_alla_@mail.ru
[1]Scientific Research Institute of Biology and Biotechnology Problems, Laboratory of Biochemistry, Kazakhstan,050040, Almaty, pr. Al-Farabi 71, b.6.
[2]Al-Farabi Kazakh National University, Kazakhstan, 050040, Almaty, pr. Al-Farabi 71, b.6.

ABSTRACT

Many microorganisms are able to accumulate and / or transform metals, petroleum hydrocarbons and other xenobiotics in large amounts. The use of biochemical features, the selection of the most stable and active cultures can intensify the process of sewage treatment and areas contaminated by various xenobiotics.
We have carried out the analysis of microbial component of active silt from treatment plant in Almaty. The collection of microorganism strains isolated from active silt has been created. Dominant species of microorganisms from this biocenosis were identified and species with hydrocarbon oxidative activity were characterized.
It was found that the active destructors of oil and diesel fuel are isolates of *Pseudomonas* bacteria and yeast genus *Candida*, which in model experiments showed a high sorption capacity with respect to the ions of zinc, copper and cobalt.
We selected the conditions for the intensification of the processes of degradation of oil and diesel fuel components and heavy metal sorption by isolates of *Pseudomonas* bacteria and yeast genus *Candida*. It was shown that increasing of bioremediation activity of these microorganisms cultures is accompanied by increased utilization of organophosphorus compounds. This fact can be of great importance in the treatment of sewage contaminated with not only oil and heavy metals, but also with a large number of phosphorus compounds, as excessive amount of phosphorus is harmful to ecosystems and leads to eutrophication.
These data suggest that the extracted isolates are perspective for the creation of biopreparations for the biological purification of sewage and areas contaminated by heavy metals, petroleum products and organophosphorus compounds.

KEY WORDS: microorganisms, hydrocarbon oxidizing activity, the sorption of heavy metals.

1. INTRODUCTION

Kazakhstan has extensive resources of hydrocarbons. Their recoverable reserves are accounted of billions of tons. The Republic of Kazakhstan occupies the leading place on the reserves of zinc, tungsten, lead, chromium, silver, manganese, phosphorus, copper, molybdenum, etc. However, the intensification of industrial and agricultural production leads to an increased threat of pollution of the hydrosphere and soil by oil and petroleum products, inorganic and organic compounds toxic influencing on living organisms. The areas with high level of oil pollutions and heavy metals are appeared. To clean the contaminated areas are widely used artificially created associations of the monoculture of microorganisms for sewage treatment - aeration systems with active silt, but they are often used in a variety of reasons not effectively, so it requires the development of a new generation of biological preparations.
Many microorganisms are able to accumulate in large quantities and / or transform metals, petroleum hydrocarbons and other xenobiotics. Use of biochemical features, the selection of the most stable and active cultures and the creation of conditions that contribute to a more complete oxidation or neutralization of toxic organic and inorganic compounds can intensify the process of sewage treatment and areas contaminated by various xenobiotics.
We used an approach based on the selection of perspective for practical use microorganisms from artificial formed ecosystems (active silt). Microorganisms of active silt used in biological treatment of municipal wastewater, exist in an aggressive and ever-changing environment, so they may have a unique, evolutionarily modified systems of absorption, concentration and processing of various pollutants (Bakulin , 2003; Baryshnikov , 2001). Selection in this direction will receive and / or to select strains that are characterized by high capacity and selectivity for many pollutants, to assess the feasibility and advisability of their use for solving practical problems for treatment of industrial fluids, sewage, contaminated soils and on this basis to create advanced products and systems for solving problems in the sphere of ecological biotechnology.

2. MATERIALS AND METHODS

The material of study was the active silt, selected in the aeration tanks of sewage treatment in Almaty, and also the strains of bacteria *Pseudomonas sp.* 409TA and the yeast *Candida sp.* 410AT, isolated from active silt. Qualitative and quantitative composition of active silt microorganisms was studied by general (standard) microbiological methods (Netrusov , 2005.).
To determine the number of microorganisms used standard culture medium. To identify the bacteria used qualifier of Berg (Zavarzin, 2001.). The dynamics of microorganism cultures growth was evaluated by nephelometric change in optical density of cell suspension (photocolorimetric at 590 nm) during cultivation in liquid synthetic medium E-8, pH 6.5 - 7.0 (on a shaker at 220 rpm). The curves of the suspension optical density on the number of microbial cells were constructed for nephelometric determination of cells number. The initial value of the optical density in the culture medium for a suspension of bacterial and yeast cells was 0.20, which corresponds to 0.98 x 10^6 cells of bacteria and yeast 18x 10^3 cells per 1 ml of culture medium. Experience options include the addition of copper to a final concentration of 1, 5, 10, 20 mg / l, zinc to a final concentration of 1, 5, 10, 20, 30, 50, 100, 200, 300 mg / l , oil at a concentration of 10 ml / l (V /V).
The residual content of heavy metals was determined by complexometric method (in the presence of indicators: murexide for copper and xylenol orange for zinc (Leites, 2004) and by atomic absorption spectrometry (Lapenko, 1986). Residual of oil content was determined by gravimetric method and by infrared spectrometry on IR - 200 (Chibisov ,1999; Utkelov, 1988). Experiments were carried out at 3-5 replications. Data processed statistically.

3. RESULTS AND DISCUSSION

Biological sewage treatment is provided by biocenosis of active silt involved in the biochemical decomposition of organic matter, dissolved and emulsified in sewage.
Active silt is a complicated biocenosis of different organisms. In the biocenosis may include both single-celled microorganisms - bacteria, protozoa and more complex multi-cellular organisms - algae, fungi, various worms, insect larvae. Each of these groups of organisms is a necessary step in the cleaning process. In samples of active silt selected for the analysis were identified the following representatives of the indicator organisms, characterizing the state of the active silt and the quality of his work: *Opercularia coastata, Carchesium lacham, Aspidisca, Epistylis plucatilis, Vorticella microstoma, Vorticella convallaria* relating to the infusorium. Furthermore, in samples of active silt were identified crustaceans, filamentous algae, worms. The presence of these organisms in active silt evidence of its satisfactory work.
In the global processes of matter conversion an ecological importance have only those microorganisms which are numerous and exhibit active life. The work on the analysis of the dominant microbial biocenosis of active silt was carried out. From the active silt samples plated on universal and selective medium have been allocated 127 colonies forming units. Morphological and cultural, physiological and biochemical characteristics (Gram stain, sporulation, mobility, cell shape, tests for catalase, oxidase, amylase activities, acid production, etc.) were studied for identify the isolated microorganisms. It was shown that in the biocenosis of active silt of Almaty sewage treatment are the dominant bacteria of the genus *Pseudomonas, Micrococcus, Flavobacterium, Bacillus, Actinomyces,* and yeast genus *Kluyveromyces, Metschnikowia, Torulopsis, Phaffi*a. 25 isolates of *Pseudomonas* bacteria and yeast genus *Metschnikowia, Candida* demonstrated the hydrocarbon oxidizing ability.
The most active and viable strains showing the hydrocarbon oxidizing activity - bacterial strain *Pseudomonas sp. 409TA* and the yeast *Candida sp. 410AT* were identified (Karpenyuk, 2006, Karpenyuk, 2010). They were able to grow on toluene, diesel fuel, gasoline, oil and showed a high degree of degradation of these compounds (Table 1).

TABLE 1: Dynamics of growth and the degree of degradation of diesel fuel by strains *Pseudomonas sp. 409TA and the yeast Candida sp. 410AT*

Strain	Time of cultivation (hours)	Number of cells /ml	Quantity of consumption of petroleum products (%)
Pseudomonas sp. 409TA	0	0,98 x 10^6	0
	24	1,8-2×10^6	56-60
	96	3,2-3,8×10^6	78-80
Candida sp. *410AT*	0	180 x 10^2	0
	24	8,7-9,2×10^4	75-80
	96	34,9×10^5	90-95

These cultures have demonstrated the ability to reproduce in the medium of cultivation in the presence of high concentrations of Cu^{2+} and Zn^{2+} (Table 2, 3).

TABLE 2: Dynamics *of Pseudomonas sp. 409TA and Candida sp. 410AT* strains growth in the presence of Cu^{2+} at concentrations of 1-20 mg / l

Time of cultivation (hours)	The index of the optical density				
	Control (medium without addition of metal)	1 mg Cu^{2+}/l	5 mg Cu^{2+}/l	10 mg Cu^{2+}/l	20 mg Cu^{2+}/l
Pseudomonas sp. 409TA					
0	0,22±0,01	0,2±0,02	0,24±0,01	0,21±0,01	0,2±0,02
24	1,37±0,07	1,34±0,2	0,34±0,04	0,25±0,01	0,23±0,03
48	2,3±0,06	6,89±1,01	1,4±0,85	1,36±0,23	0,69±0,04
72	2,5±0,08	9,5±0,91	1,58±0,91	1,52±0,15	0,89±0,03
96	3,72±0,05	10,9±1,1	1,61±0,79	1,6±0,11	1,01±0,04
Candida sp. *410AT*					
0	0,2±0,01	0,21±0,02	0,25±0,01	0,23±0,02	0,19±0,03
24	1,94±0,09	2,35±0,6	1,87±0,2	1,65±0,8	0,4±0,02
48	4,45±1,6	5,34±0,9	4,35±0,5	4,34±0,9	0,75±0,03
72	5,45±1,01	6,05±1,01	4,7±0,8	4,4±1,2	1,17±0,02
96	5,65±1,05	6,05±1,1	5,31±0,7	4,32±1,1	1,73±0,01

TABLE 3: Dynamics *of Pseudomonas sp. 409TA and Candida sp. 410AT* strains growth in the presence of Zn^{2+} at concentrations of 5-300 mg / l

Time of cultivation (hours)	The index of the optical density							
	Control (medium without addition of metal)	5 mg Zn^{2+}/l	10 mg Zn^{2+}/l	20 mg Zn^{2+}/l	30 mg Zn^{2+}/l	100 mg Zn^{2+}/l	200 mg Zn^{2+}/l	300 mg Zn^{2+}/l
Pseudomonas sp. 409TA								
0	0,21±0,01	0,21±0,01	0,23±0,03	0,24±0,04	0,22±0,02	0,23±0,02	0,21±0,01	0,2±0,01
24	0,37±0,02	0,33±0,03	0,39±0,1	0,69±0,01	0,61±0,03	0,75±0,1	0,72±0,03	0,65±0,02
48	0,44±0,03	0,39±0,03	0,47±0,1	2,38±0,2	0,64±0,02	0,87±0,09	0,9±0,02	0,49±0,1
72	1,09±0,02	1,06±0,04	1,2±0,09	-	0,72±0,03	0,59±0,2	0,95±0,01	0,28±0,9
96	2,08±0,02	2,19±0,03	2,59±0,08	2,24±0,3	2,45±0,04	0,96±0,1	1,04±0,1	0,25±0,8
Candida sp. *410AT*								
0	0,19±0,01	0,21±0,01	0,23±0,03	0,26±0,04	0,27±0,06	0,2±0,01	0,21±0,01	0,2±0,01
24	1,22±0,1	1,22±0,09	1,25±0,04	2,6±0,09	2,58±0,01	1,74±0,05	1,64±0,03	2,51±0,01
48	2,22±0,1	2,22±0,07	2,2±0,01	5,09±0,03	5,61±0,03	3,78±0,1	3,8±0,06	2,6±0,02
72	2,33±0,09	2,3±0,1	2,29±0,02	6,27±0,02	6,16±0,05	5,36±0,13	6,1±0,2	3,3±0,03
96	3,72±0,11	3,72±0,09	3,69±0,02	7,05±0,01	6,38±0,03	3,36±0,1	3,7±0,1	4,7±0,02

In model experiments we studied the processes of extraction from solutions of Cu^{2+} ions and Zn^{2+} by strains of *Pseudomonas sp. 409TA and Candida sp. 410AT.* to assess their potential for practical use in waste water treatment contaminated with both petroleum and heavy metals. The study of the kinetics of extraction of copper ions from culture medium of bacteria showed that in the first hour of cultivation the content of metal in the

medium with bacteria cells decreased by 93,25% ± 1,6, a day bacterial cells extracted from the culture medium up to 94,75% ± 1, 4 added copper ions (at a concentration of introduced copper 0,9 g / l).
Increase the concentration of copper ions up to 7,5 g/l led to an increase in the sorption of these ions by bacterial cells. Thus, during an hour the cells absorbed 99,2% ± 0,5, and a day later - 99,28% ± 0,3. Increasing the contact time of bacteria with copper almost had no effect on the sorption activity. The percentage of metal absorbed by bacterial cells, the addition of zinc ions in a concentration of 0,01g /l at 20 min amounted to 81,6% ± 0,8; 30 minutes - 83,2% ± 1,3; in an hour – 91,04% ± 1,8; a day - 99,8% ± 1,5. After contact of bacteria with zinc in a concentration of 0,3 g/l in the culture medium remains 7,6% ± 0,5; 60 minutes - 3,3% ± 1, the next day - 2,87% ± 1,1 ions of zinc.
These data suggest that in system 'microorganism cells – solution of metal salt' state of equilibrium is reached already in the first hour of incubation.
The results showed that significant differences in the amount of extracted metal between live, boiled and autoclaved yeast cells do not. Thus, living cells at a concentration of copper ions of 0,1g /l per hour absorbed 91,51% ± 0,1; boiled - 90,02% ± 1 and autoclaved - 90,89% ± 1,1. At high concentration of copper ions (7,5 g /l) living, boiled and autoclaved yeast cells absorbed, respectively 98,88% ± 0,4; 98,05% ± 1,2; 97,67% ± 1. This fact indicates the ability of bacterial and yeast cells to a rapid sorption of copper and zinc ions from solutions. With increasing concentration of copper / zinc ions in the medium in tested range the percentage of adsorbed metal by cells of bacteria *Pseudomonas sp. 409TA* and the yeast *Candida sp. 410AT* increased. Co-cultivation of bacteria and yeast cultures did not reduce the percentage of metals sorption from the culture medium.
This index characterize the yeast *Candida sp. 410AT* and bacteria *Pseudomonas sp. 409TA* as perspective biosorbent capable during the first 10-30 minutes of contact with the medium containing copper or zinc ions to absorb them from the medium in large quantities (in the range of concentrations studied in these experiments). These results indicate that the culture of these isolates can be used for sewage treatment and to create biological products based on them for cleaning water from oil products and heavy metals pollution.

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DEGRADATION OF PHENOL BY RHODOCOCCUS ERYTHROPOLIS IN PRESENCE OF MAGNETIC FIELDS

Lucie Křiklavová, Barbora Kracíková
lucie.kriklavova@tul.cz
Centre for Nanomaterials, Advanced Technologies and Innovations, Technical University of Liberec, Studentska 2, 461 17 Liberec, Czech Republic

ABSTRACT

All living organisms themselves produce and actively use the electromagnetic field, but this field is much smaller (in the range of about 10^{-12} T) than artificially generated fields that affects all live. An additional impact of an external magnetic field (up to the order of 10^{1} T) on bacterial cells might draw attention to any another interesting effects of magnetic field on living organisms in general. The aim of this study was to determine the impact of static magnetic field induction about 250 mT on organic compounds removal. The influence of magnetic field on the degradation process was observed with an initial phenol concentration about 300 mg/l. Microorganisms in the presence of magnetic field demonstrated enhanced oxidation of phenol than those in the control sample (without magnetic field). In this study, the effects of magnetic field on bacterial *Rhodococcus Erythropolis* were investigated in the batch recirculation bioreactor.

KEY WORDS: magnetic field, phenol, biodegradation, *Rhodococcus Erythropolis*

1. INTRODUCTION

The magnetic field is represented by the physical quantity called magnetic induction. Magnetic induction expresses the force effects of a magnetic field on the particle with charge or with magnetic dipole moment. Strong fields above 1 T may inhibit physiological processes of organisms (Guevorkian and Valles, 2006; Miyakoshi, 2005). On the other hand, weak field in living organisms can cause the increase of their activity that may have a positive effect, e.g. use for wastewater treatment.

Łebkowska et al. (2011) has studied the effect of static magnetic field on the biodegradation of synthetic wastewater with activated sludge. They have stated that an induction of 7 mT increases the efficiency of the wastewater treatment by 30 % compared to the control sample (without magnetic field). Effect of magnetic field on the microorganism activity in activated sludge has been also investigated by Ji et al. (2010); magnetic induction induction has been changed in the range from 0 to 500 mT. It has been found that activated sludge adaptation and organic pollutant biodegradation processes have been stimulated under magnetic field resulting in a higher efficiency of wastewater treatment. Jung and Sofer (1999) have demonstrated an enhancement of phenol oxidation at 150 and 350 mT as well as by exposure during the adaptation of free microorganisms prior to immobilization.

The issue of the influence of magnetic fields on microorganisms is still paid little attention; there is a lack of scientific work dealing with degradation of hardly biodegradable organic compounds by bacterial population of *Rhodococcus Erythropolis*. Bioremediation using various bacterial strains of this genus has proved to be a promising option for the clean-up of polluted sites (Martínková et al. 2009). Thus, the use of magnetic field could lead towards higher efficiency of wastewater treatment process as well.

2. MATERIALS AND METHODS

2.1 Microorganisms

The large genomes of rhodococci, their redundant and versatile catabolic pathways, their ability to uptake and metabolize hydrophobic compounds, to form biofilms, to persist in adverse conditions and the availability of recently developed tools for genetic engineering in rhodococci make them suitable industrial microorganisms for biotransformations and the biodegradation of many organic compounds. Bioremediation using various bacterial strains of the genus *Rhodococcus* has proved to be a promising option for the clean-up of polluted sites. The peripheral and central catabolic pathways in rhodococci are characterized for each type of aromatics (hydrocarbons, phenols, halogenated, nitroaromatic, and heterocyclic compounds; Martínková et al. 2009).

2.2 Chemicals

Phenol p.a. (C_6H_6O min 99%), Ammonium chloride (NH_4Cl min 99%), Monopotassium phosphate (KH_2PO_4 min 99%), and Sodium chloride (NaCl min 99%) were supplied by Lachner.

2.3 Static magnetic field

The specifications of permanent neodyme magnets for generation the static magnetic field are following: 18.8 kg magnetic force, magnetization N38, Nickel coated (Ni-Cu-Ni), dimensions D30 / 6 x 10 mm (needed was 4 pieces).

2.4 Analytical methods

A Hach-Lange spectrophotometer DR-2800 and the cuvette-tests Hach-Lange (LCK345 for phenol determination, LCK304 for NH_4- determination, LCK348 for PO_4- determination) were used during each test. Dissolved oxygen concentration was measured by using the oxygen probe ConOx-1.5 (probe for multiparameter device WTW Multi 350i). Optical density measuring was performed by UV/VIS spektrofotometr Lambda 35 (PerkinElmer).

2.5 Experimental set-up

All samples were stored in the refrigerator for at least a week and taken out just prior to use. Optical density of bacterial suspension was set at about 0.3 at the beginning of the experiment (wavelength of 420 nm). Furthermore, macronutrients were added as follow 60 mg/l K_2HPO_4, 100 mg/l NH_4Cl and NaCl (so that the salinity wasabout 7 mS/cm); micronutrient content was ensured by using tap water. Before the start up of the experiment (aeration in the reservoir), phenol was dosed into the bioreactor to reach its required concentration about 300 mg/l. The experiments were performed at temperature 20 ± 2 °C.

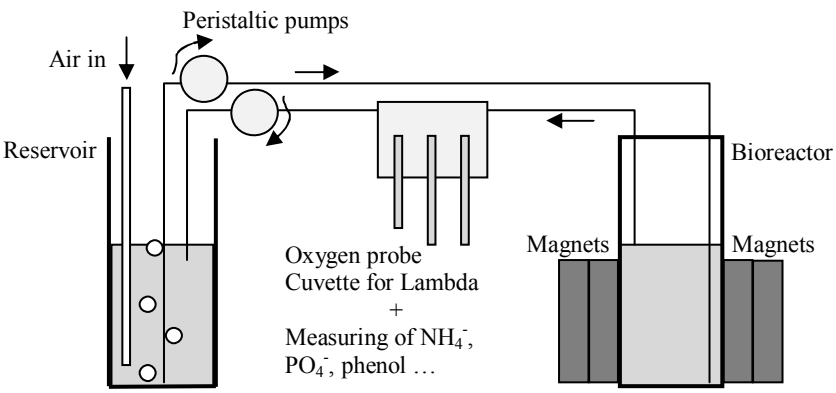

Figure 1. Experimental set-up of the batch recirculation bioreactor. Set-up of the control reactor (without magnetic field) is the same but with no magnets.

The reaction medium was recirculated between reservoir and bioreactor using a peristaltic pump. The experimental set-up is shown in Figure 1. The bioreactor was 2.7 cm in diameter and 10 cm in height, reactor wall thickness was about 0.5 mm; the reservoir was the same type. The total reaction volume was 57.3 ml (reactor 25 ml + reservoir 25 ml + volume of tubing). The recirculation flow rate was 170 ml/h (this means that the microorganisms were exposed to magnetic fields in total 3 times in one hour, duration of exposure was about 9 minutes). The magnets (250 mT) were attached to the exterior bioreactor body such that only the reactor stayed in the direct face of the magnetic field. The reservoir, the pump and probes were located at least 0.5 m from the effects of the magnet.

3. Results and discussion

Free microorganisms packed in a batch recirculation reactor with phenol as the substrate were used in this study to determine their response in the magnetic field. The batch recirculation bioreactor is a powerful tool for obtaining reaction rate information and is described in detail elsewhere. Rate of biodegradation, rate of oxygen consumption and turbidity were monitored throughout the experimental process.

3.1 Effect of static magnetic field on biodegradation of phenol

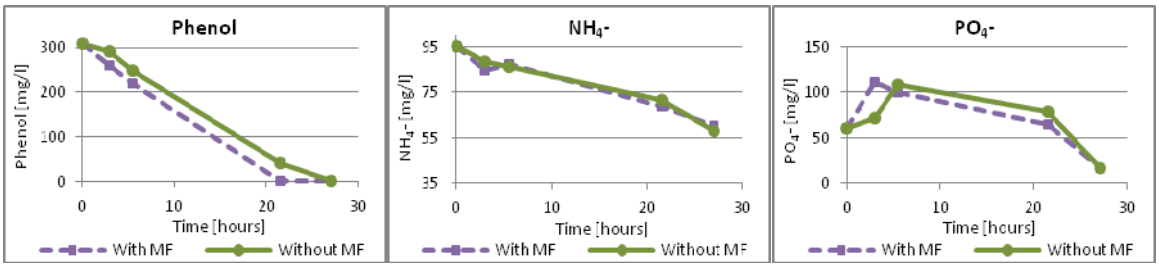

Figure 2. The course of biodegradation of phenol; the course of consumption of NH_4- and PO_4-.

The experiment demonstrated the positive effect of magnetic field within the meaning of biodegradation. The effectiveness of phenol biodegradation in magnetic field by this set-up was about 11 to 13% higher compared to set-up without magnetic field. By comparing the curves of NH_4- and PO_4- elimination (ie macronutrients, which were supplied to the system), it can be said that the magnetic field had no effect on the consumption of these nutrients. The magnetic field supported biodegradation but it did not lead to increas consumption of macronutrients.

3.2 Effect of static magnetic field on growing of *Rhodococcus Erythropolis*

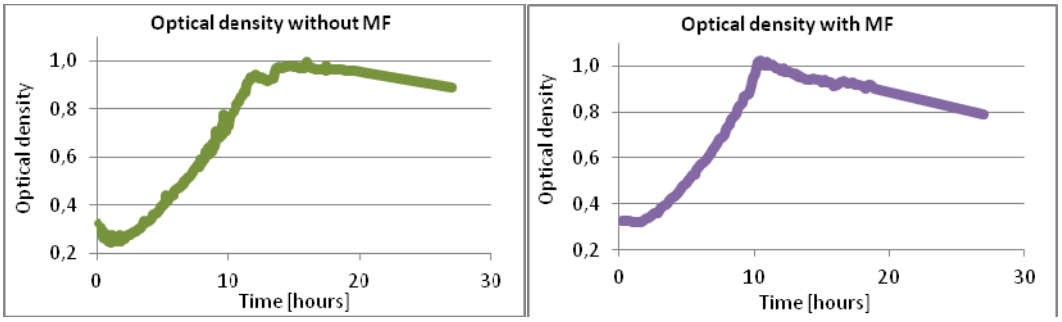

Figure 3. Monitoring of the optical density in the system without magnetic field and under the influence of magnetic field.

The experiment demonstrated the positive effect of magnetic field in terms of growth of bacterial population of *Rhodococcus Erythropolis*. The optical density reached the same values for both set-ups however in different operation time. Length of lag-phase (during which the rate of bacterial growth is zero) under the influence of the magnetic field decreased by 40% (from 1.73 hours for the sample without magnetic field to 1.02 hours for the sample with the influence of magnetic field). It is obvious that microorganisms were adapted better to the conditions in the bioreactor with magnetic field. Length of the exponential growth phase under the influence of the magnetic field decreased by 25% (from 11.9 hours for the sample without magnetic field to 9.1 hours for the sample in magnetic field). Length of death-phase under the influence of the magnetic field reduced twice (final slope of the curve was twice steeper for the benefit of the magnetic field).

3.3 Effect of static magnetic field on respiration ability of bacteria

Measurement of substrate removal contained in the wastewater is possible by identifying substrate removal rate of wastewater, or indirectly by determining rates of oxygen consumption required for substrate oxidation. Over consumption of oxygen in water is biological need for aerobic metabolism and is measured by respiration rate.

Respiration rates are classified as kinetic tests. Typically, rate of volumetric respiration r_V is measured, which relates to the volume unit of the mixture in the reactor. Volumetric respiration rate is determined from the rate of change of oxygen concentration in a given time period; expressed units are mg/(l.hod).
The experiment showed the positive effect of magnetic field in the sense of measuring respiration rate. Respiration rate of microorganisms in the experimental set-up with the magnetic field was by 12 and 33% higher compared to the sample without magnetic field after 12 and 24 hours, respectively.

4. CONCLUSIONS

The magnetic field intensity of 250 mT had a positive effect on the biodegradation activity of the bacterial population of the genus *Rhodococcus Erythropolis*. Biodegradation under the influence of magnetic field was about 13% more efficient. Under the influence of magnetic field respiration rate was also supported by 11% during the first 12 hours and by 33% during the first 24 hours and difference in consumption of macronutrients was not demonstrated. Shortening lag-phase as well as exponential phase was observed under influence of magnetic field. Moreover, downward phase was faster in the presence of magnetic field.
Optimum intensity and exposure time are important. The next step will be to verify the influence of magnetic fields in this set-up for different residence time of bacterial population and for different magnetic field intensity. Validation of the magnetic field in the system with immobilized bacterial populations in the biofilm will be performed as well.

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ENHANCEMENT OF POLYCHLORINATED BIPHENYLS BIOREMEDIATION BY SOIL ENRICHMENT WITH PLANT SECONDARY METABOLITES

Lucie Musilova[1], Ondrej Uhlik[1], Michal Strejcek[1], Vlasta Dudkova[1], Martina Mackova[1], Tomas Macek[1];
musilovu@vscht.cz
[1] Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Technicka 3, 166 28 Prague 6, Czech Republic

ABSTRACT

Polychlorinated biphenyls (PCBs) belong among recalcitrant compounds persisting in the environment. One possible way of their removal is by stimulating the activity of naturally occurring soil bacteria capable of PCB degradation. Plant secondary metabolites can serve as carbon sources for cell growth and energy production necessary among others for PCB cometabolism and/or as inducers for degradative enzymes. To investigate the effect of plant secondary metabolites, natural materials rich in common secondary metabolites were selected – lemon peels rich in limonene, grapefruit peels rich in naringin and pears rich in caffeic acid. Contaminated soil was mixed with homogenized natural materials and incubated at ambient temperature for 16 weeks. After incubation, samples were collected for further studies – determination of PCB removal using gas chromatography with ECD-detector, cultivation of bacteria on mineral medium and biphenyl as a sole carbon source and construction of microcosms for stable isotope probing. ^{13}C-4-chlorobiphenyl and ^{13}C-benzoate were used as labeled substrates in order to cover the whole pathway of chlorobiphenyl mineralization. Isolated heavy DNA was further subjected to microbial community analysis using pyrosequencing of 16S rRNA gene amplicons. Diversity of functional genes – biphenyl and benzoate dioxygenases – was also studied by amplicon pyrosequencing. Results of this study help to understand processes taking place in real environment and potentially to make bioremediation strategies more effective.
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KEY WORDS: bioremediation, PCB, amplicon pyrosequencing, bacterial community

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) represent organic contaminants widely used in industry for several decades. Despite their current prohibition PCBs persist in the environment and due to their properties and climatic conditions PCBs occur even in places they have never been used. Because of their toxicity PCBs pose risk for human health and the environment.

Elimination of PCBs using physicochemical methods is expensive and when not maintaining proper conditions for combustion, toxic products – dioxins – can occur (Totevova *et al.* 1997). Other possibility of PCB removal from the environment is bioremediation which uses metabolic capabilities of bacteria, fungi or plants (Macek *et al.* 2000; Mackova *et al.* 2006). This process is cheaper and more environmental-friendly. PCBs were considered resistant to microbial degradation for long time. But some aerobic bacterial species capable of degradation of specific PCB congeners were described. Monochlorinated biphenyls can be used as a carbon source, other congeners are transformed via cometabolism which does not provide the cell with energy. There also exist anaerobic bacteria capable of dehalogenation of more chlorinated PCBs thereby accessing them to aerobic bacteria (Totevova *et al.* 1997). Bacterial aerobic PCB degradation is facilitated by enzymes coded in biphenyl operon (*bph*) and consists of two pathways (Furukawa 2000). The so called upper pathway is a serie of chemical reactions transforming biphenyl to benzoic acid and an aliphatic acid, which is further transformed to pyruvate and acetyl coenzyme A. But these enzymes need to be induced, e. g. by biphenyl, at least in model organisms (Beltrametti *et al.* 2001; Watanabe *et al.* 2003). Biphenyl application into the environment is, however, prohibited because of its toxicity for water organisms. Therefore, there are efforts to find nontoxic inductors of *bph* operon, with one of suitable options being plant secondary metabolites.

Plants produce more than 100,000 different low molelucar-weight secondary metabolites with terpenes and phenolics being the most extended (Singer *et al.* 2003). Some of these metabolites are secreted in the form of root exudates into the rhizosphere and stimulate bacterial growth. Some secondary metabolites can also serve as stimulators of degradation activity. This is made possible by structural similarities between some secondary metabolites and pollutants. (Singer *et al.* 2004). There have been several experiments carried out in which authors focused on the effect of plant secondary metabolites on degradation of organic xenobiotics in soil

samples (Leigh 2006; Luo *et al.* 2007; Rezek *et al.* 2009; Singer *et al.* 2000). Roots are subjected to seasonal exchanges and after necrosis they provide microorganisms with other nutrients. After root decay, new space also appear for air and especially oxygen transfer, which is necessary for aerobic degradation (Leigh *et al.* 2002).

2. MATERIALS AND METHODS

2.1 Soil preparation

Long-term PCB-contaminated soil coming from dumpsite Lhenice in south Bohemia was used (Mackova *et al.* 2009; Pavlikova *et al.* 2007). The soil was amended with homogenized natural materials rich in secondary metabolites – grapefruit pericarp rich in naringin, lemon pericarp rich in limonene and pears rich in caffeic acid. Incubation time was 18 weeks and during this period the natural materials were twice re-added (every 8 weeks). After the last amendment the soil was incubated for two more weeks and after that samples were collected for PCB content determination, plate counts and metagenomic analysis.

2.2 PCB removal

Soil incubated with homogenized natural materials was dried at ambient temperature and sieved. Samples were extracted into diethylether for 6 hours and purified using activated Florisil. PCB removal was determined by gas chromatography with µECD detector.

2.3 Plate counts and identification of biphenyl utilizers

Soil rhizosphere bacteria were extracted using 1% (w/v) sodium pyrophosphate. 10 g of soil were suspended in sodium pyrophosphate solution (total volume of soil and solution was 100 ml) with glass beads for 2 hours at 28 °C. The extraction was followed by the series of 10-times dilutions into 0.85% (w/v) sodium chloride (physiological solution). 100 µl of each solution were inoculated onto plate count agars (in order to enumerate the diversity of cultivable bacteria in the sample) and mineral medium with crystals of biphenyl which evaporated and served as the only carbon source (in order to select bacteria capable of growth on biphenyl) and plates were incubated at 28 °C. Growth of colonies was statistically evaluated and pure cultures growing on biphenyl were further indetified using sequencing of 16S rRNA gene and/or MALDI-TOF mass spectrometry (Koubek *et al.* 2012).

2.4 Metagenomic analysis

Stable isotope probing (Dumont and Murrell 2005; Radajewski *et al.* 2003; Uhlik *et al.* 2009) was used to identify microorganisms potentially capable of PCB degradation. From contaminated soil incubated with natural materials, microcosms containing ^{13}C-labeled substrates were constructed. 4-chlorobiphenyl or benzoic acid were used as substrates to study the entire biphenyl pathway. Organisms deriving carbon from labeled substrates incorporate ^{13}C into their DNA ("heavy DNA") which can be further separated from DNA containing only isotope ^{12}C ("light DNA"). After incubation with labeled substrates, ^{13}C-labeled metagenomic DNA was isolated and separated.. For the analysis of phylogenetic bacterial diversity, amplicons of 16S rRNA gene were prepared and subjected to 454 sequencing (Acosta-Martínez *et al.* 2008; Uroz *et al.* 2010). In addition to 16S rRNA genes, functional genes encoding for biphenyl and benzoate dioxygenases were amplified, pyrosequenced and analyzed.

3. RESULTS AND DISCUSSION

Soil amendment with used natural material caused changes in composition of PCB congeners – some of higher chlorinated congeners were transformed into lower chlorinated ones which are usually less toxic and more suitable for further degradation. However, total PCB amount was not decreased. This means that microbial degradation occurred but some chemicals contained in natural materials could have had activity causing slower PCB metabolism and longer incubation or further treatment would be needed for PCBs to decrease..

Plate counts showed that soil amendment resulted in increased numbers of cultivable microorganisms – the largest increase was observed in soil incubated with lemon pericarp. The number of isolated microorganisms from soil samples incubated with grapefruit pericarp or pears was lower, but still higher in comparison to control soil. Identification of organisms isolated on biphenyl as a sole carbon source showed increased bacterial diversity when comparing control soil and amended soil samples. In control soil only bacteria belonging to *Arthrobacter* spp. were isolated. In soil samples incubated with natural materials more bacterial genera were identified.

Differences in structures of bacterial communities were confirmed also by the metagenomic analysis. Proteobacteria were dominant in all samples but differences at lower taxonomic levels were observed. In control soil bacteria belonging to class *Gammaproteobacteria* were dominant in contrast to amended soils where bacteria from class *Alphaproteobacteria* dominated. . Analysis of stable isotope labeled DNA showed that bacteria actively metabolizing ^{13}C-labeled substrates also differed among all samples. Bacteria capable of mineralization of PCBs (capable of deriving carbon from both 4-chlorobiphenyl and benzoic acid) were also detected. But in soil amended with lemon pericarp the labeling with ^{13}C was not sufficient probably due to inhibition of degradative activity by some compounds included in the pericarp. It is not expected that lemon pericarp inhibited bacterial growth since the number of cultivable microorganisms isolated from this soil was the highest one.

Our results confirm that the addition of natural materials has a fundamental effect on composition of bacterial communities in soil and on their activity as well. For further studies and potential use, grapefruit pericarp and pears can be recommended. Additional studies might lead to identification of natural compounds capable of making bioremediation more effective – these compounds may be added in pure form (which is expensive) or as natural materials or can even be produced by plants growing in contaminated environments. Higher efficiency can be expected when plants capable of pollutant degradation are used.

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OCCURRENCE OF *ARS* AND *AOX* GENOTYPES WITHIN AS-RESISTANT ISOLATES FROM THE BACTERIAL COMMUNITY OF A DISMISSED INDUSTRIAL AREA FORMERLY USED AS A DUMPING SITE FOR ROASTED ARSENOPYRITE

Chiara Santi, Silvia Lampis, Giovanni Vallini
chiara.santi@univr.it, silvia.lampis@univr.it, giovanni.vallini@univr.it
Universitá Degli Studi Di Verona, Dipartimento Di Biotecnologie
Strada Le Grazie N. 15, Ca' Vignal – 37134 Verona (Italia)

ABSTRACT

The present study investigates on the bacterial community structure of arsenic contaminated soil samples from an arsenopyrite dumping site near Scarlino (Tuscany, Italy). 56 different bacterial strains were isolated and phylogenetically identified through 16S rRNA sequencing analysis starting from enrichment cultures in presence of either arsenite As(III) or arsenate As(V) as selective factors. *Actinobacteria* and *Firmicutes* resulted to be the main *phyla* within the As(V) resistant bacteria community, on the other hand Gamma- and Beta-proteobacteria were the prominent classes within the As(III) resistant strains.. The As-resistant isolates were then screened through PCR to assess the occurrence of genes coding for arsenite transporters, arsenate reductases (*ars* operon), and arsenite oxidases (*aox* operon). Evidence was gained that 41% of the isolates contained at least a gene related to arsenite transporters, 37.5% a gene related to arsenate reductase, and 23% a gene related to arsenite oxidases. Moreover, two isolates cable of efficiently reducing arsenate were identified. Actually, *Deftia acidovorans* strain U and *Pseudomonas putida* strain N showed the ability to reduce 5 mM As(V) within 48h and 72h of incubation respectively. Thus, the bacterial strains here described possess promising traits for a possible exploitation in bioremediation protocols of As polluted environments.

KEY WORDS: arsenopyrite, soil bacteria, arsenate reduction, 16S rDNA, diversity

1. INTRODUCTION

Arsenic is a toxic metalloid largely distributed in soils and aquifers, released both from natural and anthropogenic sources. Its toxicity affects both plants and animals and inorganic arsenicals are proven carcinogens in humans (Mandal and Suzuki, 2002). Despite of its toxicity for living beings, the contribution of microorganisms to the biogeochemistry of arsenic in the environment is extensive and detailed as it involves various oxidation, reduction, methylation, and demethylation reactions of its dominant chemical species (Oremland and Stolz, 2003; Rosen, 2002). Both microbial arsenite oxidation and arsenate reduction were reported to influence environmental arsenic cycles. Understanding the diversity and distribution of indigenous bacterial species in arsenic-contaminated sites could be important for improvement of arsenic bioremediation. The main purposes of this study were: i) to study the bacterial community structure of arsenic-contaminated pyrite ashes discharged in Scarlino's dismissed industrial area and ii) to investigate on the arsenic related functional genes of autochthonous bacteria isolates. The fact that the arsenic-resistant bacteria characterized in this study are autochthonous enhances their uniqueness and utility in possible applications for bioremediation strategies in the polluted soils in exam. The role of bacteria in speciation, mobility, and bioavailability of As in this area has never been considered in previous studies.

2. MATERIALS AND METHODS

2.1 Isolation of arsenic-resistant strains from enrichment cultures

Six soil samples were collected in Scarlino's dismissed industrial area. In detail, one sample from the ashes disposal (MP) that consists in a cumulus of arsenopyrite ashes of 800.000 tons and 5 samples from the rhizosphere of 5 different autochthonous plants (P1, P2, P3, P4 and P5) grew adjacent to the waste disposal. The different soil samples (5 g) were incubated for two weeks in R2A liquid medium in the presence of the inorganic arsenicals (AsIII 2 mM or AsV 2mM). To isolate arsenic-resistant bacteria, appropriate dilutions of enrichment cultures were plated on R2A, Nutrient and Waksmann agarized medium and the plates were incubated at 27°C for 5 days. After incubation, colonies with different morphotypes were selected from the plates and subsequently streaked until axenic cultures were obtained.

2.2 Taxonomic characterization

Bacterial isolates were analyzed through the Amplified Ribosomal DNA Restriction Analysis (ARDRA). Initially, 16S rRNA amplicons were obtained by PCR using F8 and R11 primers (Weisburg *et al*., 1991). PCR reactions were carried out as in Andreolli *et al.* (2011). Then restriction reactions were set up with both *AluI* and *HhaI* enzymes. The isolates were then clustered in different OTUs (Operational Taxonomic Unit). A representative isolate for each OTU was selected for the sequencing of its 16S rRNA gene. Similarity comparisons of the partial 16S rDNA sequences were performed using the National Centre for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/).

2.3 Identification of arsenic resistance genes

All the isolates were screened for the presence of important genes involved in As resistance and transformation using group-specific primer pairs. To detect the arsenite transporters genes, PCR reactions were performed using 3 sets of primers targeting *arsB*, *ACR3(1)* and *ACR3(2)* genes respectively (Achour *et al*., 2007); four pairs of primers were used to detect *arsC* (As(V) reductase) gene (Macur *et al*., 2004) and three different pairs of primers for the amplification of *aox* (As(III) oxidase) genes (Inskeep *et al*., 2007 and Quemeneur *et al*., 2010).

2.4 Arsenate reduction by isolates

To test the ability of the isolates to reduce As(V) the strains were inoculated into vials each containing 30 ml of Tris Minimal Medium with As(V) 5 mM. Control flasks without inoculum were incubated to check abiotic transformation of arsenic. At each sampling time, 1 ml of suspension was removed to measure cell growth by OD600 nm and to determine As(V) and As(III) concentration by spectrophotometric analysis according to the method described by Cummings *et al*., (1999).

3. Results and discussion

3.1 Isolation of arsenic-resistant strains from enrichment cultures

A total of 109 As-resistant bacteria were isolated, 63 from As(V)- and 46 from As(III)-enriched cultures respectively. These data are consistent with the fact that As(V) is less toxic than As(III), thus more species can adapt to this form of arsenic. On the other hand, being As(V) the dominant chemical specie, more bacterial taxa have been isolated in As(V) enrichment culture than in As(III) one. The isolates were clustered in 56 OTUs through ARDRA analysis (Table 1).

Table 1. Number of strains isolated from the soil samples in presence of AsIII 2 mM or AsV 2 mM.

Sample	Number of isolates AsIII	Number of isolates AsV
MP (Matrix)	7	11
P1 (*Graminaceae*)	5	6
P2 (*Umbrelliferae*)	6	16
P3 (*Trifolium* sp.)	10	8
P4 (*Populus* sp.)	5	11
P5 (*Crassulaceae*)	13	11

16S rDNA genes representative of the different OTUs were all sequenced and a research of similarity was done. From the obtained taxonomic data we could assume some evaluations in relation to the distribution in the different bacterial *phyla* in the two enrichment cultures, illustrated in Figure 1.

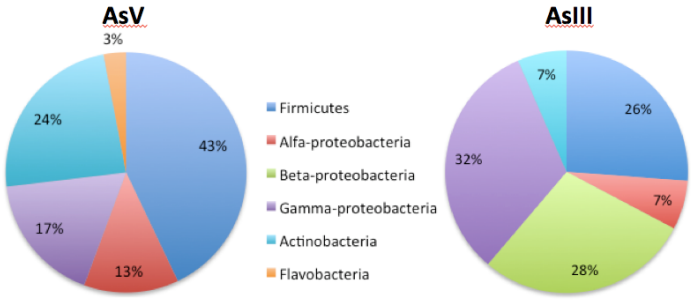

Figure 1. Distribution of the among the isolates of the different enrichment cultures.

Results obtained indicated that in As(V) enrichment cultures *Actinobacteria* and *Firmicutes* (67%), were the most abundant *phyla*. On the other hand, in As(III) cultures Gamma- and Beta-proteobacteria (Gram negative *phyla*) resulted to be the dominant classes. Having that AsV is easily adsorbed on the bacterial cell wall, mostly Gram positive (especially *Actinobacteria* like *Microbacterium* sp. and *Firmicutes* like *Bacillus cereus*) have been selected in AsV cultures. AsIII cultures show instead a typical distribution of bacterial *phyla* associated with contaminated soils, 30% of Gram + and 70% of Proteobacteria belonging to Gamma, Beta and Alpha classes (Cavalca *et al*., 2010).
Phyla distribution in the rhizosphere of the analyzed plants and in the highly contaminated matrix was also evaluated. Results indicate that in the polluted matrix the most part of the isolates belonged to *Bacillus genus* (66%). Otherwise in the rhizosphere samples (P1, P2, P3, P4 and P5) the most abundant *phyla* were those belonging to Proteobacteria, in particular to Gamma class (like *Stenotrophomonas* sp. and *Pseudomonas* sp.) and Beta class (*Delftia* sp.). Similarly *Actinobacteria phylum* appeared to be more represented among the rhizosphere isolates than in the matrix and, consequently, *Firmicutes* resulted to be less abundant in the 5 rhizosphere soils.

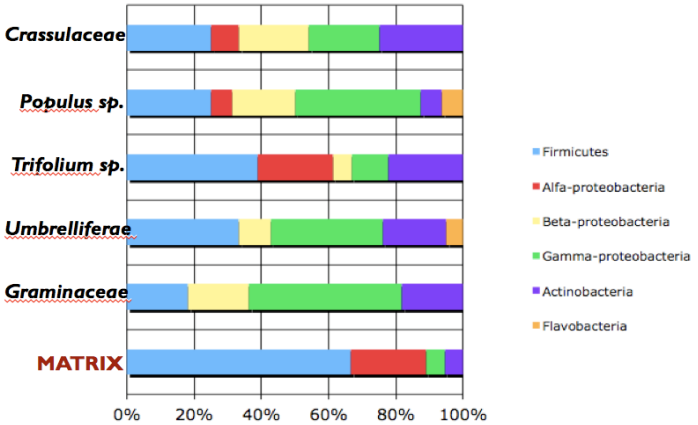

Figure 2. Distribution of the species among the isolates of the different samples.

It could be hypothesized that the preponderance of *Bacillus* spp. strains at the highest soil arsenic levels might be related to enduring the toxicity of arsenic in sporulated form or the structure itself of the bacterial cell wall of Gram + species. Whereas it's attested that Gamma-proteobacteria *phylum* includes species able to establish a symbiontic relationship with the rhizosphere environment (Sun *et al*., 2009).

3.2 Genotypic study of the mechanisms of arsenic transformation

Arsenic microbial metabolism is very complex and in this study the attention was focused on to two different reaction mechanisms. Actually, the presence of either *ars* operon or *aox* operon was investigated. The first one, in its basic components consists of a cytoplasmic arsenate reductase (AsV ® AsIII) and an arsenite efflux pump; conversely the *aox* operon contains a membrane arsenite oxidase (AsIII ®AsV) (Cai *et al*., 1998). Each isolate was tested by PCR reactions using group-specific primer pairs for the presence of these genes. In Table 2 we reported a summery of the results, showing the positive PCR grouped by soil samples.

Table 2. *Ars* and *aox* genotypes of the bacterial isolates, grouped by soil sample.

SAMPLE	*Ars* OPERON	*Aox* OPERON	BOTH OPERON
MP (Matrix)	13/13	5/13	5/13
P1 (*Graminaceae*)	3/6	1/6	1/6
P2 (*Umbrelliferae*)	6/9	3/9	2/9
P3 (*Trifolium* sp.)	4/11	3/11	3/11
P4 (*Populus* sp.)	7/8	-	-
P5 (*Crassulaceae*)	3/9	1/9	1/9
TOTAL	36/56	13/56	12/56

Considering that various PCR negative results in some of the arsenic resistant isolates may be due to sequence differences between genes and primers used in this study, some evaluations can be assumed. The *ars* operon was detected in all strains isolated from the matrix sample; on the other hands, in the strains isolated from the different plant rhizospheres, these genes are less represented. In general *ars* operon appears to be more diffused than *aox* one (none of the strains from *Populus* sp. for istance has the *aox* operon). Remembering that *ars* genes

catalyze the reduction of AsV to AsIII that is more mobile and thus more bioavailable, we can state that these isolates could be successfully utilized in designing potential arsenic bioremediation strategies.

3.3 Arsenate reduction by isolates

Some bacterial strains were tested for their ability to reduce As(V) to the more mobile specie As(III). The best performances were exhibited by *Delftia acidovorans* strain U that was capable of completely reducing 5 mM As(V) in 48 h of incubation and *Pseudomonas putida* strain N, able to totally reduce As(V) 5 mM in 72 hours of incubation under aerobic condition. These bacterial species are known for the presence of *ars* operon in their genome and thus for their ability to reduce As(V). However, to the best of authors' knowledge, the complete reduction of such a high As(V) concentration has never been reported for these species.
The ability to reduce As(V) to As(III) represents a very important parameter in a bioremediation context, As(III) is indeed more mobile and bioavailable and can be easily removed from the contaminated soils and aquifers.
In conclusion, the bacterial strains here described possess promising traits for a possible exploitation in bioremediation protocols of As polluted environments.

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DIVERSITY OF *BPHA* GENES IN CONTAMINATED AND PRISTINE SOILS

Michal Strejcek, Ondrej Uhlik, Lucie Musilova, Tomas Macek and Martina Mackova
michal.strejcek@vscht.cz
Institute of Chemical Technology Prague, Department of Biochemistry and Microbiology, Technická 3, Prague 6, Czech Republic.

ABSTRACT

The introduction of next generation DNA sequencing techniques, i.e. high throughput techniques, has elevated the value of metagenomic analyses to a new level. Such technologies enable screening for biologically and ecologically important markers. Although whole-metagenome sequencing provides ultimate genetic information of sampled system, its cost, computational demand and low coverage make it unsuitable for diversity studies. Gene-targeted metagenomics is a valuable alternative. The approach of using PCR amplicons derived from metagenomic DNA as sequencing templates significantly reduces the analytical complexity. For this task, pyrosequencing has been employed due to its capability to acquire longer reads than other sequencing technologies. For describing bacterial diversity, screening variable regions of 16S rRNA genes is the most used and the best developed method so far. Several specific procedures have been developed for 16S rRNA analysis; however, they are not all applicable to functional gene analyses.
The focus of this study is to describe *bphA* genes diversity in a pristine soil and a contaminated soil. BphA, a large subunit of biphenyl dioxygenase, plays the key role in the bacterial degradation pathway of polychlorinated biphenyls (PCBs). To correct errors derived from amplicon generation and pyrosequencing process, several bioinformatics tools were applied.
The results show a significant number of novel sequences that are not closely related to previously characterized PCB degrading bacteria.

KEY WORDS: *bphA*, pyrosequencing, diversity, functional gene analysis

1. INTRODUCTION

Microbial diversity and activity in the environment are influenced by a variety of biotic and abiotic effects. One of the most significant biotic factors that impacts on microorganisms directly is stress connected with the presence of xenobiotics. Xenobiotics have been released into the environment either intentionally, such as pesticides, or accidentally, such as polychlorinated biphenyls, leaked from industrial facilities.
The oldest remediation technologies were based on physical and chemical principles. Even though they were improved over the time, they are still economically inefficient and their application comes with a further devastation of the exposed area. A more appealing alternative is to employ living organisms in remediation processes. Such an approach is called bioremediation (Mackova *et al.,* 2006). Organisms able to grow in the presence of pollutants are monitored for the mechanisms they react to a specific contaminant. The scientific focus is particularly aimed at species with diverse metabolic pathways which are able to react easily to the changes of growing conditions, such as bacteria or fungi.
A group of widespread pollutants are polychlorinated biphenyls (PCBs). These chlorinated derivatives of biphenyl (differing in number and position of chlorine atoms) were largely produced worldwide between late 1920s and early 1980s for a large variety of industrial uses. Ever since, they have been entering the environment contaminating many sites worldwide. Several processes are involved in PCB degradation. Under anaerobic conditions, dechlorination occurs reducing the number of chlorine atoms in higher chlorinated biphenyls. In aerobic degradation of lower chlorinated biphenyls, aromatic ring is cleaved allowing further metabolism. Bacteria have been found utilizing monochlorinated (Sylvestre 1980) and rarely dichlorinated congeners (Kim and Picardal 2001) to derive carbon and energy. Other lower chlorinated PCB congeners are usually biodegraded cometabolically (Furukawa 2000; Macková *et al.,* 2010; Pieper and Seeger 2008) with biphenyl or monochlorinated biphenyls being the primary substrates. Upper biphenyl degradation pathway is encoded for in the *bph* operon. This 4-step catabolic pathway results in the formation of 2-hydroxypenta-2,4-dienoate and benzoate (or chlorobenzoate). A key role in this degradation metabolism play aromatic ring hydroxylating dioxygenases (namely biphenyl and benzoate dioxygenases – BphA and BenA, respectively). The C-terminal portion of BphA α subunit, encoded for in *bphA*, is crucial for substrate specificity and regiospecificity towards PCB congeners (Mondello *et al.,* 1997). Thus, *bphA* is regarded as a suitable genetic marker for diversity analysis of PCB-degrading bacteria.
In the past, majority of diversity experiments were based on cultivation-dependent techniques. It is known that these techniques suffer from large biases as only 1-5 % of bacterial species are expected to be cultivable (Lopez-Garcia and Moreira 2008). Metagenomics is an approach that targets entire DNA available in a sample. In

conjunction with high-troughput sequencing technologies, such as pyrosequencing (Margulies *et al.,* 2005) , it is possible to obtain whole genomes or thousands of genetic and ecological markers at a time. The latter is achieved by pyrosequencing PCR products defined by specific primers. Such a method is called gene-targeted metagenomics (Iwai *et al.,* 2011) and it is well applied in determining bacterial diversity using variable regions of 16S rRNA genes as phylogenetic markers (Sogin *et al.,* 2006).
Applying amplicon pyrosequencing has several issues that require careful attention. Both PCR and pyrosequencing processes are prone to introduce errors. Polymerase mismatches during elongation, "chimeric" sequences and incorrect base calling are major alternation to original templates, inevitably leading to overestimated diversity (Kunin *et al.,* 2010). To overcome such problems, several procedures were designed. A common method is to cluster similar sequences within an identity interval as erroneous sequences (except "chimeric") are likely to be very similar to the original ones. Several clustering strategies can be applied, each with different specifics (Huse *et al.,* 2010; Quince *et al.,* 2009).
This study aims to employ gene-targeted metagenomics for in-depth analysis of biphenyl dioxygenase genes diversity in pristine and long-term contaminated soil.

2. MATERIALS AND METHODS

2.1 Materials

In this study, two types of soil were sampled: i) long-term contaminated soil poor in nutrients containing pollutants such as PCB, PAHs, pesticides, heavy metals, etc. (Macková *et al.,* 2009; Pavlíková *et al.,* 2007); ii) pristine soil used for gardening rich in nutrients.

2.2 Sample preparation

Metagenomic DNA was extracted from both soil samples with PowerMax Soil DNA Isolation Kit (MoBio Laboratories Inc., USA) using the standard protocol with minor modifications described earlier (Uhlík *et al.,* 2009). Pyrosequencing templates were prepared as PCR products using primers ligated with pyrosequencing adaptors. Primers for the amplification of *bphA* genes, encoding for the large subunit of biphenyl dioxygenase, and PCR temperature profile were adopted from literature (Iwai *et al.,* 2010).

2.3 Mock community

A mock community was used to evaluate errors in data generation and to optimize parameters of algorithms used for data processing. The mock community was prepared as a mixture of genomic DNA isolated from these five strains: *Burkholderia xenovorans* LB400 (Bopp 1986), *Rhodococcus jostii* RHA1 (Masai *et al.,* 1995), *Pseudomonas alcaliphila* JAB1 (Ryšlavá *et al.,* 2003), *Cupriavidus necator* (formerly *Ralstonia eutropha*) H850 (Bedard *et al.,* 1987), *Pandoraea pnomenusa*(formerly *Comamonas testosteroni*) B-356 (Hurtubise *et al.,* 1995). All these strains carry *bphA* genes.

2.4 Sequencing

GS FLX+ Titanium pyrosequencing system was employed to obtain large number of *bphA* sequences.

2.3 Bioinformatic tools

All sequence processing (denoising pyrosequencing reads, sequence validation, chimera removal, etc.) and statistics were performed in MOTHUR software package (Schloss *et al.,* 2009).

2.3 Sequence filtering and data processing

Based on processing the mock community sequences, a following workflow was set up: i) pyrosequencing reads were deinosed using PyroNoise algorithm implemented in MOTHUR software package; ii) sequences were filtered allowing no mismatch in primer and barcode sequence; iii) all sequences were chopped to length of 360 bp, shorter sequences were discarded; iv) a single-linkage preclustering at 1 % distance was applied (Huse *et al.,* 2010); v) clusters were generated at 97% identity cutoff using average-linkage clustering.

3. Results and discussion

The results show that each soil system has unique *bphA* diversity. No sequences were shared in both types of soil. In contaminated soil, the most abundant sequences were closely related to sequences isolated from model PCB degraders such as *Pseudomonas alcaligenes* B-357, *Burkholderia xenovorans* LB400, *Pseudoxanthomonas spadix* BD-a59 and *Rhodococcus jostii* RHA1. These sequences represented more than 50% of all sequences isolated from the contaminated soil sample. The rest were poorly related to any know sequences and they showed 30-40% sequence identity to descibed aromatic ring hydroxylating dioxygenases (ARHD), being on the edge of protein homology. In the case of pristine soil, the majority of obtained sequences were 40-60% identical to ARHDs. The only more significant matches were sequences similar to these from *Rhodococcus jostii* RHA1 and *Rhodoccocus equi* 103S, representing less than 5% of total diversity.
These findings correspond to a common theory that the presence of contaminants forms a selective pressure on organisms. Bacteria with enzymatic capabilities that enable them to utilize these xenobiotic compounds are favored in growth and, as a result, biodegradative potential of the whole community is raised. On the other hand, the majority of detected dioxygenases in pristine soil have probably different role than primarily utilize biphenyl. One explanation could be the degradation of lignin structures in soil (Furukawa *et al.,* 2004).
In both soil samples, a vast number of uncharacterized sequences were obtained. To conclude whether these genes can be expressed into fully functional enzymes and what their true role is, further research is necessary.

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BACTERIA UTILIZING AROMATIC POLLUTANTS IN LONG-TERM CONTAMINATED SOIL AS REVEALED BY STABLE ISOTOPE PROBING AND GENE-TARGETED METAGENOMICS

Ondřej Uhlík[1], Michal Strejček[1], Lucie Musilová[1], Jiří Wald[1], Jakub Rídl[2], Miluše Hroudová[2], Čestmír Vlček[2], Martina Macková[1] and Tomáš Macek[1]
ondrej.uhlik@vscht.cz
[1] Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Technicka 3, 166 28 Prague 6, Czech Republic.
[2] Department of Genomics and Bioinformatics, Institute of Molecular Genetics, Czech Academy of Sciences, Videnska 1083, 142 20 Prague 4, Czech Republic.

ABSTRACT

Microorganisms are capable of not only degrading organic compounds of natural origin but also anthropogenic ones, contributing thereby to decontamination of the environment. Most of our knowledge on biodegradative processes, however, has still been based on highly biased results of cultivation studies. Stable isotope probing enables researchers to link metabolic activity and phylogeny without previous cultivation of microbes.
The aim of the study was to identify bacteria in contaminated soil that derive carbon from biphenyl, naphthalene, and benzoate as well as analyze diversity of functional dioxygenases potentially responsible for the biodegradation activity. In order to reach this goal, we applied DNA-based stable isotope probing with subsequent pyrosequencing of tag-encoded amplicons of 16S rRNA and functional genes. Pyrosequencing data were processed within mothur software package. In order to determine appropriate parameters for sequences processing, sample sequences were amplified, sequenced, and analyzed along with a mock community.
Our results demonstrate that, in addition to traditionally isolated genera of degradative bacteria mostly associated with *Proteobacteria*, yet-to-be cultured bacteria are important players in bioremediation. When analyzing functional dioxygenases, some novel types of biphenyl and benzoate dioxygenases were detected. Overall, this study shows the importance of cultivation-independent techniques to investigate biodegradation processes. At the same time our results show the importance of sequencing and analyzing a mock community in order to more correctly process and analyze sample sequences.

KEY WORDS: gene-targeted metagenomics, stable isotope probing, aromatic pollutants

1. INTRODUCTION

Microbial bioremediation takes advantage of certain bacteria and fungi being capable of degrading widespread environmental contaminants, such as petroleum hydrocarbons (PHC), polychlorinated biphenyls (PCBs) and \other halogenated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), pesticides, explosives, or solvents. This strategy is a much cheaper and more environmentally-sound alternative of remediation compared to physical-chemical methods (Demnerová *et al.,* 2005). In order to better understand biodegradation and bioremediation processes taking place in contaminated environemnts, it is crutial to identify microorganisms capable of degrading contaminants. However, until recently much of this information had been very difficult to elucidate and rarely achieved. Stable isotope probing (SIP) is an elegant tool which enables researchers to track isotopically labeled substances into phylogenetically and functionally informative microbial biomarkers (Radajewski *et al.,* 2000; Radajewski *et al.,* 2003). It involves tracking the incorporation of heavy stable isotopes, usually ^{13}C or ^{15}N, from specific substrates into phylogenetically informative biomarkers (DNA, RNA, fatty acids, proteins) associated with microbes that assimilate the substrate – e.g. pollutant (Chen *et al.,* 2010; Madsen 2010; Uhlík *et al.,* 2009a). SIP is thus applicable as a tool for the identification of microbes and genes that play key roles in given habitats' metabolic processes. After stable isotope labeled compounds have been pulsed into a microbial community and active cells utilizing the substrate have become heavily labeled, biomarkers (nucleic acids, fatty acids, proteins) are recovered and analyzed (Neufeld *et al.,* 2007). Currently, DNA-SIP is of interest due to recent possibilities of metagenomic analyses. Metagenomics is presently undergoing a huge development due to advances in high-throughput sequencing (e.g. 454 pyrosequencing) and constantly improving bioinformatic tools. Yet the first experiments in which shotgun sequencing was employed showed that metagenomics may be capable of revealing the metabolic potential of the community but will hardly succeed in linking individual functional genes (hence functions) to certain populations. This may be achieved

only in case of very dominant members of the community or in case of communities with a very low diversity (Chen and Murrell 2010). However, even lower abundant members of the community can play significant roles in the community functioning. Targeting metagenomics to specific subpopulations which are likely to contain the genes of interest may overcome this obstacle, as can be achieved with stable isotope probing. Alternatively, next-generation sequencing can be applied to 16S rRNA genes (or functional genes) amplicon analyses. Unlike analyzing clone libraries, amplicon pyrosequencing enables one to sample the community much more thoroughly and provides greater sensitivity (Engelbrektson *et al.,* 2010).
The aim of the study was to identify bacteria in contaminated soil that derive carbon from biphenyl, naphthalene, and benzoate as well as analyze diversity of functional dioxygenases potentially responsible for the biodegradation activity. In order to reach this goal, we applied DNA-based stable isotope probing with subsequent pyrosequencing of tag-encoded amplicons of 16S rRNA and functional genes.

2. MATERIALS AND METHODS

2.1 Soil samples

Contaminated soil from Lhenice, south Bohemia, Czech Republic (Pavlíková *et al.,* 2007) was mined from the depth of about 0.5 m in the late summer of 2008. Main contaminants of the slightly alkaline sandy soil were PCBs. Their total amounts have gradually decreased over the years (Macková *et al.,* 2009) with actual amount at the time of experiment being 36.3±10.9 $mg.kg^{-1}$ dry soil. In addition to PCBs, the soil contains PAHs (1.7±0.6 $mg.kg^{-1}$), pesticides (DDT, 1.4±0.4 $mg.kg^{-1}$, traces of hexachlorbenzene and lindane), and heavy metals (Zn, Cr, Pb, etc). The values stated are averages from three independent samples with uncertainty.

2.2 Stable isotope probing

SIP microcosms were constructed in three replicates in 100mL serum bottles (Sigma-Aldrich, USA) with 2.5 g of soil and 0.5 mg of each substrate (Sigma-Aldrich, USA) – ^{13}C-labeled biphenyl, naphthalene, and benzoate – and amended with 500 µL of mineral salt solution (Uhlík *et al.,* 2009b). Bottles were sealed with crimp seals with silicone septum and incubated at laboratory temperature. Microcosms were destructively harvested by freezing after 4 and 14 days of incubation.
DNA was extracted with a PowerMax Soil DNA Isolation Kit (MoBio Laboratories Inc., USA). Isolated metagenomic DNA (^{13}C-enriched as well as control DNA) was subjected to isopycnic centrifugation performed on a Discovery 90 Ultracentrifuge with TFT 80.2 rotor (Sorvall, USA) at conditions of 145000 × *g* for 70 hours in 2-ml cuvettes. Using a Beckman Fraction Recovery System (Beckman Coulter, USA) and Harvard Pump 11 Plus Single Syringe (Harvard Apparatus, USA), each gradient was fractionated into 50µl fractions (with flow rate 200 $\mu l.min^{-1}$). DNA retrieval and quantification were carried out as described elsewhere (Uhlík *et al.,* 2009b).

2.3 Amplicon preparation and pyrosequencing

Regions V4-V5 of 16S rRNA genes and C-terminal portions of BphA (biphenyl dioxygenase) and BenA (benzoate dioxygenase) were amplified with primers modified with sequencing adapters (454 Sequencing Application Brief No. 001-2009, Roche). The forward primers were also modified with different tags (454 Sequencing Technical Bulletin No. 005-2009, Roche) so that more samples could be pooled and sequenced at once. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and mixed together with other barcoded samples for pyrosequencing. Amplicon pool was purified using AMPure XP Beads (Agencourt, Beckman Coulter, USA) to remove residual primer-dimers according to manufacturer's instructions. Amplicons were unidirectionally sequenced from forward primer using GS FLX Titanium chemistry followed by amplicon analysis of signal processing (Roche).

2.4 Data processing

Pyrosequencing data were processed within mothur software package, version 1.23 (Schloss *et al.,* 2009). Except some minor modifications, processing was based on standardized operating procedure defined for the needs of Human Microbiome Project (Schloss *et al.,* 2011). Special attention was paid to pyrosequencing data analysis in order to eliminate the errors caused by either generation of amplicons (random errors caused by DNA polymerase, formation of chimeric sequences) or sequencing itself. Therefore, sample DNA was amplified,

sequenced, and analyzed along with the DNA of a mock community. This warranted that appropriate tools and parameters were chosen for sample sequences processing.

3. Results and discussion

The contaminated soil was predominated by bacteria from the phyla *Proteobacteria* and *Acidobacteria*. The dominant genus was *Rhodanobacter*. The retrieved sequences were mostly related to *R. spathyphili*. This population was the most abundant one to derive carbon from biphenyl. Other labeled populations included *Burkholderia*, *Dyella*, *Pandoraea*, and some other *Proteobacteria* that were not identified at the level of genus. Most of these populations were also detected to acquire carbon from labeled benzoate and naphthalene. Carbon from benzoate and naphthalene was mainly incorporated into DNA of pseudomonads, which were far less labeled by heavy carbon from biphenyl. The capability of all these taxa to derive carbon from the three substances implicate their possible use for the bioremediation of sites with mixed organic contamination (Uhlík *et al.,* 2012). Detailed information about functional gene analysis is described in this Book of Proceedings in the manuscript by Strejček M.

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REMOVAL OF NITROGEN WASTES IN AQUACULTURE SETTINGS A JOB FOR BACILLUS?

Barbara Weber, Goncalo A. Santos, Michaela Mohnl, Viviana Klose and Gerd Schatzmayr
Barbara.Weber@biomin.net
BIOMIN Research Center, 3430 - Technopark 1, Austria

ABSTRACT

Aquaculture, a fast growing industry creates large amounts of waste products, including nitrogen compounds like nitrite, nitrate and ammonia. Accumulation of these compounds is toxic for the animals and leads to low product quality and to substantial economic losses. Thus, it is crucial to take care of these waste products. One solution is the development of products for bioremediation in aquaculture.
Our strain collection was screened for bacteria that remove nitrogen compounds, that do not pose any risk on the environment and that are likely to be accepted for registration by authorities. We focused on the genus *Bacillus* and analyzed several isolates for their bioremediation properties. All tested *Bacillus* sp. removed excess amounts of nitrite. Many strains could reduce nitrate, when low amounts of oxygen were present (denitrification). Efficient nitrification was observed with several strains and required active aeration. Each *Bacillus* sp., isolate has a unique potential to remove nitrogen waste. Thus, to create an efficient product the combination of two to three strains is required. In the future, we will also focus on strains from other genera.

KEY WORDS: nitrogen, aquaculture, *Bacillus* sp.

1. INTRODUCTION

Aquaculture is the controlled farming of aquatic animals and plants, such as fish, mollusks, crustaceans or algae and involves human interference with rearing and breeding to optimize production (FAO 2006). Over the last decades the demand for seafood has increased substantially, partly because of the expanding global population and a change of consumer behavior. To meet the needs for consumption, aquaculture industry keeps growing globally and generates higher revenues year by year (Bostock et al., 2010). Similar to other industries, aquaculture creates high levels of waste products that can lead to problems inside the farms, but also in the surrounding ecosystems. At the sites of farms, large amount of organic matter, originating from feces and uneaten feed are discharged into the environment. Toxic compounds and waste products accumulate and deteriorate water quality. Poor water quality negatively impacts the health of the reared animals, which in the long run is detrimental to animal performance and will cause significant economic losses.
Nitrogen compounds, such as nitrite, nitrate and ammonium ions / ammonia are toxic when their concentrations exceed a certain level in the rearing water. Ammonium nitrogen that occurs partly in the form of ammonium ion (NH_4^+) and ammonia (NH_3^+) originates from decomposing organic waste and animal excretions in the farm. It interferes with neuronal processes and replaces K^+ ions, which prevents depolarization of the neuron that leads to too high Ca^{2+} influx and subsequently cell death. The sensitivity to ammonium nitrogen depends largely on the species. Some fish have developed strategies, e.g.: the formation of glutamine in the brain to detoxify ammonium to urea, to protect themselves from toxic ammonia levels (Randall and Tsui, 2002). Nitrite (NO_2^-) is usually present below dangerous concentrations in fresh and sea water. However, prolonged exposure to high nitrite levels, especially when oxygen is limited, leads to anoxia and slow suffocation of the animals, because nitrite changes hemoglobin into methemoglobin, a form that is not able to bind oxygen (Lewis and Morris, 1986). Nitrate (NO_3^-) is the least dangerous compound and low concentrations are not problematic. Similar to nitrite, nitrate converts hemoglobin, into a non-binder for oxygen. Permanent exposure to high nitrate levels causes weight loss and a higher occurrence of infectious diseases. To avoid these complications, excess nitrate needs to be removed to reach lower, non-toxic concentrations (Camarga et al., 2005).
Waste water management in aquaculture systems is crucial to maintain a good health status of the animals as well as to counteract the negative impacts on the environment. Bioremediation, the application of microorganisms like bacteria to remove dangerous waste products, is a promising tool for onsite treatment of waste water and contaminated sediments. For the bioremediation of nitrogenous compounds, bacteria have to perform nitrification and denitrification. Bacterial nitrification is the oxidation of ammonium / ammonia (NH_4^+, NH_3^+) to nitrate (NO_3^-) via hydroxyl amine and nitrite (NO_2^-). Denitrification describes the reduction of nitrate to nitrous oxide and finally to nitrogen gas, which returns into the atmosphere (Chávez-Crooker and Obreque-Contreras, 2010). Although a range of bacterial species are capable of nitrification and / or denitrification, not all species are applicable for bioremediation products. Development of new products involves careful characterization of the bacterial strains for bioremediation properties, for survival, proliferation and for safeness in the aquatic environment.

The purpose of this study was to screen isolates from the genus *Bacillus* for their bioremediation capacities. The individual strains showed distinct features, indicating that the development of a multi-strain product will lead to the desired bioremediation effects when applied at aquaculture sites.

2. Materials and methods

2.1 Bacterial strains and growth conditions

Bacillus sp. were routinely grown in Tryptone Soy Broth (Fluka 22092) at 30°C with aeration (120rpm). To measure nitrification and denitrification, cells were cultured in Brunner mineral medium (DSMZ number #462) that lacked $(NH_4)_2SO_4$, but was supplemented with 1g l^{-1} C (41.6 mM sodium acetate) and 1g l^{-1} yeast extract. For each type of measurement, overnight cultures were washed and diluted to an optical density of 0.1 in Brunner mineral medium that contained either 1.5 mg l^{-1} nitrite, 60 mg l^{-1} nitrate or 16 mg l^{-1} ammonium. The cultures were incubated at 30°C either with or without aeration (120rpm) for 48 hours. Culture samples to determine the concentration of the respective component were withdrawn after 0, 24 and 48 hours.

2.2 Nitrite-, nitrate and ammonium concentration measurements

In vitro test kits that rely on colorimetric measurements to determine the concentration of a said compound were obtained from MERCK (Germany): Ammonium test kit # 1.00683.0001, Nitrite test kit # 1.14776.0001 and the Nitrate test kit # 1.14942.0001. The manufacturer's instructions were followed in each case, with the difference that the proper culture volume was first centrifuged to remove the bacterial cells and then the supernatant was used for the measurements. For each compound, a linear standard curve in Brunner mineral medium was established to couple absorbance readings at certain wave lengths to specific compound concentrations.

3. Results and discussion

3.1 Nitrite removal is a common trait of all tested *Bacillus* sp.

Nitrite is created as an intermediate ion during both, nitrification and denitrification. Although it is an unstable ion that is quickly converted to more stable nitrogen compounds, an increase in concentration is detrimental for the aquatic animals. A rise in concentration occurs, when the indigenous bacteria cannot cope with the mass of waste products created in aqua culture settings or when filter systems are faulty. Thus, all *Bacillus* sp. strains were tested whether they could remove an excess of nitrite from their growth medium.
Each one of the isolate could remove nitrite from the culture medium within 48 hours, no matter whether the cultures were incubated with or without aeration. Several isolates, except isolate #1 and #7, performed better when the cultures were not aerated. Isolates #3, #5 and #8 are the most efficient nitrite removers, when taking both conditions into consideration (Figure 1A).

3.2 Only three *Bacillus* sp. isolates perform nitrate respiration

Aquatic animals handle nitrate in the water quite well. Even though this compound is not as toxic as nitrite or ammonium, permanent exposure to high nitrate levels will be detrimental to animal health. Nitrate interferes with the oxygen binding of hemoglobin and thus, impairs respiration.
The eight *Bacillus* sp. strains were tested for nitrate reduction with and without aeration. Only three out of eight isolates could significantly reduce nitrate. The other three isolates showed either very weak denitrification activity (strain #2 and #8) or were unable to do so (strains #5 - #7). Interestingly, the nitrate levels in cultures of strain 3 did also decrease under aerobic conditions. Likely, this strain can perform aerobic denitrification or better: co-respiration of nitrate and oxygen (Robertson and Kuenen, 1984). The same is true for strain #4, although here, the aerobic denitrification is less efficient than the one determined with cultures of strain #3 (Figure 1B).

3.3 Most of the *Bacillus* sp. are able to nitrify ammonium nitrogens

Ammonium ions are excretion products from aquatic animals, but also originate from unconsumed feed. Therefore, it is not easy to control the levels in aquaculture farms. The toxicity of ammonium nitrogen stems from its interference with neurological processes that are often terminal for the animals. Nitrification, the oxidation of ammonium via nitrite to nitrate was tested for all *Bacillus* sp. strains under aerobe and static (low oxygen conditions). Since nitrification requires oxygen, it was not surprising that none of the strains could oxidize ammonium when the cultures were not actively aerated. Interestingly, the ammonium nitrogen

concentration increased significantly for strain #1 and #6 within the first 24 hours, before a reduction could be observed at 48 hours. The most efficient nitrifier was strain 8, followed by isolates #5, #2, #3 and #4 (Figure 1C).

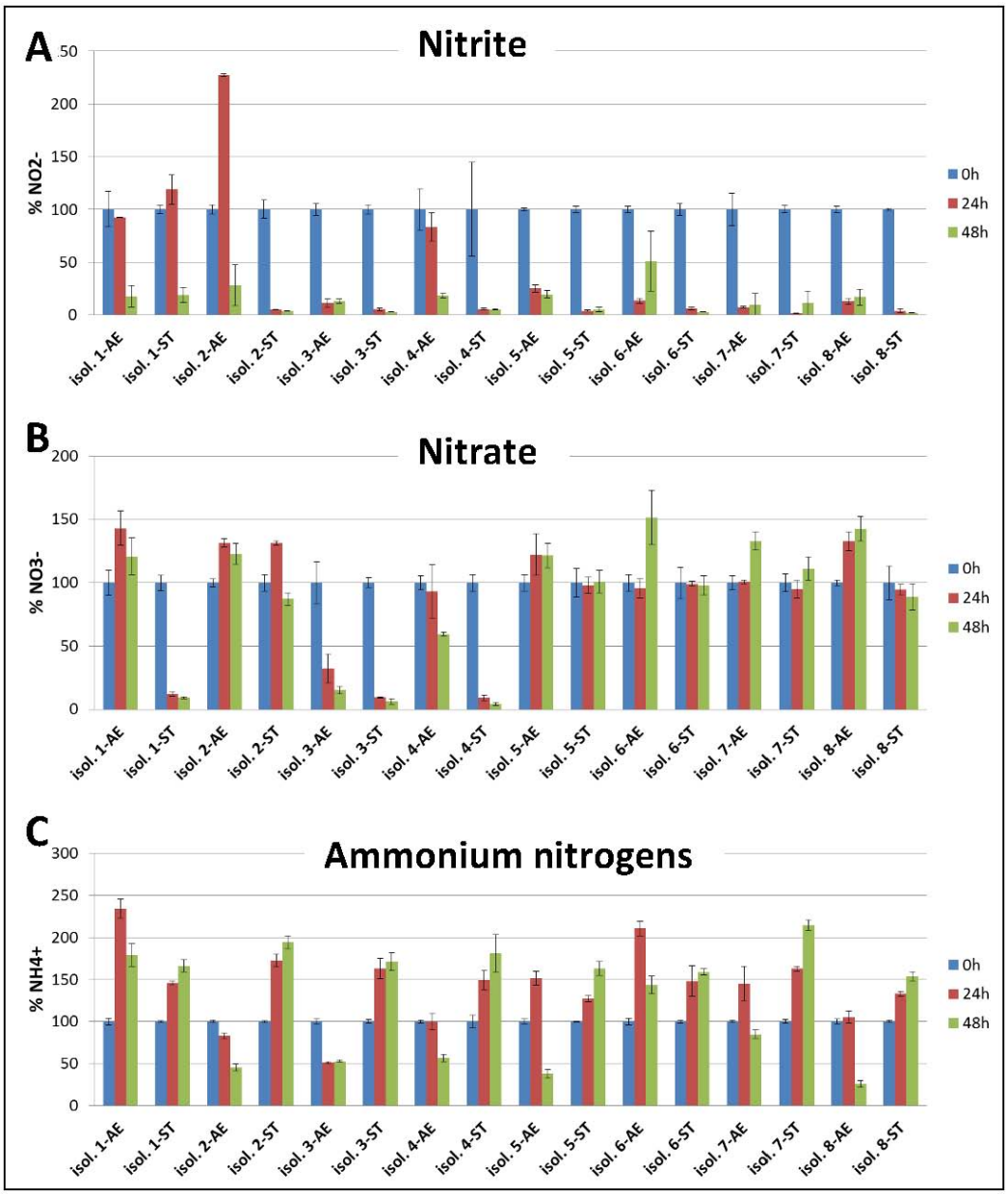

Figure 1. Nitrification and Denitrification patterns of eight *Bacillus* sp. isolates in Brunner mineral medium supplemented with either 1.5 mg l^{-1} nitrite, 60 mg l^{-1} nitrate or 16 mg l^{-1} ammonium. Nitrite (A): The cell-free culture supernatant was mixed with NO_2^- reagent-1 and incubated for 10 minutes at room temperature. The nitrite ions form a diazonium salt with sulfanilic acid that reacts to a pink azodye in the presence of N-(1-Naphthyl)-ethylendiamine dihydrochloride. The absorbance of the dye was measured at 525 nm and related to a specific nitrite concentration. Denitrification (B): The cell-free culture supernatant was mixed with NO_3^- reagent-1 and NO_3^- reagent-2, incubated for 15 minutes at room temperature. Then NO_3^- reagent-3 was added and dissolved, followed by 60 minutes incubation at room temperature. In the presence of chloride, nitrate ions build a red indophenol dye with resorcin, which is measured at a wavelength of 500 nm. Nitrification (C): The cell-free culture supernatant was mixed with NH_4^+ reagent-1 and NH_4^+ reagent-2, incubated for 15 minutes at room temperature. Ammonia, the preferred form of ammonium nitrogen in strong alkaline solutions, forms monochloramine with hypochlorite. This reacts with a substituted phenol to an indophenol dye that absorbs at a wave length of 690 nm. Thus, this test kit measures ammonium ions and disolved ammonia.
The amount of the nitrogen compound is shown as percentage left at 24 and 48 hours with the level at time point

0 set to 100%. Isol. (isolate), AE (with aeration), ST (static incubation).

Each *Bacillus* sp. has a specific ability to perform nitrification and / or dentrification *in vitro*. For example, isolate #3 has very good denitrification activities, but is not as efficient as isolate #8 regarding the oxidation of ammonium nitrogens. Strain #8 is the better nitrifier, but is deficient for denitrification.
Since a single strain, is very unlikely to harbor all desired qualities, a combination of strains that perform best for one or two compounds is more likely to ensure a stable performance. Isolates #3 and#8 complement each other in their bioremediation activities and are considered the most promising candidates for a bioremediation product.

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CREATION OF NEW MICROBAL COMPOSITIONS FOR REDUCTION OF HYDROCARBONS IMPACT ON OIL-POLLUTED SOIL IN WESTERN KAZAKHSTAN

A.A.Zhubanova, Z.A.Mansurov, I.S.Savickaya, G.Zh. Abdieva, G.K. Kaiyrmanova, A. S. Baubekova, Ye.O. Doszanov, N.Sh. Akimbekov
nur_akimbek@yahoo.com
al-Farabi Kazakh National University, Almaty, Kazakhstan

ABSTRACT

Biological approaches of purification of oil and drilling wastes are being intensively developed. Associations of active strains of hydrocarbonoxidationary microorganisms are used in these approaches. It is important for these strains to be allocated directly from soils of the polluted regions. Such approach is applied on the "Zhanazhol" field on the west of Kazakhstan. It firstly by the fact that hydrocarbon composition of crude oil and petroleum contaminations are notedly heterogeneous. Secondly, this region is characterized by acute continental climate.
Bacterial strains were allocated from oil polluted soil and identified as *P. mendocina* H3, *P. pseudoalcaligenes*, *P. stutzeri, P. alcaligenes, P. pseudoalcaligenes, P. mallei* and *Micrococcus luteus*. It has been determinated that they possess substrate specificity, which is able to oxidize different fractions of petroleum hydrocarbons.
Microorganisms in the immobilized state are effectively protected from the influens of toxic agents. They are more resistant to the extreme factors and are able to save biochemical activity in long terms. Therefore, microbal cells were attached to the surface of natural material carriers for the increase of efficiency of biologic product. The following carriers such as zeolite, straw, sawdusts, rice shell were used. These materials are able to execute the functions of ameliorator making soil mellow.
It was found that immobilization increases metabolic activity of *P. stutzeri, P. mallei, Micrococcus luteus* cultures.Among of *Pseudomonas* and *Micrococcus* genus the active oil destructors have been reveled. They can be recommended for using them as the complement for preparation-biodestructors of oil and oil products.

KEY WORDS: microbial composition, active strains, immobilized microorganisms, carriers, oil destructors, biodestructors.

1. INTRODUCTION

Biotechnological purification method of soils polluted with oil and oil products is based on the ability of microorganisms to converse oil and oil products to simple compounds, accumulate organic substance and include it in the circulation of carbon. The advantages of biopurification are ecological safety, possibility of degradation of pollutants to harmless intermediate products at fully retained structure of soil and without additional environmental pollution. Biodecomposition is mainly carried out by aerobic microflora which uses oxidation energy of constituent components of oil and oil products for its development. Use of the given technology is limited by duration of the process and dependence of natural-climatic factors (Fedorov et al., 1992).
In Russia there was developed a great amount of biopreparations used for rendering oil slimes polluted with oil and oil products of grounds, sludges and precipitates harmless, utilizing dump oil and butyric wastes such as Valentis, Olevorin, Ecodin, Devoroil, Bioset, Putidoil and ect. (Gabbasova et al., 2002). In our Republic there are also carried out investigations on using biopreparations for cleaning oil-polluted soils, microbic preparation “Munaibak” and others have been developed (Zhanburshin, 2005). Nevertheless, a question about chemical mechanism of conversion of hydrocarbons during biodegradation of oil and oil products is still unsettled (Murygina and et al., 2005).
In the last decades in many countries (Japan, the USA, Finland, Netherlands and other) for intensification of biotechnological productions, bioremediation of polluted soils and reservoirs, extractions of heavy metals from industrial waste water use the approaches of engineering enzymology. The possibility of receipt of the attached enzymes with prolonged action first reported by G.Nelson and E.Griffin in 1916, adsorbing an enzyme - invertase on coal. In the next few years in the different laboratories of the world for the receipt of heterogeneous biocatalists the different methods of immobilization were developed, but only in 1971 at the First Conference on Engineering Enzymology in Henniker (the USA) a term "immobilized enzymes" was legalized. It was pointed that this type of biocatalists compared to their native analogues such advantages as stability to the effect of hostility (higher temperature, scaling pH, presence of organic and denaturant substances), capacity for the long-term storage and regulation of enzymatic activity. The prospects of development of engineering enzymology are related to expansion of area of the use of the immobilized biocatalytic systems received by functionalazition of

different carriers by means of microbal cells. Depending on the type of the catalyzed processes system cell-carrier called biocatalists, biosorbents and biodestructors, if they contain immobilized cells of microorganisms that able to carry out catalytic processes and sorption of different substances in particular, toxic, or possess hydrocarbon oxidizing ability, for example, oxidize oil, oil products and other xenobiotics (Abisheva et al., 1999).
In this article the results of study of bioremediation processes of soils of the oilfield "Zhanazhol" in Western Kazakhstan are presented.

2. MATERIALS AND METHODS

Objects of research seven strains of bacteria allocated from soils of Tengiz deposit and water of the Caspian sea, and one active destructor of aromatic hydrocarbons P. aeruginosa, kindly provided by used PhD. Dzhusopova D.B.
Carriers for immobilization of cells these materials were chosen: Silica gel with the middle size of granules is 0,5 cm; glass marbles, diameter 1 mm, zeolite, straw and sawdust.
In model experiments as sources of carbon used: oil, diesel fuel, toluene and fuel oil.
Oil destructive microorganisms were allocated by direct sowing of sample and story culture.
Quality and quantitative composition of microorganisms allocated from oil polluted natural substrates is studied by generally accepted methodologies in microbiology.
Diagnostic tests, Bergi identification and original works were used for identification of bacteria.
Intensity of growth on agar medium was estimated by sight on five point system during the third, fifth, seventh twenty-four hours. As only source of carbon and energy oil and oil products was brought directly in a growing medium.
For the receipt of biodestructors conducted sorption immobilization of microorganisms on carriers. During immobilization determined sorption activity of sorbent on the difference of number of microbal cells in culture medium before and after immobilization after installation of sorption equilibrium.
Determination of oxidates (destructions) of oil and oil products was carried out by IR-spectrometry on IR - 200 device, Thermo electron corporation, USA in a culture medium to carrying in microorganisms and after cultivation on free and immobilized microbial cells during two days in aerobic conditions.

3. RESULTS AND DISCUSSION

Is generally known, oil and oil products are the most widespread pollutants of environment by their wide scales of booty and use. In addition, they show the most mass and dangerous anthropological factor to natural ecosystems.
Microbial destruction of oil contaminations are one of major processes assisting their elimination from water and soil ecosystems. The special biochemical organization of microbial cells provide with polyfunctional enzymatic activity due to microorganisms can use hydrocarbons of oil as a source of carbon in the process of energy and structural metabolism by destroying them. This ability of microorganisms is fixed in basis of microbiological method of purification of oil polluted objects. A leading role in realization of processes of mineralization of composite hydrocarbons to more simple and un-toxic belongs to chemical organic trophy microorganisms. Presently the search of such microorganisms-destructors can be conducted fully purposefully, if to use the microflora adapted to certain substrates. Terms for similar adaptation, for example, in oil polluted soils, activated sludge of sewage treatment plants and effluents of different chemical productions are carrying out.
By us from oil polluted soils of Tengiz deposit seven strains of bacteria identified as *P. mendocina* H3*, P. pseudoalcaligenes* H7, *P. stutzeri* H10*, P. alcaligenes* H15*, P. pseudoalcaligenes* H16*, P. mallei* 36K and *Micrococcus luteus* 37K. All strains possessed a capacity for a growth on oil and oil products as an only source of carbon.
All allocated bacteria of *Pseudomonas* genus are the active oxidants of hydrocarbons and are characterized stability to the hostility of environment.
The study of physiology and biochemistry of microorganism-destructors of hydrocarbons, large value for forming of the scientific approaches to decision of problem of purification of muddy territories has determination of optimal parameters necessary for the display of destructive activity of the allocated microorganisms. The point is that the most favorable terms are only in the certain limits of such factors, as aeration, acidity of environment, concentration of source of carbon.
As a result of conducted studies it is shown that all bacteria grow at interfusion in a liquid synthetic medium at pH 6,5-7,5, concentration of oil from 2 to 10 g/l. P. mendocina H3 at the same parameters of cultivation can grow in the static terms of cultivation at the different values of pH.
The preceding and obligatory stage in the study of processes of biodegradation of oil and oil products is determination of oil oxidizing activity of microorganisms.

For the decision of this study we conducted determination of oil oxidizing activity of *P. mendocina* H3, *P. aeruginosa* 8, *P. stutzeri* H10, *P. mallei* 36K, *Micrococcus luteus* 37K cells by IR- spectrometry. The collection strains of *P. aeruginosa* 8 was also used in our work allocated from the effluents of chemical enterprise of D.B. Dzhusupova. As an only source of carbon for addition in medium added oil, toluene or diesel fuel to the concentrations - 2 g/l.

IR-spectrum of cultivation medium received before and after the growth of microorganisms showed reduction of intensity of stripes of absorption in area of valency vibrations symmetric (υ_s) and asymmetric (υ_{as}) vibrations of C-H in the groups of -CH3 and >CH2.

IR-spectrum of samples of medium at the growth on them cultures of *P. mendocina* H3, *P. aeruginosa* 8, *P. stutzeri* H10 the considerable lowering of intensity of CH_2 and growth of valency vibrations and growth of intensity of stripe of absorption registers near-by 1745 cm^{-1}.

The relative increase of intensity of peaks in area of 3600 - 3800 cm^{-1} testifies to appearance of frequencies of valency vibrations OH the unrelated hydroxy-group. Usually the stripe of water lies within the limits of frequencies of such vibrations, what a conclusion allows to do about formation of water and carbonic acid, as a result of oxidization of acetyl-CoA in the loop of three-carboxylic acid.

The study of specificity of different microbial strains educed in relation to destruction of separate factions of petroleum hydrocarbons, that culture of *Micrococcus luteus* 37K, growing on oil and diesel fuel weaker, than other cultures, showed high destructive activity in regard to a toluene, and culture of *P. stutzeri* H10, actively consuming oil and diesel fuel, didn't show ability to utilize a toluene. These results testify that the cultures distinguished by us show high substrate specificity in relation to oxidization of different organic compounds.

The study of specificity of different microbial strains relation to destruction of separate factions of oil hydrocarbons revealed that culture of *Micrococcus luteus* 37K, growing on oil and diesel fuel weaker than other cultures and showed high destructive activity in regard to a toluene, and *P. stutzeri* H10 culture, actively consuming oil and diesel fuel, did not show ability to utilize a toluene. These results testify that the cultures allocated by us show high substrate specificity in relation to oxidization of different organic compounds.

Immobilization of microbial cells, independently of carrier types notably increased oil destructive activity of microbial cells. This phenomenon can be explained by that immobilization, firstly, gives an opportunity to work cells of microorganisms in a quorum, that is "collective", secondly, all carriers selected by us are able to sorbs petroleum hydrocarbons, facilitating access of this substrate for microorganisms. In addition, microorganisms in the immobilized state are better protected from the action of toxic agents, more steady to the extreme factors of environment and able long time to save biochemical activity. Therefore, for the increase of efficiency of biologic microbial cells registered to the surface of hard carriers from natural materials. Zeolite, straw, sawdust, rice husk were used. Such carriers are useful and that they are able to execute the functions of ameliorator and aerator-fluffier of soil, and also to be the source of mineral feed for microbial cells.

Table 1. Destruction of hydrocarbons by the immobilized cells of microorganisms

Culture	Substrate	Destraction degree, %
P. mendocina H3	Oil	89,5
	Diesel fuel	91
	Toluene	92,5
P. aeruginosa 8	Oil	87,5
	Diesel fuel	86
	Toluene	90
P. stutzeri H10	Oil	91
	Diesel fuel	91,5
	Toluene	75
P. mallei 36K	Oil	79
	Diesel fuel	87,5
	Toluene	90
Micrococcus luteus 37K	Oil	75,5
	Diesel fuel	80,5
	Toluene	92

From the table 1, most oil oxidizing activity was possessed by the cultures of *P. mendocina* H3 and *P. aeruginosa* 8, a degree of destruction of that is 87 % and 72,5 % in regard to oil, 90 % and 86 % diesel fuel, 86 % and 85 % toluene, accordingly. These cultures can be recommended for including in the complement of biologics for bioremediation of soils and reservoirs polluted oil and oil products.

In connection with high destructive activity in regard to oil and oil products cultures *P. mendocina* H3, *P. aeruginosa* 8 and *P. stutzeri* H10 were used for productive tests in the field experiments on the ground-store of LPS of "ChimPromServis-Aktobe" located in the Western Kazakhstan. According to the obtained data the free and immobilized cells of allocated microorganisms and their associations possess high oil oxidizing activity and can be recommended for including in the complement of biologic for bioremediation of oil polluted soils.
Results conducted studies are basis for development of technology of bioremediation of oil polluted territories.

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IN SITU BIOREMEDIATION OF CONTAMINATED SOIL AND GROUNDWATER

ECOTOXICITY ASSESSMENT OF DDT-CONTAMINATED SOIL AFTER NANOIRON APPLICATION

Kateřina Bobčíková[1], Yehia Sayed El-Temsah[2], Miroslav Černík[1] and Alena Ševců[1]
katerina.bobcikova@tul.cz
[1] The Institute of Novel Technologies and Applied Informatics, Advanced Technologies and Innovations, Technical University of Liberec, Czech Republic.
[2] Bioforsk, Norwegian Institute for Agricultural and Environmental Research, Soil and Environment Division, Norway

ABSTRACT

Nanosized zero-valent iron (nZVI) has been developed for remediation of several contaminants groups and seems to be particularly promising for decontamination of DDT, which poses serious risks towards human health and the environment. On the other hand, we must be aware of possible harmful effects of nZVI application to the unintended environmental effects of nZVI application.
Historically DDT-contaminated soil was treated with two types of nZVI. nZVI-B synthesized by using borohydride method and nZVI-T synthesized by gas phase reduction of iron oxides in H2. Soil was incubated in suspension with nZVI (1 g/l) shaken for 48h or filled in columns and treated for 72h. The ecotoxicity of filtrated or leachate of nZVI treatment on bacteria E. coli were determined using colony forming units counts.
DDT and its byproducts were degraded by both types of nZVI in soil suspension and soil column. However, DDT decontamination in column soil samples was significant with nZVI-B compared to control without nZVI (25% degradation of DDT). Both types of nZVI posed different level of toxic effect against E. coli. The highest toxicity to E. coli was detected in filtrates form suspension and column leachates treated with nZVI-B. The treatment with nZVI-T did not show significant negative effect in filtrates and show less negative effect in leachates. Toxicity of both nZVI in soil column was reduced after leachings. Significant negative effect of both types of nZVI was found in first and second leachates of column soil; whereas the third leachate was not toxic to bacteria.
To conclude, both nZVI types are suitable for DDT degradation in soil suspension and subsurface soil. Thus, every modification of nZVI should be carefully tested and characterized before environmental application to reduce undesired impact on indigenous microbial assemblage.

KEY WORDS: DDT, nanosized zero-valent iron, ecotoxicity

1. INTRODUCTION

An organochlorine insecticide DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane] has been used massively worldwide for three decades after World War 2 to control agricultural pests and malaria bearing mosquitos (Wong et al., 2005). For its persistence, chemical stability, bioaccumulation, biomagnification in food chains and potential toxicity to human and wildlife throughout the world, DDT was subject to an international ban in 1972 (Eggen and Majcherczyk, 2006). Although, it has been banned for three decades, DDT residues and its metabolites are still widely distributed and are frequently detected in air, water, soil, sediments, fish, birds and humans all over the world (Daly et al., 2007).
Bulk sized zero-valent iron has been used for DDT degradation in water and soil with some success (Eggen and Majcherczyk, 2006). nZVI has smaller particle size and has a very high reactivity it is well suited for injection and transport in porous media.
The widespread use of nanomaterials has led to an increasing amount of research on their toxicity and ecotoxicity. The ecotoxicological data about nZVI are still not conclusive and potential effects are unknown due to limited nZVI application in Europe. The same properties, which make nZVI undoubtedly useful for environmental remediation, such as their small size and high redox reactivity, may be potentially harmful to living organisms (Auffan et al., 2008; Crane and Scott, 2011). Although the use of nZVI represents one of the most recent innovative technologies for environmental remediations, the nanoparticles may adversely affect plant development, mortality and diversity of microbial assemblages. Such organisms are often important contributors to the remediation processes, therefore it is of high interest to increase our knowledge about their behavior after nZVI treatment.
The objectives of this research were firstly, to compare and evaluate two types of nanosized zerovalent iron, nZVI-B (synthesized using the common borohydride method) and nZVI-T (synthesized by gas phase reduction of iron oxides in H_2) with regards to efficiencies in DDT degradation in historically contaminated soil. Secondly, we aimed to examine the ecotoxicity effects of nZVI on non-target organisms.

2. MATERIALS AND METHODS

2.1 Synthesis of nanosized zero-valent iron

Nanosized zero-valent iron B (nZVI-B) was prepared using a modified borohydride method according to (He et al., 2010). Briefly, nZVI-B was prepared in carboxymethyl cellulose (CMC) solution without adding palladium (Pd). ZVI nanoparticles were formed by reducing ferrous sulphate ($FeSO_4 \cdot 7H_2O$) using a 1.9 M borohydride solution (introduced at 5 ml/min).
Nanosized zero-valent iron T (nZVI-T) was prepared from commercial product Nanofer 25P, which was obtained from NANOIRON Czech Republic. Nanofer 25P contained ZVI nanoparticles without surface modification, which were synthesized by gas phase reduction of iron oxides in H_2. Suspension of nZVI-T was prepared in a laboratory dispersing unit LD 05 (NANOIRON) enabling the processing of metal nano-powders under protective atmosphere. Dispersing unit was equipped with vacuum pump and inert nitrogen gas inlet with automatic regulation. nZVI-T was prepared in sodium salt polyacrylate acid.

2.2 DDT degradation in contaminated soil

Contaminated soil was obtained from the west coast of Norway. Historical pollution of DDT originated about 50 years ago and its concentration was 24 mg/kg.
In shaking experiment, 300 g dry weight of contaminated soil were incubated in suspension with nZVI (600 ml) and shaken on a vertical shaker (up-down shaker). Final concentration of nZVI in samples was 1 g/L and control without nZVI was included. All treatments were done in triplicate. Samples were taken after 48 h of shaking and analyzed for amount of DDT in solid phase. Slurries were separated into soil and liquid phase by centrifugation at 3622 × g and the water phase was filtered through cellulose Whatman No.5 filter (2.5 µm). The water samples (filtrates) were further used for ecotoxicity test. Three gram of dry weight soil was extracted with 10 ml hexane (≥ 95%, Sigma Aldrich) and 10 ml of acetone (≥ 99.8%, Sigma Aldrich) in glass bottles. This suspension was shaken at 175 rpm on a horizontal shaker for 1 h. After shaking, 1.5 ml of the organic phase was transferred to GC glass vial and analyzed by gas chromatography-mass spectrometry (GC–MS; Varain PC-3800).
In column experiment, glass columns (15 cm long, 8 cm diameter) were filled with 200 g of dry contaminated soil. Soil was percolated with aqueous suspension of nZVI-B or nZVI-T (1 g/L) or as a control without nZVI. All columns were prepared in triplicate. The suspension volume corresponded to 50 % of the pore volume of the soil to avoid losing the nZVI from the column before the leaching treatment started. Two days after nZVI treatment, 100 ml of distilled water was added onto the top of each columns and the leaching water was continuously collected in vials placed below the columns for 24 hours. This treatment was repeated three times. Samples of leached water from the triplicate treatments were pooled as one sample. These samples (leachates) were further used for ecotoxicity test. After 3 days of leaching, the soils were collected from columns. Soils were taken for DDT analysis as described above.

2.3 Toxicity test on *Escherichia coli*

The strain *Escherichia coli* CCM 3954 was obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). The culture was spread on agar plates with fresh Tergitol 7 medium (Modified Tergitol 7 Agar Base, Himedia, Czech Republic) and was let grown for 48 h at 37 °C. For the toxicity tests, 5 bacterial colonies were transferred from agar plates using a sterile inoculation loop into 50 ml of Soya extract (Soyabean Casein Digest Medium, Himedia, Czech Republic). The bacterial culture was carefully shaken to acquire homogeneous inoculum.
The cytotoxicity test involved mixing freshly prepared inoculum of *E. coli* (0.5 ml) with filtrate from shaking experiment or leachate from column experiment (4.5 ml) obtained from the DDT polluted soils treated with nZVI-B or nZVI-T. Control samples with bacterial inoculum and filtrate or leachate without nZVI were prepared. All treatments were performed in duplicate and the samples were incubated for 24 hours at 37 °C. Samples were diluted in decimal steps with sterile physiological solution (8.5% NaCl) after incubation. From each dilution, 1 ml of sample was transferred onto a Petri dish and covered with liquid Plate Count Agar (Biorad, Czech Republic). Duplicate samples were incubated for 48 hours at 37 °C and the colony forming units (CFU) of *E. coli* were counted.

3. Results and discussion

3.1 DDT degradation in soil treatment with nZVI-B or nZVI-T

The degradation efficiency of DDT and its metabolites (both isomers of DDE and DDD) in the soil treated with nZVI-B or nZVI-T in slurry after 48 h of shaking or in column after 3 pore volume water leachings is shown in Table 1. The initial soil concentration of total DDT was up to 24.7 mg/kg. There was no significant difference between the total DDT degradation in soil treated in suspension containing nZVI-B and nZVI-T. On the other hand, there was a significant effect on DDT-pp degradation by nZVI-B or nZVI-T compared with control after 48 h shaking. In soil column, the addition of nZVI-B and 3 pore volume water leachings significantly increased the degradation of total DDT compared with the control. While, there was no significant effect on total DDT degradation in soil treated with nZVI-T compared with control treatment.

Table 1. DDT degradation in soil [mg/kg] treated with nZVI-B or nZVI-T after 2 day in slurry and after 3 day in column. Statistical differences between treatments and control were calculated using one way ANOVA (*p<0.05, n=3).

	Slurry		Column	
	DDT	DDE, DDD	DDT	DDE, DDD
Control	17.4	7.3	16.9	7,7
nZVI-B	13.5 *	6.5 *	12.6 *	6,5 *
nZVI-T	15.8 *	7.5	16	7,7

It was evident that nZVI has a potential effect on DDT degradation in soil compared to the control which was DDT-soil without nZVI. Treatment with nZVI at concentration of 1 g/L after 48 h shaking with nZVI-B or nZVI-T resulted degradation of DDT in soil in over 22 and 9 %, respectively. Degradation of DDT in column after 3 pore volume water leaching in same concentration of nZVI were 25 and 5 % of DDT degradation, respectively. nZVI-B has more degradation effects on DDT than nZVI-T in both experiments.

3.2 Effect of filtrates and leachates with nZVI treatments on Escherichia coli

The toxicity tests with *E. coli* were performed 4 months after the nZVI-B and nZVI-T treatment of historically contaminated DDT soil. The highest nZVI ecotoxicity toward *E. coli* was detected in filtrates from samples treated with nZVI-B (p<0.01), the filtrates treated with nZVI-T did not show significant negative effect (Fig. 1). In leachates, both types of nanoparticles seemed to have negative effect on *E. coli*, though nZVI-B was again significantly more toxic (p<0.01) than nZVI-T (p<0.05). The significant negative effect was found in first and second leachates, while in the third leachate the CFU bacteria numbers were not different from control sample without nZVI.

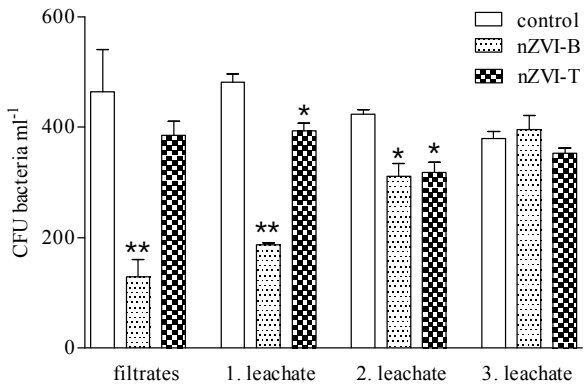

Figure 1. Effect of nZVI-B and nZVI-T in filtrates (n=3) and three leachates (n=2) on *E. coli* determined in duplicate samples. Error bars show SD. Statistical differences between treatments and control were calculated using non parametric t-test (**p<0.01, *p<0.05).

Generally, nZVI-T has less adverse effects on grow of bacteria than nZVI-B in filtrates. Adding nZVI-B had high significant negative effects on bacteria, this may be due to anoxic conditions which resulted from the reductive reactions with excess of nZVI. While nZVI-T had much less negative effects for to slow oxidation

process of nZVI-T. Oxidation and leaching of both nZVI-T and nZVI-B in soil column after 3 day exposure significantly reduced their toxicity effects.

To conclude, both types of nanosized zero-valent iron have been used as a potential reducing agent for DDT degradation former contaminated soil. The effectiveness of nZVI on DDT degradation was evaluated under aerobic conditions in shaking and column experiments. The results showed that both nZVI-B and nZVI-T are effective in degradation of DDT in soil. The negative effect of nZVI residues on bacteria were observed in filtrates and in first and second leachates. nZVI-B had more negative effects on tested organisms.

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ISOLATION AND CHARACTERIZATION OF SURFACE ACTIVE COMPOUNDS PRODUCED BY HALOPHILIC BACTERIA

Omneya Ahmed Osman, and Stefan Bertilsson
omneya.ahmed@ebc.uu.se
Uppsala University, Department of ecology and genetics, Limnology, Norbyvägen 18D, SE-75236, Uppsala, Sweden

ABSTRACT

Biosurfactants or bioemulsifiers produced by microorganisms is considered an essential step in hydrocarbon biodegradation in the marine environment. Four bacterial strains isolated from Maruit lake, Egypt displayed hemolytic activity when grown on blood agar. 16S rRNA sequencing revealed that these strains were affiliated to *Halomonas*, *Chromohalobacter and Halobacillus*. Bacterial adhesion to benzene, xylene and lubricant motor oil ranged between 60-80 % indicating the presence of surface active compounds. The 4 strains had the capacity to produce surface active compounds when grown with 1% of motor oil, rapeseed oil and olive oil as sole carbon source and in the presence of different NaCl concentrations (0.0, 0.5 and 1.0 M). Most of the strains showed enhanced growth in the presence of 0.5 and 1.0 M NaCl, emphasizing the importance of NaCl in controlling the growth of halophilic bacteria. Drop collapse test was positive in all strains. Surface tension measurements ranged between 72- 58 mN/m for motor oil, olive oil and rapeseed oil. All the isolates exhibit emusification stability of 69-100% with the tested oils. Resultant emulsion found stable without any phase separation. We concluded that these strains have a potential application as a bioemulsifiers which can grow in different types of oils.

KEY WORDS: halophilic bacteria, surface active compound, biodegradation

1. INTRODUCTION

Synthetic surfactants used to increase contaminant solubility are often toxic, representing an additional source of contamination (Bognolo, 1998). Microbially produced surface-active compounds have similar technical properties but are less toxic, biodegradable and can be produced in situ, at the contaminated site (Cha, 2000). Microorganisms capable of emulsifying and solubilizing hydrophobic contaminants in situ may have a distinct advantage over competitors in contaminated systems, therefore samples from such sites are often rich in microorganisms with desired characteristics for both in situ and ex situ bioremediation processes (Cassidy and Hudak, 2001). Biosurfactants with proven potential for remediation of contaminated sites include surfactin, produced by *Bacillus subtilis* and the rhamnolipids from *Pseudomonas aeruginosa* (Mulligan et al., 2001). Surface-active compounds produced by microorganisms are of two main types, the low-molecular-mass biosurfactants which lower surface and interfacial tensions, whereas the higher molecular mass bioemulsifiers are more effective at stabilizing oil-in-water emulsions. At present few biosurfactants or bioemulsifiers have been used on an industrial scale because of the lack of cost effective production processes (Makkar and Cameotra, 1997). Therefore, the search continues for biosurfactant-producing microorganisms that can be grown economically in an industrial scale. Biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environments that can potentially increase their stability in the harsh environments in which they are to be applied in biotechnology. There are only few reports on biosurfactant producers in hypersaline environments (Margesin and Schinner, 2001). Halophiles, which have a unique lipid composition (phytanylglycerol), may have an important role to play as surface-active agents (Yakimov et al., 1995). The objectives of this study were to isolate microorganisms with elevated potential for production of biosurfactants and/or bioemulsifiers and to characterize the surface-active properties of the metabolites produced.

2. MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

Halophilic bacteria were isolated from water samples collected from the heavily polluted Maruit lake, located SW of Alexandria in Egypt, by culturing strains on nutrient medium used in a previous study (Osman et al., 2009). The isolated strains were identified using 16S rRNA gene sequence similarity. Bacteria were grown 48 h on nutrient agar at 35ºC. DNA was extracted from single colonies using Ultra clean DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Partial 16S rRNA gene were amplified with PCR using 27F (´5-AGAGTTTGATCMTGGCTCAG -3´) and 1492R (´5- GGTTACCTTGTTACGACTT-3´) primers. PCR product

were run on agarose gel and extracted using the QIAGEN PCR purification kit. The DNA sequences were analyzed by capillary electrophoresis on an ABI3730XL DNA Analyzer (Perkin Elmer, Wellesley, MA, USA). The obtained sequences were compared to the NCBI database using the megaBLAST nucleotide alignment (http://blast.ncbi.nlm.nih.gov) and closely related strains with high similarities were considered.

2.2 Screening for biosurfactant and bioemulsifier producing isolates

The composition of the medium used for cultivation of biosurfactant-producing bacteria was (g/l): Na_2HPO_4, (2.2); KH_2PO_4, (1.4); $MgSO_4.7H_2O$, (0.6); $(NH4)_2SO_4$, (3); yeast extract, (1); NaCl, (0.05); $CaCl_2.7H_2O$, (0.02); $FeSO_4.7H_2O$, (0.01); trace elements, 1 ml. 1% (v/v) motor oil, rapeseed oil or olive oil was used as a sole carbon source. Cultures were grown aerobically in a shaking water bath (200 rpm) at 35ºC for 5 days. After incubation, the cultures were filtered, and the culture filtrates were used in the additional experiments.

2.2.1 Hemolytic assay

Fresh single colonies from the isolated cultures were retrieved and transferred to Blood agar plates. The plates were incubated for 48-72 hours at 35ºC. This clearing zone around the colonies indicates the presence of biosurfactant producing organisms.

2.2.2 Bacterial adhesion to hydrocarbons (BATH)

Cell hydrophobicity was measured by BATH assay according to the method described by Rosenberg et al., 1980. Hydrophobicity is expressed as the percentage of cell adherence to motor oil calculated as follows: 100 x (1-OD of the aqueous phase/ OD of the initial cell suspension). A few drops of INT solution was added to the BATH assay culture broth and observed under light microscope. The INT turned red if it was reduced inside the cells, indicating the viability and adherence of cells to motor oil droplets.

2.2.3 Drop-collapse test

The bacterial strains were cultured in minimal medium with 1% motor oil for 48 h. Motor oil was used in this test, 2 µl of oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 h. 5 µl of the culture supernatant was transferred to the oil-coated well regions and drop size was observed 1 min later with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the drop diameter was at least 1mm larger than that produced by deionized water (negative control).

2.2.4 Surface tension and emulsification capacity

Surface tension of the culture filtrate was measured using the Du-Nouy Tensiometer (NY, USA). For the emulsification assay, 1 ml of motor oil, rapeseed oil or olive oil was respectively added to the same amount of cell-free filtrate in a glass test tube with 15 mm diameter, and mixed for 2 min through a vortex mixer. The height of the emulsion in the tube was noted after 1 min of aging and periodically thereafter up to 24 h to monitor the stability of the formed emulsion. Each experiment was carried out in triplicate. All glass and plastic devices were acid washed (HCl, 0.1M). The emulsion volume (emulsion height ×cross-section of tube) was quantified as percent of emulsified volume (% EV), emulsion stability (% EV-24), was calculated by dividing emulsion volume at 24 h on emulsion volume at 0 time. An emulsion was defined as stable if it maintains at least 50 % of the original volume after 24 h.

2.2.5 Estimation of growth at different NaCl concentration

The isolates were grown in minimal medium with 1% of tested oil and different NaCl concentration (0.0, 0.5, and 1.0 M). Growth was monitored by measuring the turbidity at 610 nm (OD_{610}) with a Beckman DU 640 spectrophotometer (CA, USA). The turbidity of the cultures was measured after appropriate dilution of the samples with the medium when the OD610 was more than 1.

Table 1. Bacterial adhesion to motor oil, xylene and benzene and the predicted strain identification

BATH (%)				Strain identity (%)
Strain #	Motor oil	Benzene	xylene	
EG 7	15.70	23.17	53.79	*Halomonas elongata* strain Qphe 1 (100%)
EG 9	62.65	83.90	65.56	*Halobacillus* sp. 109 (99%)
EG 20	91.43	28.89	57.80	*Halomonas elongata* , strain ATCC 33173 (99%)
EG 22	86.02	38.62	69.75	*Halomonas elongata* DSM 2581 (100%)
EG 23	8.13	27.91	62.50	Bacterium N5 (2011) strain N5 (99%)
EG 24	85.89	41.20	58.66	*Chromohalobacter* sp. HS2 (100 %)
EG 25	87.97	45.31	73.24	*Halomonas elongata* (100%)

3.RESULTS AND DISCUSSION

3.1 Halophilic bacteria and biosurfactant/ bioemulsifier production

Twenty five halophilic strains were isolated from Maruit lake, Egypt and tested for hemolytic activity on blood agar. Seven strains displayed high hemolytic activity with maximum activity particularly for strains EG 7 and EG 25 (1.5-1.0 cm clear zone). Carrillo et al. (1996) found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production. However, in some cases the hemolytic assays have led to the exclusion of many potent biosurfactant producers. Hence in the present investigation the BATH assay and drop collapse test with motor oil were included to confirm biosurfactant production.

Table 2: Surface tension (ST) and emulsification stability (ES-24) of halophilic strains grown on different oils*.

Strain #	ST (mN/m)			ES-24 (%)		
	Rapeseed oil	Olive oil	Motor oil	Rapeseed oil	Olive oil	Motor oil
EG9	57.00	62.84	72.00	68.50	87.83	93.25
EG23	65.41	63.00	71.00	80.23	91.56	99.57
EG24	71.49	68.00	71.90	75.92	92.33	98.65
EG25	67.00	56.58	70.62	87.84	83.60	99.17

*Surface tension of the control medium was 73.5 mN/m.

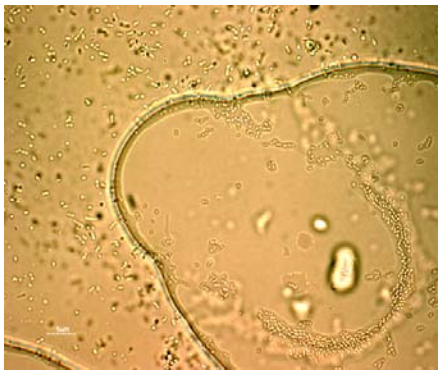

Figure .1 Light micrograph showing the cells of strain EG 25 adhering to motor oil droplet.

The BATH assay results revealed that strain EG 20, EG 22, EG 24 and EG 25 had high affinity for motor oil. Strain EG9 feature slightly lower affinity for benzene and xylene (TABLE 1). Visualization of bacterial cells adhered to motor oil using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride staining, also confirmed the BATH results (Figure. 1). All the strains showed a positive drop collapse activity with motor oil indicating the presence of surface active compounds. 16S rRNA sequencing revealed that these strains were affiliated to *Halomonas*, *Chromohalobacter and Halobacillus*.

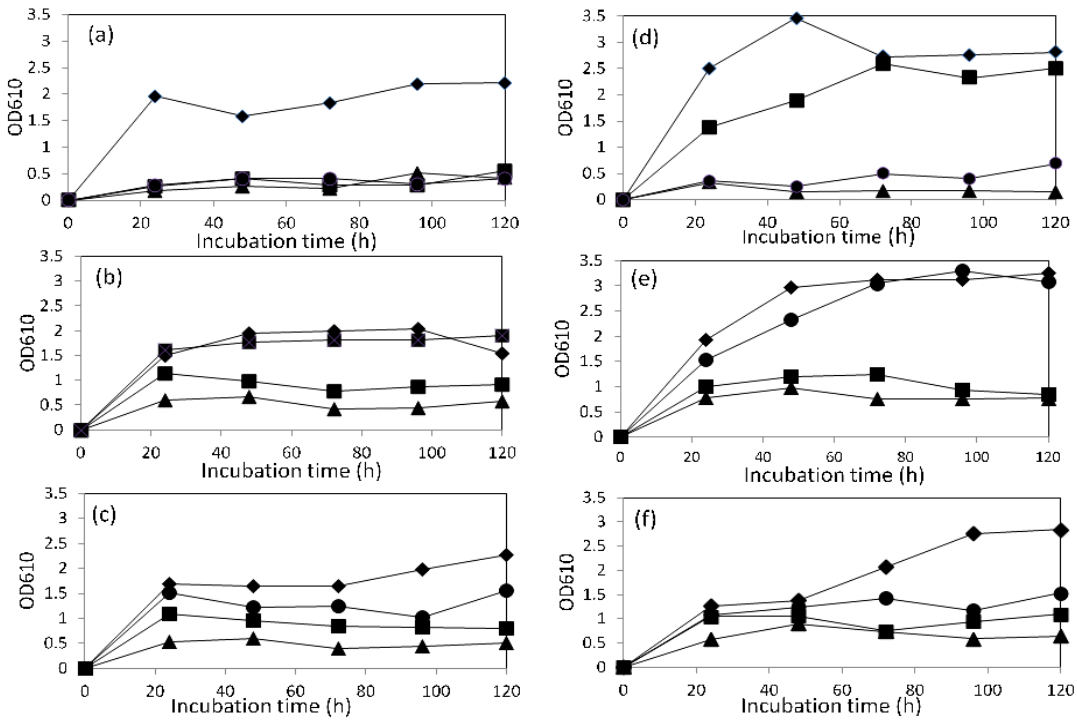

Figure. 2 Growth of halophilic strain in rapeseed oil (a-c) and olive oil (d-f). ♦, strain EG9; ●, strain EG23; ▲, strain EG24; ■, strain EG25. a and d represent 0.0 M NaCl, b and e represent 0.5 M NaCl, and c and f represent 1.0 M NaCl .

3.2 Characterization of surface active compounds and its salt dependence

Four strains EG 9, EG 23, EG 24 and EG 25 were selected for further study. To determine the role of NaCl in controlling their growth, the 4 strains were grown on motor oil, rapeseed oil and olive oil and 0.0 - 1.0 M NaCl. Strain EG9 had an enhanced growth when grown on rapeseed oil and olive oil at 0.0 M NaCl, while the other strains displayed enhanced growth at 0.5 and 1.0 M NaCl (figure. 2). All the strains formed scum when grown on motor oil and had a relatively enhanced growth in the presence of high NaCl concentration (0.5-1.0 M, data not shown). These results indicate the importance of NaCl on enhancing growth of halophilic strain and surface active compound production.

The surface tension was slightly decreased in all tested conditions as shown in TABLE. 2. The emulsion volume reached between 80-70%. Among the three oils tested, motor oil was found to be emulsified the most while cooking oil was least emulsified by all halophilic strains. These results indicate that these compounds have a potential use as a bioemulsifiers and also retained more than 50 % emulsion stability with no breakage even after one month. We concluded that these halophilic strains can utilize all tested substances as a sole carbon source, which suggested that the direct contact between the cells and immiscible substrates is the first step for the degradation. Hydrocarbon degrading bacteria increase the contact area between bacteria and water-insoluble hydrocarbons through the emulsification of hydrocarbons. High salt tolerance and flexibility might be useful for the bioremediation in hydrocarbon contaminated environments, pharmaceutics, and food industries (Banat et al., 2010). Nakano et al., 2011, reported a wax ester-like compounds isolated from *Dietzia maris* WR-3 which had no effect on the surface tension when grown on motor oil, olive oil and rapeseed oil. The isolated compounds might be related to such compounds and further investigations to identify and characterize these compounds are in progress.

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ACCELERATED DIURON MINERALISATION IN SOIL USING CYCLODEXTRIN SOLUTIONS

J. Villaverde, M. Rubio-Bellido, R. Posada-Baquero, E. Morillo
jvillaverde@irnase.csic.es
Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC)Apartado 1052, Sevilla, SPAIN

ABSTRACT

The phenylurea herbicide diuron is widely used in a broad range of herbicide formulations, and consequently, it is frequently detected as a major water contaminant in areas where its use is extensive. Diuron has the unfortunate combination of being both, slowly degraded in the environment mobile, what makes this herbicide potentially leachable to groundwaters. Diuron seem to be biodegraded in soil, but it must be kept in mind that this degradation could lead to accumulation of very toxic derived compounds, such as 3,4-dichloroaniline. In order to find procedures that might result in an enhancing of diuron bioavailablity, biodegradable molecules as cyclodextrins, which are capable to form inclusion complexes in solution with hydrophobic compounds were used. The inclusion of diuron in different selected cyclodextrins in solution was studied by phase solubility, and the best complexation parameters were obtained for Hydroxypropyl-BCD (HPBCD), with a constant complexation value of 207.70 M^{-1}. Diuron bioavailability was tested in a soil which had been managed with diuron for a number of years, and supported microbial populationthat have some specificity for diuron. Natural soil attenuation for diuron was confirmed by monitoring the $^{14}CO_2$ production where the endogenous flora together with a micronutrients solution (trace elements) was capable to mineralise diuron reaching 44%. This mineralisation was improved when a HPBCD solution was employed as bioavailability enhancer, and micronutrients as biostimulant, obtaining 63% of mineralisation and reducing the presence of 3,4-DCA toxic and persistent metabolite in the bioremediation process.

KEY WORDS: Diuron, natural attenuation, biostimulation, bioremediation, cyclodextrin, contaminated soil.

1. INTRODUCTION

Diuron is considered a Priority Hazardous Substance by the European Commission since its degradation in soil lead to 3,4-dichloroaniline, a very toxic compound, which was shown to accumulate in the medium (Widehem et al, 2002; Sorensen et al., 2008, 2009). Pollution of water and soil by diuron has become a serious problem due to the formation of 3,4-DCA subjected to leaching and bioaccumulation. Consequently, diuron has been included in the European Commission's list of priority substances for European freshwater resources (Directive 2000/60/EC) and in the U.S. Contaminant Candidate List 3 (Environmental Protection Agency, 2011). Then, there is a need to understand fully the movement, the degradation processes and the toxicological characteristics of diuron and its principal metabolite, 3,4-DCA. Based on this diuron fate and behaviour knowledge background, one objective of this work was to know the effect of the different soil colloidal particles and soils of contrasting textures on diuron adsorption-desorption. On the other hand, studies on degradation and mineralisation were carried out with the aim of monitoring the presence of the principal diuron metabolite 3,4-DCA.

The bioavailability of organic pollutants governs their ecotoxicology and degradation in contaminated soils. Environmental microbiologist defines bioavailability as: "the contaminant fraction which represents the accessibility of a chemical to a living organism for assimilation, degradation and ecotoxicology expression". Consequently, the bioavailability of contaminants varies with soil type, nature of contaminants and environmental factors. Factors such as sorbent type, the residence time, desorption rate and nature of microorganisms influence bioavailability and consequently biodegradation and toxicity of sorbed compounds. Biodegradation is by far the best and probably the only environmentally mean of organic contaminants detoxification in soils and waters. However, despite environmental conditions favouring degradation, contaminant degradation is often limited by its non availability in the aqueous phase. Numerous studies suggest that only pesticides present in aqueous phase are instantaneously available for degradation, and that the sorbed phase must first be desorbed into aqueous phase for any significant degradation. Sorption appears to be one of the crucial factors limiting the intracellular degradation of pesticides.

In this sense, in this present work studies were conducted to find procedures that might result in an increase in the bioavailablity of diuron in a contaminated soil, through solubility increasing using biodegradable molecules as cyclodextrins whose main important structural feature is their toroidal shape, with a hydrophobic interior

cavity and hydrophilic faces (Sezjtli, 1982). It is well-known that they are capable of forming inclusion complexes both in solution and in solid state with a variety of guest molecules, which are placed in their hydrophobic interior cavity (Nakai et al., 1987). The application of CDs as solubility-enhancing agents has been investigated. A number of papers in which the complexation of CDs with pesticides that present problems can be found, from both agricultural and environmental points of view. Most of pesticide-CD complexes have been prepared to improve their solubility in water (Pérez-Martínez et al., 2000; Morillo et al., 2001, Villaverde et al., 2004; Villaverde, 2007). However, no works have been reported with the aim of founding correlations between this increasing in solubility, desorption percentage from soil, and bioavailability by means of mineralising assays, confirming the complete degradation of the pesticides in the environment.
The development of an in situ and environmental friendly soil decontamination technique, which could give rise to a complete diuron mineralisation by means of increasing the bioavailability of the pollutant employing specific chemical bacterium degraders, would involve an improvement from both, economical and environmental point of view.

2. MATERIALS AND METHODS

2.1. MATERIALS

Technical grade (98%) diuron [N-(3,4-cichlorophenyl)-N,N-dimethyl-urea] was provided by PRESMAR S. L. Radiolabelled [ring-U-14C]-diuron was purchased from Institute of Isotopes Co., Ltd., Budapest, Hungary (specific activity 36 mCi mmol-1, chemical purity 99.9% and radiochemical purity, 100%). The cyclodextrins (CDs) employed were: β-CD, hydroxypropyl-β-CD (HPBCD), γ-CD, hydroxypropyl-γ-CD (HPγCD) and polymethylated-β-CD (RAMEB) (all of them from Cyclolab, Budapest, Hungary and with a chemical purity >97%).
The investigated soil was taken from the superficial horizon (0-20 cm) from southwestern Spain, named Soil I, from agricultural land. Soil was air-dried for 24 h to enable them to be sieved through 2 mm, in order to remove stones, plant material and facilitate mixing. Soil was analyzed for particle size distribution, measured by a Bouyoucos densimeter, organic matter, measured by K2Cr2O7 oxidation, pH determined in the 1:2.5 soil/water extract, and total carbonate content, measured by the manometric method. The characteristics of this soil are: pH 8.7; CaCO3 6.9%; organic matter 1.0%; sand 82.3%; silt 4.1%; clay 13.5% and with a textural classification as a loamy sand soil.

2.2. METHODS

2.2.1. Solubility studies in the aqueous phase in the presence of different CDs.

The phase solubility studies were performed according to the method reported by Higuchi and Connors (Higuchi and Connors, 1965). An excess of diuron (5 mg) was added to aqueous solutions (20 ml) containing various concentrations of CDs (0-0.05 M for β-CD and γ-CD; 0-0.1 M for HPBCD, HPGCD). The flasks were shaken at 25ºC for a week. After that the suspensions were filtered through a 0.22 µm Millipore glass fibre membrane and the concentration of diuron was determinated by HPLC. The apparent stability constants of the different diuron-CDs complexes (Kc) were determined from the straight line obtained in the phase solubility diagram, following the equation proposed by Higuchi and Connors (Higuchi, 1965): Kc = slope/S0 (1 – slope) Eq. (1), where S0 is the diuron equilibrium concentration in aqueous solution in the absence of CD, and slope is the slope of the phase solubility diagram. Another parameter that can be obtained from the data of the solubility diagram is the solubilisation efficiencies (Se), defined as the increment of diuron apparent solubility at a fixed concentration of CD of 100 mM with respect to its solubility.

2.2.2. Diuron Adsorption-Desorption Studies on the Soil Studied.

Triplicate batch adsorption experiments were performed in batch experiments by mixing 5 g of the soil with 10 mL of 0.01 M $Ca(NO_3)_2$ solution, containing various concentrations (5, 10 and 15 mg L^{-1}) of diuron, in 50 mL polypropylene centrifuge tubes. The samples were shaken for 24 h at 20 ± 1 °C. This time of reaction was chosen from preliminary kinetic studies (not shown), which showed that adsorption had reached pseudoequilibrium. After shaking (on an orbital shaker), the suspensions were centrifuged, and the concentration of diuron in the supernatant was determined by using a Shimadzu HPLC equipped with a UV detector. The difference in herbicide concentration between the initial and final equilibrium solutions was assumed to be due to sorption, and the amount of diuron retained by the adsorbent was calculated. Desorption experiments were

performed after adsorption equilibrium had been reached for the different diuron initial concentrations by removing half of the supernatant after centrifugation, replacing it by 5 mL of the extractant solution, allowing equilibration for an additional 24 h period, and after that, operating as in the adsorption experiment. This process was repeated twice. Desorption experiments were carried out using 0.01 M $Ca(NO_3)_2$ solution with and without CD (HPBCD 50 mM). The percentage of diuron desorbed (%D) with respect to that previously adsorbed during adsorption process (%D) was calculated for all the desorption experiments.

2.2.3. Mineralization assay.

Mineralisation of ^{14}C-labelled diuron assays were carried out in repirometers: modified 250 mL Erlenmeyers, into which, 10 g of soil and 50 mL of mineral salts medium (MMK) were placed. Acetone stock solution containing ^{14}C-labeled and unlabeled diuron was added to Soil I to obtain a final concentration of 50 mg kg^{-1} and a radioactivity of approximately 900 Bq per flask. The flasks were closed with Teflon-lined stoppers, and incubated at 20 ± 1 ºC. Production of $^{14}CO_2$ was measured as radioactivity appearing in the alkali trap of the biometer flasks. The trap contained 1 mL of 0.5 M NaOH. Periodically, the solution was removed from the trap and replaced with fresh alkali. The NaOH solution was mixed with 5 mL of liquid scintillation cocktail (Ready safe from PerkinElmer, Inc., USA) and the mixture kept in darkness for about 8 h for dissipation of chemiluminescence.

2.2.4. Cyclodextrin and micronutrients application on soil mineralisation and biodegradation.

A HPBCD solution, CD selected from the phase solubility experiment, with a concentration corresponding to 10 times the millimoles of the diuron previously added in the soil degradation experiments flasks (50 mg kg^{-1}), was employed to enhance the herbicide bioavailability and increase its biodegradation rate. CD solution was added after 17 days of having added diuron to the suspension experiment with the aim of observing the effect after an aging period. The micronutrient solution used was a 1 mL of a trace element solution which contained $CaSO_4$ $2H_2O$, $ZnSO_4$ $7H_2O$, $Al_2(SO_4)_3$ $16H_2O$, $NiCl_2$ $6H_2O$, $CoCl_2$ $2H_2O$, KBr, KCl, $MnCl_2$ $4H_2O$, $SnCl_2$ $2H_2O$, $FeSO_4$ $7H_2O$.

2.2.5. Model of mineralisation kinetics.

Mineralisation data (expressed as the percentage [P] of the initial activity converted to $^{14}CO_2$ as a function of time [t] were fitted to a first-order production equation of the following form (Guerin and Boyd, 1992):$P = P_{max}(1-e^{-kt})$. Nonlinear regression analysis (Sigmaplot v. 8.0) was used to estimate the parameters Pmax (overall extent of ^{14}C mineralisation) and k (first-order mineralisation rate). The parameters derived from this model accurately describe the mineralisation kinetics of nonsorbed diuron in systems under equilibrium (instantaneous sorption and/or desorption) and pseudoequilibrium (desorption rates much slower than degradation rates) conditions.

3. RESULTS AND DISCUSSION

3.1. Proposal of an in situ decontamination technique using cyclodextrin solutions as diuron bioavalability enhancers.

3.1.1 Diuron solubility studies in aqueous phase in the presence of different cyclodextrins.

The phase solubility diagrams of diuron in the presence of the different CDs used in this work are shown in Figure 1. The initial purpose of this experiment was to test whether the interaction of diuron with the different CDs produced the formation of inclusion complexes in solution. The increase in diuron hydrosolubility in the presence of CDs indicates that the herbicide forms inclusion complexes with them. A solubility limit could not be obtained in the range of CD concentrations used in any of the cases, which would be in agreement with an A_L classification, according to Higuchi and Connors (1965). Inclusion complexation parameters for all CDs tested are shown in Table 1. The straight lines of the phase solubility diagrams presented a slope less than 1, which could be ascribed to the formation of a 1:1 complex stoichiometry in solution. The apparent formation constants of the inclusion complexes formed (Kc) were calculated according to Eq. (1). A comparative study of the Kc shows as the highest value was obtained when HPBCD, and BCD were used. The lowest solubilisation efficiency (Se) and Kc values corresponded to GCD and its derivative HPGCD. This reflects the effect of the size of the CD cavity (BCD and GCD containing 7, and 8 α-(1,4)-linked glucose units, respectively, forming the toroidal ring), on the formation of the different inclusion complexes, since the bigger size of the internal cavity diameter of GCD would result in a easy get out of the diuron molecule, from the GCD cavity. Although Kc value

for BCD was high, indicating high tendency to form complex with diuron, however, Se value is very low due to the low solubility of this CD (16 mM).

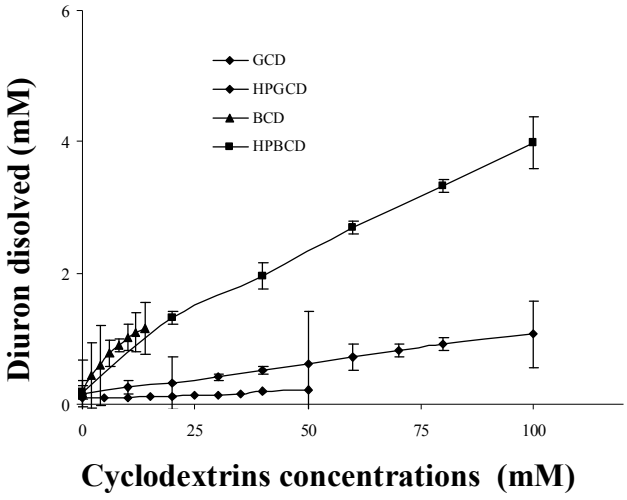

FIGURE 1: Phase solubility diagrams of diuron in the presence of the cyclodextrins studied.

TABLE 1: Diuron apparent stability constants (Kc) and solubilisation efficiency (Se) obtained from the phase solubility diagrams.

CDs	Se	Kc (M^{-1})	R^2
HPBCD	23,27 ± 1.33	207,70 ± 3.55	0,9886
BCD	6,76 ± 1.02	375,86 ± 4.21	0,9577
HPGCD	6,79 ± 0.88	58,68 ± 1.11	0,9336
GCD	2,27 ± 0.36	25,74 ± 2.85	0,9985
RAMEB	73,53 ± 2.21	669,60 ± 6.66	0,8924

3.1.2. Diuron desorption experiments using HPBCD or $Ca(NO_3)_2$ as extractant solutions.

The desorption percentages (%D) values obtained for the soil studied when the HPBCD or $Ca(NO_3)_2$ solution were employed as extractant, are shown in Table 2.

TABLE 2: Amount of diuron adsorbed and percentage of diuron desorbed from the soils and selected colloidal soil components studied.

	Diuron initial concentrations (mg L^{-1})	Diuron adsorbed (µmol kg^{-1} soil) (µmol g^{-1} colloids)	Diuron initial concentrations (mg L^{-1})	Extractant Solutions (0.01 M)	
				Ca $(NO_3)_2$	HPBCD
Soil I	5	7.71 ± 1.01	5	51,58 ± 0.66	94,64 ± 2.01
	10	21.46 ± 2.22	10	84,01 ± 1.02	89,79 ± 3.33
	15	31.59 ± 0.37	15	87,20 ± 2.55	100 ± 1.09

The results obtained indicate the high extracting power of HPBCD towards the herbicide previously adsorbed on the soils in comparison to the percentages extracted with $Ca(NO_3)_2$ solution, due to the formation of water-soluble inclusion complexes between diuron and HPBCD. In general, low-polarity pesticides have a high tendency to be adsorbed on soil surfaces, leading to their inactivation and low bioavailability and, sometimes, to soil contamination. If these pesticides are able to form inclusion complexes with CD and, as a consequence, to increase their solubility, the application of CD solutions to soils containing a high concentration of pesticide residues adsorbed can increase their removal and pass to the soil solution where they become bioavailable.

3.3.3. Diuron mineralisation experiments in the presence of HPBCD and/or micronutrients.

In Figure 2, diuron mineralisation curves obtained by natural soil attenuation, in the presence of micronutrients and/or HPBCD solution, from soil slurries are shown. A significant increase in diuron mineralisation rate was observed when micronutrients were applied (Table 3) what indicates the presence of diuron sensitive endogenous microorganisms in Soil I, managed with this herbicide during years. On the other hand, when HPBCD solution was applied plus the nutrient solution to the soil slurry a drastic increment in the extent of mineralisation was observed, reaching a value of 66% and the mineralisation rate was 1.94 d^{-1}.

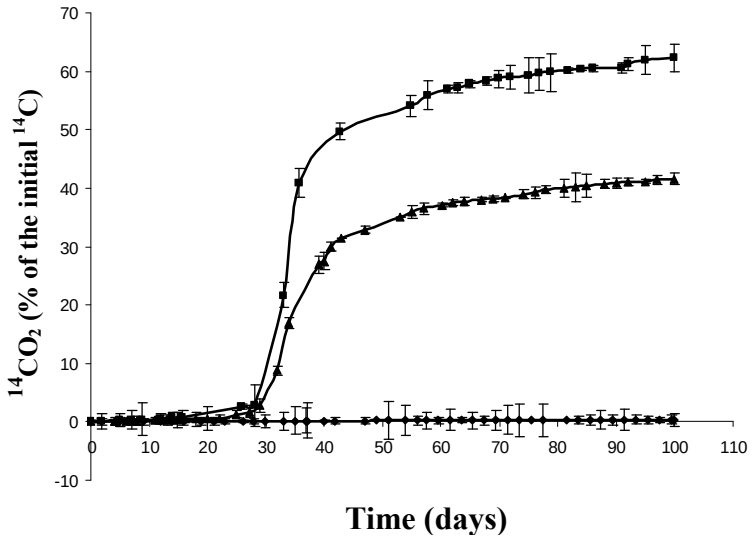

FIGURE 2: Mineralisation of 14C-labeled diuron in Soil I amended with different treatments: no treatment (♦); micronutrients (▲); HPBCD and micronutrients (■).

TABLE 3: Effect of HPCB and/or micronutrients on natural soil attenuation.

Treatment	Mineralisation rate 10^2 (d^{-1})	Extent of mineralisation (%)
Micronutrients	0.87 ± 0.01	66.02 ± 2.56
HPBCD and micronutrients	1.94 ± 0.32	44.15 ± 3.44

In conclusion and based on these results, this study shows that the indigenous microbial communities in a soil managed with diuron during years have the potential to actively and extensively degrade target herbicide. Futhermore, by enriching the microbial activity with micronutrients, results essential for natural soil attenuation. The use of a HPBCD solution at a very low concentration of only 10 times the diuron equimolar concentration in soil will act as an bioavailability enhancer, accelerating the pass of the diuron desorbing fraction from the soil particle surface to the soil solution, and improving the microorganism accessibility to the herbicide, provoking an increasing in the diuron mineralised percentage, and therefore, reducing the presence in the soil solution of its main, toxic and also persistent metabolite 3,4-DCA.

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HEAVY METAL PROTEIN TRANSPORTERS AND THEIR PHENOTYPICAL EFFECTS IN EUKARYOTIC MODELS

Jachym Suman, Martina Novakova, Pavel Kotrba, Martina Mackova and Tomas Macek
sumanj@vscht.cz
Institute of Chemical Technology Prague, Department of Biochemistry and Microbiology,
Technicka 5, 16628 Prague, Czech Republic

ABSTRACT

The work is focused on two bacterial genes *metA* and *metT* encoding for putative Cd^{2+}/Zn^{2+} ion transporters originating in gramnegative soil bacterium *Achromobacter xylosoxidans* A8. Impact of these genes on the heavy metal associated phenotype in eukaryotic cells was studied. In order to determine the effect of their expression on yeast phenotype, yeast expression plasmids were constructed bearing either *metA* or *metT* genes under the control of yeast constitutive promoter. These plasmids were transferred into *Saccharomyces cerevisiae* DTY 168 strain. It was demonstrated that the constitutive expression of *metA* gene affects Cd^{2+} resistance of heavy metal hypersensitive *S. cerevisiae* strain. Transgenic yeast cells carrying *met* genes were also shown to alter the Cd and Zn intracellular accumulation. Subcellular localization of *metA* and *metT* protein products was revealed by the emission of fluorescence signal after the expression of their 3'-fusion genes with *egfp* (Enhanced Green Fluorescent Protein) coding sequence in yeasts. Further, *metA* and *metT* expression was also studied in a plant model *Nicotiana tabacum*.

KEY WORDS: heavy metals, heavy metal protein transporters, genetically modified organisms, bioremediation

1. INTRODUCTION

Some of the most widespread inorganic pollutants of the environment are heavy metals. Some of them are essential factors for the living organisms (for example Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} and Co^{2+}). In excess, however, they act as serious contaminants with extensive impact on the environment along with unequivocally toxic ions (such as Cd^{2+}, Pb^{2+}, Hg^{2+}, etc.). In addition, unlike the majority of organic pollutants, heavy metals cannot be effectively chemically or biologically degraded (Ghosh and Singh 2005, Williams et al 2000). Decontamination of heavy metals on the level of single cell is therefore based merely on their export from the cell (in case of prokaryotes), on their binding by certain chelation agents or on their deposition in "sewage" departments such as vacuoles (Dembitsky and Rezanka 2003, Hashim et al 2011, Macek et al 2004).

The term phytoremediation stands for the use of plants for the decontamination or retention of both organic and inorganic contaminants in polluted environments. Phytoremediation provides some advantages in comparison with classical physical-chemical decontamination methods. Their application can be performed *in situ*, usually no excavation of contaminated material is needed and decontamination can be achieved without any negative impact on soil conditions. Phytoremediation also does not depend on employment of heavy machinery. Therefore, the decontamination process is more friendly to the environment and cheaper than classic methods (Cunningham and Ow 1996, Cherian and Oliveira 2005, Kumar et al 1995, Macek et al 2008).

For the purposes of phytoremediation of heavy metals, the plant species are capable of high heavy metal rhizoextraction efficiency as well as their effective translocation to aerial parts together with high biomass production (Ghosh and Singh 2005). However, naturally occurring heavy metal hyperaccumulators are usually bound to specific biotops or do not produce sufficient amounts of biomass. These two features make them useless for extensive application in phytoremediation processes. The use of genetically modified plant species with noncomplicated agrotechnological properties, deep and wide rooting as well as high biomass production seems to be promising (Banuelos et al 2002, Clemens et al 2002, Macek et al 2008).

Heavy metal detoxification in plants is represented by two main mechanisms: *(i)* toxic heavy metal ions chelation by sulphhydryl groups-rich ligands such as glutathione, phytochelatines and metallothioneins and *(ii)* intracellular compartments sequestration of free toxic ions or their complexes with organic acids and chelation agents mentioned above. On the level of a single plant root-to-shoot heavy metal ions translocation plays significant role in terms of toxic ions being transported from roots to aerial tissues and sequestrated there (Kotrba et al 2009, Williams et al 2000). Number of model studies shows that heterologous expression of certain genetic elements can lead to altered heavy metal translocation and distribution pattern in plants, thus making these genetic modifications perspective for possible preparation of transgenic plants with enhanced phytoextraction efficiency (Macek et al 2008, Williams et al 2000). In this study, two bacterial genes *metA* and *metT* are studied encoding for two putative heavy metal transporters: the P-ATPase (*metA*) and a homologue (*metT*) of PbrT protein from *Cupriavidus metallidurans* CH34 (transporting Pb^{2+} ions, TCDB no. 9.A.10.2.1), respectively. Both *metA* and *metT* genes were identified on megaplasmid pA81 from gramnegative soil bacterium *Achromobacter*

xylosoxidans A8 (Jencova et al 2008). Based on the results of another study of P-ATPase *zntA* gene from *Escherichia coli* (Lee et al 2003), we assume that the introduction of *met* genes into plants could alter their metal-associated phenotype in favor of their possible use for efficient heavy metal phytoextraction. Considering their prokaryotic origin, initially phenotypic studies of *met* genes and determination of subcellular localization of appropriate protein products were performed in the yeast model *Saccharomyces cerevisiae,* a heavy metal hypersensitive strain. Afterwards, we proceeded to expression studies in the plant model *Nicotiana tabacum*.

2. EXPERIMENTAL DESIGN AND RESULTS

2.1 Expression studies in yeasts

In order to study their expected impact on heavy metal resistance phenotype of eukaryotic cell, *metA* and *metT* genes were expressed under the control of constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase gene (Mumberg et al 1995) in cells of *S. cerevisiae* DTY168 (Cd^{2+}/Pb^{2+}-hypersensitive strain) (Szczypka et al 1994). By means of heavy metal resistance assays on solid medium containing 1µM $CdCl_2$ it was demonstrated that the expression of *metA* gene severely affects cadmium resistance of transformed yeast lines. To the contrary, the expression of *metT* had no influence on heavy metal resistance.
Changes in the ability to accumulate cadmium and zinc ions in transformed cells were studied in liquid growth medium containing 10µM $CdCl_2$ or 100µM $ZnCl_2$. From these heavy metal accumulation assays the effect of both *metA* and *metT* expression on cadmium and zinc accumulation in yeasts can be concluded.
The subcellular localization of appropriate protein products of *met* genes was determined after expression of fusion genes composed of single *met* and *egfp* (encoding for Enhanced Green Fluorescent Protein, Clonetech) coding sequences in yeast *S. cerevisiae* W303 strain. The localization of resultant fusion proteins on the subcellular level was studied using fluorescence microscopy. According to these observations, both products of fusion genes *metA-egfp* and *metT-egfp* are localized in cellular membranes in yeast cells.

2.2 Expression in tobacco

From the previous experiments it could be assumed that the product of *met* coding sequences can mediate heavy metal transport in eukaryotic cells. Therefore the vector usable for heterologous expression of *met* genes *in planta* was constructed. A pair of plasmids based on the commercial plasmid pGreen0029 enabling introduction of *metA* and *metT* genes to the tobacco genome was prepared. The *metA* and *metTs* genes were fused with *egfp* gene and inserted into the pGreen0029 backbone under the control of CaMV 35S constitutive promoter. Successful expression in plants was performed *via* transient expression (particle bombardment method) in tobacco leaves (*N. tabacum* cv. Wisconsin 38). Fluorescence microscopy was used to observe the signal of appropriate protein products of fusion genes *metA-egfp* and *metT-egfp*. These constructs enabling constitutive expression of genes of interest will be subsequently used for stable transformation of *N. tabacum* cv. Wisconsin 38 plants.

3. DISCUSSION

From the achieved findings, we conclude that *metA* and *metT* can be potentially utilized for facilitating phytoextraction or phytoaccumulation of heavy metals. Our model anticipates that these genes constitutively expressed *in planta* should enhance the uptake of heavy metals end their deposition inside the cells. Further research will be aimed to prepare stable transgenic tobacco plants possessing single studied genes. These plants will be used for subsequent testing of metalloresistance and metalloaccumulation profiles.
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