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The Role of Microorganisms in Dispersion of Thallium Compounds in the Environment

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Abstract

Thallium is a highly toxic element and very rarely studied in the context of environmental hazards connected with zinc and non-ferrous metal industry. Microorganisms naturally existing in post-flotation and smelt wastes can participate in thallium release from waste deposits and can contribute to its dispersion in the environment. Twenty-one isolates were obtained from wastes of a non-ferrous smelter in Southern Poland characterised by high heavy metal contamination. Ten isolates showed high activity in thallium leaching from wastes (post-flotation and smelt wastes) as well as from pure thallous sulphide. Additionally, cadmium and lead were bioleached from wastes. The isolated bacteria indicated thallium resistance at a concentration up to 100 mg/l and some of them were able to survive in good condition at a concentration of up to 4 g/l. The same bacteria were isolated from rivers and wastewater in this region. A preliminary characterisation of isolates was performed. It was shown that some petroleum products *i.e.* asphalt-base crude occasionally used for waste immobilisation at the edge of pond or flotation surfactants partially stopped the activity of sulphide oxidising bacteria.

K e y w o r d s: thallium, metal bioleaching, environmental pollution

Introduction

The role of microbes in the dispersion of inorganic metal salts (especially sulphides) has been known for years. Oxidation of these compounds is the way of gaining energy, needed in many biochemical processes such as CO_2 fixation *etc.* Metal ions are unused products of reactions, which can penetrate into the environment. Since the 70's it has been known, that thiobacilli (today the genus *Thiobacillus* is divided into a few genera, belonging to other subclasses of *Proteobacteria* (Kelly and Wood, 2000)) can divide thallous sulphide, to obtain sulphide ions – the energy source (Galizzi and Ferrari, 1976). Free thallous ions can penetrate into the environment, and take part in many biogeochemical cycles. Microbes can methylate thallium ions, producing dimethylthallium – Me_2Tl^+. The estimation of this compound concentration in environmental samples was described by Schedlbauer and Heumann (2000). The mechanism of this process is still unknown.

Thallium is very rarely studied in the context of environmental hazards connected with zinc and non-ferrous metal industry. Research carried out in Southern Poland enabled the identification of several regions, which are seriously threatened by thallium as well as indication of direct sources of pollution (Dmowski *et al.*, 2002) Polluted regions include mainly the surroundings of zinc smelter and flotation waste ponds. Flotation wastes contain mainly thallous sulphide (Tl_2S).

Experimental

Materials and Methods

Collecting and storing of samples. Samples of wastes of a non-ferrous smelter in Southern Poland were collected in October 2001 and May 2002. The material was stored in disposable, sterile plastic tubes. Microbiological analysis was carried out within 24 hours. The remaining material was stored at –20°C.

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Bacterial strains. Ten strains were isolated from wastes. Non-modified strains of *Halothiobacillus neapolitanus* and *Paracoccus versutus* received from The Department of Bacterial Genetics, Warsaw University were used as a reference.

Isolation of strains. 5 g of fresh wastes were added to 50 ml of sterile Beijerinck's medium. The mixture was incubated on rotary shaker, at room temperature. After 24 hours, the culture was transferred to solid medium and cultivated at 28°C for 4 days. Single colonies were inoculated on fresh solid medium every 5 days.

Culture media. a. Beijerinck's medium with 10 g of $Na_2S_2O_3 \times 5\ H_2O$, as the only energy source and 2 ml of Tuovinen's salts (Tuovinen and Kelly, 1973). Solid medium contained 25 ml of 3 % phenol red's solution, pH = 7.5. b. Modified LB medium (Skłodowska *et al*., 1996) with 20 g of NaCl, pH = 7.5. c. Modified LB medium with thiosulphate (Skłodowska *et al*., 1996) with addition of 20 g $Na_2S_2O_3 \times 5\ H_2O$, pH = 7.5. d. Davis medium with glucose, as carbon source. e. Modified Beijerinck's medium with 10g of Tl_2S instead of thiosulphate. f. Modified Beijerinck's medium: 60 g of wastes, dried to dry weight at 105°C instead of thiosulphate, and without Tuovinen's salts.

Estimation of colony forming units (CFU) number in wastes. 5 g of fresh wastes were added to 50 ml of sterile NaCl solution (0.9%) and stored on laboratory rotary shaker for ½ hour. Solutions of this culture were then inoculated on solid media: Beijerinck, modified LB with NaCl and Davis medium. Plates were stored at 28°C for 7 days. After incubation the colonies on every plate were counted.

Characterisation of isolates. Isolated strains were inoculated on solid media to test their ability to grow in different conditions. Beijerinck's for autotrophs and modified LB, modified LB with $Na_2S_2O_3$ and Davis media for heterotrophs and mixotrophs were used. All isolates were stained using Gram method.

The resistance of strains to thallous ions was tested. Bacterial strains were inoculated on solid modified LB medium with $Na_2S_2O_3$, containing Tl^+ (as $TlNO_3$) in concentrations: 25, 50, 75, 100 ppm, or solid Beijerinck's medium with analogous ratios of thallium. *Paracoccus versutus* and *Halothiobacillus neapolitanus* were inoculated on the same media.

Preparation of a mixture of strains. Fresh cultures of all isolated strains on Beijerinck's medium were prepared. After 5 days of incubation at room temperature on a laboratory shaker 1 ml of each culture was added to sterile medium without thiosulphate. This mixture was used as an inoculum in further experiments.

Thallium bioleaching from thallous sulphide. 100 ml of Beijerinck's medium with 10 g of Tl_2S (Sigma-Aldrich) was inoculated with mixture of bacterial strains. Experiment was conducted in Erlenmayers' flasks (300 ml) on a laboratory shaker at room temperature. Non-inoculated medium, cultivated under the same conditions as the cultures was the control for this experiment. Two series of culture (designated 1st culture and 2nd culture) were prepared. Every day pH, Tl ions concentration and the number of bacterial cell (indirectly, by estimation of a number of CFU in culture liquid) were measured.

Thallium bioleaching from wastes. Beijerinck's medium (200 ml) with 60 g of wastes (previously dried at 105°C to dry weight) was inoculated with mixture of strains. Incubation was carried out in Erlenmayer flasks (500 ml), on a shaker at room temperature. Non-inoculated medium served as a control for this experiment. Two series of culture (designated 1st culture and 2nd culture) were prepared. Wastes for experiments were collected in different places of tailing ponds. In 1st culture crude wastes were used and for 2nd culture wastes from the edge of pond, which were treated by asphalt-base crude for immobilisation. The concentration of thallium, cadmium and lead in waste was estimated. Additionally, the pH and the number of bacterial cell were assessed.

The number of bacterial cells was estimated after staining with 4,6-diamidino-2-phenilinindole (DAPI) and counting on filters under epifluorescence microscope.

Chemical analysis. The concentration of metals in acidified supernatant and mineralised wastes was measured using Flame Atomic Absorption Spectrometer SOLAAR M6.

Before the analysis wastes were dried at 105°C and mineralised in Millestone Ethos Plus Laboratory Microwave System with 65% HNO_3 and 36% H_2O_2 (9:1).

The analysis of thallium in soils was carried out with diisopropylether extraction, according to Asami *et al.* (1996). Mineralised wastes were filtered and distilled water was added, giving the total volume of 100 ml in the flask 50 ml were moved to Erlenmayer flask, 6 ml of HBr (36%) and 1 ml of 0.1% solution of $CeSO_4 \times 4\ H_2O$ was added. After 15 minutes the total volume was placed into a separator (200 ml), 5 ml of ether was added and then shaken for 5 minutes. Organic phase was collected and evaporated. The sample was then dissolved in 5 ml of 3% HNO_3.

Results and Discussion

Concentration of heavy metals and pH of wastes of a non-ferrous smelter were measured and are presented in Table I.

Table I
Heavy metals concentration in wastes and pH of wastes

pH	Concentration of heavy metals (mg/kg d.w.)			
	Tl	Cd	Pb	Zn
7.00–7.10	40–50	120–130	18000–21000	4500–5000

The bacterial cell number isolated from wastes able to grow on different media was estimated. For all media (Beijerink's, modified LB and Davis) similar results were observed: 10^4–10^5 cells per g of wet weight of wastes. Ten bacterial strains were isolated from wastes on Beijerinck's medium. All isolates were

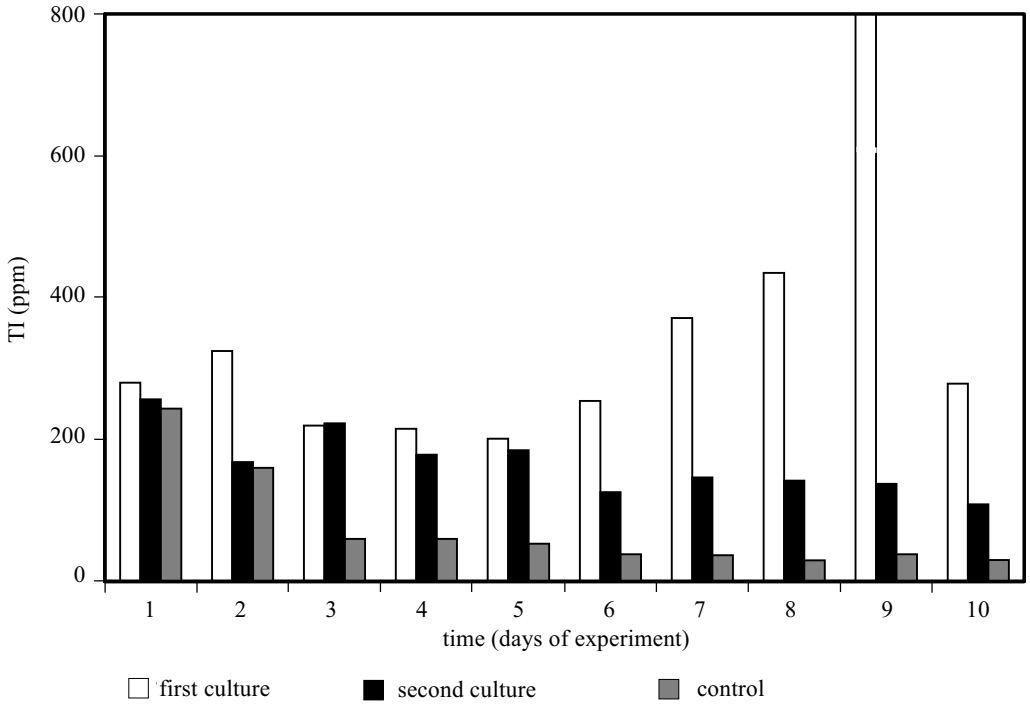

Fig. 1. Bioleaching of thallium from Tl_2S – concentration of Tl ions in leaching solution

gram-negative or gram-variable (young cells were negative, and after 10 days of incubation-positive). Eight of the strains were rods, and two of them were too small to identify their morphology under the light microscope. The isolated bacteria showed thallium resistance at a concentration up to 100 mg/l and some of them were able to survive in good condition at a concentration of up to 4 g/l.

The ability of isolates to leach thallium from pure Tl_2S was tested in the first experiment. Sulphide ion was the only energy source for microorganisms. The concentration of Tl^+ in the supernatant indicated the rate of the leaching process (Fig. 1). In the 2nd culture the highest concentration was observed at the beginning of the experiment – 250 ppm. From the 5th day it dropped and reached 110 ppm on the last day of cultivation. Throughout the experiment the highest concentration in the 1st culture was noticed. For the first five days the concentration was stable (210–320 ppm), then it started to increase, reaching 1624 ppm on the 9th day in the 1st culture. This phenomenon may be explained by the irregular structure of the crystal, which was visible, or some impurities in the crystal net. In both cultures brown sediment, localised on flask walls, under the liquid line was observed.

Table II
Characteristics of isolates

Strain	Gram	Morphology	Beijerinck	Growth on medium:		
				Modified LB	Davis	Modified LB with $Na_2S_2O_3$
1.	negative	rod	+	–	–	+
2.	negative	rod	+	–	–	–
3.	negative	rod	+	–	–	–
4.	variable	rod	+	+	+	+
5.	variable	rod	+	–	–	+
6.	variable	rod	+	–	–	+
7.	variable	n/e	+	–	–	–
8.	negative	rod	+	–	+	+
9.	negative	rod	+	–	–	+
10.	negative	n/e	+	+	+	+

n/e – not estimated

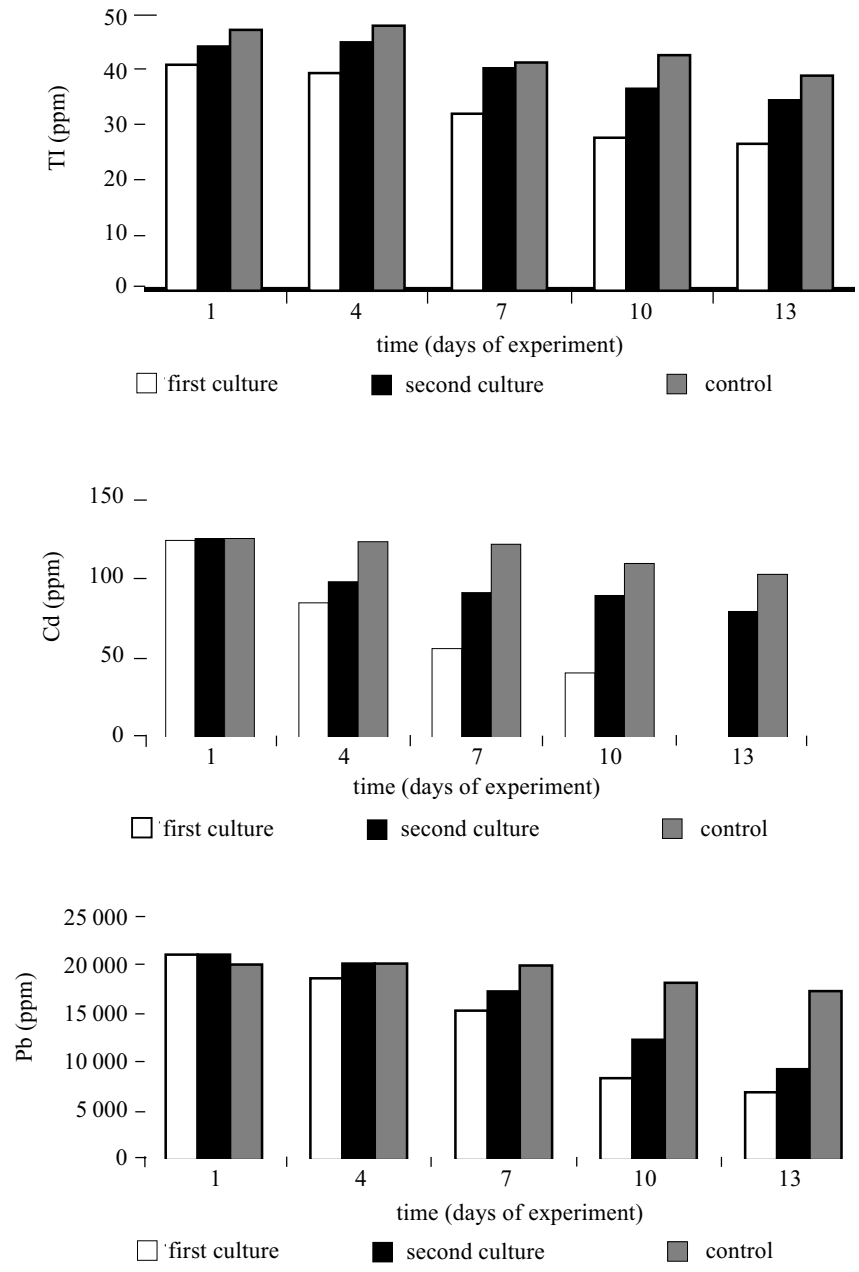

Fig. 2. Heavy metal concentration in waste during bioleaching experiment (non bioleached metals): A. Thallium concentration in wastes; B. Cadmium concentration in wastes; C. Lead concentration in wastes

Bacterial cell number systematically increased in both cultures throughout the experiment. For both series of cultures similar results were obtained – from about 1×10^4/ml to about 7×10^5/ml.

In both cultures pH decrease of about 1 unit was observed. In the first culture the decrease from 7.40 to 6.50 and in the 2nd one from 7.00 to 6.20 was observed while in the control from 7.29 to 7.00. In both cultures pH was stable for the first 5 days, then it decreased.

To check the ability of the isolates to leach heavy metals from wastes, an experiment using modified Beijerinck's medium supplemented with waste was carried out. Thallium concentration in wastes (mg/kg d.w.) was measured and its decrease showed the efficiency of the process. Additional parameters of process were: bacterial cell number, pH changes. The rates of cadmium and lead bioleaching were also measured. Apart from the fouling of culture medium, no other differences between cultures and control images were observed during the experiment.

In the first culture 40% of thallium was leached. This was more than in the second culture, where the result was only 25%. In the control less than 10% of thallium was leached. At the beginning of the experiment the concentration of thallium was different for both cultures and the control (Fig. 2A). The wastes are

Table III
Resistance of freshly isolated bacterial strains to different concentration of thallium ions

Strain	Growth on medium with thallium in concentration:			
	25 ppm	50 ppm	75 ppm	100 ppm
1.	+	+	+	+
2.	+	+	+	+
3.	+	+	+	+
4.	+	+	+	+
5.	+	+	+	–
6.	+	+	+	+
7.	+	+	+	+
8.	+	+	+	+
9.	+	+	+	–
10.	+	+	+	+
H. neapolitanus	+	+	–	–
P. versutus	+	–	–	–

non-homogeny blend with unspecified ratio of different compounds. Cadmium was bioleached in 100% in the 1st culture and more than in 70% in the 2nd one. In the control flask 17% decrease of the concentration was noticed (Fig. 2B). For lead 70% decrease of the concentration for the 1st culture and more than 50% for the 2nd one was obtained at the end of the experiment, while in the control about 25%. For lead, differences in the concentration at the beginning of the experiment, were smaller than for thallium and cadmium (Fig. 2C).

Bacterial cell number during the experiment was similar for both cultures: from 3.20×10^6 to 8.05×10^7 per ml in the 1st one and $5.40 \times 10^6 - 5.00 \times 10^7$ for the 2nd one. Lag-phase was longer in the 2nd culture, than in the 1st one.

Figure 3 presents a mixture of isolates cultivated in mineral medium containing wastes. The bacterial cells stained with DAPI attached to particle of wastes are visible.

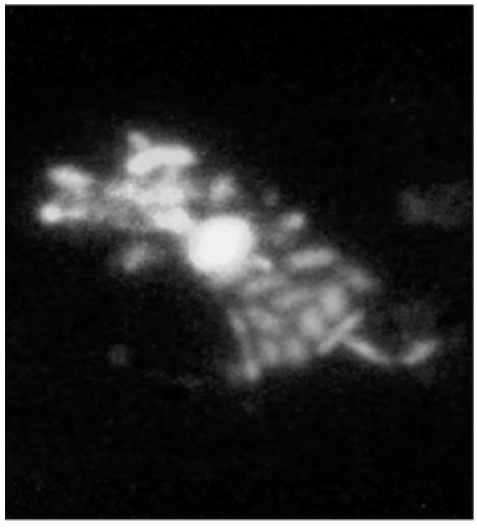

Fig. 3. Bacterial cells stained with DAPI adhered to particle of wastes during bioleaching process (magn. 800x)

Throughout the experiment the pH was stable due to buffer capacity of wastes, which includes a significant amount of carbonate. The maximal ratio of changes was 0.8 unit in the 1st culture, 1 unit in the 2nd one and only 0.1 unit in the control.

Isolated strains are able to leach thallium from both pure salts and ores. The isolates are very interesting due to their ability to grow in high concentrations of thallium and other heavy metals, which are thought to be highly toxic. It was shown that activity of microorganisms might be one of the reasons of thallium contamination near ores treatment plants and near flotation wastes deposits. It was shown that some petroleum products *i.e.* asphalt-base crude occasionally used for waste immobilisation at the edge of the pond partially stopped the activity of sulphide oxidising bacteria (2nd culture).

The obtained results are significant for environmental risk assessment methods and clearly indicate that potential microbial activity is an important factor in hazardous pollution dissemination, especially in a groundwater. Additionally, a new kind of environmental contamination was shown. Contamination by thallium compounds in the vicinity of the centre of zinc and non-ferrous metal industry was not observed and described till 2002 (Dmowski *et al*., 2002) as well as it is not included in environmental regulations. This research should be continued to understand the problem of the taxonomy and physiology of these isolates, and to find the possibilities of microorganisms activity inhibition in the deposits.

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