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# Characterization of Microbial Communities in Acidified, Sulfur Containing Soils

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### Abstract

Over a period of three years, microbial communities in acidified soil with high sulfur content were analyzed. In soil water extracts ureolytic, proteolytic, oxidoreductive, and lipolytic activity were detected. The presented results indicate that the enzymatic activity of soil microbial communities varied considerably over time. Isolated 26 (80%) bacterial strains belonged to genus *Bacillus* sp. and were identified by cultivation and 16S rRNA methods. The commercially available procedures for bacterial DNA isolation from acidified soil failed, therefore a new, specific DNA isolation method was established. Ureolytic activity, detected in soil extracts as well as in isolated *Bacillus* sp. strains may be considered as a tool for the bioremediation of acidified soils with high sulfate content.

K e y   w o r d s: acidified soil microflora, DNA isolation specific method, soil bacteria, soil biochemical activity

## Introduction

Soil is a highly heterogeneous microbiome habitat due to the varied physical and chemical conditions of microniches (Ettema and Wardle, 2002; Xu *et al.*, 2014). The enzymatic activity found in the soil is mainly of microbial origin, being derived from intracellular, cell-associated or free enzymes. The biochemical activity of soil is a key factor in its natural remediation, and microbial activity acts as a biomarker of soil condition (Kumar *et al.*, 2013; Wang *et al.*, 2014).

Sulfur mines have a destructive influence on the surrounding biocenoses (González *et al.*, 2011). The contamination of soil with sulfur results in a change of groundwater chemistry and plant growth inhibition (Li *et al.*, 2006). Technogenic soils derived from sulfur mines present a challenge in terms of rehabilitation processes. Ureolytic activity is essential for the nitrification process, determining the amount of nitrogen compounds available to plants (Cheng *et al.*, 2013). In acidic soils, nitrification may be restricted to microniches where active ureolytic bacteria are present at higher pH levels. pH is one of the most essential factors for the growth of soil microorganisms as well as the availability of carbon and nitrogen sources and metal solubility. In another studies of acidified soils, fungi prevail over bacteria and artificial fertilization may increase the urease activity of soils (Rousk *et al.*, 2009; Krzywy-Gawrońska, 2012). Kang and co-authors showed the possibility of using ureolytic bacteria for lead removal by biomineralization (Kang *et al.*, 2015). Ureolytic *Bacillus* strains may be applied for soil solidification by enhancing the precipitation of $CaCO_3$ (Shirakawa *et al.*, 2011).

In the present study we investigated soil samples collected from the vicinity of the former Grzybów sulfur mine located in south-western Poland. The Grzybów sulfur mine extracted sulfur by the hot water method from ore 180 m below sea level in the years 1966–1996 (Zieliński and Wałek, 2012). The high content of sulfur (4%) resulted in lower hygroscopic and capillary capacity of the soil (Zieliński and Wałek, 2012). In 1966 the Grzybów sulfur mine was closed and the surrounding area was subjected to rehabilitation, but those efforts failed and plant vegetation is still absent. Up to date microbial communities of sulfate, acidified soil (pH 4.0) were not investigated.

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Aims of presented studies were to characterize soil microbial communities by their biochemical activities and strains identification. It was done by over the period of three years.

In aqueous extracts from acidified soil samples ureolytic, oxidoreductive, and lipolytic activity were determined. From soil samples, isolated 26 (80%) bacterial strains belonged to genus *Bacillus* sp. that were identified by cultivation and 16S rRNA methods.

## Experimental

### Materials and Methods

**Site description and chemical determination of soil samples**. The studied area was located near the town of Staszów (Fig. 1). Samples were collected in October 2010, 2011, and 2012 from the floor of a pine forest and from a plant-free site (sample 5). Weather conditions during sample collection are presented in Table I. The prevalent rocks in the studied region include Miocene limestone, gypsum, clay, and sand, which are covered by Quaternary layers. Terrain is slightly undulating and

Table I
Coordinates of sampling sites and weather conditions during sample collection*

| Sample number | Sampling place | Elevation (MASL) | Latitude | Longitude |
|---|---|---|---|---|
| 1 (G1) | Rzędów | 238 | 50°55.039' | 21°03.223' |
| 2 (G2) | Donica | 208 | 50°55.432' | 21°21.053' |
| 3 (G3) | Dziki Staw | 182 | 50°52.405' | 21°17.190' |
| 4 (G4) | Jasny | 215 | 50°57.542' | 21°24.507' |
| 5 (G5) | Grzybów Pustynia | 235 | 50°52.817' | 21°07.396' |

| October | 2010 | 2011 | 2012 |
|---|---|---|---|
| Monthly average temperature (°C) | 5.8 | 7.8 | 7.9 |
| Monthly sum of precipitation (mm) | 7.2 | 24.4 | 102 |
| Monthly humidity (%) | 84 | 85 | 87 |

* data from Hydro-Meteorological Station Sukow 19B, 26–021 Daleszyce, Poland, sampling was done in October of each year.

dotted with karst funnels on top of non-karst layers. In spite of drainage by the river Czarna Staszowska, many small lakes and bogs are present in this area due to karst phenomena. Silty-boggy soils and black soils are found in depressions. Samples of 0–7 cm topsoil were
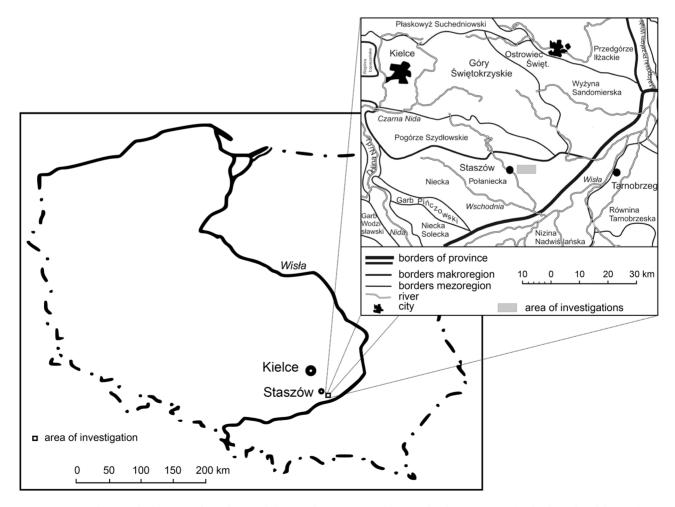


Fig. 1. Map showing the location of Grzybów and the sampling sites. Based on Kondracki maps, 1994 (Zielinski and Walek, 2012).

collected and kept for 24 hours at 4°C under humid conditions till experiments were performed. pH was determined by two methods: in water and in the presence of KCl. Selected physicochemical parameters (pH in $H_2O$ and in KCl, soil organic matter, and carbonate content) were determined in soil samples using standard procedures (Van Reeuwijk, 2006). To determine element concentrations in soil, the samples were dried at ambient temperature, sieved (< 2.00 mm), and disaggregated with a Fritsch mill to pass a 0.063 mm sieve. Homogenized samples were placed in small seal-top polyethylene sample bags and analyzed using a portable XRF analyzer (Niton XL3t from Thermo Scientific) with a geometrically optimized large drift detector and a 50 kV X-ray tube with Ag anode. The soil analytical mode was selected for analysis and each measurement was done in triplicate. The following elements were determined with precision expressed as 2 standard deviations of the mean from three measurements (in parentheses): S (± 200 mg/kg), K (± 200 mg/kg), Ca (± 100 mg/kg), Ti (± 200 mg/kg), V (± 10 mg/kg), Cr (± 20 mg/kg), Fe (± 100 mg/kg), Zn (± 40 mg/kg), As (± 10 mg/kg), Rb (± 0.00), Sr (± 0.00), Zr (± 20 mg/kg), Pb (± 10 mg/kg), Mn (± 10 mg/kg). A certified reference material NIST 2709a (San Joaquin Soil) was used for quality control. Detailed chemical analysis of five sampling sites, over a period of three years are presented in Table III.

**Total soil DNA isolation.** Total DNA from soil samples was isolated according to the procedure given below: 2 ml of phosphate buffer was added to 1 g of freeze-dried soil and mixed. The mixture was supplemented with 0.4 ml of 5% SDS and 0.04 g of PVPP. Humic acids were precipitated by adding $CaCl_2$ to a final concentration of 2% (w/v). Samples were incubated for 1 h in an incubator at 65°C and centrifuged at 8,000 × g for 10 min at 10°C. The supernatant was collected into new tubes. Then, 570 µl of TE buffer, 30 µl of 10% SDS, and 3 µl of proteinase K (20 mg/ml) were added. The tubes were incubated at 37°C for 1 h. Then, 100 µl of 5 mol/l NaCl was added, followed by vigorous stirring. Subsequently, 80 µl of CTAB solution (10% hexadecyltrimethylammonium bromide in 0.7 mol/l NaCl) was added. In the next step, 700 µl of phenol-chloroform was added, mixed, and supplemented with 30 µl of isoamyl alcohol. The mixture was centrifuged at 20,000 × g at ambient temperature for 3 min. After centrifugation, the aqueous phase was collected and added to the mixture of 700 µl of phenol-chloroform and 30 µl of isoamyl alcohol. The mixture was centrifuged at 20,000 × g at ambient temperature for 3 min. The supernatant was transferred to a new tube and 420 µl of isopropanol was added. The mixture was centrifuged at 20 000 × g at ambient temperature for 3 min. The supernatant was decanted (a mixture of phenol-

chloroform and ethanol) and remaining supernatant was removed with a pipette. Next, 100 µl of 70% alcohol was added. The mixture was centrifuged at 15,000 revolutions (20 000 × g) at ambient temperature for 3 min and the supernatant was evaporated. Finally, 100 µl of TE buffer was added.

A second method for total soil DNA isolation was also used. DNA isolation was performed according to the procedure described by Tsai and Olson (Tsai and Olson, 1991). Isolation was followed by DNA purification on a column filled with Sepharose 4B-CL (Sigma) and elution in three centrifugation steps 1000 × g for 2 min with TE buffer. Additionally, total DNA isolation was performed using the commercial kit Genomic Mini AX SOIL Spin (AA Biotechnology).

**Ribosomal intergenic spacer analysis (RISA).** Primers designed for RISA are complementary to the 16S and 23S rRNA conservative sequences. PCR amplifies the non-coding sequence between those genes. This non-coding sequence accumulates mutations and has different length in each species. RISA analysis was performed on isolated DNA templates from five soil samples. The intergenic spacers between the small- and large-subunit rRNA genes were amplified using the primers S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGCGGCTGGATCCCCTCCTT- 3') and L-D-Bact-132-a-A-18 (eubacterial rRNA large subunit, 5'-CCGGGTTTCCCCATTCGG-3') (Ranjard *et al.*, 2000). Amplification was performed in an Eppendorf Mastercycler EP at 94°C for 3 min, followed by 25 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min; extension of incomplete products was conducted at 72°C for 5 min (Ranjard *et al.*, 2000).

**DGGE of 16S rRNA fragments.** The 16S rDNA fragment was amplified in PCR with primers for the conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were as follows: V3F 5'-CCTACGGGAGGCAGCAG-3'; V3R 5'-ATTACCGC GGCTGCTGG-3'; and V3FGC 5'-CGCCCGCCGCG CGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGCAG-3'. The PCR procedure was performed according to the protocol described by Muyzer (Muyzer *et al.*, 1993).

**Biochemical activity water extracts of soil samples**. Determination of the total biochemical activity water extracts of soil samples were performed. Ureolytic activity was determined at 37°C, 25°C and 4°C based on the detection of ammonium ions in a phenol-hypochlorite assay (Moreno *et al.*, 2001). Lipolytic activity was determined using p-nitrophenyl butyrate (pNPB) as a substrate (Margesin *et al.*, 2002), proteolytic activity was measured in the presence of sodium caseinate (Subrahmanyam *et al.*, 2011), oxidoreductase activity with dimethyl-p-phenylenediamine and alpha-naphtol (Kumar *et al.*, 2013). Activities were determined at

25°C. Due to the lack of some soil samples not for all periods of time were tests done.

**Ureolytic assay**. Determination of the ureolytic activity of the bacterial strains isolated from soil samples: liquid Christensen media (pH 4, 5, 6 and 7) were inoculated (1:50) with a bacterial suspension (in sterile saline, McFarland standard 1) and incubated at an appropriate temperature. Change of color (arising as a result of urease activity) was measured with a spectrophotometer ($\lambda = 560$ nm) *vs.* a non-inoculated medium.

**Soil bacteria isolation**. Isolation of soil bacteria: 1 g of each soil sample was suspended in sterile Winogradsky salt solution (0.4 g of $K_2HPO_4$, 0.13 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of NaCl, 1.52 mg of $MnSO_4 \cdot H_2O$, and 0.5 g of $NH_4NO_3$) and stirred intensively using a magnetic stirrer for 15 min. After sedimentation of soil particles, the soil solution was diluted to $10^{-6}$ and spread on the surface of soil extract agar (SEA – 0.5% glucose, 0.5% peptone and 1% soil extract). Each soil extract and its dilutions were plated on separate SEA plates. Plates were incubated at 25°C and 4°C for up to 1 and 2 weeks, respectively, and the number of aerobic bacteria was determined. Bacterial strains with different macro-

scopic morphologies were isolated and investigated. The nomenclature of the isolated strains in Table II should be read as follows: G1/1 – sample 1, strain 1 with the plate with colonies of similar morphology.

**Bacterial DNA isolation**. DNA was isolated from bacterial cultures using a Genomic DNA Mini Kit (AandA Biotechnology) according to the manufacturer's instructions (temperature of incubation of a sample with lysis buffer was raised from 37°C to 56°C).

**Identification of bacterial strains.** Bacteria were identified by sequencing an amplified 16S rDNA fragment. Com1: 5'-CAGCMGCCGCGGTAATWC and Com2: 5'-CCGTCAATTCMTTTRAGTTT primers (Schwieger and Tebbe, 1998) were used for PCR at a concentration of 0.2 μM each. Approximately 0.1 ng of bacterial DNA was used as a template. The reaction was done with a DreamTaq™ Green Master Mix (Fermentas). Reaction mixtures were placed at 94°C for 5 min and then subjected to 30 cycles of amplification by incubation at 94°C for 2.5 min, 55°C for 30 s and 72°C for 2 min. Finally, incubation was conducted at 72°C for 5 min. The quality of the obtained PCR products was tested by electrophoresis on 2% agarose, followed by staining

Table II
Ureolytic activity of bacterial strains isolated from acidified, sulphate soils.

| Year | Soil | Temperature +25°C | | | | | | | | |
|------|------|------|------|------|------|------|------|------|------|------|
| 2011 | G1 | G1/1 | G1/2a | G1/2b | G1/3 | G1/4 | G1/5 | G1/6 | G1/8 | G1/17 |
| | | G1/9 | G1/10 | G1/11 | G1/12 | G1/13 | G1/14 | G1/15 | G1/16 | |
| | G2 | G2/2 | | | | G2/4 | | | | |
| | G3 | G3/1 | | G3/2a | | | G3/2b | | | |
| | | G3/3 | | G3/4 | | | G3/5 | | | |
| | | G3/6 | | G3/7 | | | G3/8 | | | |
| | G4 | G4/2 | | G4/9 | | G4/11 | | G4/15 | | |
| | | G4/17 | | G4/18 | | G4/20 | | G4/21 | | |
| | G5 | G5/1 | | | | G5/2 | | | | |
| 2012 | G1 | G1/25 | | G1/26 | G1/28 | | G1/29 | G1/30 | G1/31 | |
| | | G1/33a | | G1/33b | G1/34a | | G1/34b | G1/35 | G1/36 | |
| | | G1/38 | | G1/39 | G1/40 | | G1/41 | G1/43 | G1/46 | |
| | G2 | 2/14 | | | | | | | | |
| | G3 | G3/10 | | G3/12 | | G3/16 | | G3/21 | | |
| | G4 | G4/36 | | G4/40 | | G4/43 | | G4/46 | | |
| | G5 | - | | | | | | | | |

Legenda:

| Ureolytic activity | Scale |
|------|------|
| None | |
| Weak | |
| Moderate | |
| High | |

with ethidium bromide. Subsequently, PCR products were sequenced using a Beckman-Coulter CEQ™ 8000 Genetic Analysis System. 16S rDNA sequences were identified based on data from the Ribosomal Database Project, release 10 (http://rdp.cme.msu.edu/).

Determination of the total ureolytic activity of soil samples: ureolytic activity was determined based on detection of ammonium ions in a phenol-hypochlorite assay.

**Influence of ureolytic soil bacteria on soil pH.** To 5 g of an autoclaved soil sample, 2.5 ml of *Staphylococcus* sp. (sample soil G1) suspension (0.5 McFarland) in sterile saline with 0.4% of urea was added. Parallel samples without bacteria were used as a controls. After incubation for 2 weeks at 25°C, 5 ml of distilled water was added to each sample and pH was measured.

## Results

**Soil chemical properties.** In the tested soil samples, pH varied from 4 (sample 1) to 2.4 (sample 5). In both methods used ($H_2O$ and KCl) the pH of soil samples was found to be highly acidic (Table III). Organic matter content was the highest in sample 3 (27.6%). The highest level of organic carbon detected in sample 3 (near Donica Lake) may be connected to the lay of the land as this site is located in a deep karst funnel and the steeply inclined slope facilitates the retention of organic matter (Tables I and Fig. 1). This was confirmed by the lowest ash content among the sampling sites (Gąsiewicz *et al.*, 2012). Sulfate content was below 1% and varied within sampling sites. For example, over a period of 3 years, in sample 1 it was 0.2% in the 1st year, followed by a tenfold decrease in the 2nd and 3rd years. This variation was not observed in sample 4. As concerns metal content, calcium was the most abundant, and again it varied over time, but less significantly than sulfates. Trace amounts of other metals, including chromium, manganese, and vanadium, were detected (Table III).

**Biochemical activity of water soil extracts.** Ureolytic activity was present in almost all samples (except from sample 5 in the 3rd year – see Table IV). Ureolytic activity was measured at three different temperatures – 37°C, 25°C and 4°C. The highest overall 13.1 nM $NH_4$ $g^{-1}$ dry soil $h^{-1}$ ureolytic activity was observed in the 1st year, in sample 1 (G1) at 37°C. At 25°C and 4°C water soil extracts ureolityc activities were low in range 2.1 nM $NH_4$ $g^{-1}$ dry soil $h^{-1}$ (data not shown). Proteolytic activity was detected in all five samples in the 2nd year, but only in sample 1 in the 3rd year. Also lipolytic activity was observed in all samples except for samples 3 and 4 in the 1st year. Differences in enzymatic activities of soil water extracts might be resulted by different monthly precipitation rates in studied areas – see Table I.
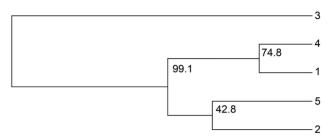


Fig. 2. Phylogenetic tree illustrating the genetic distance between DNA fragments of five soil samples subjected to RISA.

**Soil DNA isolation and the presence of a urease operon fragment in soil samples.** The commercially available kit did not make it possible to obtain bacterial DNA from acid sulfate soils at an amount and purity suitable for PCR reactions. After several attempts, a modified method was established. A combination of several techniques led to obtaining non-degraded, high molecular weight DNA suitable for molecular analysis (see the Materials and Methods section). To confirm the presence of a urease operon fragment in the tested soil samples, PCR was performed using primers (UreUnR and UreUnF) recognizing the urease-conservative fragments of Gram-negative bacteria. Electrophoresis confirmed the presence of a product of expected length of 440 bp in all soil samples, except for 4 data not shown.

**Ribosomal intergenic spacer analysis (RISA).** Ribosomal intergenic spacer analysis allows one to estimate the genetic distance between the tested organisms based on the number of and distance between PCR products (Fig. 2). Out of the three DNA isolation methods used, only one method (with PVPP) gave templates sufficiently free from humic acids to be used for PCR. Sample 3 is more distant from other samples, which are clustered in a different clade (Fig. 2). There is a small genetic distance between samples 2 and 5, which suggests that their composition is very similar. Also samples 1 and 4 exhibited a low genetic distance.

**DGGE of 16S rRNA fragments.** Denaturing gradient gel electrophoresis (DGGE) makes it possible to distinguish between products of the same length but with small different in sequences. Figure 3 presents 16S rRNA analysis from five soil samples. These results confirm RISA analysis that microbiome communities of tested soils are very homogenous and only from 1 to 3 PCR products are detected (Fig. 3).

**Isolation, identification and biochemical activity of bacterial species**. Over a period of three years, 118 bacterial strains were isolated by the plate method supplemented with a water soil extract. After isolation, only 26 strains were successfully cultivated for a prolonged period of time. The isolated strains were subjected to 16S rRNA analysis. The majority of these strains belong to the family *Bacillaceae* (Fig. 4). Isolated

Table III
Chemical analysis of soil sampling in 2010 (white), 2011 (grey) and 2012 (dark grey)

| Sample | pH $H_2O$/KCl | $M_{org}$ | P | $C_{org}$ | $CaCO_3$ | [%] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | S | K | Ca | Ti | V | Cr | Fe | Zn | As | Rb | Sr | Zr | Pb | Mn |
| 1 | | | | | | 0.226±0.03 | 0.587±0.018 | 0.692±0.01 | 0.159±0.06 | 0.006±0.02 | 0.002 | 0.698±0.01 | 0.08±0.01 | 0.002±0.01 | 0.002 | 0.004 | 0.011 | 0.011 | 0.031 |
| | 4.00/3.37 | 2.9 | 97.1 | 1.55 | 0 | <0.015 | 0.929±0.021 | 0.187±0.01 | 0.281±0.01 | 0.008 | 0.003 | 0.344±0.01 | 0.002 | <0.001 | 0.003 | 0.004 | 0.033 | <0.001 | 0.009 |
| | | | | | | <0.015 | 1.01±0.02 | 0.114±0.01 | 0.311±0.01 | 0.008 | 0.004 | 0.478±0.01 | 0.002 | <0.001 | 0.004 | 0.004 | 0.046 | 0.002 | <0.001 |
| 2 | | | | | | 0.067±0.02 | 0.784±0.019 | 0.291±0.01 | 0.230±0.01 | 0.006 | 0.002 | 0.480±0.01 | 0.004 | 0.001 | 0.002 | 0.003 | 0.022 | 0.003 | 0.022 |
| | 3.7 0/3.35 | 6.5 | 93.5 | 4.35 | 0 | 0.068±0.04 | 0.802±0.026 | 1.89±0.01 | <0.05 | 0.009 | 0.005 | 1.27±0.02 | 0.007 | 0.001 | 0.003 | 0.006 | 0.014 | 0.003 | <0.001 |
| | | | | | | <0.015 | 1.06±0.02 | 0.167±0.01 | 0.318±0.01 | 0.009 | 0.004±0.01 | 0.461±0.01 | 0.003 | <0.001 | 0.004 | 0.004 | 0.039 | 0.003 | 0.009 |
| 3 | | | | | | 0.094±0.02 | 0.496±0.009 | 0.370±0.009 | 0.102 | 0.003 | <0.001 | 0.361±0.01 | 0.004 | 0.001 | 0.001 | 0.002 | 0.005 | 0.001 | 0.008 |
| | 3.50/3.59 | 27.6 | 72.4 | 16–27.1 | 0 | 0.056±0.03 | 0.537±0.021 | 0.297±0.01 | 0.198±0.01 | 0.005 | <0.001 | 0.322±0.01 | 0.004 | 0.01 | 0.001 | 0.001 | 0.004 | 0.02 | <0.001 |
| | | | | | | 0.067±0.03 | 0.753±0.024 | 0.091±0.01 | 0.3±0.01 | 0.013 | 0.006±0.02 | 0.706±0.01 | 0.003 | 0.001 | 0.003 | 0.002 | 0.008 | 0.003 | <0.001 |
| 4 | | | | | | 0.229±0.03 | 0.356±0.014 | 1.41±0.02 | 0.075 | 0.004 | 0.009 | 0.590±0.01 | 0.015 | 0.001 | 0.001 | 0.003 | 0.004 | 0.004 | 0.025±0.01 |
| | 3.59/3.2 | 4.7 | 95.3 | 11.1 | 0 | 0.057±0.02 | 0.383±0.016 | 0.094±0.01 | 0.098 | 0.004 | <0.001 | 0.433±0.01 | 0.003 | <0.001 | 0.001 | 0.002 | 0.007 | 0.003 | <0.001 |
| | | | | | | 0.057±0.02 | 1.32±0.03 | 0.131±0.01 | 0.333±0.01 | 0.008 | <0.001 | 0.373±0.01 | 0.002 | 0.001 | 0.004 | 0.004 | 0.025 | 0.004 | <0.001 |
| 5 | | | | | | 0.666±0.04 | 0.849±0.018 | 0.395±0.01 | 0.292±0.01 | 0.006 | 0.002 | 0.112±0.01 | <0.001 | <0.001 | 0.002 | 0.005 | <0.001 | 0.001 | <0.001 |
| | 2.41/2.29 | 1.8 | 98.2 | 1.07 | 7.3 | 7.81±0.1 | 0.883±0.02 | 0.220±0.01 | 0.158±0.01 | 0.004 | 0.01 | 0.171±0.01 | <0.001 | <0.001 | 0.004 | 0.006 | 0.009 | 0.006 | >0.001 |
| | | | | | | 0.550±0.04 | 0.938±0.02 | 0.2±0.01 | 0.302±0.01 | 0.007 | 0.004 | 0.18±0.01 | <0.001 | <0.001 | 0.004 | 0.005 | 0.037 | 0.005 | <0.001 |

$M_{org}$ – organic matter content; P – ash content; $C_{org}$ – organic carbon content
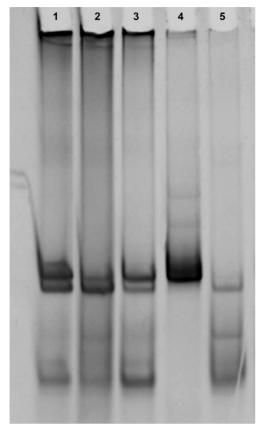
Fig. 3. PCR products of 16S rRNA on a template of five soil DNA isolates (wells 1–5) separated by denaturing gradient polyacrylamide gel electrophoresis (DGGE).

of samples from the 2nd and 3rd years indicated that sample 1 contained the greatest proportion of strains with urease activity (12 strains). Overall, 29 out of 118 strains possessed measurable ureolytic activity. The ureolytic activities of isolated strains indicate that detected in water soil extracts urea decompositions are due to bacterial biochemical activities.

**Influence of ureolytic soil bacteria on soil pH.** Soil samples inoculated with *Staphylococcus* sp. strain (previously isolated from a soil sample G1) were fertilized with urea and incubated for two weeks. pH levels were measured before and after incubation. In all samples bacteria survived low soil pH and started to decompose urea to carbon dioxide and ammonia, which resulted in an overall weak pH increase in all the soil samples studied, from 0.02 to 0.16 for soil sample G1, G3, respectively.

### Discussion

One of the most important environmental factors determining soil bacterial activity is pH. The presented study analyzed soil samples of pH ranging from 4 to 2 from the area of a former sulfur mine, in south-western Poland. The biochemical activities of the bacteria were detected in tested soil samples. Those activities varied, depending on the year of sampling and sample type. The ureolytic activity of soil extracts as well as isolated soil strains was investigated in more detailed due to the expectation that urea decomposition may help to increase very low pH of sulfate soils. The isolated strains that exhibited ureolytic activity were present in all tested soil samples. Low pH is a crucial environmental factor affecting the process of nitrification and inhibiting the decomposition of organic matter as a source of soluble soil nitrogen for plant vegetation (Cui *et al.*, 2013). Data suggest that inhibition of microbial species was observed below pH 4.5, which is probably attributable to increased inhibitory effects caused by the release of free aluminum or decreasing

bacteria were grown at both 4°C and 25°C. Staining of the strains isolated in 2011 and 2012 revealed similar results: 75% were Gram-positive and 25% were Gram-negative bacteria. Morphological analysis of Gram-positive bacteria revealed that 60% of them were rod-shaped and 7% were coccoid. *Streptomyces* cells were identified in 2% of samples. The highest number of different species was isolated from sample 1.

It was found that 29 of the tested strains possessed ureolytic activity (Table II). Only one strain could decompose urea at +4°C. Soil sample 5 exhibited a lack of living bacteria with ureolytic activity. A comparison

Table IV
Biochemical activities of water extracts of soil samples

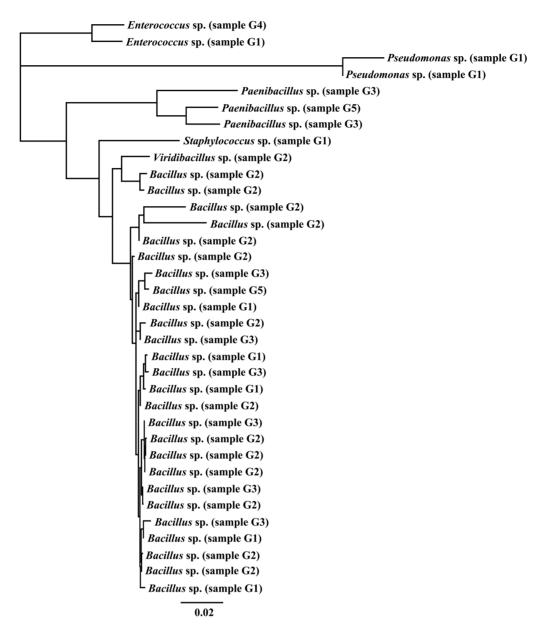| | Lipolytic activity ($\mu$g p-nitrophenyl butyrate g$^{-1}$ h$^{-1}$) | | | Proteolytic activity ($\mu$g tyrosine g$^{-1}$ h$^{-1}$) | | Oxidoreductase activity (U)* | Ureolytical activities (nM NH$_4$ g$^{-1}$ dry soil h$^{-1}$ at 37°C) | | |
|---|---|---|---|---|---|---|---|---|---|
| Year | 1st | 2nd | 3rd | 2nd | 3rd | 2nd | 1st | 2nd | 3rd |
| Sample 1 (G1) | 39.1 | 17.6 | 536.9 | 59.3 | 21.0 | 0.16 ± 0.05 | 13.1 | 2.2 | 0.2 |
| Sample 2 (G2) | 33.1 | 1299.8 | 536.8 | 80.2 | 0 | 0.22 ± 0.02 | 0.7 | 3.1 | 0 |
| Sample 3 (G3) | 0 | 38.6 | 291.7 | 77.8 | 0 | 0.22 ± 0.04 | 11.1 | 1.8 | 0.1 |
| Sample 4 G4) | 0 | 2299.3 | 240.6 | 76.3 | 0 | 3.28 ± 0.39 | 1.7 | 2.8 | 0.1 |
| Sample 5 (G5) | 94.2 | 2455.8 | 0 | 99.5 | 0 | 1.63 ± 0.12 | 0 | 2.4 | 0 |

* standard deviation

Fig. 4. Identification and phylogenetic tree of bacterial strains isolated from acidic soil samples based on 16S rDNA.

plant productivity (Rousk *et al.*, 2009). Bacterial communities in acidic soils are more strongly influenced by low pH than fungal communities (Rousk *et al.*, 2010). That might be one cause of failed attempts to rehabilitate the soil by liming the surface, leading to the formation of carbonates and the absence of vegetation in the sampling sites, which is still observed (Zieliński and Wałek, 2012). The majority of bacterial strains isolated from acidified sulfate soils belong to the family *Bacillaceae*. This may indicate that under harsh conditions involving acidic pH and a high content of sulfur only spore-forming bacteria may be able to survive. That observation was confirmed by other studies (van Elsas *et al.,* 2008; Blagodatskaya and Kuzyakov, 2013; Valenzuela *et al.*, 2006). Moreover, *Bacillus* spp. are ureolytic strains which can survive acidic conditions (Mols and Abee, 2008). The RISA test was conducted for the iso-

lated soil DNA by our new established method. The low number of DNA bands indicated very low heterogeneity of bacterial populations in acid sulfate soil. As it was aptly stated by Fierer and Jackson, "the diversity and richness of soil bacterial communities differed by ecosystem type, and these differences could largely be explained by soil pH" (Fierer and Jackson, 2006). In general, bacterial diversity in acidic soils is significantly lower than in neutral soils (Fierer and Jackson, 2006). The highest sulfur concentration (7.81% in sample 5 in the 2nd year) exceeded the highest content of this element found by Martyn *et al.* in the soils of the former Basznia sulfur mine in eastern Poland (1.56%) (Martyn *et al.*, 2004). The results of sulfur determination in soil samples in conjunction with pH values were similar to those obtained by Sołek-Podwika and Ciarkowska in their comprehensive study of soils in the Grzybów

mining area (Sołek-Podwika and Ciarkowska, 2012). Those authors also observed increasing sulfur concentrations with sampling depth. The relatively low concentrations of metals that were found in the soil samples examined may be explained by the migration of ions mobilized in an acidic environment.

## Conclusions

Biochemical activities, based on presence of bacteria, were detected in acidifed sulfate soils. DNA analysis of soil samples indicate on very small diversity of bacterial population, reduced to spore forming *Bacillus* sp. In all the tested samples soil ureolytic activities, the presence of ureolytic bacteria and urease genes were detected. Our study indicates that *Bacillus* sp., strains isolated from acidified sulfate soils may be considered tools for biomineralization that will led to pH increase by urea decomposition.

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