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

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13

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18 Mine for providing access and permission to sample on the site.

19 **Abstract**

20 An arsenic-resistant fungal strain, designated WKC-1, was isolated from a waste roaster pile in
21 a historical tin mine in Cornwall, UK and successfully identified to be *Acidomyces acidophilus*
22 using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass
23 spectrometry (MALDI-TOF/TOF MS) proteomic-based biotyping approach. WKC-1 showed
24 considerable resistance to As⁵⁺ and Sb⁵⁺ where the minimal inhibitory concentration (MIC) were
25 22500 mg L⁻¹ and 100 mg L⁻¹ respectively on Czapek-Dox Agar (CDA) medium; it was
26 substantially more resistant to As⁵⁺ than the reference strains CBS 335.97 and CCF 4251. In a
27 modified CDA medium containing 0.02 mg L⁻¹ phosphate, WKC-1 was able to remove 70.30 %
28 of As⁵⁺ (100 mg L⁻¹). Sorption experiment showed that the maximum capacity of As⁵⁺ uptake
29 was 170.82 mg g⁻¹ dry biomass as predicted by the Langmuir model. The presence of Sb⁵⁺
30 reduced the As⁵⁺ uptake by nearly 40%. Based on the Fourier-transform infrared spectroscopy

31 (FT-IR) analysis, we propose that Sb is competing with As for these sorption sites: OH, NH, CH,
32 SO₃ and PO₄ on the fungal cell surface. To our knowledge, this is the first report on the impact
33 of other Group 15 elements on the biosorption of As⁵⁺ in *Acidomyces acidophilus*.

34 **Keywords**

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

36

37 **Introduction**

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

50

51 In natural environments, compounds of metalloids such as As and Sb are widely dispersed as a
52 consequence of anthropogenic and geological activities. As and Sb are by-products of tin-mining
53 activities during the smelting process, where As is primarily found in the arsenopyrite (FeAsS)
54 form (Telford *et al.*, 2009). The continuous disposal of arsenic trioxide (As₂O₃), a by-product in
55 the furnace channel during the roasting process in tin mining activities, has been reported to cause
56 serious contamination to surrounding soils and waters in proximity of mining sites. For instance,
57 the concentration of As in soil adjacent to the Ron Phibun district tin mine in the Nakorn Si
58 Thammarat province of southern Thailand was reported to be as high as 11000 mg kg⁻¹
59 (Francesconi *et al.*, 2002). To fully appreciate the toxic effects transitional metals exert on
60 biological systems, it is important to analyse their bioavailability by determining the uptake of
61 these metals from soil by microorganisms within a given time span (Olaniran *et al.*, 2013).

62

63 Geevor Tin Mine is a disused historical mine in the St Just mining district, one of the oldest
64 mining districts in Cornwall (Yim, 1981). This tin mine was first established in the 1910s but
65 due to the low global demand for tin and the high cost of operations, it was closed down in early
66 1990s. Upon closure of the mine, all the waste piles were abandoned on the site, the soil pollutants
67 were contained and access to the site was restricted. According to Pirrie *et al.*, (2002), transitional
68 metal and metalloids contamination is very common in Cornwall and it was estimated that
69 approximately 1000 km² of Southwest of England are still contaminated with elevated
70 concentrations of toxic metals and arsenic (Abrahams and Thornton, 1987; Camm *et al.*, 2004;
71 Van Veen *et al.*, 2016). Metals bioavailability analysis of these soil samples will help to fully
72 understand the actual amount of these metals available for uptake by microorganisms and their
73 toxicity (Olaniran *et al.*, 2013).

74

75 Biosorption using fungal biomass has been receiving attention from many researchers globally
76 as an alternative method to remove heavy metal/metalloid(s) from contaminated water and soil.
77 It offers many advantages such as high efficiency, reduced operating cost, minimal usage of
78 chemicals and low production of toxic chemical sludge (Gadd, 2009; Vijayaraghavan *et al.*,
79 2006). The transitional metals and metalloids present in the soil can be either already available
80 or made available for uptake by microorganisms or plants, where they will be accessible for the
81 sorption process (Peijnenburg and Jager, 2003; Del Giudice *et al.*, 2013; Antonucci *et al.*, 2017).
82 It has also been established that ions from the same group in the periodic table could compete
83 with each other during the biosorption process (Tsezos *et al.*, 1996).

84

85 A number of extremophile fungi have been successfully isolated from adverse environmental
86 conditions. One of the most well-known is *Acidomyces acidophilus*, formerly known as
87 *Scytalidium acidophilum*, and also known as the black fungi. It is a pigmented ascomycete
88 capable of growing in extremely acidic conditions (Sigler and Carmichael, 1974). Its melanin-
89 containing cell walls offer the fungus protection from adverse environmental conditions such as
90 extreme pH, temperature and toxins (Jacobson *et al.*, 1995; Martin *et al.*, 1990; Tetsch *et al.*,
91 2006; Hujšlová *et al.*, 2013). This protection also provides the fungus a certain level of resistance
92 to oxidative stress (Jung *et al.*, 2006). The enzymes produced by this fungus are of great interests
93 as they can function at low pH and high temperatures and could have potential applications for a
94 variety of industries (Polizeli *et al.*, 2005; Hess, 2008; Selbmann *et al.*, 2008). So far, there are
95 no reports on the use of *A. acidophilus* for metalloids bioremediation.

96

97 This paper reports the isolation and characterisation of a highly resistant *A. acidophilus* WKC-1
98 strain from the disused mine in Cornwall that can tolerate high levels of As⁵⁺. The ability of this
99 isolate to remove As⁵⁺ is being investigated and sorption analyses carried out to determine its
100 maximum adsorption capacity. The influence of Sb⁵⁺ and PO₄³⁻ on this isolate's capacity to
101 remove As⁵⁺ has been studied to provide a better understanding of the relationship between its
102 As-resistance and the presence of other chemicals in soil. Finally, the potential of using resistant
103 fungi to bioremediate metalloids from polluted soil in historical sites is discussed.

104

105 **Materials and Methods**

106 **Site description**

107 The Geevor Tin Mine is located in the St Just District, Cornwall at 50°09' 06.43" N 5°40' 34.96"
108 S, in the Southwest of England. It was the only tin mining site in the district after the closure of
109 Levant Mine in 1930 (Noall, 1973) and ceased its operation in 1991 (Camm *et al.*, 2003). The
110 site covers an area of 67 acres (270,000 m²) and it is now on the European Route of Industrial
111 Heritage sites, an important tourist attraction in Cornwall.

112 **Soil sampling**

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

117 **Soil analysis**

118 Soil samples were air-dried for 72 h, ground finely using a pestle and mortar and sieved through
119 a 2 mm sterile mesh prior to analysis. The pH of the soil samples, suspended in deionised water
120 (soil:deionised water 1:2 w/v), was measured using a calibrated pH meter (Jenway, Model 3505).
121 The soil organic matter (OM) content was determined using the ASTM (American Society of
122 Testing and Materials) standard procedure (ASTM, 2000) and the cation exchange capacity
123 (CEC) was analysed using the protocol recommended by Gillman and Sumpter (1986). The
124 concentrations of As and Sb in each of the six soil samples were analysed using a three-step
125 sequential extraction method for exchangeable (F1), weakly bound organic bound (F2) and
126 residual (F3) fractions (Carapeto and Purchase, 2000). All the extracts (F1, F2 and F3) were

127 analysed using inductively coupled plasma optical emission spectrometry iCAP 1600 (ICP-OES)
128 and the operating parameters summarised in Table S1. All the analyses were carried out in
129 triplicates and the ICP-OES generated three readings per analysis. The percentage of
130 bioavailability of both As and Sb was calculated by division of the summed fractions 1 and 2 by
131 the total (F1+F2+F3) of each metalloid from the three-step sequential extraction. For analytical
132 accuracy, the percentage recoveries (R) of all soil trace elements of interest were performed in
133 soil certified reference materials (CRM) (#SQC001, lot 011233 and lot 017309, RTC, Laramie,
134 WY, USA).

135 **Enumeration and isolation of arsenic-tolerant fungi**

136 Soil samples containing high As and Sb concentrations were used for screening of arsenic-
137 resistant fungi. A ten-fold serial dilution was carried out using one gram of soil sample and plated
138 out on to 2% malt extract agar (MEA; Oxoid Ltd., UK), supplemented with 100 mg L⁻¹ of
139 chloramphenicol to prevent bacteria growth. The inoculated plates were incubated for 7 – 21
140 days at 25 °C and fungal viable counts determined. Colonies were sub-cultured, purified by
141 passaging for ten times, screened in 2 % MEA at pH 1 containing As⁵⁺ (1000 – 25000 mg L⁻¹),
142 prepared from sodium arsenate (Na₂HAsO₄). Fungal strains that survived the highest As-stress
143 were considered as a potential candidate and maintained using the same MEA conditions with or
144 without 100mg L⁻¹ of As⁵⁺.

145 **Molecular identification of isolated fungi**

146 Fungal isolates were grown on Potato Dextrose Agar (PDA) (CM0139, Oxoid Ltd, UK), pH 1 at
147 25 °C for 21 days. Mycelia were collected by pipetting Triton X-100 on the same colony spot for
148 several times and transferring into a sterile tube. DNA was extracted using cetyl
149 trimethylammonium bromide (CTAB) following the protocol by Stirling (2003) with a minor
150 modification, DNA extraction was carried out twice on the samples at 65 °C for 50 min followed
151 by bead milling. The extracted DNA was dissolved in 20 µl ultrapure water and stored at 4 °C.

152 The internal transcribed spacer (ITS) nuclear region of 18S-ITS1-5.8S-ITS2-28S rRNA of the
153 fungal isolate was amplified by PCR using three sets of primers based on published sequences
154 (White *et al.*, 1990; Martin and Rygiewicz, 2005). The first PCR used ITS1 forward and ITS2
155 reverse primers, the second used ITS5 forward and ITS4 reverse primers and the third used ITS1
156 forward and ITS4 reverse primers, all were obtained from Sigma-Aldrich and PCR amplifications
157 performed (ITS1-ITS2 PCR: 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 63 °C for 2 min, 72
158 °C for 1 min; followed by 72 °C for 10 min; ITS5-ITS4 and ITS1-ITS4 PCRs: 94 °C for 4 min;

159 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).
160 The PCR products were analysed by 2.0% (w/v) agarose gel electrophoresis and capillary
161 electrophoresis on the MCE-202 MultiNA system (Shimadzu) in “on-tip analysis” mode
162 following the protocol recommended by the manufacturer using the DNA 1000 reagent kit
163 (Shimadzu) to quantify their concentrations and to confirm the results of the agarose gel
164 electrophoresis. The PCR products were sequenced by GATC biotech (London, UK) and
165 sequences analysed by nucleotide BLAST (NCBI) analysis. Based on the generated DNA
166 sequences of the isolate and other reference sequences of fungi obtained from NCBI databases,
167 a phylogenetic dendrogram from the evolutionary distance via the neighbour-joining method was
168 constructed using bootstrap method of 1000 replications using Molecular Evolutionary Genetics
169 Analysis 6 (MEGA6) software (Tamura *et al.*, 2013).

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

171 Three reference strains of *A. acidophilus* were obtained from Centraalbureau Schimmelcultures
172 (CBS), Netherlands (strain CBS 335.97) and Culture Collection of Fungi (CCF), Czech Republic
173 (strains CCF4251 and CCF3679). Reference strains and the isolated fungal strain were grown in
174 liquid salt medium (LSM) containing 2% dextrose, 0.1% (NH₄)₂SO₄, 0.001% K₂HPO₄, 0.05%
175 MgSO₄×7 H₂O, 0.0026% FeSO₄ and 0.008% CaCl₂ with a final pH of 4.0-4.2 using a horizontal
176 rotator (SB2 rotator, Stuart) for 72 h at room temperature. The sample preparation and extraction
177 of proteins and peptides of *A. acidophilus* and the three reference strains were performed
178 according to the Bruker fungi sample preparation protocol. Each extracted sample was analysed
179 using a MALDI ground steel plate and six different sample spots (replicates) to generate six
180 combined mass spectra (MSP) per fungal isolate. The reference strains for *A. acidophilus* CBS
181 335.97, CCF4251 and CCF3679 were analysed to generate reference spectra and used to create
182 an in house supplementary new database library for *A. acidophilus* fungal strains identification.
183 The identification of the isolated fungal strain through comparison with reference strains and
184 visualization of the mass spectra was performed with MALDI Biotyper software 3.0 (Bruker
185 Daltonics).

186 **Determination of As minimum inhibitory concentration (MIC)**

187 The MIC for the isolated fungal strain and two *A. acidophilus* reference strains (CBS 335.97 and
188 CCF 4251) were determined using solid acidic culture medium of modified Czapek dox agar
189 (CDA) with either 1 mg L⁻¹ or 100 mg L⁻¹ PO₄³⁻ at pH 1, containing As⁵⁺ concentrations ranged
190 from 1000 to 25000 mg L⁻¹. To allow polymerization of agar in culture medium at pH 1, double
191 concentration of agar was added, and pH was adjusted after sterilization. The fungal mycelia

192 plugs were removed using a sterile pipette and placed in the middle of the agar. The plates were
 193 incubated for 21 days at 25 °C and the diameter of the each of the fungal colony was measured
 194 and MIC calculated from the average of the triplicate results.

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

196 The effect of pH on the isolated *A. acidophilus* WKC-1 was determined using solid culture
 197 medium of MEA containing 1000 mg L⁻¹ of As⁵⁺ with pH ranged from 0.5 to 5, the desired pH
 198 was adjusted using NaOH (0.1M) or HCl (0.1M). The plates were incubated for 21 days at 25 °C
 199 and the diameter of the each of the fungal colony was recorded.

200 **Arsenic removal efficiency**

201 The efficiency of arsenic removal by *A. acidophilus* WKC-1 and three *A. acidophilus* reference
 202 strains were studied in a 0.15 mL centrifugal tube using LSM containing 1 g L⁻¹ of viable wet
 203 fungal biomass in pH 1 and supplemented with 100 mg L⁻¹ As⁵⁺, the cultures were cultivated
 204 using a horizontal rotator (SB2 rotator, Stuart) at 120 rpm for 21 days at room temperature and
 205 the final concentration As⁵⁺ in each filtrate was measured every 7 days using ICP-OES. All
 206 experiments were carried out in triplicates. The arsenic removal efficiency by all studied *A.*
 207 *acidophilus* strains was calculated using the following equation:

$$208 \quad R = [(C_i - C_f) / C_i] \times 100$$

209 where:

R = Percentage As⁵⁺ removal;

C_i = Initial concentration of As⁵⁺ (mg L⁻¹);

C_f = Final concentration of As⁵⁺ (mg L⁻¹) after 21 days.

210

211 **Biosorbent preparation and analysis of As biosorption**

212 The *A. acidophilus* WKC-1 was inoculated in LSM for 21 days at 25 °C with constant shaking
 213 at 110 rpm using an orbital shaker (Minitron, Infors HT). The fungal biomass was harvested by
 214 filtration through Whatman No.11 filter paper, cleaned three times with deionised water to ensure
 215 the removal of all the excessive media residuals, freeze-dried (ScanVac CoolSafe, Labogene) for
 216 24 h and grounded in mortar and pestle to fine powder. Each of the 1000 mg L⁻¹ As⁵⁺ and Sb⁵⁺
 217 stock solution was prepared by dissolving Na₂HAsO₄ × 7 H₂O and KSb(OH)₆ (Sigma-Aldrich)
 218 in deionised water.

219 All adsorption tests were carried out in 50 mL conical flasks containing 20 mL of As⁵⁺ and/or
 220 Sb⁵⁺ solution at 25 °C on an orbital shaker at 120 rpm. Biosorption isotherms were formulated

221 through investigating the effect of pH and biomass loading capacity on the fungal cell as
 222 previously performed by Xu *et al.* (2012). In order to identify the pH effect on As⁵⁺ biosorption,
 223 two sets of experiments were carried out. Firstly, biosorption using fixed 0.5 g L⁻¹ dried fungal
 224 biomass in a range of As⁵⁺ (100 - 600 mg L⁻¹) and different pH range (1.0 - 6.0) was examined.
 225 Secondly, biosorption of a range of fungal biomass (0.5 - 5 g L⁻¹) using fix concentration of As⁵⁺
 226 (500 mg L⁻¹) at different pH range was investigated. In order to investigate optimum contact time
 227 for the biosorption of As⁵⁺, samples were collected at different times (5, 15, 30, 60, 120 and 180
 228 min) and filtered through Whatman No.11 filter paper. All filtrates were analysed for residual of
 229 As⁵⁺ concentration using ICP-OES. The uptake of As⁵⁺ by *A. acidophilus* WKC-1 was calculated
 230 using the following equation:

$$231 \quad q_{eq} = \frac{V (c_i - c_{eq})}{m}$$

232 where:

- q_{eq} = As⁵⁺ uptake in mg per g biomass;
- V = Volume of As⁵⁺ used in mL;
- c_i = Initial concentration of As⁵⁺ (mg L⁻¹);
- c_{eq} = Equilibrium concentration of As⁵⁺ (mg L⁻¹);
- m = Amount of dry biosorbent (g).

233 FT-IR studies

234 The detection of vibration frequency changes in *A. acidophilus* WKC-1 for the untreated and
 235 As/Sb-treated biomass samples before and after the As⁵⁺ and Sb⁵⁺ biosorption were analysed
 236 using Fourier transform infrared spectroscopy (Travel IR, Perkin Elmer) and the attenuated total
 237 reflection (ATR) technique in the same experimental conditions as described for the biosorption
 238 experiment. Each biomass was freeze-dried and was placed on the single reflection diamond
 239 ATR crystal. The infra-red spectra were collected using the ATR-FTIR ranged from 400 to 4000
 240 cm⁻¹ (Guibaud *et al.*, 2003).

241 Statistical analyses

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

248

249 **Results**250 **Physical and chemical properties of the soil samples**

251 Results showed that all of the soil samples were acidic and the pH range varied sites with soil
 252 sample from site 3 being the most acidic (pH 1.13) and site 5 being the least acidic (pH 5.25).
 253 The highest and lowest CEC were 32.14 ± 4.31 meq/100g for soil sample 3 and 12.09 ± 3.71
 254 meq/100g for soil sample 4 respectively, the mean CEC for all soil samples was 19.96 ± 3.57 .
 255 The highest and lowest OM contents were observed in soil samples 1 with 14.60 % and 4 with
 256 3.34 % respectively. Results for the chemical and physical characterisation of the soil samples
 257 are summarised in Table 1.

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

261

262 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 |

263

264

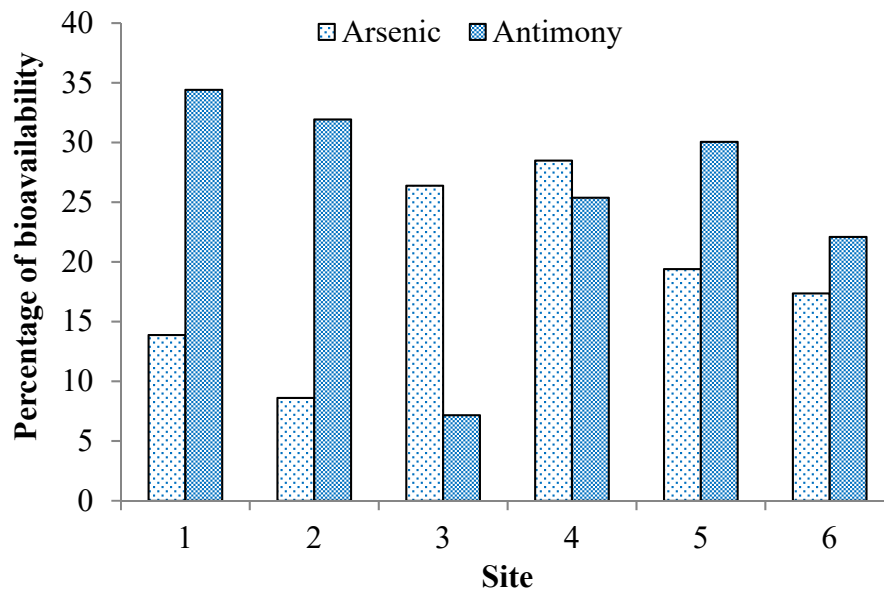
265 The total metal/metalloid concentration analysis showed that As levels exceeded those of Sb in
 266 all sampling sites (Table 2). The highest concentrations of As ($18043.50 \text{ mg kg}^{-1}$) and Sb (213.69
 267 mg kg^{-1}) were detected in soil sample 3, collected from the location of the roaster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

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

275

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

280



281

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

Table 2: Mean As and Sb concentration ($\text{mg kg}^{-1} \pm$ 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 | $\Sigma(\text{F1} + \text{F2} + \text{F3})$ | F1 | F2 | F3 | $\Sigma(\text{F1} + \text{F2} + \text{F3})$ | |
| 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).

285 Identification and isolation of fungal strains

286 A total of 31 strains were isolated from soil samples collected from six different locations were
 287 exposed to As⁵⁺ ranged from 1000 to 22500 mg L⁻¹. Only one fungus from the most acidic and
 288 polluted soil in site 3 was able to grow on the medium containing the highest As⁵⁺ concentration
 289 (Table 3). It was selected for identification and further experiments.

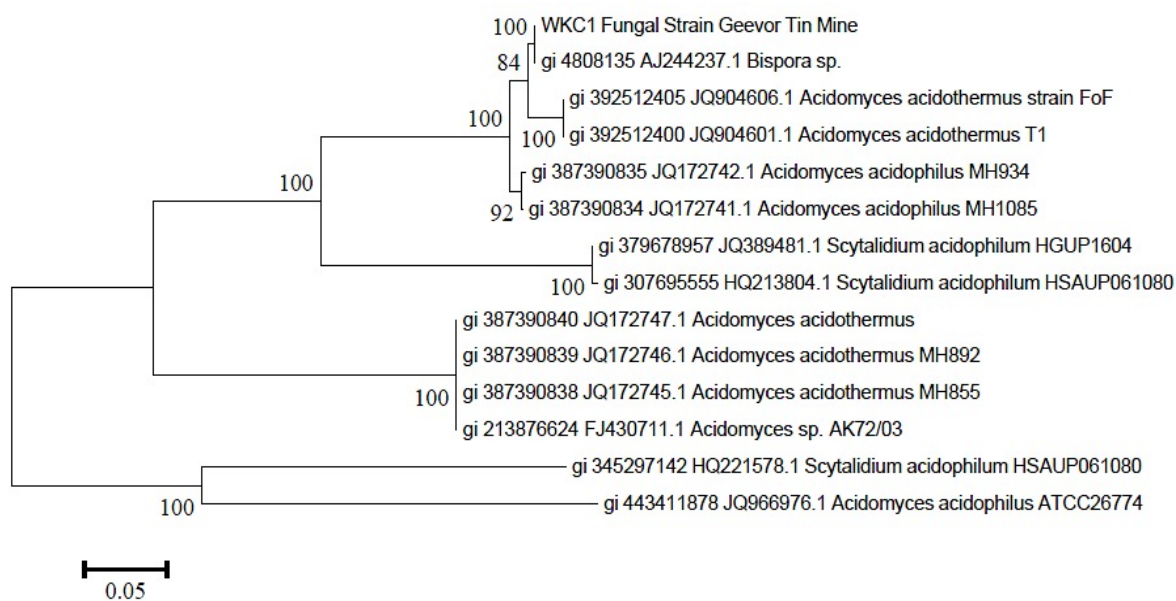
290 The colony and micro-morphological features of the isolated fungal strain WKC-1 which was
 291 highly resistant to arsenic are presented in Figure S2 This strain was slow growing, achieving
 292 diameters of 22 to 45 mm in 21 days at 25 °C. The colonies appeared compact and dark greenish
 293 in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

295

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

302

303



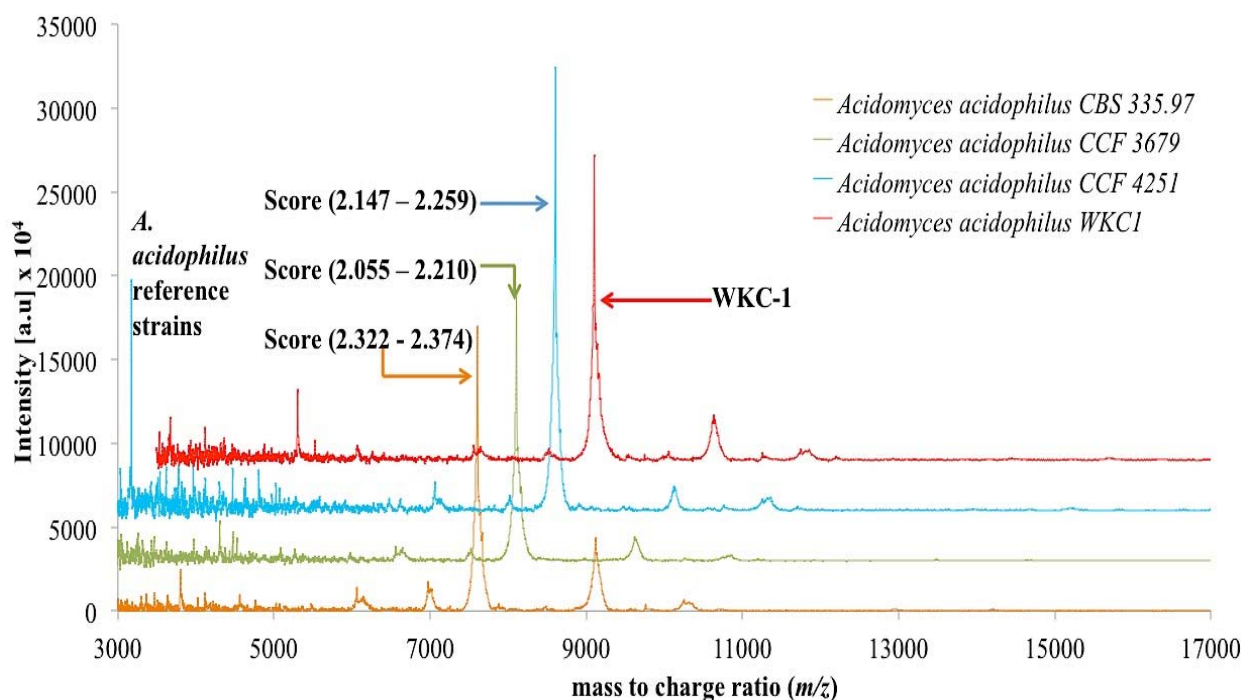
304

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

307

308 Prior to the analysis of the isolated WKC-1 strain by MALDI TOF/TOF MS, the mass spectra of
 309 the three *A. acidophilus* reference strains were generated and inserted to the in-house database to
 310 create an *A. acidophilus* database library, since there is currently no database available for this
 311 species. The identification of *A. acidophilus* WKC-1 against three *A. acidophilus* reference strains
 312 showed that the isolated WKC-1 strain belongs to the *A. acidophilus* species with highly probable
 313 species identification to CBS 335.97 strain followed by secure genus identification against CCF
 314 4251 and CCF 3679 strains (Figure 3).

315



316

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

319 *2.300-3.000 indicates high probable species identification; 2.000-2.299 indicates secure genus
 320 identification.

321 Table 3: The minimum inhibitory concentration (cm ± standard error) of As⁵⁺ by *A. acidophilus*
 322 WKC-1 and the effect of low and high phosphate concentration. Data shown are the mean of
 323 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*** |

324 Asterisks indicate statistical significance of differences tested by 2-sample t-test where * $p < 0.05$, ** $p <$
325 0.01 , *** $p < 0.001$ compared to *A. acidophilus* WKC-1 containing 1 mg L⁻¹ of PO₄³⁻.

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

327 The MIC at pH 1 of isolated *A. acidophilus* WKC-1 reflects an extremely high tolerance for
328 arsenate, the strain could tolerate up to 22500 mg kg⁻¹ of As⁵⁺ in solid media. Two reference
329 strains of *A. acidophilus* (CBS 335.97 and CCF 4251) were tested for their tolerance to As⁵⁺ and
330 found to tolerate up to 10000 and 7500 mg L⁻¹ of As⁵⁺, respectively 2.5 times lower than the MIC
331 of the isolated WKC-1 strain (Table S3).

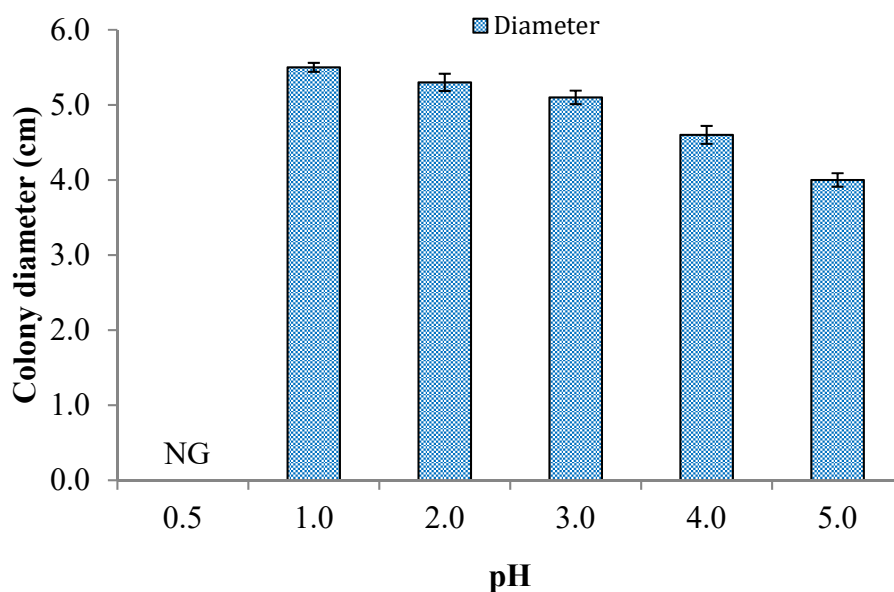
332

333 The CDA media containing 100 mg L⁻¹ of phosphate did have an effect on As⁵⁺ growth profile,
334 which resulted in increased resistance to As⁵⁺ (Table 3). The MIC between *A. acidophilus* WKC-
335 1 grown with 1 mg L⁻¹ of PO₄³⁻ and 100 mg L⁻¹ of PO₄³⁻ showed a statistical significant difference
336 in all media containing As⁵⁺ concentrations ranging from 1000 mg L⁻¹ to 22500 mg L⁻¹ ($p < 0.05$).

337

338 **Effect of pH on fungal growth**

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



342

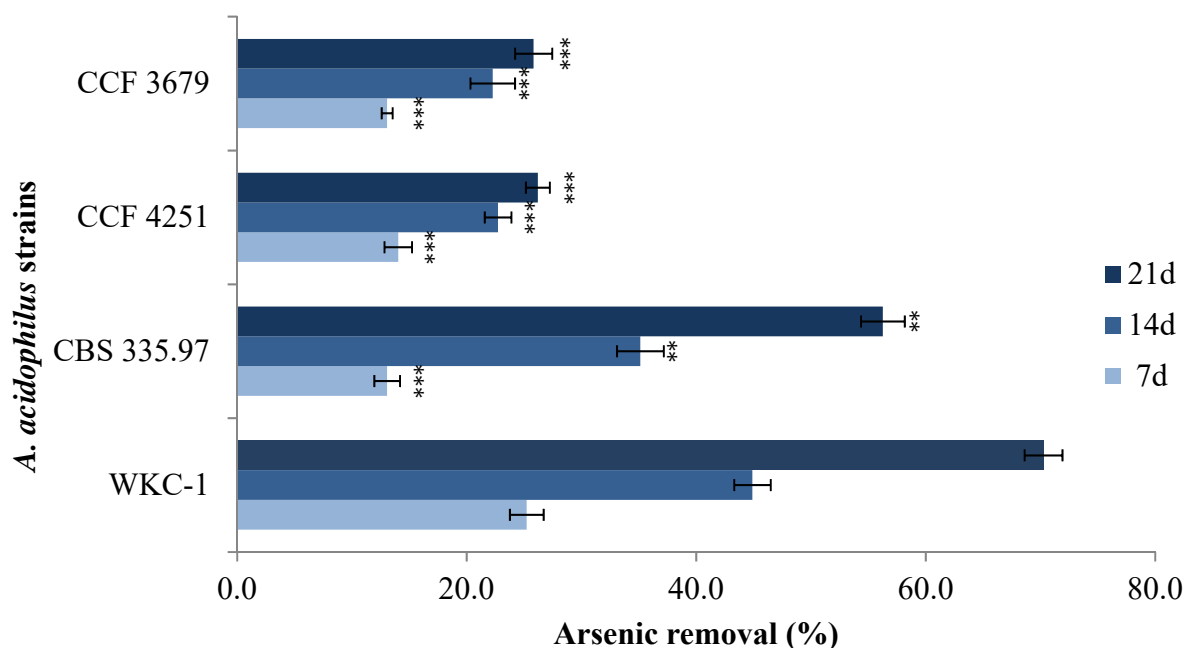
343 Figure 4: Colony diameter, representing a measurement of growth (cm \pm standard error) of
 344 isolated *A. acidophilus* WKC-1 at the minimum inhibitory concentration of As^{5+} , at different pH,
 345 at room temperature, on MEA. Data shown are the mean of three replicates.

346 * NG indicates no growth

347

348 **Arsenate removal efficiency**

349 In Figure 5, the mean percentages of arsenic removal by *A. acidophilus* WKC-1 and three *A.*
 350 *acidophilus* reference strains show that WKC-1 achieves a significantly higher percentage As^{5+}
 351 removal after 7, 14 and 21 days periods of cultivation compared to the *A. acidophilus* CBS
 352 335.97, CCF4251 and CCF3679 reference strains.



353

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

359

360 There is a significant difference in As removal between the cultivation days ($p < 0.001$) for all four
 361 strains except for *A. acidophilus* CCF 4251, where there is no significant difference between 14
 362 days and 21 days cultivation. The percentage removal of As⁵⁺ by *A. acidophilus* WKC-1 is 70.30
 363 % after 21 days of cultivation compared to 56.30 %, 26.20 % and 25.80 % achieved with the
 364 reference strains CBS 335.97 and CCF 4251 and CCF 3679 respectively.

365

366 Biosorption of As

367 The summary of the effect of initial pH in the As⁵⁺ solution on the biosorption process of As⁵⁺
 368 by *A. acidophilus* WKC-1 showed that there was an increase from 0.01 to 0.09 mg mg⁻¹ of the
 369 amount of As⁵⁺ absorbed by isolated *A. acidophilus* WKC-1 as the pH increased from 1.0 to 4.0
 370 (Figure 6a). However, as the uptake started to decrease above pH 4.0, the optimum pH for the
 371 biosorption analysis of As⁵⁺ was set at pH 4.0.

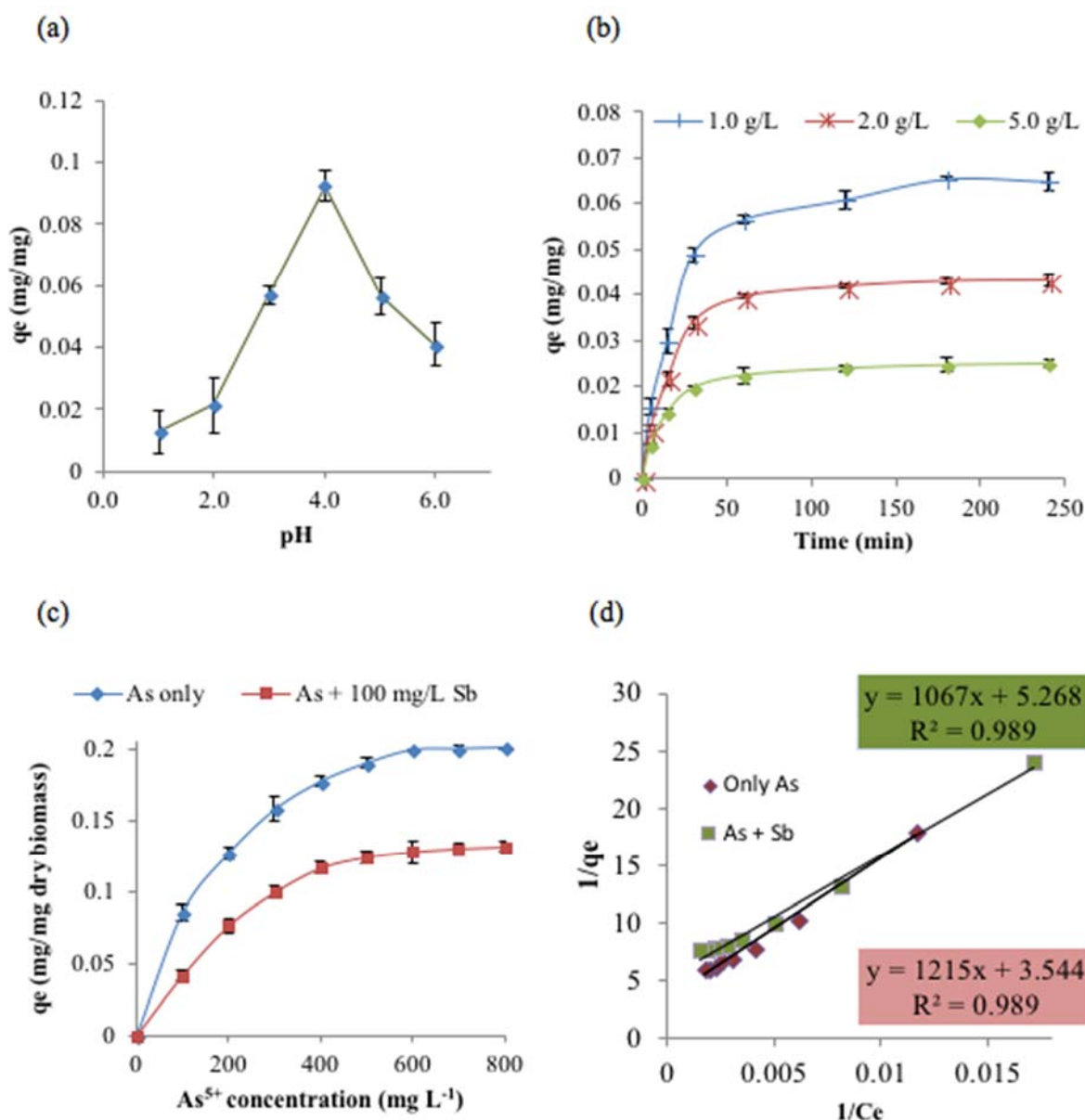
372

373 The biomass loading with increased contact time was studied and it was found that the absorption
374 of As^{5+} rapidly increased in the first 30 min (Figure 6b). After 120 min, the sorption of As^{5+} by
375 *A. acidophilus* WKC-1 reached equilibrium and remained constant ($p > 0.05$). Therefore, the time
376 for the biosorption analysis for both As^{5+} and Sb^{5+} loaded biomass was set at 120 min. The effect
377 of biomass loading is summarized in Figure 6b. The sorption capacity by *A. acidophilus*
378 decreased as the biomass loading increased from 1 g L^{-1} to 5.0 g L^{-1} . In the presence of competing
379 Sb^{5+} ions, the As^{5+} uptake by fungal biomass is significantly affected ($p < 0.05$) as shown in Figure
380 6c.

381

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

387



388

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

393

394 FT-IR analysis

395 FT-IR spectrum range of 4000-400 cm^{-1} was used to detect vibration frequency of changes in the
 396 functional group of isolated *A. acidophilus* strain before and after As^{5+} and Sb^{5+} loading (Figure
 397 7). For control biomass spectrum (vibrational frequencies of bio-molecular functional groups), a
 398 broad band at 3306.55 cm^{-1} indicates -OH bonds stretching vibration at high concentration and

399 weak to medium of the -NH stretching (secondary amines). The peaks appearing in the 2921.74
400 and 2852.67 cm^{-1} region can be attributed to the strong asymmetric and symmetric stretching
401 vibration of CH_2 , respectively. Strong stretching vibrations of $\text{C}=\text{O}$ (esters) and $\text{C}=\text{O}$ (amide I
402 band) observed at peak 1744.18 and 1640.06 cm^{-1} respectively. The peak at 1640.06 cm^{-1} also
403 indicated variable symmetric stretching variations of $\text{C}=\text{C}$. The peak at 1544.68 cm^{-1} was
404 assigned to a motion of -NH bending (amide II) while the peak at 1456.46 cm^{-1} indicated medium
405 CH_2 and CH_3 deformation. O-H bending (in-plane) and strong stretching vibrations of C-F
406 appeared at the peak 1373.94 cm^{-1} . Medium to strong stretching vibrations of C-O and medium
407 C-N stretching of amine groups was observed at both 1238.95 and 1148.40 cm^{-1} peak.

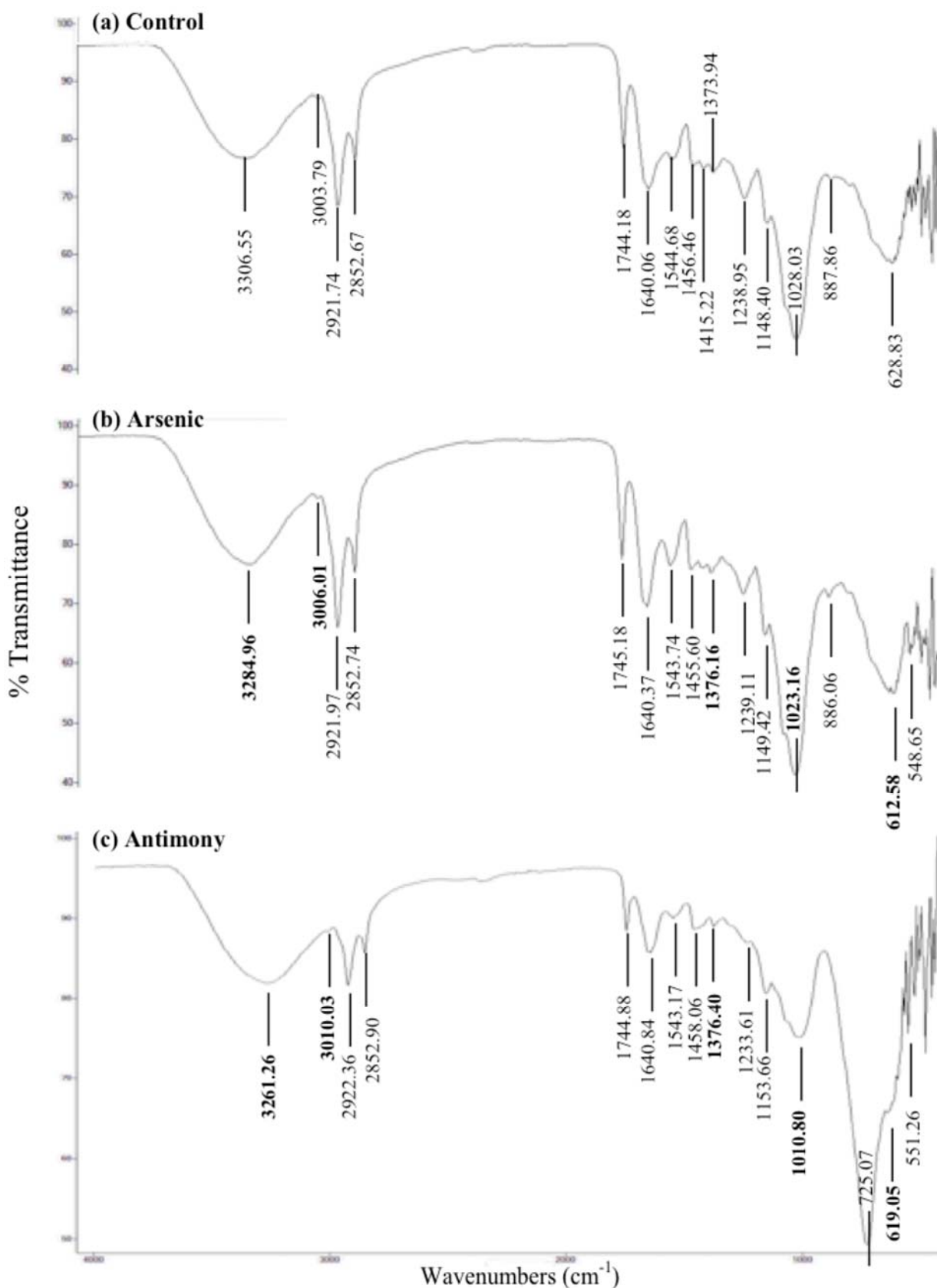
408

409 A strong peak at 1028.03 cm^{-1} indicates P-OR (esters) as well as Si-OR groups. A NH_2 and N-H
410 wagging (shifts on H-bonding), C-H bending and ring puckering and a strong $=\text{C}-\text{H}$ & $=\text{CH}_2$
411 bending was observed at peak 887.86 cm^{-1} . The 'finger print' zone of the spectra, ranging from
412 500-700 cm^{-1} usually represents phosphate or sulfur functional groups.

413

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

418



419

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

422

423 Discussion

424 Soil abiotic characteristics and their interaction with the fungal isolates

425 Due to the igneous geology of Geevor tin mine, its activities generated various metal by-products
426 such as Zn, Cu, As and Sb (Adriano, 1986; Hamilton, 2000). A process called roasting using a
427 Brunton Calciner (burning furnace) where cassiterite (tin ore) containing As, Sb and other
428 minerals such as Fe and Cu were burnt was used in Geevor tin mine. Large amount of roasting
429 waste was deposited near to the production facilities. The contamination of As (Langdon *et al.*,
430 2009) and Sb (Flynn *et al.*, 2003) found in the soil samples were most likely to come from the
431 by-products of the roasting process used to obtain tin.

432

433 The three-step sequential extraction method provided information about the metals and
434 metalloids potential mobility, bioavailability and amount bound to different soil fractions
435 (Carapeto and Purchase, 2000; Lei *et al.*, 2010). This detail information is important for the
436 evaluation of toxicity and bioavailability of metals and metalloids in soil from the mining dump
437 as well as the feasibility of their remediation (Chen *et al.*, 2007). Total concentration of As and
438 Sb were comparable to previously published data for mining sites in Cornwall by Peterson *et al.*
439 (1979) and Dybowska *et al.* (2005) which presented concentration of As at 20 and 40 mm depth
440 in the soils as high as 20,000 and 40000 mg As kg⁻¹, respectively. In addition, over 100 mg kg⁻¹
441 of Sb levels in the soil have been recorded in close proximity to where the mining operations
442 were carried out in Derbyshire, England (Li and Thornton, 1993). Most of the As was found in
443 the residual fraction, which is not readily available and the metalloids present in this fraction can
444 be used as a measurement of the degree of environmental pollution in soil. The higher the metals
445 present in this fraction, the lower the degree of pollution (Howari and Banat, 2001).

446

447 The sum of concentrations in exchangeable and weakly organically bound fractions can be used
448 to determine the bioavailability of transitional metals and metalloids in soils (Carapeto and
449 Purchase, 2000). Geevor tin mine soils also contained high level of iron between 30000 and
450 270000 mg kg⁻¹ (results not shown). According to Drahota and Filippi (2009), acidic conditions
451 (pH<6) with relative abundance of iron oxide (Fe-oxide) may decrease the bioavailability of As
452 in the soil with the formation of iron arsenates such as scorodites and pharmacosiderite in the
453 mining soils (Jacobs *et al.*, 1970).

454

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

456 *Acidomyces acidophilus* (Selbmann *et al.*, 2008) was first isolated by Starkey and Waksman
457 (1943) in an extremely acidic, sulphate containing industrial water. Subsequently, more
458 *Acidomyces acidomyces* strains were isolated in various extreme environments such as acid
459 drainage (Germany), soil near sulfur pile (Canada), volcanic soil (Iceland), acidic industrial water
460 (The Netherlands) and acid mine drainage water (USA) (Selbmann *et al.*, 2008). The
461 morphologies of *Acidomyces* species were not easily described using microscopy because of its
462 tendency to convert to meristematic growth, produce reluctantly disarticulating clumps of cells,
463 or tend to appear to be entirely hyphal without any conidiation (Selbmann *et al.*, 2005; Selbmann
464 *et al.*, 2008; Hujslová *et al.*, 2013).

465

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

475

476 *Penicillium* species was successfully identified using MALDI-TOF MS by Chen and Chen (2005)
477 directly from intact fungal spores. Hettick *et al.*, (2008) obtained abundant peaks in the range
478 5000-20000 m/z by using bead beating in the extraction process, the fungal samples and the
479 MALDI-TOF MS in their experiment have identified all the 12 *Penicillium* species correctly.
480 This study also show that the MALDI-TOF/TOF MS is a robust, cost saving and powerful system
481 in fungal identification and characterization as suggested by Wieser *et al.* (2012).

482

483 However, the use of MALDI-TOF for identification of fungi has a few limitations. The spectral
484 signal generated by MALDI-TOF is strongly influenced by the fungal growth medium as well as
485 the protein extraction methods (Santos *et al.*, 2010). Due to the cell wall structure of fungi, protein
486 extraction requires an additional step such as bead beating, to yield high quality spectra that

487 enable a valid identification (Croxatto *et al.*, 2012). The lack of reference spectra available in the
488 database is the main disadvantage in using MALDI-TOF MS to identify fungal species and work
489 like this current study can contribute to the development of a fungal database. The use of MALDI-
490 TOF/TOF MS described in this paper has demonstrated that this method is capable to identify
491 environmental fungi species provided that the correct sample preparation methods are being used.

492

493 **Tolerance and removal efficiency of As**

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

499

500 *A. acidophilus* WKC-1 exhibits high As⁵⁺ removal efficiency even in extreme pH conditions. This
501 indicates that *A. acidophilus* WKC-1 has great cellular detoxification mechanisms in toxic
502 metalloids tolerance. The unique composition of fungal cell wall containing excellent metal-
503 binding properties offers great advantage in metal removal either by entrapment in extra-cellular
504 capsules and precipitation of metals (Gupta *et al.*, 2000). Previous study by Su *et al.* (2010)
505 observed the intracellular uptake of As⁵⁺ in *Tichoderma asperellum* and *Fusarium oxysporum* can
506 be as efficient as extracellular sorption in many fungi where the intracellular As⁵⁺ accumulation
507 accounted for 82.2% and 63.4% of the total accumulated As⁵⁺.

508

509 **Biosorption of As**

510 The As⁵⁺ uptake by fungal biomass is significantly affected by the presence of competing ions,
511 in this case Sb⁵⁺ (Figure 6). These ions compete for active binding sites due to the non-specificity
512 of the functional groups present on the fungal cell surface. As a result, it is often found that
513 specific transitional metal/metalloid(s) uptake from mixed solutions is lower than those in a
514 solution containing the single transitional metal/metalloid.

515

516 The pH has profound effect on As⁵⁺ uptake by *A. acidophilus* WKC-1. The *A. acidophilus* WKC-
517 1 As⁵⁺ sorption capacity increased with increasing pH from 1 to 4 and showed optimum As⁵⁺

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

528

529 Higher absorption of As^{5+} was observed with increased contact time due to the abundant binding
530 sites available on the fungal cell surface for the metal sorption by *A. acidophilus* WKC-1. The
531 biomass loading of *A. acidophilus* WKC-1 for As^{5+} sorption was found to be optimal at 1 mg L^{-1} .
532 ¹. The optimum biomass loading results support the hypothesis by Gupta and Rastogi (2008) that
533 an increase in biomass loading could exert a shell effect by protecting the active binding sites
534 from being occupied by the metal, resulting in the decrease of metal sorption. A similar effect of
535 high biomass loading resulting in low sorption was observed by Bishnoi *et al.* (2007) in Cr (VI)
536 removal by *Trichoderma viride*. In the presence of competing ions, metal uptake from mixed
537 solutions is often found to be lower than those in a single-species system (Chong and Volesky,
538 1995).

539

540 In general, metal uptake by fungus increases as the ionic radius of the metal cation increases, thus
541 metals with higher ionic charge show greater binding to biomass. However, as the concentration
542 of other competing metalloid cations present within the same biosorption process increases, the
543 uptake of another metalloid further decreases. Chemical interactions between two metal species
544 as well as biomass may take place, resulting in competition for sorption sites on the surface (Akar
545 *et al.*, 2005). Sari and Tuzen (2009) reported that maximum biosorption capacity of As^{5+} by
546 *Inonotus hispidus* biomass was found to be 59.6 mg g^{-1} . Plant biomass prepared from sawdust of
547 *Picea abies* has the maximum As^{5+} sorption capacity of 1.369 mg g^{-1} (Urik *et al.*, 2009). The As^{5+}
548 adsorption capacity of zirconium (IV) loaded phosphoric chelate adsorbent, synthesized by
549 radiation induced graft polymerization, was 149.8 mg g^{-1} (Seko *et al.*, 2004). *A. acidophilus*
550 WKC-1 showed a greater As^{5+} adsorption capacity than previously studies fungal strains, where

551 the predicted maximum capacity of fungal strain uptake of As^{5+} by WKC-1 was 170.82 mg g^{-1}
552 dry biomass and has potential to be used in bioremediation transitional metals in soils. The fate
553 of As^{5+} after being adsorbed into the fungal cell might be broken down to less toxic species by
554 powerful secondary enzymes produce intracellularly by the fungal itself or undergo a number
555 detoxification pathways within the fungal cell which include the reduction to As^{3+} by arsenate
556 reductases, followed by exclusion or sequestration of As^{3+} (Sharples *et al.*, 2000; González-
557 Chávez *et al.*, 2002). Further study is required to elucidate and understand the detoxification
558 mechanisms of As^{5+} of *A. acidophilus*.

559

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

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

566

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

572

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

582

583 Based on the spectra from FT-IR generated, it suggests that As^{5+} and Sb^{5+} might compete for the
584 binding sites of OH, -NH, -CH, -SO₃ and PO₄ functional groups on the surface of the isolated *A.*
585 *acidophilus* WKC-1 strain. Previous study by Dixon (1997) showed that As^{5+} reacting in a similar
586 way as phosphate in which it has the ability to form ester linkages with hydroxyl groups. A study
587 carried out by Parascondola (1977) found out that Group 15 elements in the periodic table in both
588 pentavalent and trivalent state can interact with sulphur (formation of As-S complexes), thus this
589 supports the analysis from the FT-IR analysis that -SO₃ functional group could involve as a
590 binding site of As^{5+} and Sb^{5+} . Some other functional groups such as C-O, C-N and CH₂ may also
591 compete to a lesser extent by these two metalloids to bind on the surface of *A. acidophilus* WKC-
592 1.

593

594 **Conclusions**

595 In conclusion, metal analysis showed that 26.40% of As^{5+} is bioavailable in the soil samples at a
596 level below the MIC of *A. acidophilus* WKC-1, suggesting a good potential to apply this strain
597 to remediate As polluted soil. The presence of phosphate decreases the toxicity of As^{5+} whereas
598 Sb^{5+} significantly reduces the As removal ability of WCK-1. The -OH, -NH, -CH, -SO₃, and PO₄
599 functional groups have been identified as the key competitive binding sites between As^{5+} and
600 Sb^{5+} . The isolate WKC-1 showed a high resistance and high percentage As^{5+} removal, one of the
601 highest reported in *A. acidophilus* species. Our study also demonstrated that MALDI-TOF/TOF
602 MS could provide a faster and cheaper way to identify environmental fungal strains. The
603 tolerance of the isolated *A. acidophilus* WKC-1 strain to low pH and high As concentration
604 together with its capacity to remove approximately 170 mg As^{5+} per gram dry biomass, made it
605 an potential candidate to be used in bioremediation of As.

606

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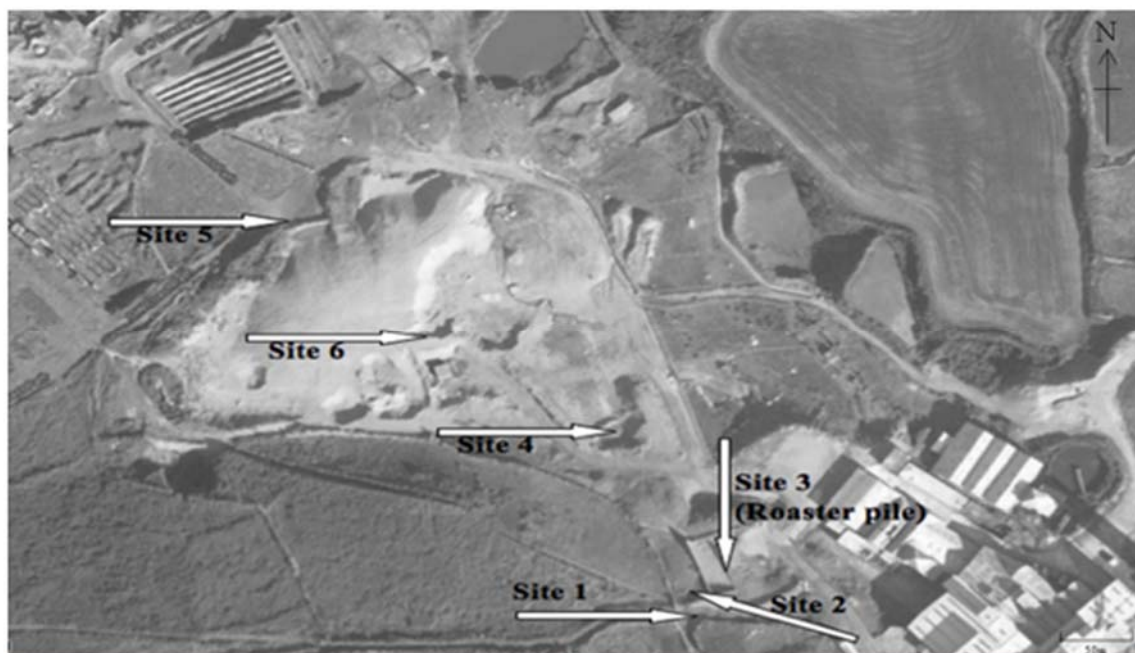
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885 Supplementary materials



886

887 Fig. S1: An aerial photograph of the Geevor tin-mine in Pendene, Penzance, Cornwall, UK and

888 locations of soil sampling sites (Greevor Tin Mine was viewed on 17 July 2013.

889 <https://www.google.co.uk/maps/places/Greevor+Tin+Mine/@50.1519033,-5.6744307,1404m>).

890

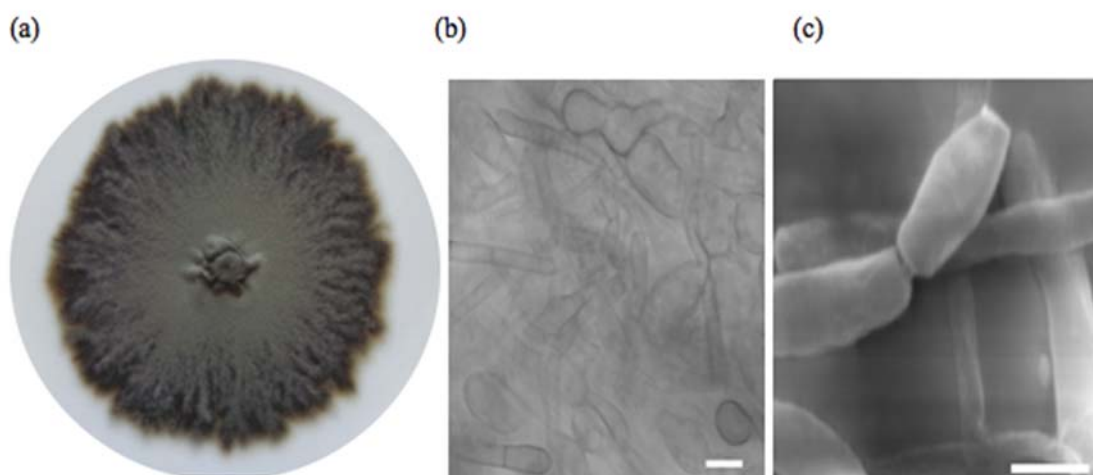


Figure S2: Morphological features the fungus of (a) colony of the isolated fungal strain in CDA medium, (b) Hyphae of the strain observed by light microscope at a magnification of 400x and (c) scanning electron microscope (SEM) at a magnification of 1000x (b) and 2200x (c), scale bar in (b) and (c) =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

891

892 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 |

893

894 Table S2: Recovery of As and Sb (mg kg⁻¹) metal using certified reference material, SRM 2710a
895 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 |

896 Table S3: The diameter measurement of the minimum inhibitory concentration of As⁵⁺ by
 897 isolated *A. acidophilus* and two positive control *A. acidophilus* type strains

| As ⁵⁺ concentration | Diameter (cm) | | |
|-----------------------------------|---|-------------------------------------|-----------------------------------|
| | Isolated <i>A. acidophilus</i> strain | Positive control | |
| | | <i>A. acidophilus</i> CBS 335.07 | <i>A. acidophilus</i> 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 |

898 * NG indicates no growth