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Effect of copper upon the actions of sulphate-reducing bacteria isolated from soil contaminated by crude oil and heavy metals

Wpływ miedzi na aktywność bakterii redukujących siarczany wyizolowanych z gleby zanieczyszczonej ropą naftową i metalami ciężkimi

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Słowa kluczowe: bakterie redukujące siarczany, metale ciężkie, środowiska zanieczyszczone

Abstract
In the present study, copper tolerance by a mixed culture of sulphate-reducing bacteria (SRB) were evaluated. These sulphidogenic microbial communities were isolated from soils polluted by crude oil, oil-derived products and heavy metals (from selected areas of crude oil mines in south-eastern Poland).
Copper tolerance of SRB was tested in modified Postgate C medium with ethanol and lactate as the sole carbon source and copper chloride at concentrations ranging from 0 to 1500 mg/l. Bacterial growth and sulphate reduction were possible between 100 and 1500 mg/l of initial copper concentration. Active sulphate reduction – maximum of 53% was observed in the cultures.
Molecular analysis indicated not only the presence of SRB but also other microorganisms that are capable of living in environments contaminated by heavy metals. The high environmental sulphide concentrations produced by SRB lead to the precipitation of any biogenic mineral phases such as metal sulphides. As a result, soluble metal ion concentrations in the microenvironment of SRB are, therefore, extremely low. This process allows SRB to grow in environments containing high levels of toxic metals. Studies on SRB tolerance to heavy metals are extremely important because of the possibility of using this group of microorganisms for the bioremediation and microbial revitalisation of areas contaminated by heavy metals.

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Streszczenie
Przedmiot badań stanowiły sulfidogenne zespoły mikroorganizmów wyizolowane z gleby zanieczyszczonej ropą naftową i metalami ciężkich. Próbki gleby pochodziły z obszarów wybranych kopalni ropy naftowej w południowo-wschodniej Polsce. Celem badań było określenie wpływu stężenia metali ciężkich (miedzi) na aktywność wyselekcjonowanych zespołów mikroorganizmów.
Podczas selekcji mikroorganizmów z grupy bakterii redukujących siarczany (BRS) zastosowano zmodyfikowane podłoże Postgate'a C z etanolem i mleczanem sodu, jako jedynymi źródłami węgla. Do hodowli prowadzonych na danym źródle węgla dodawano $CuCl_2$ w ilościach odpowiadających końcowemu stężeniu jonów miedzi: 0-1500 mg/l. W hodowlach odnotowano aktywną redukcję siarczanów, na poziomie 53%.
Na podstawie wyników analizy molekularnej wyizolowanych mikroorganizmów stwierdzono obecność nie tylko typowych przedstawicieli bakterii redukujących siarczany, ale także innych mikroorganizmów, które posiadają zdolność życia i rozwoju w środowiskach zanieczyszczonych metalami ciężkimi. Wysokie stężenie jonów siarczkowych, uwalnianych do środowiska w wyniku aktywności metabolicznej BRS może stymulować powstawanie wielu faz mineralnych, takich jak siarczki metali, np. kowelinu. Proces ten prowadzi do spadku toksyczności lub wręcz stężenia metali ciężkich w danym środowisku, poprzez ich unieruchomienie w postaci nierozpuszczalnych siarczków.
Badania dotyczące tolerancji BRS na metale ciężkie stanowią niezwykle ważny aspekt ze względu na możliwość wykorzystania tej grupy mikroorganizmów w procesach bioremediacji i rewitalizacji terenów zanieczyszczonych metalami ciężkimi.

1. INTRODUCTION

One of the elements of the environment that have a crucial impact on the growth and development of all living organisms is the presence of macro- and micronutrients, especially those already in very small quantities determine the course of biological processes. These include metals participating in the biochemical reaction cells, such as iron, manganese, zinc, cobalt, nickel, and molybdenum. Many trace elements is classified as heavy metal groups that are part of the minerals that build the Earth's crust, acting integral part of the natural environment. Although heavy metals are natural wealth, they also create a complex environmental problem. The remains of many industries are responsible for irreversible changes in terrestrial and aquatic ecosystems [Kabata-Pendias and Pendias 1999]. Heavy metals, above a certain concentration level, can have toxic impact

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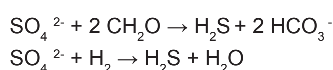
on organisms that are at different trophic levels, including microorganisms [Hattori 1992, Durska 2006].

Quantitative and qualitative changes of microorganisms are observed in soils with high content of heavy metals [Badura 1984]. Literature data indicate the varying response of microorganisms to heavy metals, including copper. Defensive response of microorganisms on the toxicity of these elements is primarily due to species differences and different enzyme systems [Nies 1999, Słaba and Długoński 2002].

Microorganisms are also capable of extracellular secretion of organic or inorganic compounds that can react with metals present in the solution, leading inter alia to changes in pH, resulting in the formation of compounds with low solubility. An example of a group of microorganisms showing adaptability to life in the presence of heavy metals are the microorganisms involved in biogeochemical sulphur cycle, sulphate-reducing bacteria (SRB) [Jong and Parry 2003].

SRBs are separated, based on the type of cellular respiration, group of anaerobic microorganisms [Fauque et al. 1991]. All organisms in the group of SRB can use oxidised sulphur compounds (sulphates, thiosulphates, etc.) as the final electron acceptor [Postgate 1984]. The final product of this process is toxic hydrogen sulphide, which is released into environment. Brief record of sulphate reduction reaction is shown as follows [Shen and Buick 2004]:



$$SO_4^{2-} + 2\,CH_2O \rightarrow H_2S + 2\,HCO_3^{-}$$
$$SO_4^{2-} + H_2 \rightarrow H_2S + H_2O$$

In the literature, there are many reports concerning the toxicity of heavy metals (including copper) on SRBs' growth. The negative effects of these elements on the SRB may appear as deactivation of enzymes, denaturation of proteins and competitive competition with other cations [Cabrera et al. 2006].

Adapting the SRBs' growth and development in the presence of certain metals concentrations bases on the immobilization toxic elements as insoluble sulphides, which locally reduces its concentration. The presence of these microorganisms in an environment leads to the accumulation of S^{2-} ions, which are the products of dissimilatory sulphate reduction. Sulfide ions in the presence of metal ions, under appropriate conditions, participate in the reaction, wherein the product is a sulfide of the metal. As a result, the process of bacterial sulphate reduction may contribute to the precipitation of various metal sulfides [Ehrlich 2001]:

$$S^{2-} + Me^{2+} \rightarrow MeS\downarrow$$

In the time scale, these minerals are accumulated and sulfide deposits are formed. Because of the fact that the chemical and microbiological processes occur simultaneously or alternately in an environment, it must be suspected that the microorganisms participate in the formation of precious deposits of metal sulfides, such as copper, iron, zinc and lead deposits [Schen and Buck 2004].

The aim of this study was to determine the effect of different concentrations of copper on the activity of SRB selected from soil contaminated by crude oil and heavy metals.

2. MATERIAL AND METHODS OF STUDY

2.1. Origin of the sulphidogenic microbial communities

Microorganisms were isolated from soil contaminated with oil and heavy metals from the area of an oil field in the Carpathians (SE Poland). A sample of soil was collected using Egner's sticks, with a depth of 40 cm, aproximately. 4 m from the mouth of the hole and tear. A soil sample was collected in triplicate and mixed. The isolated microorganisms were grown using the *microcosms* method, that is, enriched cultures with optimal conditions for the presence and development of particular microbial communities. The modified Postgate C medium [Postgate 1984], with ethanol (1.44 g/l) and sodium lactate (3.82 g/l) as the sole carbon sources, was used in the analyses. The prepared medium was distributed into 100 mL bottles (100 mL medium and 1 g soil as the source of microorganisms). These cultures were sealed and incubated in the dark for 6 weeks at 25 °C.

Anaerobic stationary culture was carried out in glass bottles with a capacity of 0.5 l, tightly sealed with a rubber stopper pierced with a needle with syringes, which served to introduce the inoculum and sampling. The inoculum-to-medium ratio was 1:10. Copper tolerance of SRB was tested in this medium with the addition of copper chloride at varied concentrations of copper: 100; 200; 500; 700; 800; 1000; 1200; 1500 mg/l. Anaerobic, stationary cultures of the studied microbial associates were incubated at 20 ºC without access of light.

2.2. Chemical determinations in stationary cultures

Sulphates were determined using the spectrophotometric method (λ = 420 nm), with $BaCl_2$ as the reagent [Moosa et al. 2002].

2.3. Molecular analysis of microorganisms

Bacterial genomic DNA was extracted from anaerobic cultures using the Genomic Mini Prep isolation kit (A&A Biotechnology) according to the manufacturer's instructions. The purity and concentration of DNA preparation were determined spectrophotometrically at 260 nm, and the DNA was used as a template for polymerase chain reaction (PCR). Universal primers for eubacterial 16S rRNA, 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5′-GGTTACCTTGTTACGACTT-3' were used to amplify a 1,540-bp segment from the 16S rRNA gene [Collins et al. 1991].

PCR was performed using the Gene Amp PCR reagent kit and AmpliTaq DNA polymerase (Invitrogen). Fifty microlitre of PCR mixture contained 5 μl of template DNA (approximately 100 ng of DNA), 5 μl of 10xPCR buffer, 25 mM of $MgCl_2$, 200 mM mix of each dNTP (Sigma), 20 pmol each of forward (27F) and reverse (1492R) primer, and 2.0 U of Taq polymerase. All PCR reactions were carried out using the Mini Cycler (MJ Research). For 16S rRNA primers, the PCR conditions were (1) 94°C for 5 min, (2) 94°C for 1 min, (3) 55°C for 1 min, (4) 72°C for 2 min. All PCR reactions were performed in 35 cycles. An initial denaturation step was at 94°C for 5 min and a final extension at 72°C for 5 min. Subsequently, amplification products were column-purified using the Wizard Purification System (Promega) and analysed

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by electrophoresis in 1% (wt vol−1) agarose gel in a 1xTBE (Tris–borate–ethylenediaminetetraacetic acid) running buffer containing ethidium bromide (0.5 μg· m^{-1}) at 4.8 V· c^{-1} for 1 h. A 100-bp DNA ladder (Invitrogen) was used as a size marker. Gels were photographed using a Syngene gel documentation system.

2.3. Sequencing and Analysis

The PCR products were sequenced directly in both directions with primers F 5'-GCGATTACTAGCGATTCC-3' and R 5'-CGTGCTTAACACATGCAAG-'', corresponding to the conserved regions of the 16S rRNA gene sequence using the ABI 3730 Genetic Analyser (Applied Biosystems) and the BigDye terminator ready reaction cycle sequencing kit, Version 3.1 (Perkin Elmer), according to the manufacturer's instructions. All gene specific primers were synthesised according to standard procedures using the Applied Biosystems synthesiser (Oligonucleotide Synthesis Laboratory, Institute Biochemistry and Biophysics PAS, Warsaw, Poland). The sequences obtained were compared to bacterial sequences available in the GeneBank database by using the Blast 2.0 program of the National Centre for Biotechnology Information (NCBI) and showed 99% homology with the corresponding sequences amongst different anaerobic species.

2.4. Analysis of the mineral composition of residues

The mineral composition of residues was determined by X-ray diffraction (XRD) using the XPERT-PRO MPD diffractometer (in Bragg-Bentano system). Residues were separated by centrifuging the culture solution; the residue was dried at 28ºC and ground in an agate mortar.

Results were analyzed using PANalytical X'pert HigtScore Plus software.

3. RESULTS AND DISCUSSION

3.1. Isolation and selection of anaerobic SRB communities

In all established *microcosms*, microbial activity marked as hydrogen sulphide release and blackening of the medium was noted.

All communities were subject to further analysis. They were passaged in 4 repetitions in order to obtain the highest activity of sulphate reduction. The incubation of each passage lasted for 14 days.

3.2. Molecular analysis of isolated microorganisms

The next stage of studies was focused on the molecular analysis of the isolated microorganisms (Table 1).

The presence of typical representative SRBs (Table 1) of the genera *Desulfotalea*, *Desulfovibrio*, *Desulfomicrobium*, *Desulfohalobium* [Castro et al. 2000] and the accompanying optionally anaerobic microorganisms: *Shewanella baltica*, *Shewanella loihica*, *Anaeromyxobacter* sp., and obligatorily aerobic – *Metallosphaera sedula* [Sanford et al. 2002; Auernik and Kelly 2010] were noted amongst the microorganisms selected from soil.

Table 1. Genetic identification of sulphidogenic microorganisms in cultures with the addition of copper at varied concentrations

Carbon source	Cu (II) concentrations [mg/l]	Microorganisms selected on Postgate C medium with different concentrations of Cu(II)	Similarity [%]
Ethanol	0	*Shewanella loihica*	98
	100	*Methanococcus maripaludis* C7	96
	200	*Myxococcus xanthus*	100
	500	*Pelobacter carbinolicus* DSM 2380	95
	700	*Solibacter usitatus*	97
	800	*Metallosphaera sedula*	99
	1000	*Gramella forsetii*	99
	1200	*Shewanella baltica*	99
	1500	*Shewanella benthica*	99
Lactate	0	*Anaeromyxobacter sp.*	99
	100	*Desulfomicrobium baculatum* DSM 4028	99
	200	*Desulfohalobium retbaense* DSM 5692	100
	500	*Desulfovibrio vulgaris*	99
	700	*Desulfotalea psychrophila*	98
	800	*Desulfovibrio salexigens*	99
	1000	*Desulfovibrio desulfuricans*	100
	1200	*Desulfovibrio vulgaris* DP4	100
	1500	*Sulfurihydrogenibium sp.*	100

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In addition to the presence of microorganisms associated with the biogeochemical sulphur cycle, we managed to find part of microorganisms such as *Pelobacter carbinolicus* DSM 2380 [Lovley, 1995], *S. baltica* and *Anaeromyxobacter* sp. [Petrie et al. 2003], whose metabolism is linked with the process of dissimilatory reduction of metals, especially iron (III) and manganese (IV).

In cultures maintained with the addition of ethanol as a carbon source, we were also able to identify the methanogenic archaea, including *Methanococcus maripaludis* C7, which requires NaCl for its growth, with optimal concentrations of 0.5–4% (wt vol^{-1}).

Some of the identified strains of microorganisms, including *M. sedula* exhibit exceptional resistance to high concentrations of toxic metals [Huber et al. 1989].

Based on the results of molecular analysis, it was found that the tested microorganisms are in most obligate or facultative anaerobes, whose metabolism is associated mostly with the use of elemental sulphur and/or its oxidised form [Castro et al. 2000]. Conditions created in the cultures also enabled the growth of microorganisms associated with the biogeochemical circulation of iron and manganese.

3.3. Activity of selected SRB assemblages on media containing sulphate ions

In all established cultures, sulphate reduction was noted (Fig. 1).

In the cultures of selected sulphidogenic microbial communities with ethanol as the sole carbon source, the maximum sulphate reduction was noted in the culture with the initial copper concentration of 800 mg/l. It reached 1668 mg/l of sulphates, which corresponds to 53% of the reduced SO_4^{2-}. The lowest activity (37% of reduced SO_4^{2-}) was noted in microbial communities in cultures with copper ion concentration of 1000 mg/l.

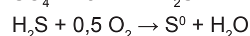
In cultures maintained on lactate as the sole carbon source, the highest reduction of sulphate (43%) was found in the culture in which the initial copper concentration was 1200 mg/l. The lowest level of sulphate reduction was observed in the culture with an initial copper ion concentration of 100 mg/l and it corresponds to 27% of reduced SO_4^{2-}.

3.4. Diffractometric analysis of the residues

After incubation, the residues were subject to diffractometric analysis in order to determine their mineral composition (Fig. 2 a, b). In post-culture residues, piypite was noted. In cultures with lactate addition, the presence of posnjakite was confirmed. Both of these phases are formed by abiotic way as a result of changes in the concentration of appropriate sulphate and copper ions in the culture medium.

In all cultures with ethanol as carbon source, the presence of elemental sulphur was confirmed. Undoubtedly, this mineral phase is the result of bacterial activity. It is believed that the sulphur of biological origin are most frequently stage process according to the reaction:

$$SO_4^{2-} + 8\,H^+ \rightarrow H_2S + 2\,H_2O + 2\,OH^-$$



$$H_2S + 0{,}5\,O_2 \rightarrow S^0 + H_2O$$

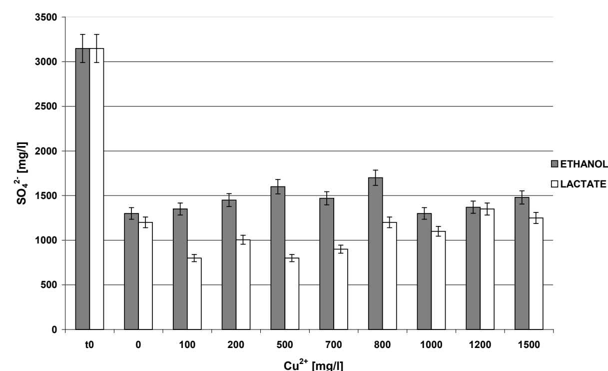


Fig. 1. Degree of sulphate reduction in cultures of selected microorganisms communities on Postgate C medium with ethanol and lactate. t_0, time of culture establishment; 0–1500, the concentration of copper ions (mg/l)

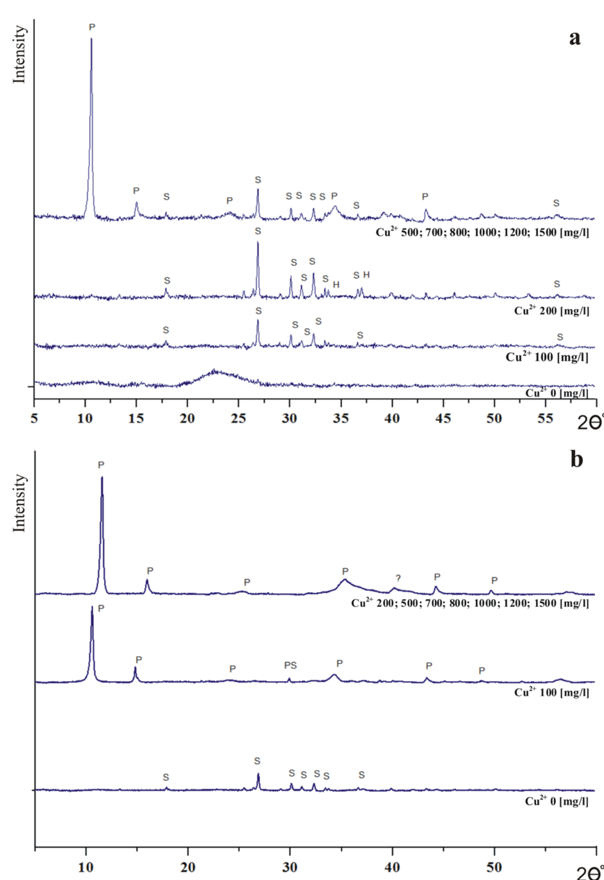


Fig. 2. Diffractometric analysis of residues from cultures on a Postage C medium with (a) ethanol and (b) lactate as the sole carbon source. S, sulphur; P, piypite; H, halite; PS, posnjakite

In the first stage of this reaction, SRB play the main role. These microorganisms reduce SO_4^{2-} to sulphide ions in the process of dissimilatory sulphate reduction. Sulphide ions can be oxidised to elemental sulphur. The oxidation of sulphide may occur under anaerobic conditions involving green and purple sulphur photosynthetic bacteria [Wolicka and Borkowski 2007].

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The results of the XRD analysis did not reveal the presence of copper sulphide, despite the high activity of sulphidogenic microbial communities.

The basic problem in the determination of sulphide mineral phases in residues could be their amorphous nature. Compounds precipitated as amorphous are often aggregated with the exopolymeric compounds, which are produced by SRBs. Exopolymeric compounds create a kind of shell around the bacterial cells, allowing their aggregation [Wolicka 2010]. Then, identifying the presence of such complexes by XRD analysis becomes impossible.

4. CONCLUSIONS

1. Genetic analysis of isolated microorganisms confirmed the presence of representatives of both SRB and accompanying aerobic microflora in studied cultures.
2. The addition of copper to the cultures had no significant effect on the microbial sulphate reduction, but in the cultures with lactate as a carbon source, this process was less efficient.
3. The decrease in the concentration of copper ions during the time of experiment was noted. There is insufficient evidence to determine whether the sole cause of this phenomenon was the precipitation of metal in the form of insoluble sulphides.
4. The mineral composition analysis of post-culture residues showed the presence of elemental sulphur, which presumably is the result of SRB activity. The presence of copper sulphides were not detected. The results of XRD analysis because of the limitations of the method (no possibility of determining the amorphous phases) is not sufficient evidence to conclude that the crystallisation of copper sulphides occurred during the cultivation of SRB.

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