Isolation and application of siderophore producing bacteria from Finnish wetland samples for treatment of mining water effluents

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Abstract Microbial bioremediation is used for the treatment of mining wastewaters via binding of metals with extracellular siderophore molecules or via precipitation of metals by reducing sulfate to sulfides. In the present research wetland samples were screened for microbes that possess useful traits regarding such applications. Two isolates, identified as Pseudomonas sp., were investigated regarding their capacity for growth and siderophore production under various substrates. The isolates were immobilized on sawdust, and tested for the binding of nickel from aqueous solutions in batch experiments. Further research is addressed for the characterization and improvement of immobilization and metal binding mechanisms.

Key words bioremediation, mining effluent, wastewater treatment, biosorption, siderophore

Introduction

Metals belong to the most problematic pollutants released by the mining industry, causing a wide variety of health and environmental problems (Amini *et al.* 2008). Conventional methods for metal removal from mining effluents, such as chemical precipitation, electrochemical treatment, membrane separation, and adsorption on activated carbon are available. These methods often fail in treatment of large amounts of mining wastewaters with very low concentrations of pollutants. Further problems such as production of large amounts of demanding secondary pollutants and by-products whose further processing generates high costs are encountered.

Microbial bioremediation is one option to substitute or complement existing wastewater treatment technologies. Microbial cells may be used for direct binding or complexing of soluble metals. Complexing owes to specific low molecular weight (500–1500 Da) compounds excreted into surrounding environment by microbes, referred to as siderophores, exhibiting an extremely high affinity towards iron (K_f>10^{30}) (Hider and Kong 2010). As the concentration of bioavailable iron is very low in nature, siderophores have been evolved to fulfill the need for soluble iron critical for vital metabolic processes (Neilands 1981), and are therefore produced by majority of microbes (Hider and Kong 2010, Nejlands *et al.* 1987). Siderophores has been successfully used for bioremediation of contaminated soils by solubilizing metals (Ahmed and Holmström 2014). In wastewater treatment applications, complexes could be removed by solid liquid separation after binding or complexing the metal.

The removal of metals from solution may be enhanced by stimulating the siderophore production by applied microbes. This may be achieved via control of soluble iron, pH, temperature, nature of carbon and nitrogen source, availability of phosphorus and oxygen transfer (Villegas 2007). As the cultivation media affects largely the costs of microbial processes, it is especially advisable to seek for suitable inexpensive carbon and nitrogen sources.

The purpose of the research presented herein was to find efficient siderophore-producing microorganisms whose siderophores might be applicable in biosorption of metal-containing wastewaters. The aim was to isolate and characterize bacterial strains, and to assess their capability to produce siderophores under various carbon and nitrogen sources. Moreover, the applicability of isolated bacteria for bioremediation purposes was addressed in initial investigation of their immobilization on an inert carrier (sawdust) and use of the immobilized bacteria for removal of nickel from aqueous solution.

Methods

The soil samples were taken nearby Lake Pyykösjärvi, Oulu, Finland (coordinates 65.048533, 25.491656) in November 2016. Portion of soil was taken into an aseptic container and stored at +4 °C prior use. Approximately 1 g of soil was transferred to 10 mL of sterile 0.9% NaCl solution and the sample was shaken on the rotary shaker at the room temperature for 2 hours. The soil suspension was decanted to remove solid particles and 1 mL of solution was transferred to the aseptic Eppendorf – type tube. Subsequently, serial dilutions in 0.9% NaCl solution (100 µL of dilutions 10^{-5}–10^{-8}) of the sample were applied onto the Petri dishes containing SCA medium (Starch Casein Agar: 10.0 g soluble starch, 0.3 g casein, 2.0 g KNO_3, 0.05 g $MgSO_4 \cdot 7H_2O$, 2.0 g K_2HPO_4, 2.0 g NaCl, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 18.0 g agar L^{-1} H_2O) supplemented with 50 µg mL^{-1} nystatin to prevent growth of fungi and incubated at 27°C for 1 week. Finally two colonies were picked (referred later on to as Strains 1 and 2) for further research.

The strains were characterized by metagenome sequencing. A portion of the 16S small-subunit ribosomal gene was amplified with primers F519 (5-CAGCMGCCGCGGTAATWC-3) and R926 (5-CCGTCAATTCCTTTRAGTTT-3). The F519 primer contained an Ion Torrent adapter sequence A, a 9-bp unique barcode sequence, and one nucleotide linker. R926 primer contained an Ion Torrent adapter trP1 sequence. Polymerase chain reaction (PCR) assays were performed in 25-µL reactions in two 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: 28 cycles of 98 °C, 10 s; 64 °C, 20 s; 72 °C, 20 s. After PCR amplification reactions were purified using the AMPure XP reagent (Agencourt Bioscience, CA, USA). Amplicon concentration of purified samples was measured on a Bioanalyzer DNA-1000 chip (Agilent Technologies, CA, USA) and individual samples were pooled in equivalent amounts. A pooled sample was further purified with Ampure XP and sequencing was performed with Ion Torrent PGM on a 316 chip using Ion View chemistry (ThermoFisher Scientific, USA).

Various carbon and nitrogen sources were tested for both strains to find efficient and low-cost substrates to promote siderophore production. The carbon sources were glucose, glycerol, skimmed milk (lactose), fructose and succinic acid. Glutamic acid (L-Glu), asparagine (L-Asn· H_2O), urine and ammonium sulphate were investigated as nitrogen sources. Compositions of the media are given in Table 1. Strains were cultivated first for 2 days with iron supplement (0.01 g L^{-1} $FeSO_4$) after which the cell pellet was collected with centrifugation, washed three times with 0.9% NaCl and incubated for another 2 days with the same media without iron. Incubation was carried out in microtiter plates at room temperature (RT, approximately 22 °C). All the media components were obtained from Sigma – Aldrich (Saint Louis, USA) and were of molecular biology grade. After cultivation the supernatants were subjected to the evaluation of siderophore production efficiency using modified CAS assay initially described in Schwyn and Neilands (1987).

Table 1 *Media used in the assessment of carbon and nitrogen sources. The compound concentrations are presented as g L^{-1} unless otherwice stated.*

Comp.	G-ASN	G-GLU	GLY-ASN	GLY-GLU	M-ASN	M-GLU	F-ASN	F-GLU	G-U	SA
L-Asn· H_2O	2.0	-	2.0	-	2.0	-	2.0	-	-	-
L-Glu	-	2.0	-	2.0	-	2.0	-	2.0	1.0	-
Urea	-	-	-	-	-	-	-	-	0.85	-
$(NH_4)_2SO_4$	-	-	-	-	-	-	-	-	-	1.0
Glycerol [mL]	-	-	5.55	5.55	-	-	-	-	-	-
Glucose	7.0	-	-	-	-	-	-	-	10.0	-
Skimmed milk	-	-	-	-	7.0	7.0	-	-	-	-
Fructose	-	-	-	-	-	-	7.0	7.0	-	-
Succinic acid	-	-	-	-	-	-	-	-	-	4.0
K_2HPO_4	-	-	-	-	-	-	-	-	0.56	6.0
Na_2HPO_4	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	-	-
KH_2PO_4	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	-	3.0
$MgSO_4·7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

The immobilization of the strains on sawdust, and the capacity of immobilized cells to bind heavy metals were also initially investigated. Sawdust was selected as support material due to its weak metal binding capacity. The strains were first precultured overnight and then inoculated into subculture at initial optical density at 600 nm (OD_{600}) of approximately 0.001 in LB medium (Luria Bertoni broth: 10.0 g peptone, 5.0 g yeast extract, 10.0 g NaCl L^{-1} H_2O), in baffled shake flasks at RT and shaking 120 rpm (Infors Multitron, Infors HT). The growth was monitored by measuring the OD_{600} hourly. At OD_{600} of approximately 0.2–0.3,

i.e. at early exponential growth phase, a 100 mL portion of each subculture was transferred to a 0.05 g (0.5 g L^{-1} dosage) portion of autoclaved Scots pine (*Pinus sylvestris*) sawdust (a size fraction of 90–250 μm) and incubated at RT for 20 hrs. The adsorption of cells was assessed based on the OD_{600} of the supernatant before and after immobilization. The capacity of immobilized cells to bind nickel was studied by incubation of the suspension in presence of nickel at pH 6.1 (5 mg L^{-1}, prepared from $Ni(NO_3)_2$) at RT and 100 rpm shaking for 24 hours. The nickel reduction was analysed by inductively coupled plasma mass spectrometry (ICP-MS) according to standardized method SFS-EN ISO 17294-2:2005.

Results

Based on the sequencing results both strains represent *Pseudomonas* sp. which are common soil bacteria found in various ecological niches. They are especially known as adaptive bacteria with high diversity of iron uptake systems (Lin *et al.* 2002), which is a positive indication of their applicability for biosorption purposes.

Both strains were able to grow in all tested media, although differences were observed in the turbidity of cultures after 2 days of cultivation. However, it is known that medium appropriate for microorganism growth is not always the one in which siderophores production efficiency is the highest, and thus the interpretation of data was focused on the last mentioned (fig. 1). Regarding the production efficiency of siderophores, Strain 1 was superior as it was able to synthesize at least a low amount of siderophores in each media. However, large variations in siderophore production were observed between the provided carbon and nitrogen sources (tab. 1).

The highest siderophore productivity for Strain 1 was observed in media G-ASN, with glucose and asparagine as sole carbon and nitrogen sources, respectively. This is in line with previously reported observations (e.g. Duffy and Defago 1999), suggesting that glucose is a proper choice as carbon source for the purpose. It is, however, a relatively expensive substrate for any low-cost applications. Skimmed milk (lactose) was another well suitable carbon source. This finding is in line with our previous experience (data not published), and would be advantageous for the process economy. Based on literature fructose and succinic acid should also stimulate siderophore production by *Pseudomonas* sp. (Duffy and Defago 1999, Rachid and Ahmed 2005). This was not confirmed in the present research, although moderate siderophore production was observed by Strain 2 on fructose. Regarding the nitrogen sources, asparagine and glutamate were the most suitable substrates. It is unsurprising as amino acids generally stimulate siderophores biosynthesis. Siderophores synthesis was clearly weaker when other nitrogen sources were applied.

The possibility for successful immobilization of the cells is an important aspect for any bioremediation application of the isolated strains. Therefore, the immobilization of the strains was investigated using sawdust as carrier material, which is an inert and abundant lignocellulosic side stream, well suitable for the purpose (Obuekwe and Al-Muttawa 2001). In the initial experiments it was observed that after the sawdust addition the turbidity of the supernatants did not grow exponentially, as would be expected under exponential growth

phase, but the OD was only moderately increasing during the 2 hours incubation (increase of 1.2–1.4 -fold). This was assumedly due to successful immobilization of the cells. The future interest would be to find suitable low-cost carrier materials that would serve both as immobilization medium for cells and as a medium towards which metal-siderophore complex has a high affinity.

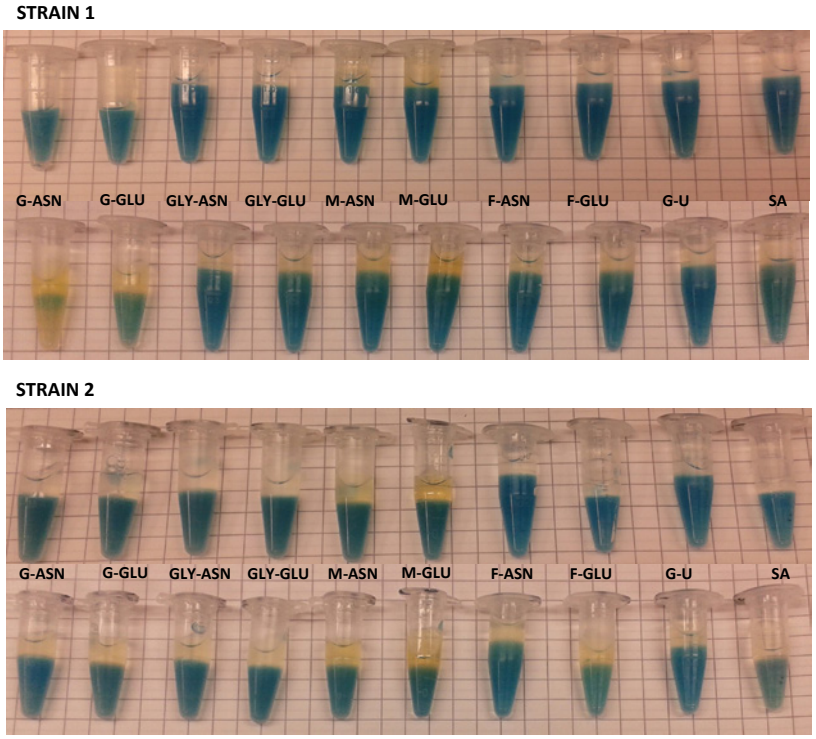

Figure 1 *CAS assays for Strains 1 and 2. References at the beginning of the assay and the colour changes after 24 hours are presented in upper and lower figures, respectively.*

Table 2 *Siderophore production efficiency of investigated strains in selected media based on colorimetric CAS assay. +++ high, ++ moderate, + weak, – no siderophores detected.*

Strain	G-ASN	G-GLU	GLY-ASN	GLY-GLU	M-ASN	M-GLU	F-ASN	F-GLU	G-U	SA
1	+++	++	+	+	++	++	+	+	+	+
2	+	+	+	+	+	-	+	++	+	+

After immobilization, the cell-sawdust –suspensions were used for the binding of nickel from aqueous solutions in order to make an initial assessment of their applicability in bioremediation. Strain 1 showed promising nickel binding ability, reducing its concentration from 5.11 mg L^{-1} to 4.69 and 4.72 in duplicate reactions (reduction by 7.9±0.41%). The CAS assay analysis performed on the treated solutions yielded weak positive results, indicating

the formation of metal-siderophore complexes in the treatment solution (data not shown). The sorption of metal-siderophore complexes is highly dependent on the pH and the medium used. For example, Karimzadeh *et al.* 2013 showed that the metal (Pb, Zn, Cd) sorption on zeolite was increased in the presence of siderophores under slightly acidic to neutral conditions. The future aim is to optimize the system and study the mechanism of nickel uptake.

Conclusions

The isolated *Pseudomonas* strains were able to grow and produce siderophores under various substrates. Regarding the siderophore production, the most promising results were achieved with Strain 1 when cultivated on glucose and asparagine as carbon and nitrogen sources, respectively. This result was confirmed in initial metal binding experiments, using Strain 1 cells immobilized on Scots pine sawdust for the removal of nickel from aqueous solution. The present results indicate potential of Strain 1 for the bioremediation purposes. Further development is focused on understanding and improvement of metal binding mechanisms in this system, to find the most suitable solid carrier material, evaluate proper dosages for the bacteria and carrier, and to develop feasible post-treatment method after metal binding.

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