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Competition of As and other Group 15 elements for surface binding sites of an extremophilic *Acidomyces acidophilus* isolated from a historical tin mining site

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Abstract

An arsenic-resistant fungal strain, designated WKC-1, was isolated from a waste roaster pile in a historical tin mine in Cornwall, UK and successfully identified to be *Acidomyces acidophilus* using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) proteomic-based biotyping approach. WKC-1 showed considerable resistance to As^{5+} and Sb^{5+} where the minimal inhibitory concentration (MIC) were 22500 mg L^{-1} and 100 mg L^{-1} respectively on Czapex-Dox Agar (CDA) medium; it was substantially more resistant to As^{5+} than the reference strains CBS 335.97 and CCF 4251. In a modified CDA medium containing 0.02 mg L^{-1} phosphate, WKC-1 was able to remove 70.30 % of As^{5+} (100 mg L^{-1}). Sorption experiment showed that the maximum capacity of As^{5+} uptake was 170.82 mg g^{-1} dry biomass as predicted by the Langmuir model. The presence of Sb^{5+} reduced the As^{5+} uptake by nearly 40%. Based on the Fourier-transform infrared spectroscopy

(FT-IR) analysis, we propose that Sb is competing with As for these sorption sites: OH, NH, CH, SO_3 and PO_4 on the fungal cell surface. To our knowledge, this is the first report on the impact of other Group 15 elements on the biosorption of As^{5+} in *Acidomyces acidophilus*.

Keywords

Acidomyces acidophilus, arsenic pollution, biosorption, bioremediation, MALDI-TOF/TOF-MS

Introduction

Due to the legacy of coal, tin and precious metals mining, abandoned mines constitute one of the most significant pollution hazards in Great Britain (Hudson-Edwards *et al*., 2008). Mining operations disposed residues, often with high levels of transitional metals and metalloids, in the mining sites and these were often dispersed by water and/or wind resulting in far-reaching pollution concerns (Asklund and Eldvall, 2005; Wang and Mulligan, 2006). The major sources for transitional metals and metalloids in the mining industries are milling, grinding, concentrating ores, disposal of tailings operations as well as milling wastewater discharge (Adriano, 1986; Razo *et al*., 2004). Roaster piles, tailing ponds and waste rock piles were some of the wastes left behind after mining operations ceased. Constant piling of such mine wastes resulted in an elevation of transitional metals and metalloids concentrations in the surrounding areas. The high soil contents of arsenic (As), iron (Fe), antimony (Sb) and zinc (Zn) and these have significant effect on the flora and fauna as well as human health (Dos Santos *et al*., 2013).

In natural environments, compounds of metalloids such as As and Sb are widely dispersed as a consequence of anthropogenic and geological activities. As and Sb are by-products of tin-mining activities during the smelting process, where As is primarily found in the arsenopyrite (FeAsS) form (Telford *et al*., 2009). The continuous disposal of arsenic trioxide (As_2O_3), a by-product in the furnace channel during the roasting process in tin mining activities, has been reported to cause serious contamination to surrounding soils and waters in proximity of mining sites. For instance, the concentration of As in soil adjacent to the Ron Phibun district tin mine in the Nakorn Si Thammarat province of southern Thailand was reported to be as high as 11000 mg kg^{-1} (Francesconi *et al.*, 2002). To fully appreciate the toxic effects transitional metals exert on biological systems, it is important to analyse their bioavailability by determining the uptake of these metals from soil by microorganisms within a given time span (Olaniran *et al*., 2013).

Geevor Tin Mine is a disused historical mine in the St Just mining district, one of the oldest mining districts in Cornwall (Yim, 1981). This tin mine was first established in the 1910s but due to the low global demand for tin and the high cost of operations, it was closed down in early 1990s. Upon closure of the mine, all the waste piles were abandoned on the site, the soil pollutants were contained and access to the site was restricted. According to Pirrie *et al.,* (2002), transitional metal and metalloids contamination is very common in Cornwall and it was estimated that approximately 1000 km^2 of Southwest of England are still contaminated with elevated concentrations of toxic metals and arsenic (Abrahams and Thornton, 1987; Camm *et al*., 2004; Van Veen *et al*., 2016). Metals bioavailability analysis of these soil samples will help to fully understand the actual amount of these metals available for uptake by microorganisms and their toxicity (Olaniran *et al*., 2013).

Biosorption using fungal biomass has been receiving attention from many researchers globally as an alternative method to remove heavy metal/metalloid(s) from contaminated water and soil. It offers many advantages such as high efficiency, reduced operating cost, minimal usage of chemicals and low production of toxic chemical sludge (Gadd, 2009; Vijayaraghavan *et al*., 2006). The transitional metals and metalloids present in the soil can be either already available or made available for uptake by microorganisms or plants, where they will be accessible for the sorption process (Peijnenburg and Jager, 2003; Del Giudice *et al*., 2013; Antonucci *et al*., 2017). It has also been established that ions from the same group in the periodic table could compete with each other during the biosorption process (Tsezos *et al*., 1996).

A number of extremophile fungi have been successfully isolated from adverse environmental conditions. One of the most well-known is *Acidomyces acidophilus*, formerly known as *Scytalidium acidophilum*, and also known as the black fungi. It is a pigmented ascomycete capable of growing in extremely acidic conditions (Sigler and Carmichael, 1974). Its melanin-containing cell walls offer the fungus protection from adverse environmental conditions such as extreme pH, temperature and toxins (Jacobson *et al*., 1995; Martin *et al*., 1990; Tetsch *et al*., 2006; Hujslová *et al*., 2013). This protection also provides the fungus a certain level of resistance to oxidative stress (Jung *et al*., 2006). The enzymes produced by this fungus are of great interests as they can function at low pH and high temperatures and could have potential applications for a variety of industries (Polizeli *et al*., 2005; Hess, 2008; Selbmann *et al*., 2008). So far, there are no reports on the use of *A. acidophilus* for metalloids bioremediation.

This paper reports the isolation and characterisation of a highly resistant *A. acidophilus* WKC-1 strain from the disused mine in Cornwall that can tolerate high levels of As^{5+}. The ability of this isolate to remove As^{5+} is being investigated and sorption analyses carried out to determine its maximum adsorption capacity. The influence of Sb^{5+} and PO_4^{3-} on this isolate's capacity to remove As^{5+} has been studied to provide a better understanding of the relationship between its As-resistance and the presence of other chemicals in soil. Finally, the potential of using resistant fungi to bioremediate metalloids from polluted soil in historical sites is discussed.

Materials and Methods

Site description

The Geevor Tin Mine is located in the St Just District, Cornwall at 50˚09' 06.43" N 5˚40' 34.96" S, in the Southwest of England. It was the only tin mining site in the district after the closure of Levant Mine in 1930 (Noall, 1973) and ceased its operation in 1991 (Camm *et al*., 2003). The site covers an area of 67 acres (270,000 m^2) and it is now on the European Route of Industrial Heritage sites, an important tourist attraction in Cornwall.

Soil sampling

Six sampling points were selected as shown in Figure S1. Approximately 1 kg of surface soil samples from a depth of up to 0.5 m were collected randomly from each sampling point into sampling bags using a sterile trowel and spade. The soil samples were transported in an insulated cool box at 4 °C back to the laboratory within 24 h and stored in a refrigerator.

Soil analysis

Soil samples were air-dried for 72 h, ground finely using a pestle and mortar and sieved through a 2 mm sterile mesh prior to analysis. The pH of the soil samples, suspended in deionised water (soil:deionised water 1:2 w/v), was measured using a calibrated pH meter (Jenway, Model 3505). The soil organic matter (OM) content was determined using the ASTM (American Society of Testing and Materials) standard procedure (ASTM, 2000) and the cation exchange capacity (CEC) was analysed using the protocol recommended by Gillman and Sumpter (1986). The concentrations of As and Sb in each of the six soil samples were analysed using a three-step sequential extraction method for exchangeable (F1), weakly bound organic bound (F2) and residual (F3) fractions (Carapeto and Purchase, 2000). All the extracts (F1, F2 and F3) were analysed using inductively coupled plasma optical emission spectrometry iCAP 1600 (ICP-OES) and the operating parameters summarised in Table S1. All the analyses were carried out in triplicates and the ICP-OES generated three readings per analysis. The percentage of bioavailability of both As and Sb was calculated by division of the summed fractions 1 and 2 by the total (F1+F2+F3) of each metalloid from the three-step sequential extraction. For analytical accuracy, the percentage recoveries (R) of all soil trace elements of interest were performed in soil certified reference materials (CRM) (#SQC001, lot 011233 and lot 017309, RTC, Laramie, WY, USA).

Enumeration and isolation of arsenic-tolerant fungi

Soil samples containing high As and Sb concentrations were used for screening of arsenic-resistant fungi. A ten-fold serial dilution was carried out using one gram of soil sample and plated out on to 2% malt extract agar (MEA; Oxoid Ltd., UK), supplemented with 100 mg L^{-1} of chloramphenicol to prevent bacteria growth. The inoculated plates were incubated for 7 – 21 days at 25 °C and fungal viable counts determined. Colonies were sub-cultured, purified by passaging for ten times, screened in 2 % MEA at pH 1 containing As^{5+} (1000 – 25000 mg L^{-1}), prepared from sodium arsenate (Na_2HAsO_4). Fungal strains that survived the highest As-stress were considered as a potential candidate and maintained using the same MEA conditions with or without 100mg L^{-1} of As^{5+}.

Molecular identification of isolated fungi

Fungal isolates were grown on Potato Dextrose Agar (PDA) (CM0139, Oxoid Ltd, UK), pH 1 at 25 °C for 21 days. Mycelia were collected by pipetting Triton X-100 on the same colony spot for several times and transferring into a sterile tube. DNA was extracted using cetyl trimethylammonium bromide (CTAB) following the protocol by Stirling (2003) with a minor modification, DNA extraction was carried out twice on the samples at 65 °C for 50 min followed by bead milling. The extracted DNA was dissolved in 20 μl ultrapure water and stored at 4 °C.

The internal transcribed spacer (ITS) nuclear region of 18S-ITS1-5.8S-ITS2-28S rRNA of the fungal isolate was amplified by PCR using three sets of primers based on published sequences (White *et al*., 1990; Martin and Rygiewicz, 2005). The first PCR used ITS1 forward and ITS2 reverse primers, the second used ITS5 forward and ITS4 reverse primers and the third used ITS1 forward and ITS4 reverse primers, all were obtained from Sigma-Aldrich and PCR amplifications performed (ITS1-ITS2 PCR: 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 63 °C for 2 min, 72 °C for 1 min; followed by 72 °C for 10 min; ITS5-ITS4 and ITS1-ITS4 PCRs: 94 °C for 4 min;

30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min; followed by 72 °C for 10 min). The PCR products were analysed by 2.0% (w/v) agarose gel electrophoresis and capillary electrophoresis on the MCE-202 MultiNA system (Shimadzu) in "on-tip analysis" mode following the protocol recommended by the manufacturer using the DNA 1000 reagent kit (Shimadzu) to quantify their concentrations and to confirm the results of the agarose gel electrophoresis. The PCR products were sequenced by GATC biotech (London, UK) and sequences analysed by nucleotide BLAST (NCBI) analysis. Based on the generated DNA sequences of the isolate and other reference sequences of fungi obtained from NCBI databases, a phylogenetic dendrogram from the evolutionary distance via the neighbour-joining method was constructed using bootstrap method of 1000 replications using Molecular Evolutionary Genetics Analysis 6 (MEGA6) software (Tamura *et al*., 2013).

Proteomics identification of fungal strain using MALDI-TOF/TOF MS

Three reference strains of *A. acidophilus* were obtained from Centraalbureau Schimmelcultures (CBS), Netherlands (strain CBS 335.97) and Culture Collection of Fungi (CCF), Czech Republic (strains CCF4251 and CCF3679). Reference strains and the isolated fungal strain were grown in liquid salt medium (LSM) containing 2% dextrose, 0.1% $(NH_4)_2SO_4$, 0.001% K_2HPO_4, 0.05% $MgSO_4 \times 7 H_2O$, 0.0026% $FeSO_4$ and 0.008% $CaCl_2$ with a final pH of 4.0-4.2 using a horizontal rotator (SB2 rotator, Stuart) for 72 h at room temperature. The sample preparation and extraction of proteins and peptides of *A. acidophilus* and the three reference strains were performed according to the Bruker fungi sample preparation protocol. Each extracted sample was analysed using a MALDI ground steel plate and six different sample spots (replicates) to generate six combined mass spectra (MSP) per fungal isolate. The reference strains for *A. acidophilus* CBS 335.97, CCF4251 and CCF3679 were analysed to generate reference spectra and used to create an in house supplementary new database library for *A. acidophilus* fungal strains identification. The identification of the isolated fungal strain through comparison with reference strains and visualization of the mass spectra was performed with MALDI Biotyper software 3.0 (Bruker Daltonics).

Determination of As minimum inhibitory concentration (MIC)

The MIC for the isolated fungal strain and two *A. acidophilus* reference strains (CBS 335.97 and CCF 4251) were determined using solid acidic culture medium of modified Czapek dox agar (CDA) with either 1 mg L^{-1} or 100 mg L^{-1} PO_4^{3-} at pH 1, containing As^{5+} concentrations ranged from 1000 to 25000 mg L^{-1}. To allow polymerization of agar in culture medium at pH 1, double concentration of agar was added, and pH was adjusted after sterilization. The fungal mycelia plugs were removed using a sterile pipette and placed in the middle of the agar. The plates were incubated for 21 days at 25 °C and the diameter of the each of the fungal colony was measured and MIC calculated from the average of the triplicate results.

Analysis of pH-effect on *A. acidophilus* WKC-1 growth

The effect of pH on the isolated *A. acidophilus* WKC-1 was determined using solid culture medium of MEA containing 1000 mg L^{-1} of As^{5+} with pH ranged from 0.5 to 5, the desired pH was adjusted using NaOH (0.1M) or HCl (0.1M). The plates were incubated for 21 days at 25 °C and the diameter of the each of the fungal colony was recorded.

Arsenic removal efficiency

The efficiency of arsenic removal by *A. acidophilus* WKC-1 and three *A. acidophilus* reference strains were studied in a 0.15 mL centrifugal tube using LSM containing 1 g L^{-1} of viable wet fungal biomass in pH 1 and supplemented with 100 mg L^{-1} As^{5+}, the cultures were cultivated using a horizontal rotator (SB2 rotator, Stuart) at 120 rpm for 21 days at room temperature and the final concentration As^{5+} in each filtrate was measured every 7 days using ICP-OES. All experiments were carried out in triplicates. The arsenic removal efficiency by all studied *A. acidophilus* strains was calculated using the following equation:

$$R = [(C_i - C_f) / C_i] \text{ x } 100$$

where:

R = Percentage As^{5+} removal;

C_i = Initial concentration of As^{5+} (mg L^{-1});

C_f = Final concentration of As^{5+} (mg L^{-1}) after 21 days.

Biosorbent preparation and analysis of As biosorption

The *A. acidophilus* WKC-1 was inoculated in LSM for 21 days at 25 °C with constant shaking at 110 rpm using an orbital shaker (Minitron, Infors HT). The fungal biomass was harvested by filtration through Whatman No.11 filter paper, cleaned three times with deionised water to ensure the removal of all the excessive media residuals, freeze-dried (ScanVac CoolSafe, Labogene) for 24 h and grounded in mortar and pestle to fine powder. Each of the 1000 mg L^{-1} As^{5+} and Sb^{5+} stock solution was prepared by dissolving $Na_2HAsO_4 \times 7\ H_2O$ and $KSb(OH)_6$ (Sigma-Aldrich) in deionised water.

All adsorption tests were carried out in 50 mL conical flasks containing 20 mL of As^{5+} and/or Sb^{5+} solution at 25 °C on an orbital shaker at 120 rpm. Biosorption isotherms were formulated through investigating the effect of pH and biomass loading capacity on the fungal cell as previously performed by Xu *et al*. (2012). In order to identify the pH effect on As^{5+} biosorption, two sets of experiments were carried out. Firstly, biosorption using fixed 0.5 g L^{-1} dried fungal biomass in a range of As^{5+} (100 - 600 mg L^{-1}) and different pH range (1.0 - 6.0) was examined. Secondly, biosorption of a range of fungal biomass (0.5 - 5 g L^{-1}) using fix concentration of As^{5+} (500 mg L^{-1}) at different pH range was investigated. In order to investigate optimum contact time for the biosorption of As^{5+}, samples were collected at different times (5, 15, 30, 60, 120 and 180 min) and filtered through Whatman No.11 filter paper. All filtrates were analysed for residual of As^{5+} concentration using ICP-OES. The uptake of As^{5+} by *A. acidophilus* WKC-1 was calculated using the following equation:

$$q_{eq} = \frac{V\,(c_i - c_{eq})}{m}$$

where:

q_{eq} = As^{5+} uptake in mg per g biomass;

V = Volume of As^{5+} used in mL;

c_i = Initial concentration of As^{5+} (mg L^{-1});

c_{eq} = Equilibrium concentration of As^{5+} (mg L^{-1});

m = Amount of dry biosorbent (g).

FT-IR studies

The detection of vibration frequency changes in *A. acidophilus* WKC-1 for the untreated and As/Sb-treated biomass samples before and after the As^{5+} and Sb^{5+} biosorption were analysed using Fourier transform infrared spectroscopy (Travel IR, Perkin Elmer) and the attenuated total reflection (ATR) technique in the same experimental conditions as described for the biosorption experiment. Each biomass was freeze-dried and was placed on the single reflection diamond ATR crystal. The infra-red spectra were collected using the ATR-FTIR ranged from 400 to 4000 cm^{-1} (Guibaud *et al*., 2003).

Statistical analyses

All the experiments were performed in triplicates and the data obtained were calculated as mean plus/minus standard errors (mean ± SE). The statistical analysis on the difference in percentage of the bioavailability of As and Sb in soil samples was performed using 2-sample t-test. The As removal was compared between the isolated *A. acidophilus* WKC-1 strain and two *A. acidophilus* reference strains (CBS 335.97 and CCF 4521) using analysis of variance (one-way ANOVA). All the statistical analyses were performed using Minitab version 16.

Results

Physical and chemical properties of the soil samples

Results showed that all of the soil samples were acidic and the pH range varied sites with soil sample from site 3 being the most acidic (pH 1.13) and site 5 being the least acidic (pH 5.25). The highest and lowest CEC were 32.14 ± 4.31 meq/100g for soil sample 3 and 12.09 ± 3.71 meq/100g for soil sample 4 respectively, the mean CEC for all soil samples was 19.96 ± 3.57. The highest and lowest OM contents were observed in soil samples 1 with 14.60 % and 4 with 3.34 % respectively. Results for the chemical and physical characterisation of the soil samples are summarised in Table 1.

Quality control data on the recovery of metal/metalloid(s) is shown in Table S2. The results showed good recovery with percentage recovery of As and Sb of more than 92% and 84% respectively using the two different certified metals in soil reference materials.

Table 1: Chemical and physical characteristics of soil samples from Geevor Tin Mine.

Site	Textural class	pH	% OM	CEC (meq/100 g)
1	Fine sand	3.75 ± 0.14	14.60%	19.85 ± 2.79
2	Fine sand	3.11 ± 0.07	13.55%	25.72 ± 4.16
3	Medium sand	1.13 ± 0.06	7.15%	32.14 ± 4.31
4	Clay	5.22 ± 0.12	3.34%	12.09 ± 3.71
5	Coarse sand	5.25 ± 0.09	10.68%	13.74 ± 3.08
6	Medium sand	3.26 ± 0.12	4.40%	16.22 ± 3.44

The total metal/metalloid concentration analysis showed that As levels exceeded those of Sb in all sampling sites (Table 2). The highest concentrations of As (18043.50 mg kg^{-1}) and Sb (213.69 mg kg^{-1}) were detected in soil sample 3, collected from the location of the roaster pile. Arsenic levels in all soil samples exceeded the UK Category 4 Screening Levels (C4SL) for commercial site of 640 mg kg^{-1} (Defra, 2014). The As concentration in soil sample from site 3 (obtained from the main roaster dump pile) exceeded the C4SL for the organically bound fractions (4511 mg kg^{-}

[1]; Table 2). Since currently there is no C4SL values for Sb, the Dutch Guideline intervention values for soil remediation (Dutch Environment Ministry, 2013) was used to assess the extent of contamination (15 mg kg^{-1}). Only two soil samples (site 2 and site 3) were found to exceed the intervention limit.

The average percentages bioavailability of As and Sb in the soil samples from all six sites are presented in Figure 1. The bioavailability of As and Sb in all the soil samples were below 50%. However, the percentage of bioavailability of As was significantly higher than Sb in soil sample collected at site 3.

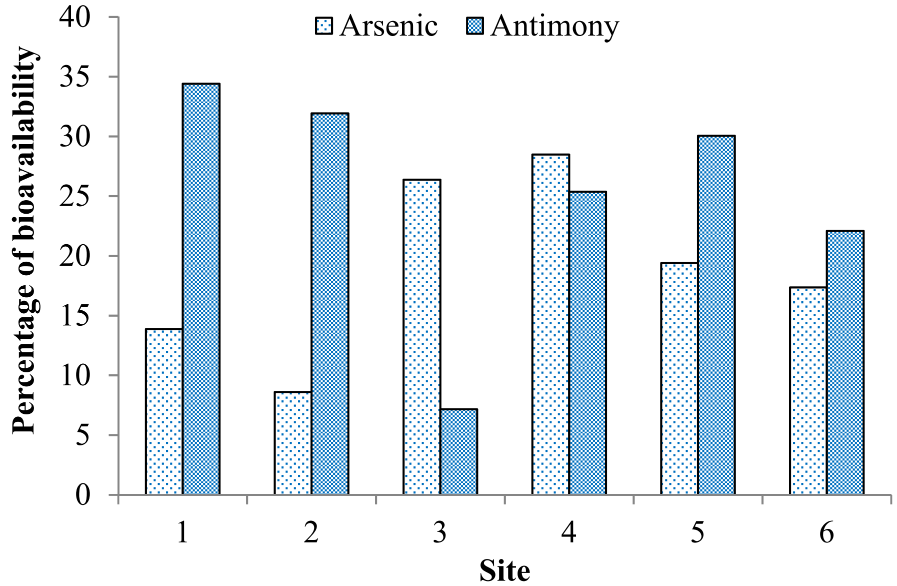

Figure 1: Percentage bioavailability of arsenic and antimony of soil from the sampling sites obtained from the summation of fraction 1 and 2 of the three-step sequential extraction.

Table 2: Mean As and Sb concentration (mg kg^{-1} ± standard error) in the soil samples from the sampling sites using the three-step sequential extraction method. Data shown are the mean of three replicates.

Element	**As**				**Sb**			
Site	F1	F2	F3	∑(F1 + F2 +F3)	F1	F2	F3	∑(F1 + F2 +F3)
1	1.13 ± 0.17	300.48 ± 7.41	1872.39 ± 44.22	2174.00 * +	0.10 ± 0.02	2.34 ± 0.88	4.65 ± 1.01	7.09
2	0.66 ± 0.09	485.20 ± 1.54	5157.31 ± 17.38	5643.17 * +	0.96 ± 0.09	14.24 ± 2.19	32.4 ± 2.08	47.60 +
3	249.20 ± 6.00	4511.30 ± 78.70	13283.00 ± 42.80	18043.50 * +	1.33 ± 0.03	13.96 ± 1.03	198.40± 4.03	213.69 +
4	2.17 ± 0.08	325.60 ± 16.79	822.88 ± 21.02	1150.65 * +	0.12 ± 0.02	0.923 ± 0.14	3.067 ± 0.17	4.11
5	11.64 ± 0.13	298.24 ± 5.10	1488.13 ± 18.98	1598.04 * +	ND	1.01 ± 0.22	2.35 ± 0.087	3.36
6	0.28 ± 0.07	200.98 ± 3.81	955.82 ± 5.41	1158.88 * +	ND	2.39 ± 0.64	8.43 ± 0.66	10.82

F1 Fraction 1 (exchangeable fraction), *F2* Fraction 2 (organically bound fraction), *F3* Fraction 3 (residual fraction), *ND* not detectable.

* Indicates exceeded the guideline values set by UK C4SL (for commercial site) and + indicates exceeded the intervention value limit set by the Dutch Guideline (Dutch Environment Ministry., 2013).

Identification and isolation of fungal strains

A total of 31 strains were isolated from soil samples collected from six different locations were exposed to As^{5+} ranged from 1000 to 22500 mg L^{-1}. Only one fungus from the most acidic and polluted soil in site 3 was able to grow on the medium containing the highest As^{5+} concentration (Table 3). It was selected for identification and further experiments.

The colony and micro-morphological features of the isolated fungal strain WKC-1 which was highly resistant to arsenic are presented in Figure S2 This strain was slow growing, achieving diameters of 22 to 45 mm in 21 days at 25 °C. The colonies appeared compact and dark greenish in colour. Under the microscope, the mycelium composed of septate, scarcely branched with thick-walled hyphae.

The ITS rDNA sequence of the fungal isolate WKC-1 found in soil 3, conforms to phylogenetic lineage identical to the species *Acidomyces acidophilus,* CBS 335.97 (ex-type AJ 244237.1, FJ430711), which has previously been isolated from various highly acidic environments (Selbmann *et al*., 2008; Hujslová *et al*., 2013). The isolated fungal strain is designated *A. acidophilus* strain WKC-1 and has been given a GenBank accession number, KT727926 and the strain is deposited in DSMZ, Germany (DSM 105253) (Figure 2).

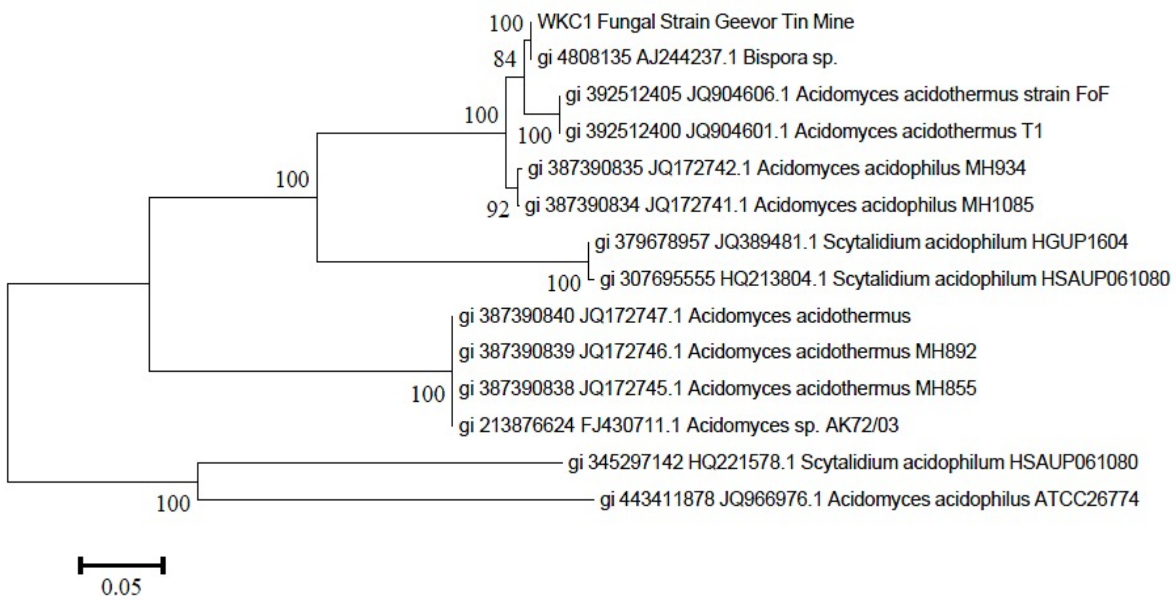

Figure 2: Phylogenetic dendrogram (scale bar = 5 represented nucleotide substitutions per 100 nucleotides. Numbers given at the nodes represent bootstrap values of 1000 replications).

Prior to the analysis of the isolated WKC-1 strain by MALDI TOF/TOF MS, the mass spectra of the three *A. acidophilus* reference strains were generated and inserted to the in-house database to create an *A. acidophilus* database library, since there is currently no database available for this species. The identification of *A. acidophilus* WKC-1 against three *A. acidophilus* reference strains showed that the isolated WKC-1 strain belongs to the *A. acidophilus* species with highly probable species identification to CBS 335.97 strain followed by secure genus identification against CCF 4251 and CCF 3679 strains (Figure 3).

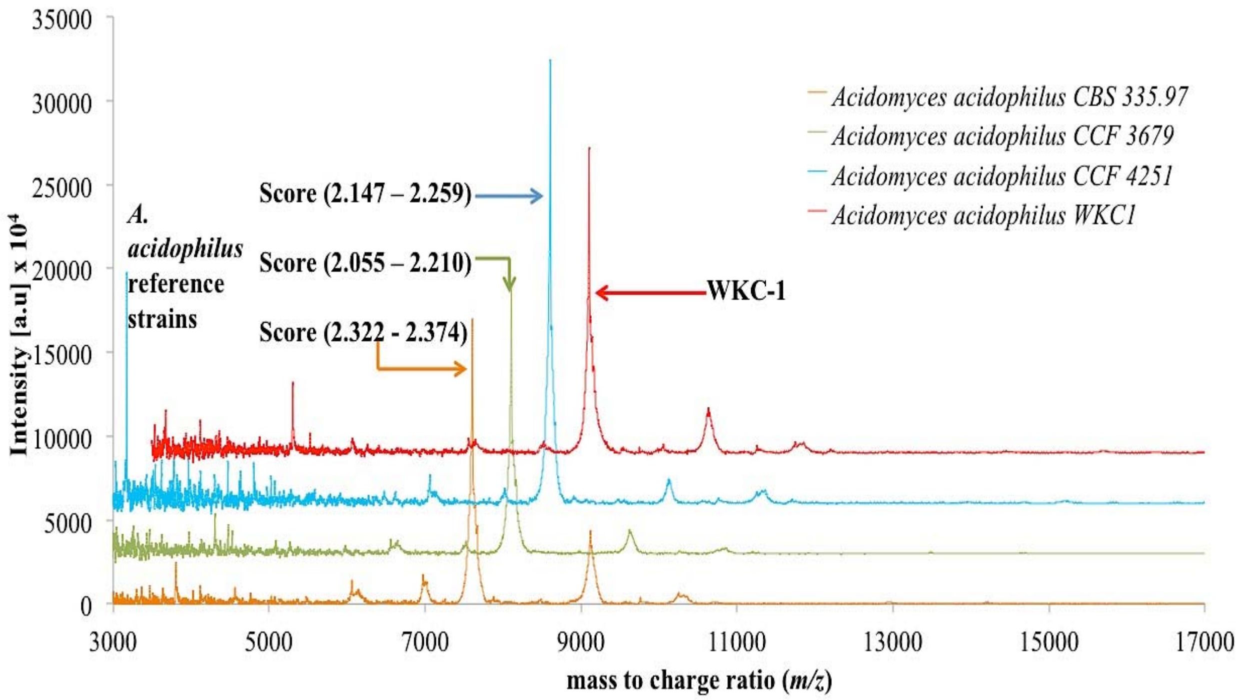

Figure 3: Representation of mass spectra of isolated fungal strain WKC-1 and three *A. acidophilus* reference strains using MALDI-TOF/TOF MS.

*2.300-3.000 indicates high probable species identification; 2.000-2.299 indicates secure genus identification.

Table 3: The minimum inhibitory concentration (cm ± standard error) of As^{5+} by *A. acidophilus* WKC-1 and the effect of low and high phosphate concentration. Data shown are the mean of three replicates.

Concentration of As^{5+}	Colony diameter (cm)	
	Medium containing 1 mg L^{-1} of PO_4^{3-}	Medium containing 100 mg L^{-1} of PO_4^{3-}
0	4.7 ± 0.3	5.3 ± 0.3***
1000	4.5 ± 0.2	4.9 ± 0.2**
7500	4.1 ± 0.2	4.5 ± 0.2*
15000	3.7 ± 0.2	4.3 ± 0.1***
20000	2.7 ± 0.1	4.0 ± 0.3***
22500	2.2 ± 0.2	3.6 ± 0.2***

Asterisks indicate statistical significance of differences tested by 2-sample t-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to *A. acidophilus* WKC-1 containing 1 mg L^{-1} of PO_4^{3-}.

Minimum inhibitory concentration (MIC) of As for *A. acidophilus*

The MIC at pH 1 of isolated *A. acidophilus* WKC-1 reflects an extremely high tolerance for arsenate, the strain could tolerate up to 22500 mg kg^{-1} of As^{5+} in solid media. Two reference strains of *A. acidophilus* (CBS 335.97 and CCF 4251) were tested for their tolerance to As^{5+} and found to tolerate up to 10000 and 7500 mg L^{-1} of As^{5+}, respectively 2.5 times lower than the MIC of the isolated WKC-1 strain (Table S3).

The CDA media containing 100 mg L^{-1} of phosphate did have an effect on As^{5+} growth profile, which resulted in increased resistance to As^{5+} (Table 3). The MIC between *A. acidophilus* WKC-1 grown with 1 mg L^{-1} of PO_4^{3-} and 100 mg L^{-1} of PO_4^{3-} showed a statistical significant difference in all media containing As^{5+} concentrations ranging from 1000 mg L^{-1} to 22500 mg L^{-1} ($p<0.05$).

Effect of pH on fungal growth

Figure 4 presents the effect of pH on the growth characteristics of *A. acidophilus* WKC-1 colonies. The diameter of colony growth appeared to decrease as the pH increased and the *A. acidophilus* WKC-1 strain can grow in extremely low pH of 1.

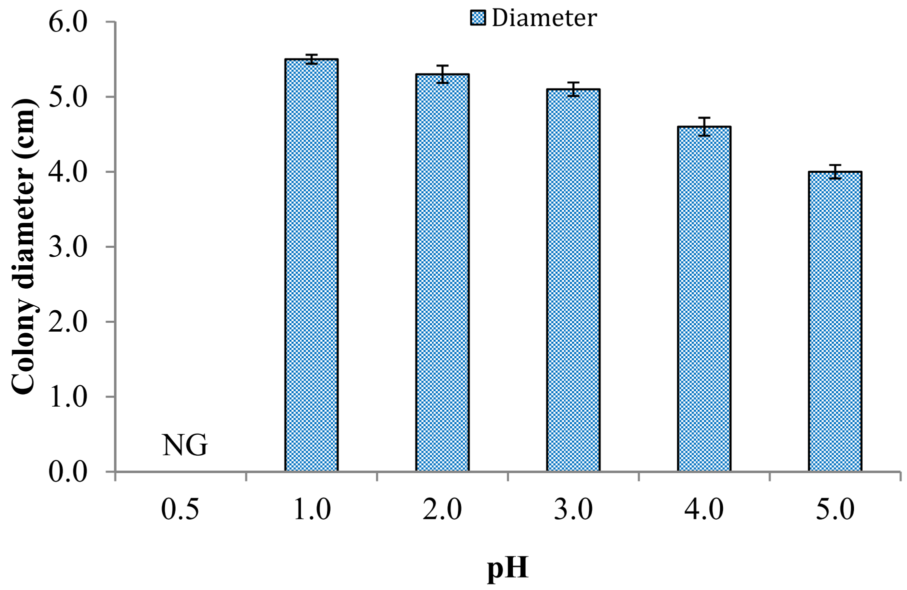

Figure 4: Colony diameter, representing a measurement of growth (cm ± standard error) of isolated *A. acidophilus* WKC-1 at the minimum inhibitory concentration of As^{5+}, at different pH, at room temperature, on MEA. Data shown are the mean of three replicates.

* NG *indicates no growth*

Arsenate removal efficiency

In Figure 5, the mean percentages of arsenic removal by *A. acidophilus* WKC-1 and three *A. acidophilus* reference strains show that WKC-1 achieves a significantly higher percentage As^{5+} removal after 7, 14 and 21 days periods of cultivation compared to the *A. acidophilus* CBS 335.97, CCF4251 and CCF3679 reference strains.

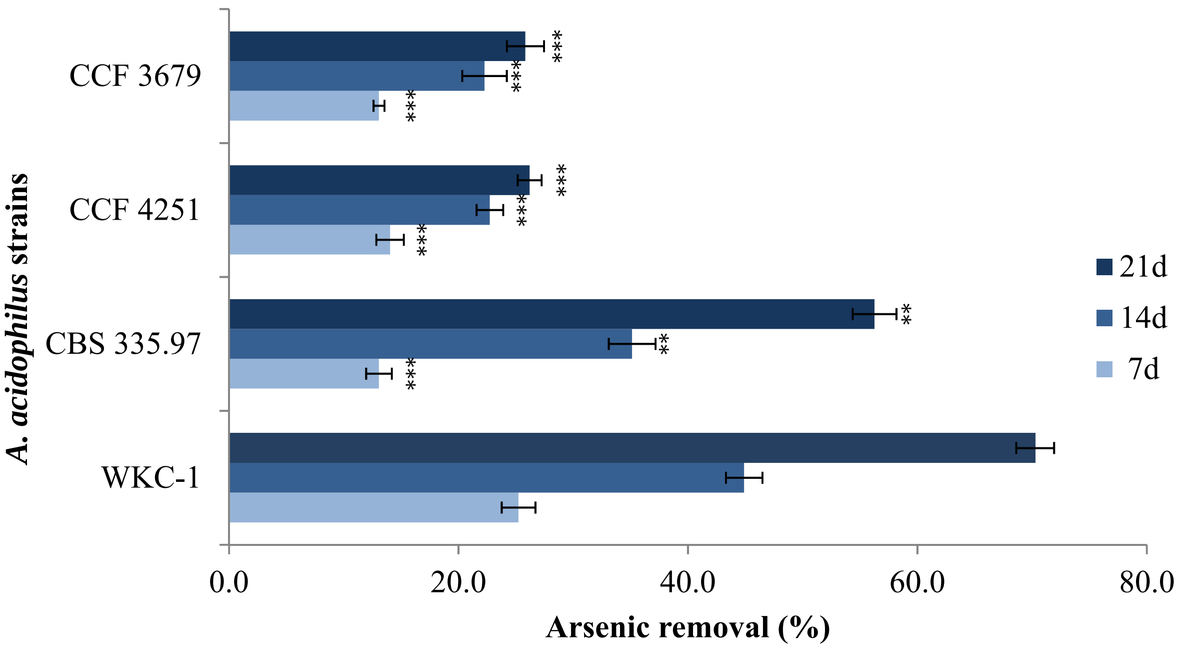

Figure 5: Percentage of arsenate removal by *A. acidophilus* WKC-1 and *A. acidophilus* reference strains after 7, 14 and 21 days cultivations of initial arsenate concentration of 100 mg L^{-1}. The error bars indicate the standard error of the mean of three replicates. Asterisks indicate statistical significance of differences tested by ANOVA where ** $p < 0.01$, *** $p < 0.001$ compared to *A. acidophilus* WKC-1.

There is a significant difference in As removal between the cultivation days ($p<0.001$) for all four strains except for *A. acidophilus* CCF 4251, where there is no significant difference between 14 days and 21 days cultivation. The percentage removal of As^{5+} by *A. acidophilus* WKC-1 is 70.30 % after 21 days of cultivation compared to 56.30 %, 26.20 % and 25.80 % achieved with the reference strains CBS 335.97 and CCF 4251 and CCF 3679 respectively.

Biosorption of As

The summary of the effect of initial pH in the As^{5+} solution on the biosorption process of As^{5+} by *A. acidophilus* WKC-1 showed that there was an increase from 0.01 to 0.09 mg mg^{-1} of the amount of As^{5+} absorbed by isolated *A. acidophilus* WKC-1 as the pH increased from 1.0 to 4.0 (Figure 6a). However, as the uptake started to decrease above pH 4.0, the optimum pH for the biosorption analysis of As^{5+} was set at pH 4.0.

The biomass loading with increased contact time was studied and it was found that the absorption of As^{5+} rapidly increased in the first 30 min (Figure 6b). After 120 min, the sorption of As^{5+} by *A. acidophilus* WKC-1 reached equilibrium and remained constant ($p > 0.05$). Therefore, the time for the biosorption analysis for both As^{5+} and Sb^{5+} loaded biomass was set at 120 min. The effect of biomass loading is summarized in Figure 6b. The sorption capacity by *A. acidophilus* decreased as the biomass loading increased from 1g L^{-1} to 5.0 g L^{-1}. In the presence of competing Sb^{5+} ions, the As^{5+} uptake by fungal biomass is significantly affected ($p<0.05$) as shown in Figure 6c.

The relationship between metalloid uptake capacity q_e, and equilibrium metal ion concentration C_e, was evaluated based on the Langmuir model. The data from current study fitted the Langmuir isotherm model well, with regression coefficient (R^2) of 0.989 (Figure 6d). Small *b* values (0.01) imply strong binding of arsenic ions to *A. acidophilus* WKC-1. The predicted maximum capacity of fungal strain uptake of As^{5+} by *A. acidophilus* WKC-1 was 170.82 mg g^{-1} dry biomass.

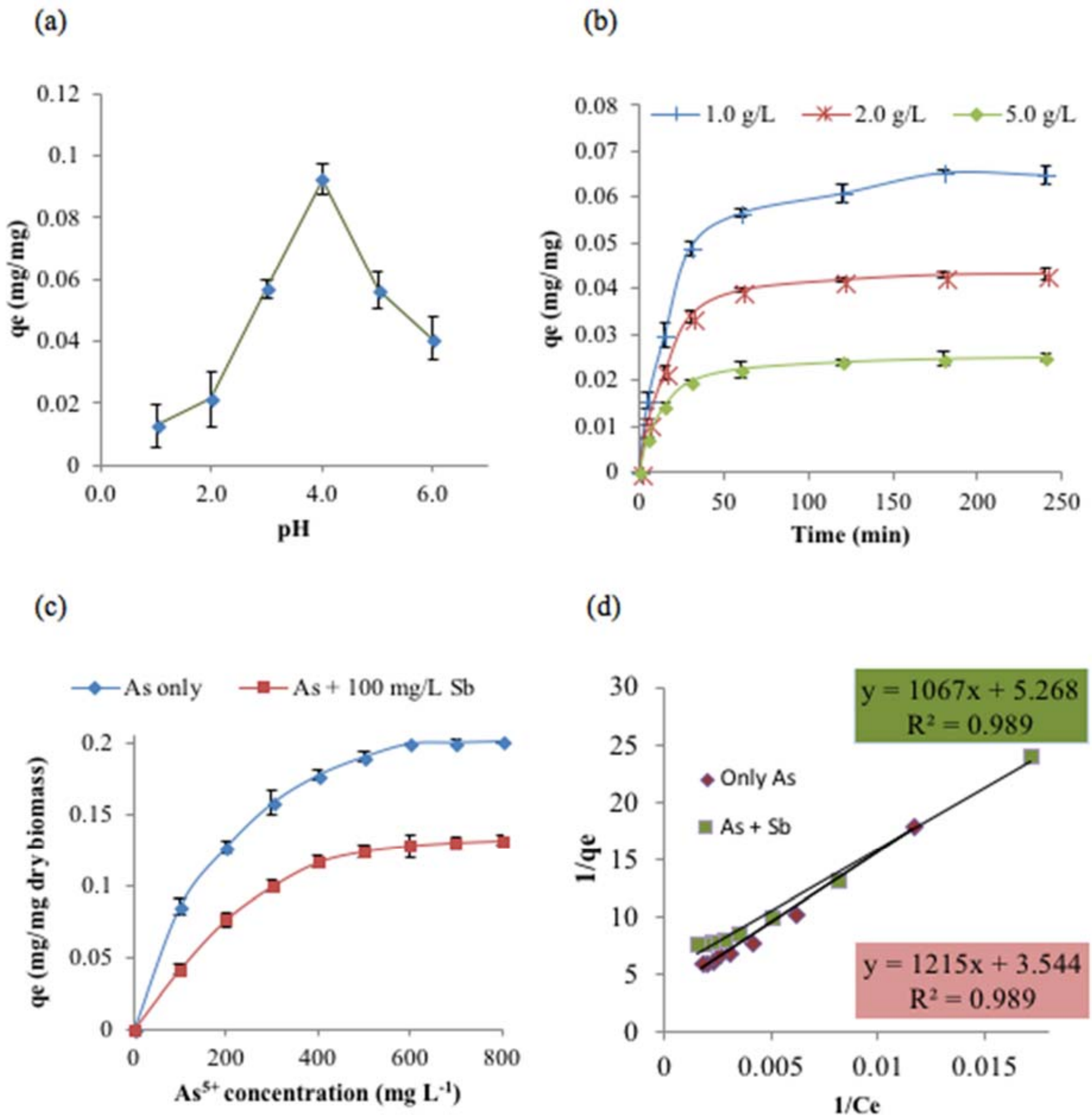

Figure 6. Biosorption of As^{5+} by *A. acidophilus* WKC-1; (a) the effects of pH on biomass loading; (b) the effect of contact time on As^{5+} biosorption at different concentration; (c) the effect of As^{5+} uptake in the presence and absence of Sb^{5+}; and (d) the Langmuir isotherm plot of As^{5+} biosorption by *A. acidophilus* WKC-1 in the presence and absence of As^{5+}.

FT-IR analysis

FT-IR spectrum range of 4000-400 cm^{-1} was used to detect vibration frequency of changes in the functional group of isolated *A. acidophilus* strain before and after As^{5+} and Sb^{5+} loading (Figure 7). For control biomass spectrum (vibrational frequencies of bio-molecular functional groups), a broad band at 3306.55 cm^{-1} indicates -OH bonds stretching vibration at high concentration and weak to medium of the -NH stretching (secondary amines). The peaks appearing in the 2921.74 and 2852.67 cm^{-1} region can be attributed to the strong asymmetric and symmetric stretching vibration of CH_2, respectively. Strong stretching vibrations of C=O (esters) and C=O (amide I band) observed at peak 1744.18 and 1640.06 cm^{-1} respectively. The peak at 1640.06 cm^{-1} also indicated variable symmetric stretching variations of C=C. The peak at 1544.68 cm^{-1} was assigned to a motion of -NH bending (amide II) while the peak at 1456.46 cm^{-1} indicated medium CH_2 and CH_3 deformation. O-H bending (in-plane) and strong stretching vibrations of C-F appeared at the peak 1373.94 cm^{-1}. Medium to strong stretching vibrations of C-O and medium C-N stretching of amine groups was observed at both 1238.95 and 1148.40 cm^{-1} peak.

A strong peak at 1028.03 cm^{-1} indicates P-OR (esters) as well as Si-OR groups. A NH_2 and N-H wagging (shifts on H-bonding), C-H bending and ring puckering and a strong =C-H & =CH_2 bending was observed at peak 887.86 cm^{-1}. The 'finger print' zone of the spectra, ranging from 500-700 cm^{-1} usually represents phosphate or sulfur functional groups.

For both As^{5+} and Sb^{5+} loaded spectra, significant shifts (weak/strong) were observed at absorbance peaks 3306.55 cm^{-1}, 3003.79 cm^{-1}, 1373.94 cm^{-1}, 1028.03 cm^{-1} and 612.58 cm^{-1} either by stretching vibrations, formation of new absorbance peaks and sharpening or lowering of the shoulder peaks. These are the functional groups of -OH, -NH, -CH, $-SO_3$, P-OR(esters) and PO_4.

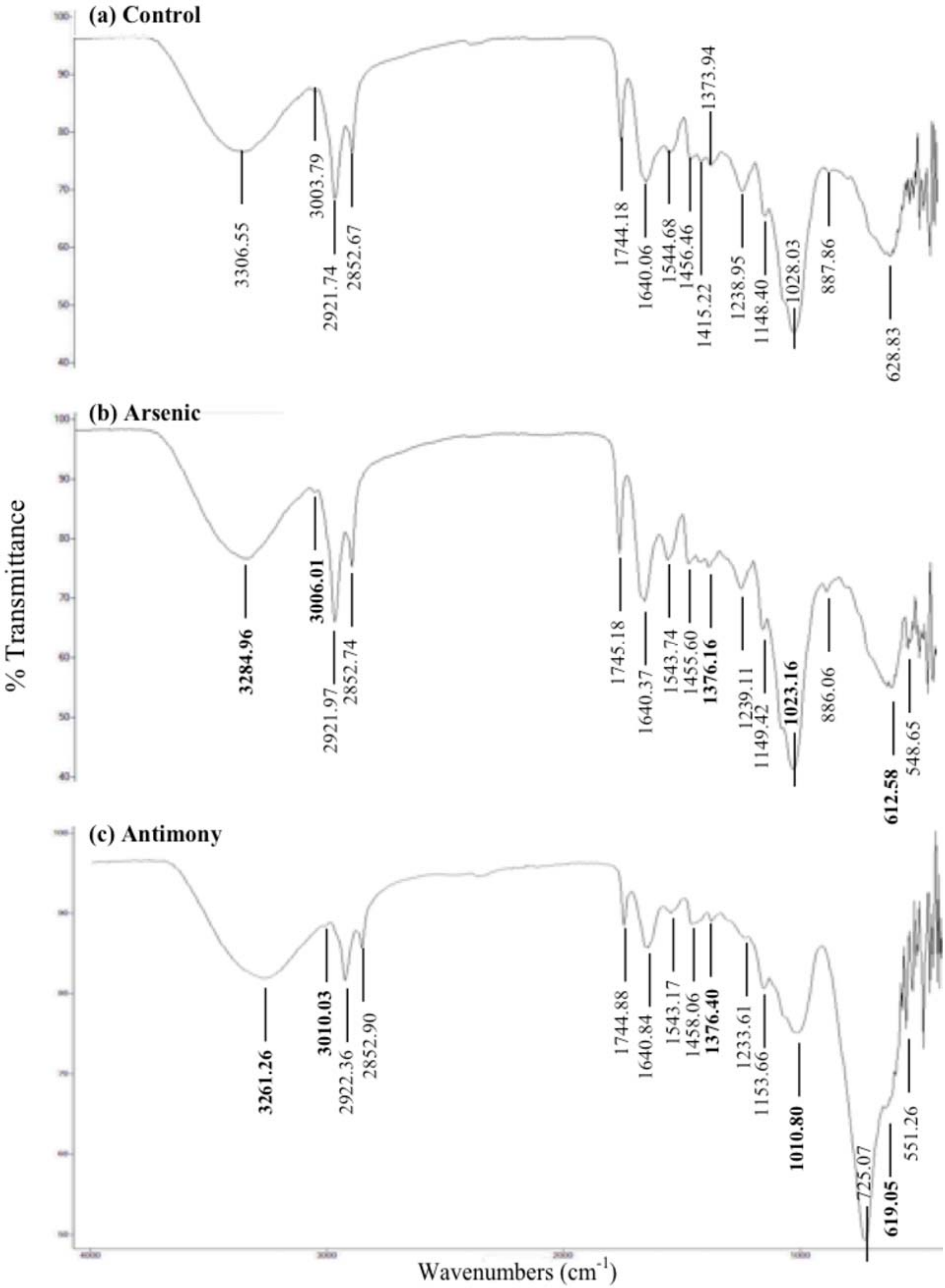

Figure 7: FT-IR spectra of *A. acidophilus* WKC-1 biomass (a) control, (b) As^{5+} loading and (c) Sb^{5+} loading. **Bold** indicates strong spectra shifting against the control.

Discussion

Soil abiotic characteristics and their interaction with the fungal isolates

Due to the igneous geology of Geevor tin mine, its activities generated various metal by-products such as Zn, Cu, As and Sb (Adriano, 1986; Hamilton, 2000). A process called roasting using a Brunton Calciner (burning furnace) where cassiterite (tin ore) containing As, Sb and other minerals such as Fe and Cu were burnt was used in Geevor tin mine. Large amount of roasting waste was deposited near to the production facilities. The contamination of As (Langdon *et al*., 2009) and Sb (Flynn *et al*., 2003) found in the soil samples were most likely to come from the by-products of the roasting process used to obtain tin.

The three-step sequential extraction method provided information about the metals and metalloids potential mobility, bioavailability and amount bound to different soil fractions (Carapeto and Purchase, 2000; Lei *et al*., 2010). This detail information is important for the evaluation of toxicity and bioavailability of metals and metalloids in soil from the mining dump as well as the feasibility of their remediation (Chen *et al*., 2007). Total concentration of As and Sb were comparable to previously published data for mining sites in Cornwall by Peterson *et al.* (1979) and Dybowska *et al*. (2005) which presented concentration of As at 20 and 40 mm depth in the soils as high as 20,000 and 40000 mg As kg^{-1}, respectively. In addition, over 100 mg kg^{-1} of Sb levels in the soil have been recorded in close proximity to where the mining operations were carried out in Derbyshire, England (Li and Thornton, 1993). Most of the As was found in the residual fraction, which is not readily available and the metalloids present in this fraction can be used as a measurement of the degree of environmental pollution in soil. The higher the metals present in this fraction, the lower the degree of pollution (Howari and Banat, 2001).

The sum of concentrations in exchangeable and weakly organically bound fractions can be used to determine the bioavailability of transitional metals and metalloids in soils (Carapeto and Purchase, 2000). Geevor tin mine soils also contained high level of iron between 30000 and 270000 mg kg^{-1} (results not shown). According to Drahota and Filippi (2009), acidic conditions ($pH<6$) with relative abundance of iron oxide (Fe-oxide) may decrease the bioavailability of As in the soil with the formation of iron arsenates such as scorodites and pharmacosiderite in the mining soils (Jacobs *et al*., 1970).

Identification of the isolated *A. acidophilus* WKC-1

Acidomyces acidophilus (Selbmann *et al*., 2008) was first isolated by Starkey and Waksman (1943) in an extremely acidic, sulphate containing industrial water. Subsequently, more *Acidomyces acidomyces* strains were isolated in various extreme environments such as acid drainage (Germany), soil near sulfur pile (Canada), volcanic soil (Iceland), acidic industrial water (The Netherlands) and acid mine drainage water (USA) (Selbmann *et al*., 2008). The morphologies of *Acidomyces* species were not easily described using microscopy because of its tendency to convert to meristematic growth, produce reluctantly disarticulating clumps of cells, or tend to appear to be entirely hyphal without any conidiation (Selbmann *et al*., 2005; Selbmann *et al*., 2008; Hujslová *et al*., 2013).

A. acidophilus WKC-1 was identified by DNA sequencing and by MALDI-TOF/TOF MS. The latter is a robust method that is widely used in the identification of fungal species, especially clinical strains (Nenoff *et al*., 2013). The growth period of *A. acidophilus* WKC-1 was significantly reduced (from 28 to 3 days) by culturing the fungal strain in liquid medium and incubating on a tube rotator. In order to obtain a trustworthy positive identification, the culture period for fungi should be no more than 10 days (De Respinis *et al*., 2013). Since *A. acidophilus* is a black fungus, the pigment from the strain could inhibit the analysis using MALDI-TOF/TOF MS as the pigment will generate noise to the spectra produced (Buskirk *et al*., 2011). However, such inhibition of obtaining spectra was not observed during the identification analysis.

Penicillium species was successfully identified using MALDI-TOF MS by Chen and Chen (2005) directly from intact fungal spores. Hettick *et al*., (2008) obtained abundant peaks in the range 5000-20000 *m/z* by using bead beating in the extraction process, the fungal samples and the MALDI-TOF MS in their experiment have identified all the 12 *Penicillium* species correctly. This study also show that the MALDI-TOF/TOF MS is a robust, cost saving and powerful system in fungal identification and characterization as suggested by Wieser *et al*. (2012).

However, the use of MALDI-TOF for identification of fungi has a few limitations. The spectral signal generated by MALDI-TOF is strongly influenced by the fungal growth medium as well as the protein extraction methods (Santos *et al*., 2010). Due to the cell wall structure of fungi, protein extraction requires an additional step such as bead beating, to yield high quality spectra that enable a valid identification (Croxatto *et al*., 2012). The lack of reference spectra available in the database is the main disadvantage in using MALDI-TOF MS to identify fungal species and work like this current study can contribute to the development of a fungal database. The use of MALDI-TOF/TOF MS described in this paper has demonstrated that this method is capable to identify environmental fungi species provided that the correct sample preparation methods are being used.

Tolerance and removal efficiency of As

The soil condition where *A. acidophilus* WKC-1 was isolated is extremely hostile and inhabitable to most living organisms. However, the extreme acidity (pH 1) in the soil is a crucial factor for the growth of this acidophile. The ability of *A. acidophilus* to resist and survive in such acid and toxic environment is thought to be due to the presence and protection of a melanin-containing cell wall (Martin *et al*., 1990).

A. acidophilus WKC-1 exhibits high As^{5+} removal efficiency even in extreme pH conditions. This indicates that *A. acidophilus* WKC-1 has great cellular detoxification mechanisms in toxic metalloids tolerance. The unique composition of fungal cell wall containing excellent metal-binding properties offers great advantage in metal removal either by entrapment in extra-cellular capsules and precipitation of metals (Gupta *et al*., 2000). Previous study by Su *et al*. (2010) observed the intracellular uptake of As^{5+} in *Tichoderma asperellum* and *Fusarium oxysporum* can be as efficient as extracellular sorption in many fungi where the intracellular As^{5+} accumulation accounted for 82.2% and 63.4% of the total accumulated As^{5+}.

Biosorption of As

The As^{5+} uptake by fungal biomass is significantly affected by the presence of competing ions, in this case Sb^{5+} (Figure 6). These ions compete for active binding sites due to the non-specificity of the functional groups present on the fungal cell surface. As a result, it is often found that specific transitional metal/metalloid(s) uptake from mixed solutions is lower than those in a solution containing the single transitional metal/metalloid.

The pH has profound effect on As^{5+} uptake by *A. acidophilus* WKC-1. The *A. acidophilus* WKC-1 As^{5+} sorption capacity increased with increasing pH from 1 to 4 and showed optimum As^{5+}

adsorptions at pH 4 (Figure 6a). The pH of the solution affects the solubility of metalloid ions and the ionization state of the functional groups on the fungal cell wall by either interfering or enhancing with biosorption process (Fourest and Roux, 1992; Lopez *et al*., 2000; Bayramoğlu *et al*., 2003). Absorption of As^{5+} by WKC-1 at low pH is noticeably lower than at higher pH, this might be due to the competition between As^{5+} and H^+ or H_3O^+ ions present in the solution, for the negatively charged biosorbent binding sites (Gadd, 1994). It is likely that the high mobility and concentration of H^+ ions are preferentially adsorbed by the fungi cells than the studied metalloid ions. As the pH increases and the H^+ ion concentration in the solution decreases, a greater number of ligands (such as carboxyl, sulphhydryl, phosphate groups) with negative charges become available, thus increasing biosorption capacity (Feng *et al*., 2011).

Higher absorption of As^{5+} was observed with increased contact time due to the abundant binding sites available on the fungal cell surface for the metal sorption by *A. acidophilus* WKC-1. The biomass loading of *A. acidophilus* WKC-1 for As^{5+} sorption was found to be optimal at 1 mg L^{-1}. The optimum biomass loading results support the hypothesis by Gupta and Rastogi (2008) that an increase in biomass loading could exert a shell effect by protecting the active binding sites from being occupied by the metal, resulting in the decrease of metal sorption. A similar effect of high biomass loading resulting in low sorption was observed by Bishnoi *et al*. (2007) in Cr (VI) removal by *Trichoderma viride*. In the presence of competing ions, metal uptake from mixed solutions is often found to be lower than those in a single-species system (Chong and Volesky, 1995).

In general, metal uptake by fungus increases as the ionic radius of the metal cation increases, thus metals with higher ionic charge show greater binding to biomass. However, as the concentration of other competing metalloid cations present within the same biosorption process increases, the uptake of another metalloid further decreases. Chemical interactions between two metal species as well as biomass may take place, resulting in competition for sorption sites on the surface (Akar *et al*., 2005). Sari and Tuzen (2009) reported that maximum biosorption capacity of As^{5+} by *Inonotus hispidus* biomass was found to be 59.6 mg g^{-1}. Plant biomass prepared from sawdust of *Picea abies* has the maximum As^{5+} sorption capacity of 1.369 mg g^{-1} (Urik *et al*., 2009). The As^{5+} adsorption capacity of zirconium (IV) loaded phosphoric chelate adsorbent, synthesized by radiation induced graft polymerization, was 149.8 mg g^{-1} (Seko *et al*., 2004). *A. acidophilus* WKC-1 showed a greater As^{5+} adsorption capacity than previously studies fungal strains, where the predicted maximum capacity of fungal strain uptake of As^{5+} by WKC-1 was 170.82 mg g^{-1} dry biomass and has potential to be used in bioremediation transitional metals in soils. The fate of As^{5+} after being adsorbed into the fungal cell might be broken down to less toxic species by powerful secondary enzymes produce intracellularly by the fungal itself or undergo a number detoxification pathways within the fungal cell which include the reduction to As^{3+} by arsenate reductases, followed by exclusion or sequestration of As^{3+} (Sharples *et al.*, 2000; González-Chávez *et al.*, 2002). Further study is required to elucidate and understand the detoxification mechanisms of As^{5+} of *A. acidophilus*.

The effect of other group 15 elements and phosphate on As removal

The effect of Sb^{5+} in reducing the As^{5+} sorption could be due to the competition of active binding sites as shown in the FT-IR analysis. Benjamin and Leckie (1981) showed that the adsorption of cadmium, copper, zinc and lead on amorphous iron oxyhydroxide were reduced in the presence of all the metals at the same time, as the availability of the active sorption binding sites decreased, which also lead to a decrease in the apparent adsorption equilibrium constants.

Similarly in the As^{5+} resistance experiment it was observed that PO_4^{3-} reduces As^{5+} toxicity on *A. acidophilus* WKC-1. According to Hughes (2002), PO_4^{3-} and As^{5+} are both tetrahedral oxy-anions and have similarity between structure, synthesis and hydrolysis thus PO_4^{3-} can chemically mimics and acts as a substitute to As^{5+} in biochemical processes by incorporated into the metabolic pathways of *A. acidophilus* unlike the As removal process.

These shifts in absorbance peaks of -OH/-NH as well as in phosphorus functions could indicate that alcohols/phenols, carboxylic acids and its derivatives, amine II and phosphate groups could be vital sites for the binding of As^{5+} ions. In the spectra of the As^{5+} loaded biomass, the shoulder peaks of 2852.74, 1745.18 and 1023.16 cm^{-1} became sharper. Such observations could indicate that these related functional groups could be involved during the biosorption process. As seen in the 'fingerprint' region of the As^{5+} loaded biomass, multiple sharp peaks can be seen compared to the non-treated biomass. It was also noted that the absorbance of this region was much lower than the control sample. Phosphate and sulphur functional groups are indicating a possible interaction of the As^{5+} during biosorption process.

Based on the spectra from FT-IR generated, it suggests that As^{5+} and Sb^{5+} might compete for the binding sites of OH, -NH, -CH, $-SO_3$ and PO_4 functional groups on the surface of the isolated *A. acidophilus* WKC-1 strain. Previous study by Dixon (1997) showed that As^{5+} reacting in a similar way as phosphate in which it has the ability to form ester linkages with hydroxyl groups. A study carried out by Parascondola (1977) found out that Group 15 elements in the periodic table in both pentavalent and trivalent state can interact with sulphur (formation of As-S complexes), thus this supports the analysis from the FT-IR analysis that $-SO_3$ functional group could involve as a binding site of As^{5+} and Sb^{5+}. Some other functional groups such as C-O, C-N and CH_2 may also compete to a lesser extent by these two metalloids to bind on the surface of *A. acidophilus* WKC-1.

Conclusions

In conclusion, metal analysis showed that 26.40% of As^{5+} is bioavailable in the soil samples at a level below the MIC of *A. acidophilus* WKC-1, suggesting a good potential to apply this strain to remediate As polluted soil. The presence of phosphate decreases the toxicity of As^{5+} whereas Sb^{5+} significantly reduces the As removal ability of WCK-1. The -OH, -NH, -CH, $-SO_3$, and PO_4 functional groups have been identified as the key competitive binding sites between As^{5+} and Sb^{5+}. The isolate WKC-1 showed a high resistance and high percentage As^{5+} removal, one of the highest reported in *A. acidophilus* species. Our study also demonstrated that MALDI-TOF/TOF MS could provide a faster and cheaper way to identify environmental fungal strains. The tolerance of the isolated *A. acidophilus* WKC-1 strain to low pH and high As concentration together with its capacity to remove approximately 170 mg As^{5+} per gram dry biomass, made it an potential candidate to be used in bioremediation of As.

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Supplementary materials

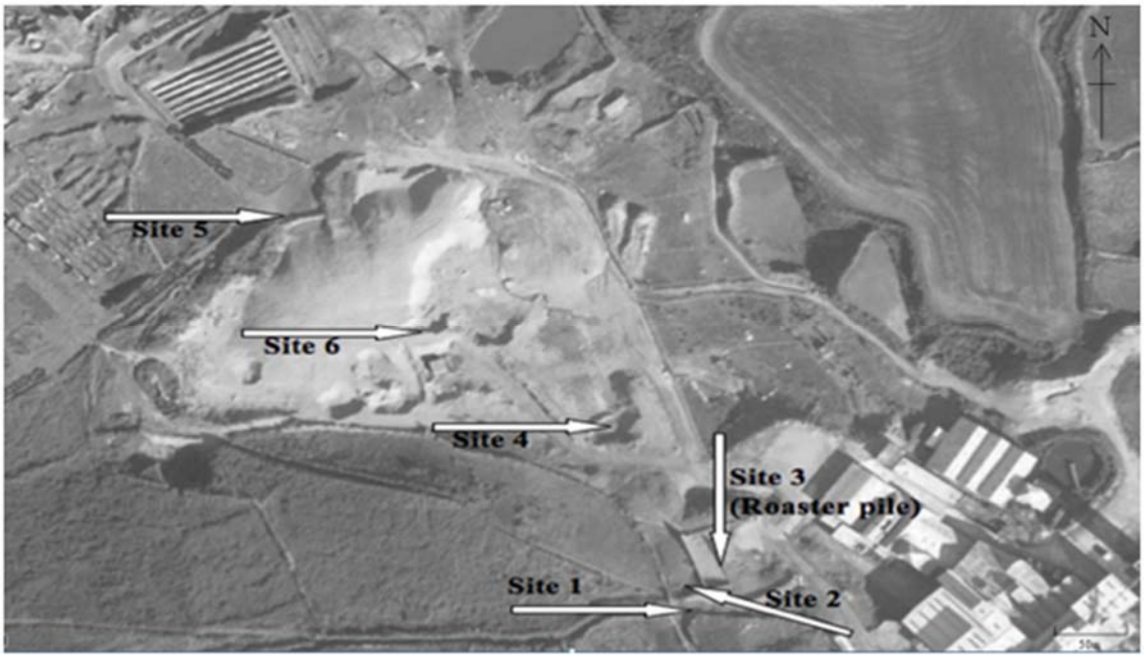

Fig. S1: An aerial photograph of the Geevor tin-mine in Pendene, Penzance, Cornwall, UK and locations of soil sampling sites (Greevor Tin Mine was viewed on 17 July 2013. https://www.google.co.uk/maps/places/Greevor+Tin+Mine/@50.1519033,-5.6744307,1404m).

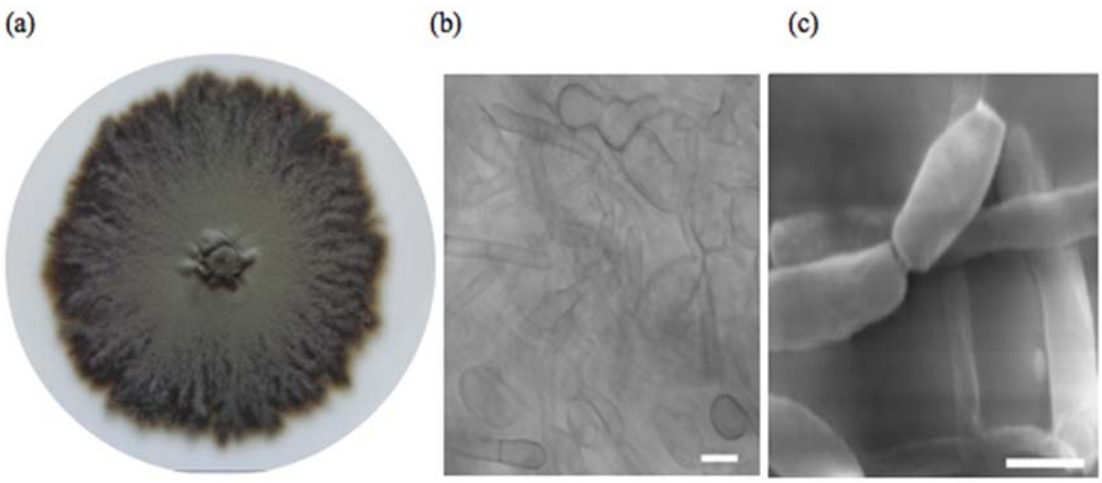

Figure S2: Morphological features the fungus of (a) colony of the isolated fungal strain in CDA medium, (b) Hyphae of the strain observed by light microscope at a magnification of 400x and (c) scanning electron microscope (SEM) at a magnification of 1000x (b) and 2200x (c), scale bar in (b) and (c) = 2 μm.

Table S1: Operating parameters of ICP- OES (iCAP 1600)

Operating parameters of the thermos ICP-OES (iCAP 1600)	
Power (W)	1150
Auxiliary gas flow (L/min)	0.5
Nebuliser gas flow (L/min)	0.75
Coolant gas flow(L/min)	12
View	Axial
Purge gas flow	Normal
Flush pump rate (rpm)	100
Analysis pump rate (rpm)	50
Camera temperature	-47
Optics temperature	38

Table S2: Recovery of As and Sb (mg kg^{-1}) metal using certified reference material, SRM 2710a Montana Soil using acid digestion method. Data shown are the mean of three replicates.

Element	Certified value	Mean obtained value	Average % recovery
Reference material Lot 011233			
As	61.10 ± 2.08	57.05 ± 0.20	93.37
Sb	73.7 ± 10.50	62.19 ± 2.03	84.38
Reference material Lot 017309			
As	202.00 ± 17.70	186.30 ± 12.32	92.23
Sb	125.00 ± 13.53	109.9 ± 12.90	87.92

Table S3: The diameter measurement of the minimum inhibitory concentration of As^{5+} by isolated *A. acidophilus* and two positive control *A. acidophilus* type strains

	Diameter (cm)		
As^{5+} concentration	Isolated A .acidophilus strain	Positive control	
		A .acidophilus CBS 335.07	A .acidophilus CBS 4251
Control	4.7 ± 0.2	3.9 ± 0.4	4.2 ± 0.2
1000	4.5 ± 0.1	3.8 ± 0.1	3.9 ± 0.4
7500	4.3 ± 0.2	2.7 ± 0.2	2.4 ± 0.1
10000	4.1 ± 0.2	1.4 ± 0.1	NG
12500	3.9 ± 0.1	NG	NG
15000	3.7 ± 0.3	NG	NG
17500	3.4 ± 0.1	NG	NG
20000	2.7 ± 0.2	NG	NG
22500	2.2 ± 0.2	NG	NG
25000	NG	NG	NG

* NG *indicates no growth*