Development of a process for microbial sulfate reduction in cold mining waters — Cold acclimation of bacterial consortia from an Arctic mining district

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**ABSTRACT**

Biological sulfate removal is challenging in cold climates due to the slower metabolism of mesophilic bacteria; however, cold conditions also offer the possibility to isolate bacteria that have adapted to low temperatures. The present research focused on the cold acclimation and characterization of sulfate-reducing bacterial (SRB) consortia enriched from an Arctic sediment sample from northern Finland. Based on 16S rDNA analysis, the most common sulfate-reducing bacterium in all enriched consortia was Desulfobulbus, which belongs to the δ-Proteobacteria. The majority of the cultivated consortia were able to reduce sulfate at temperatures as low as 6 °C with succinic acid as a carbon source. The sulfate reduction rates at 6 °C varied from 13 to 42 mg/L/d. The cultivation medium used in this research was a Postgate medium supplemented with lactate, ethanol or succinic acid. The obtained consortia were able to grow with lactate and succinic acid but surprisingly not with ethanol. Enriched SRB consortia are useful for the biological treatment of sulfate-containing industrial wastewaters in cold conditions.

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1. Introduction

Sulfate and metals are the most common pollutants in mining and metallurgical wastewaters (Huisman et al., 2006). Sulfate-containing wastewaters are also produced by the pulp and paper, food, and petroleum industries (Wolicka, 2008; Lens et al., 2003). Sulfate can be removed from water by membrane or ion exchange processes, or by chemical precipitation as gypsum for instance. These methods are efficient but have some disadvantages such as fouling of membranes, the need for pre-precipitation or downstream treatment, high costs in the case of membrane processes, and production of sludge in large volumes in the case of chemical precipitation (Silva et al., 2012). Gypsum precipitation is suitable for sulfate concentrations of several grams per liter and the achieved residual concentrations are around 1500–2000 mg/L (Geldenhuys et al., 2003), whereas limits for sulfate concentrations in mining effluent discharge in Finland range from 1000 to 4000 mg/L (AVI, 2013; AVI, 2015; AVI, 2016; AVI, 2017).

Sulfate-reducing bacteria (SRB) are anaerobic microbes that use sulfate as an electron acceptor for the oxidation of organic compounds, which results in the production of sulfide (de Matos et al., 2018). Some SRB are able to oxidize organic compounds completely resulting in the production of CO2; however, some of the bacteria can perform only partial oxidation of organic compounds which leads to the production of acetate (Gibson, 1990). According to Sulaiman et al. (2008), the most effective biological sulfate reduction by SRB is achieved when the initial sulfate concentration is 2500 mg/L, but bacterial growth inhibition is clearly detected when the sulfate concentration reaches 4000 mg/L. Biological sulfate reduction can also be used for much higher sulfate concentrations, but the method is economically more viable with moderate sulfate concentrations. The achieved residual sulfate concentrations can be even lower than 100 mg/L (Runtti et al., 2018). In the bioremediation process by SRB, the removal of sulfate and organic substances and the precipitation of base metals occur simultaneously, which improves the cost-effectiveness of the method.

SRB can be exploited in biological sulfate removal either as pure cultures or as mixed consortia with other bacteria unable to utilize sulfate as an electron acceptor. The use of diverse bacterial consortia in the microbial sulfate reduction process has some
advantages compared to the use of pure cultures, such as a lower risk of contamination by other organisms and enhanced adaptation to changes in conditions (Boothman et al., 2006; White et al., 1998). In addition, in mixed consortia the utilization of substrates is more comprehensive than in single strain cultures, for instance the acetate produced by SRB in the partial oxidation of organic compounds can act as a carbon and energy source for other microorganisms. A high diversity of bacteria also allows for the degradation of a larger variety of organic contaminants (Boothman et al., 2006; White and Gadd, 2000).

In boreal areas, the cold climate causes problems in biological wastewater treatment related to the slower metabolism and nutrient uptake of bacteria. Microbes are found in a wide variety of environmental conditions and are able to adapt rather quickly to environmental changes. The relative content of various bacterial genera is strongly dependent on the origin of the soil and the climate. Bacterial consortia used for the treatment of cold wastewaters can be screened from samples collected from Arctic areas where the microbial populations have already adapted to the cold climate. For example, Knoblauch et al. (1999) have reported that the SRB isolated from Arctic marine sediments had significantly higher metabolic rates than the comparable mesophilic strains at low temperatures. SRB are able to tolerate temperatures from −5 °C to 75 °C and they adapt easily to changes in temperature (Cocos et al., 2002). However, for the majority of SRB, the optimal growth temperature is from 28 to 30 °C (Hao, 2003) and the metabolic rates of the bacteria are highly affected by temperature. It is known that rather than being actually psychrophilic, the majority of cold-tolerant bacteria are mesophilic strains with the ability to grow also in cold temperatures, referred to as psychrotolerant bacteria or psychrotrophs (Morita, 1975; Russell, 1990).

The purpose of the present research was to enrich bacterial consortia with the ability to reduce sulfate efficiently also at cold temperatures, as the performance of biological sulfate reduction could be a major concern in boreal areas with a cold climate. Since only limited information on psychrophilic and psychrotolerant non-marine SRB is available, the aim was also to identify the enriched bacteria and the most important factors affecting their performance at cold temperatures. Even though some SRB consortia have been successfully cultivated at temperatures as low as 4–6 °C (Nielsen et al., 2018; Tsukamoto et al., 2004), in this study, cold acclimation occurred more quickly. In addition, this study examined the effect of different carbon sources and temperature on the composition of microbial communities. Thus, the present study provides new and significant information about low-temperature experiments and the cold acclimation of SRB consortia.

A sediment sample from wetlands near a mine site operating in an Arctic environment was used as the SRB source. The soil type of the sampling site indicates a possibly high content of humic substances, which can serve as carbon and nitrogen sources for the soil microbiota. The capability of the enriched SRB consortia to utilize different carbon sources was investigated. Succinic acid and ethanol were chosen as alternative carbon sources since they are economically more viable than sodium lactate, which is the most commonly utilized carbon source in SRB cultivation. Succinic acid is also one of the main degradation products of soil humic acids (Tsutsumi and Kuwatsuka, 1979) and thereby closely related to the likely nutrient sources of the microbial community in the sediment sample. In addition, ethanol is widely used in commercial applications (Bomberg et al., 2017) and, furthermore, there is a lack of knowledge on succinic acid as a carbon source in SRB cultivations. To the best of our knowledge, this is the first study to test succinic acid in the cultivation of SRB at low temperatures.

2. Material and methods

2.1. Enrichment of sulfate-reducing bacteria

A Postgate medium is commonly used for SRB cultivation (Barbosa et al., 2014; Ismail et al., 2014; de Matos et al., 2018). The medium used in this study was modified from DSMZ Desulfovibrio (Postgate) Medium (Leibniz-Institut DSMZ GmbH, 2017). A more detailed composition and preparation of the medium is presented in the supplementary material.

The mixed cultures used in this study were enriched from a sediment sample which was collected in September 2017 from a ditch near a mine located in the Lapland region of northern Finland. At the time, the average temperature in the area was around 7 °C, while the average annual temperature variation is from −15 to 15 °C. Approximately 50 mL of the sediment sample was suspended in 450 mL of 0.9% w/v saline solution, which was first sparged with N2 gas to exclude oxygen. When the suspension was settled, aliquots of 3 mL were transferred into six 40 mL glass vials containing 30 mL of the modified Postgate medium with an initial sulfate concentration of approximately 1.7 g/L. The vials were then sealed with screw caps with septa and incubated at three different temperatures: 6 °C, 16 °C and RT (approximately 22 °C).

Sulfate concentrations were determined using Hach Lange Sulfate cuvette tests LCK 353 or LCK 153 and a UV/Vis Spectrophotometer DR 2800. The principle of the cuvette tests is that sulfate ions react with barium chloride and form poorly soluble barium sulfate thereby causing turbidity, which can be measured photometrically at 450 nm.

2.2. Sulfate reduction using alternative carbon sources and cold acclimation of the enriched consortium

After one month of incubation of the enrichment cultures at 16 and 22 °C, 1 mL aliquots were inoculated into 35 mL of fresh culture media. Four new cultures were inoculated from each vial (6 samples from enrichment), making a total of 24 cultures. The media were prepared as described above, but sodium lactate was replaced with succinic acid or ethanol, both at a concentration of 30 mM. Incubation was continued at the same temperature for 10 days. After the 10-day incubation, 1 mL aliquots from the succinic acid cultivations were again inoculated into 35 mL of fresh culture media and the incubation temperatures were decreased first from 16 to 14 °C and from 22 to 20 °C (= initial temperatures in cold acclimation tests). Two new cultures were inoculated from every vial where growth was clearly detected, i.e. from all 12 succinic acid cultivations. Samples were collected periodically through the septa with a syringe and needle. When sulfate reduction was observed, the temperatures were again decreased a few degrees at a time, until a temperature of 6 °C was reached for all the cultures. Fresh medium was fed with a syringe and needle to the culture vials two times during the cold acclimation. The points at which the medium was added are shown in Fig. 4.

2.3. Identification of the enriched cultures by 16S rDNA sequencing and data analysis

The enriched cultures, with the exception of the cultivations with ethanol and the cultivations at 6 °C without acclimation, were characterized by 16S rDNA sequencing at the Biocenter Oulu Sequencing Center. All 32 samples for 16S rDNA sequencing analysis were taken at the end of all the parallel cultivations: enrichment with lactic acid — 6 samples, cultivation with succinic acid — 12 samples, and cold acclimation — 14 samples from the consortia that grew at 6 °C. The genomic DNA of these strains was isolated.
from the cell pellets using the standard protocol (QIAGEN, 2017). A portion of the 16S small sub-unit ribosomal gene was amplified with standard primers F519 (5'-CAGCMGCCGCGGTAATWC-3') and R926 (5'-CGTCAATTCTTTRAGTTT-3') modified by the addition of barcodes, namely: the F519 primer contained an Ion Torrent adapter sequence A, a 9-bp unique barcode sequence, and one nucleotide linker whereas the R926 primer contained an Ion Torrent adapter trP1 sequence. Polymerase chain reaction (PCR) assays were performed in 25 µL reactions in three replicates, each containing 1 × Phusion GC master mix (Thermo Scientific, Espoo, Finland), 0.4 µM of forward and reverse primers and 20 ng of genomic DNA as the template. After an initial 3-min denaturation at 98 °C, the following cycling conditions were used: 20 cycles of 98 °C, 10 s; 64 °C, 20 s; 72 °C, 20 s. After PCR amplification reactions, the samples were purified using the AMPure XP reagent (Agencourt Bioscience, CA, USA). The amplicon concentration of the purified samples was measured on a Bioanalyzer DNA-1000 chip (Agilent Technologies, CA, USA) and individual samples were pooled in equivalent amounts. The pooled sample was further purified with Ampure XP and sequencing performed with Ion Torrent PGM on a 316 chip using Ion View chemistry (ThermoFisher Scientific, USA). The obtained sequences were compared with those present in GenBank using a BLAST tool (National Center for Biotechnology Information, U.S. National Library of Medicine, USA).

3. Results

3.1. Culture enrichment

In the first step of the enrichment, after two weeks of incubation at 16 and 22 °C, the growth of SRB was observed by the formation of black iron sulfide precipitate in the vials (Fig. 1). However, a clear decrease in sulfate concentration was detected only after three weeks of incubation (Fig. 2). After 28 days, the sulfate had decreased by approximately 500 mg/L (30% removal). There was no significant difference in sulfate removal between samples incubated at 16 and 22 °C. At 6 °C without cold acclimation, sulfate removal of 500 mg/L was attained only after 84 days of incubation (Fig. S1).

3.2. Effect of alternative carbon sources on sulfate reduction

After one week of incubation with alternative, economically more viable carbon sources (ethanol and succinic acid), a clear difference in sulfate reduction rates was detected (Fig. 3). At 16 °C, sulfate concentrations had decreased by approximately 10% with ethanol as a carbon source and 40% with succinic acid as a carbon source. At 22 °C, sulfate removals were 20% with ethanol and 65% with succinic acid. The average sulfate reduction rates (SRR) were also higher with succinic acid (98 mg/L/d at 16 °C and 169 mg/L/d at 22 °C) than in the enrichment step with lactic acid (70 mg/L/d at 16 °C and 46 mg/L/d at 22 °C) as a carbon source at both temperatures. Therefore, the cultivations were continued with succinic acid.

3.3. Cold acclimation of the enriched sulfate-reducing cultures

Fig. 4 shows the sulfate consumption during cold acclimation with succinic acid as a carbon source. The sulfate reduction rates were more repeatable in the cultures with an initial temperature of 14 °C (Fig. 4b) than in the cultures with an initial temperature of 20 °C, which had more diversity (Fig. 4a). Both test series with different initial temperatures followed a similar trend at the beginning. The sulfate decreased to a level of about 600 mg/L but, after the second substrate addition, the sulfate concentration increased in the cultures with an initial temperature of 20 °C due to the low temperature and slower kinetics. Since the cultures also contained other bacteria than SRB, the other reason for the slight
increase in sulfate concentration could be bioleaching of sulfates from the precipitated iron sulfide. However, the sulfate concentration continued to decrease with a delay in reaching the previously obtained sulfate level for both test series. The cultures with a lower initial temperature reached a somewhat lower sulfate level. At 6°C, the average SRRs were 22 mg/L/d and 31 mg/L/d for initial temperatures of 20°C and 14°C, respectively.

3.4. Identification of the enriched cultures

Most of the bacteria in all the experiments belonged to the phyla Bacteroidetes, Proteobacteria and Firmicutes (Fig. 5). Some differences in bacterial distribution were detected after the change of substrate and the cold acclimation. At both temperatures (22 and 16°C) after sodium lactate was replaced with succinic acid, the amount of Firmicutes increased and the amount of Proteobacteria and Bacteroidetes decreased, but during the cold acclimation the amount of Firmicutes decreased again and the amount of Bacteroidetes increased. Statistically significant changes (according to t-test, p-value < 0.05) were the increase in Bacteroidetes during the cold acclimation from 16 to 6°C, and the decrease in Proteobacteria after the substrate replacement at 22°C, as well as changes in the amount of Firmicutes except during the cold acclimation from 22 to 6°C.

After the cold acclimation, the majority of the Proteobacteria present in the cultures were sulfate reducers. All the SRB found in the cultivations were members of δ-Proteobacteria. The most common sulfate reducer in all cultivations was Desulfobulbus (Fig. 6). Also other members of the family Desulfobulbaceae were present. Other genera detected were Desulfovibrio and Desulfotignum. The highest amount of sulfate reducers, 19% of all bacteria present in the consortium, was detected after cold acclimation with an initial temperature of 16°C, which signifies that a lower temperature is more favourable to SRB than to the other bacteria present in the consortia. In particular, the amount of Desulfobulbus spp. grew during cold acclimation and the increase was also statistically significant (p-values < 0.05). When comparing the Desulfobulbus spp. amounts between 22 and 16°C, it can be stated that the amounts show distinct homogeneity (p-value > 0.05). With succinic acid as a carbon source, the amount of Desulfovibrio spp. was significantly higher (p-value < 0.05) at 16°C than at 22°C as well as after cold acclimation with the initial incubation temperature of 16°C rather than 22°C as the initial temperature.

4. Discussion

In the present research, SRB were enriched from an environmental sample using a liquid Postgate medium, which is selective towards Desulfovibrio species. In addition to lactate, the performance of succinic acid and of ethanol as carbon sources was also investigated. The detected diversity of SRB was low in all cultivations and SRB accounted for 6–19% of the bacterial consortia. The results are in agreement with Boothman et al. (2006) who detected only three different SRB species in their consortium, constituting 21% of the consortium. In addition, Bomberg et al. (2017) detected
In the present study, the dominating SRB genus was Desulfovibrio and the second most abundant genus was Desulfobulbus, both of which are gram-negative mesophiles (Barbosa et al., 2014). Desulfobulbus propionicus and various Desulfovibrio spp. have also been found to be microaerophilic (Dannenberg et al., 1992). Desulfotignum spp. were detected only in the sediment sample; however, some bacteria belonging to the same Desulfo bacteraceae family were detected in cultivations conducted at 22 °C. Desulfotignum spp. are strictly anaerobic, growing in temperatures ranging from 10 to 42 °C (Kuever et al., 2005). Desulfobulbus spp. prevailed over Desulfovibrio spp. probably because they were already present in larger amounts in the original sediment sample. The enrichment of Desulfobulbus spp. has also been detected in other studies on sediment samples collected from an urban pond in Brazil (Barbosa et al., 2014) and an area impacted by drainage water discharge from tailings in northern Siberia (Karnachuk et al., 2005).

For most SRB the optimal growth temperature is from 28 to 32 °C. Optima of 24–28 °C have been observed with some Desulfobacterium and Desulfbacter strains (Hao, 2003). In addition, some psychrophilic strains with optima below 20 °C have been found from Arctic marine sediments (Knoblauch et al., 1999). Many Desulfobulbus strains have an optimal growth temperature of around 30 °C, but this study confirmed that they are able to grow at temperatures as low as 6 °C. In comparison, D. aggregans (Kharrat et al., 2017), D. mediterraneus (Sass et al., 2002), D. propionicus (Widdel and Pfennig, 1982) and D. rhabdoformis (Lien et al., 1998) are able to grow at a minimum temperature of 10°C.

Sulfate reductions rates at different temperatures are summarized in Table 1. Biological sulfate reduction has been investigated on reactor scale at a temperature of 9 °C with H2 as the electron donor (Nevatalo et al., 2010), at 8 °C with ethanol as the electron donor (Sahinkaya et al., 2007), at 6 °C with ethanol or methanol combined with spent manure as the electron donor (Tsukamoto et al., 2004) and at 4–8 °C with molasses as carbon source (Nielsen et al., 2018). Low temperature batch experiments have been conducted at 15 °C with acetate as an electron donor (Qian et al., 2019a) and at 5 °C with methanol or ethyl glycol as carbon sources (Nielsen et al., 2019). Tsukamoto et al. (2004) acclimated the SRB to cold conditions by lowering the temperature from 22 to 6 °C for 266 days, whereas in the present research cold acclimation from 22 to 6 °C was conducted in 61 days using succinic acid as a carbon source. In addition, in the study made by Nielsen et al. (2019), adaptation to the temperature of 5 °C lasted over 80 days before any significant sulfate reduction occurred. The result was quite similar compared to the cultivation at 6 °C without cold acclimation in the present study (Fig. S1).

Consequently, sulfate reduction rates also depend on temperature. Sulfate reduction may increase over 3-fold with a 10 °C increase in temperature (Nielsen, 1987). The present research achieved quite similar results, i.e. much lower reduction at lower temperature, which signifies that the SRB obtained are not psychrophilic but more likely psychrotolerant. As a result, there is a noticeable difference in the duration of the lag phase even between 16 and 22 °C with succinic acid as a carbon source. At 22 °C sulfate reduction starts faster but the difference in reduction rates seems to decrease in the long term (Figs. 3 and 4). Even though psychrophilic SRB could be more efficient sulfate reducers at low temperatures, there may still be some advantages to the use of psychrotolerant cultures. In boreal areas, the seasonal temperature variation is wide and in summer the metabolism of psychrophilic bacteria might be affected once again when the temperature increases above 20 °C. The results obtained in cultivations under cold temperatures cannot be directly compared with those achieved in other studies at higher temperatures and in reactor experiments (Table 1). In the reported low temperature bioreactor experiments, substrates were fed to the reactors continuously (Nevatalo et al., 2010; Tsukamoto et al., 2004; Sahinkaya et al., 2007; Nielsen et al., 2018), so that the SRBs were unaffected by any nutrient limitations in contrast to the small-scale batch experiments in this study. SRB adapt rather...
easily to changes in temperature (Cocos et al., 2002), which was also observed in the present study in the cold acclimation step. Bacterial growth was detected each time within a few days of the temperature changes and, interestingly, all parallel cultures also reduced sulfate at a remarkably similar rate even during cold acclimation (Figs. 2–4). This finding shows that cold acclimation is an effective method to adapt SRB to low temperatures.

Ethanol is widely used as a carbon and electron source in commercial applications of biological sulfate reduction and it is a relatively economical option (Bomberg et al., 2017). However, it is even more economical to use ethanol-containing wastes (Costa et al., 2009; Martins et al., 2009). In addition to ethanol, other economically viable substrates suitable for the growth of SRB have been investigated, such as acetate (Qian et al., 2019a; Qian et al., 2019b), animal manure and cellulose materials (Gibert et al., 2004; Cocos et al., 2002), sewage sludge (Ristow et al., 2018), and ethanol oxidation, acetate should also be oxidized to produce bi-

Table 1
Sulfate reduction rates reported for various temperatures and electron donors.

<table>
<thead>
<tr>
<th>SRB source</th>
<th>T (°C)</th>
<th>Electron source</th>
<th>SRR (mg/d)</th>
<th>System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched from sediment near mining district</td>
<td>22</td>
<td>Succinic acid, yeast extract</td>
<td>169</td>
<td>Batch</td>
<td>This study</td>
</tr>
<tr>
<td>Enriched from sediment near mining district</td>
<td>16</td>
<td>Succinic acid, yeast extract</td>
<td>98</td>
<td>Batch</td>
<td>This study</td>
</tr>
<tr>
<td>Enriched from sediment near mining district</td>
<td>6</td>
<td>Succinic acid, yeast extract</td>
<td>13–42</td>
<td>Batch</td>
<td>This study</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>15</td>
<td>Acetate, glucose, yeast extract</td>
<td>1300</td>
<td>Batch</td>
<td>Qian et al. (2019a)</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>NA</td>
<td>Acetate, glucose, yeast extract</td>
<td>2487</td>
<td>SRUSB</td>
<td>Qian et al. (2019b)</td>
</tr>
<tr>
<td>Creek sediment near mining district</td>
<td>5</td>
<td>Methanol</td>
<td>26.7</td>
<td>Batch</td>
<td>Nielsen et al. (2019)</td>
</tr>
<tr>
<td>Creek sediment near mining district</td>
<td>5</td>
<td>Ethylene glycol</td>
<td>4.1^</td>
<td>Batch</td>
<td>Nielsen et al. (2019)</td>
</tr>
<tr>
<td>Mine impacted waters</td>
<td>4–8</td>
<td>Molasses</td>
<td>0–22</td>
<td>PBR</td>
<td>Nielsen et al. (2018)</td>
</tr>
<tr>
<td>Psychrotolerant enrichment culture obtained</td>
<td>9</td>
<td>H2</td>
<td>451–663</td>
<td>MBR</td>
<td>Nevalato et al. (2010)</td>
</tr>
<tr>
<td>from cold temperature mining areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psychrotolerant enrichment culture obtained</td>
<td>8</td>
<td>Ethanol</td>
<td>250^</td>
<td>FBR</td>
<td>Sahinkaya et al. (2007)</td>
</tr>
<tr>
<td>from cold temperature mining areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spent manure</td>
<td>6</td>
<td>Ethanol, spent manure</td>
<td>961–1345</td>
<td>AF</td>
<td>Tsukamoto et al. (2004)</td>
</tr>
<tr>
<td>Spent manure</td>
<td>6</td>
<td>Methanol, spent manure</td>
<td>1057–1441</td>
<td>AF</td>
<td>Tsukamoto et al. (2004)</td>
</tr>
<tr>
<td>AMD affected creek sediment</td>
<td>22</td>
<td>Wood chips, leaf compost, poultry manure</td>
<td>103.7</td>
<td>Batch</td>
<td>Cocos et al. (2002)</td>
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<tr>
<td>Creek sediment</td>
<td>21</td>
<td>Compost</td>
<td>ND</td>
<td>Batch</td>
<td>Gilbert et al. (2004)</td>
</tr>
<tr>
<td>Creek sediment</td>
<td>21</td>
<td>Oak leaf</td>
<td>24</td>
<td>Batch</td>
<td>Gilbert et al. (2004)</td>
</tr>
<tr>
<td>Creek sediment</td>
<td>21</td>
<td>Sheep manure</td>
<td>24</td>
<td>Batch</td>
<td>Gilbert et al. (2004)</td>
</tr>
<tr>
<td>Creek sediment</td>
<td>21</td>
<td>Poultry manure</td>
<td>7</td>
<td>Batch</td>
<td>Gilbert et al. (2004)</td>
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<tr>
<td>Sewage sludge</td>
<td>35</td>
<td>Sewage sludge</td>
<td>184</td>
<td>CSTR</td>
<td>Ristow et al. (2018)</td>
</tr>
<tr>
<td>Isolated from soil and wastewater containing</td>
<td>30</td>
<td>Dairy wastewater</td>
<td>74</td>
<td>Batch</td>
<td>Wolicka (2008)</td>
</tr>
<tr>
<td>petroleum residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched from soil polluted by oil-derived</td>
<td>25</td>
<td>Whey, yeast extract</td>
<td>88</td>
<td>Batch</td>
<td>Wolicka and Borkowski (2009)</td>
</tr>
<tr>
<td>products</td>
<td></td>
<td></td>
<td>40</td>
<td>Batch</td>
<td>Wolicka and Borkowski (2009)</td>
</tr>
<tr>
<td>Enriched from soil polluted by oil-derived</td>
<td>25</td>
<td>Whey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>products</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

^ Average SRR without the lag phase and sulfate-limited phase.

^ Maximum SRR.

2CH3CHOHCOO^- + SO4^2- → 2CH3COO^- + 2HCO3^- + H2S

(1)

2CH3CHOHCOO^- + SO4^2- → 2CH3COO^- + H2S + 2H2O

(2)

4(CH2)2(CO0^-)2 + 3SO4^- + 6H+ → 4CH3COO^- + 4HCO3^- + 3H2S + 4CO2

(3)

CH3COO^- + SO4^2- + H+ → 2HCO3^- + H2S

(4)

Higher bicarbonate alkalinity production may be one reason why better sulfate reduction rates were observed in cultivations with succinic acid as a carbon source than with ethanol as a carbon source (Fig. 3), even though ethanol is known to be suitable for various SRB species. Moreover, succinic acid contains more organic carbon compared to ethanol. In addition, Karnachuk et al. (2005) reported that, in their experiments, Desulfobulbus spp. were the most dominant SRB at 4°C and pH 7.2 with lactate as a carbon source as well as at 28°C and pH 7.2 with ethanol as a carbon source. However, at pH 7.2, Desulfobulbus cells were not detected at 28°C with lactate or at 4°C with ethanol as a carbon source. Moreover, at acidic pH values, Desulfobulbus spp. were not detected at 4°C with lactate as a carbon source. In the present study, the most likely cause of the enrichment of Desulfobulbus spp. is production of propionate, which is known to be readily utilized by Desulfobulbus spp. In the cultivation medium lactate, succinate and amino acids provided by yeast extract can be fermented to propionate by the other bacteria present (Stams et al., 1998).

All the cultivations in the present study were dominated by the phyla Bacteroidetes, Proteobacteria and Firmicutes (Fig. 5). This is unsurprising since Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Acidobacteria are usually widespread in soil and are almost always present in any type of soil (Lauber et al., 2009). The lack of Actinobacteria in the enriched cultures is self-evident, considering that most of the bacteria belonging to this phylogenetic group are aerobic. In addition, the majority of Acidobacteria...
are acidophilic and cultivations in this study were conducted at neutral or slightly alkaline pH.

The majority of the Firmicutes found in sulfate-reducing consortia belonged to the class Clostridia – commonly known fermentation organisms. The most dominant genus within Clostridia was Acidaminococcus, which are anaerobic cocci that utilize amino acids, especially glutamic acid (Rogosa, 1969). Their amount increased significantly after the change of substrate from lactate to succinic acid (Fig. 5). The Postgate medium was supplemented with 1 g/L yeast extract, which contains glutamic acid, and has probably enhanced the growth of the Acidaminococcus spp. The genus is also able to produce propionate as a fermentation product (Shimizu et al., 2018). Another common family within Clostridia was Peptostreptococcaceae, including the genus Proteocatella. For example, P. sphensis was found to be a psychrotolerant organism able to ferment yeast extract (Pikuta et al., 2009). Most of the Bacteroidetes present in the enriched cultures belonged to the family Porphyromonadaceae, and one common genus detected within this family was Peludibacter. Propionate is one of their major fermentation end-products (Sakamoto, 2014) and most likely further utilized by the Desulfobulbus spp. Oxidation of ethanol to propionate by Acidaminococcus spp. or Porphyromonadaceae is very unlikely, which would explain why sulfate reduction was insignificant in the case of ethanol as carbon source. Since the enriched consortia included rather high amounts of different fermenting bacteria, utilization of more complex substrates by the consortia would be very likely and will be examined in further studies.

The most common genus within β-Proteobacteria was Dechloromonas. According to the literature, D. agitata utilizes acetate, lactate and succinate while reducing chloride, otherwise sulfide and ferrous iron can act as alternative electron donors for D. agitata and be oxidized to elemental sulfur and ferric iron oxide, respectively (Achenbach et al., 2001). Consequently, the change of substrate did not have an effect on the amount of Dechloromonas spp., but the genus was more abundant at 22 °C and the amount reduced significantly after cold acclimation. In addition to sulfate reducers, one common genus within δ-Proteobacteria was Geobacter. Usually Geobacter spp. reduce iron and other metals, even uranium (Anderson et al., 2003), and oxidize organic compounds and contaminants (Childers et al., 2002). For instance, G. sulfurreducens is known to oxidize acetate and reduce sulfur and iron (Caccavo et al., 1994). However, Geobacter spp. died out in the cultivations almost completely after the substrate change. Interesting genera were also found within the phylum Spirochaetes, including Sphaerchaeta spp., which are able to reduce ferrous iron (Ritalahti et al., 2012). They were found first only at 22 °C although the amount increased after the substrate change and was still detectable after cold acclimation (Fig. 5). Consequently, the consortia contained a high diversity of different anaerobic bacteria with specific functions in oxidizing and reducing the compounds of the cultivation media.

5. Conclusions

The enriched sulfate-reducing cultures were able to grow and reduce sulfate at as low as 6 °C with rates varying from 13 to 42 mg/L/d. As can be expected, the sulfate reduction rates were not as high as the values reported in continuously operated bioreactor experiments at low temperatures, but they give a promising start for further investigation. Interestingly, the cultures were not able to grow with ethanol as a carbon source. As an alternative, succinic acid was found to be an effective substrate for the cultivated consortia. The dominant SRB genus in all the cultivations was Desulfobulbus, which, as an incomplete oxidizer, produces acetate as an oxidation product. Acetate can be further utilized by other microorganisms in the consortia. Further research is required to find suitable low-cost carbon sources for the sulfate-reducing bacterial consortia enriched in this research. Use of a low growth temperature, an easily available low-cost carbon source and comprehensive utilization of substrate by the consortium make it possible to develop a cost-effective biological method for sulfate reduction. With further development, the enriched consortia could be applicable for sulfate management in cold mining waters.

Compliance with ethical standards

Declarations of interest

None.

Ethical approval

This article did not entail any studies using human participants or animals by any of the authors.

Availability of data and material

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

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