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BIO-PRECIPITATES PRODUCED BY TWO AUTOCHTHONOUS BORON TOLERANT *STREPTOMYCES* STRAINS

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Abstract

Boron is widespread in the environment. Although contaminated soils are hard to recover different strategies have been investigated in the recent years. Bioremediation is one of the most studied because it is eco-friendly and less costly than other techniques. The aim of this research was to evaluate whether two *Streptomyces* strains isolated from boron contaminated soils in Salta, Argentina, may help remove boron from such soils. For this, they were grown in different liquid media with two boric acid concentrations and their specific growth rate and specific boric acid consumption rate were determined. Both strains showed great capacity to remove boron from the media. Increasing boric acid concentrations affected negatively the specific growth rate, however the specific boric acid consumption rate was superior. Boron bio-precipitates were observed when the strains grew in the presence of boric acid, probably due to an adaptive response developed by the cells to the exposure, for which many proteins were differentially synthetized. This strategy to tolerate high concentrations of boron by immobilizing it in bio-precipitates has not been previously described, to the best of our knowledge, and may have a great potential application in remediating soils contaminated with boron compounds.

Keywords

Streptomyces; boric acid; boron; biomineralization; bioremediation; contaminated soils

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1. Introduction

Salta province, in the northwest of Argentina, is the third most important natural reserve of boron mineral around the world. Boron mining activity is economically important in this province, not only for the foreign currency income but also for both the direct and indirect employment it brings. Although many methods are used to minimize or avoid contamination produced in the industrialization and production processes, the intense activity impacts strongly the environment.

Many methods based on different physical or chemical principles have been developed to decontaminate polluted environments around the world. Also, because of the important ecological and economic advantages, various strategies related to the use of microorganisms have been developed [1]. Microorganisms can develop different strategies to survive in the presence of metals such as the excretion of chelating compounds to the exocellular media that allows to immobilize them (ie, the exopolysaccharides production), the interaction and bonding of metals to the cell membrane to prevent entry (biosorption) [2], the development of transport and incorporation systems (bioaccumulation), chemical transformation (reduction) reactions and metal mineralization, forming a primary reservoir (biomineralization) [3], among others.

Biomineralization is the process by which organisms produce minerals. Microorganisms are the second most important group and can produce a great variety of different minerals through two processes following differing mechanisms: Biologically Controlled Mineralization (BCM) and Biologically Induced Mineralization (BIM) [4]. In BCM, minerals are normally formed on or inside organic matrices or vesicles within the cell. The organism exerts a meaningful degree of control over the nucleus formation and mineral growth process and, thus, over the intracellular composition, size, and location of the particles [5]. Instead, in the BIM the mineral formation happens as a consequence of changes in the supersaturated system due to the intake or excretion of different metabolites (active mechanism) [6] and to the contribution of substances that can act as nuclei of crystallization, such as cell surfaces (cell wall, membranes, excreted organic compounds, cell debris from lysis) which favour precipitation (passive mechanisms) [6]. This is the way how microorganisms modify their closest extracellular microenvironment, thus generating the required conditions for the mineral to precipitate [7]. Two surface BIM processes have been distinguished: passive and active [8, 9]. The first refers to simple non-specific binding of cations and recruitment of solution anions, resulting in surface nucleation and growth of minerals. The second, among other things, implies direct redox transformation of surface-bound metal ions.

Minerals formed by induced mineralization are almost always deposited outside the inducing organism. Therefore, mineralization happens in an open environment for this purpose and not inside a delimited intracellular space [5]. Nor is there a specialized cell or macromolecular machinery with a specific role in the biomineralization process. On the whole, biominerals count with calcium as major cation, iron being the second most common, while phosphates, oxides, and carbonates happen to be the most numerous anions

[4]. There is evidence of numerous bacterial genera which produce biominerals, such as *Pseudomonas* [10] *Bacillus* [11] and *Vibrio* [12] that mediate the precipitation of calcite under well-defined conditions, sulphate reducing bacteria and cyanobacteria involved in dolomite formation [13], *Myxobacteria* that allows obtaining struvite and calcite crystals in presence of the cellular membrane fraction [14], *Halobacillus* which precipitates carbonates at different salt and magnesium/calcium ratios concentrations [15], *Shewanella* capable of inducing extracellular precipitation of magnetite [16] and *Streptomyces* associated with hydromagnesite and needle-fiber aragonite deposits [17] and struvite biominerals in which Mg is replaced by Ni [18, 19]. Biominerals are increasingly being exploited in bio-nanotechnology, focused on exploiting ferritins [20], magnetite nanoparticles as Magnetic Resonance Imaging (MRI) contrast agents, ferrofluids, magnetic recording materials, therapeutic delivery vehicles [21], and on the remediation of other contaminants such as chromium [22], nickel [18] and cobalt [23]. *Streptomyces* are involved both in calcite biomineralization processes in marine sediments and extreme environments, [17] as well as in the biomineralization of a new phase isostructural with struvite, in which Mg is replaced by Ni [18].

In previous studies, we isolated bacteria from soils naturally containing high concentrations of boron and from others anthropogenically contaminated with the element in the Salta province, northwest of Argentina [24]. We also characterized the isolated strains genetically and studied their ability to grow in the presence of boron and their tolerance to different boric acid concentrations [24]. As the results were promising a couple of *Streptomyces* strains were selected to conduct further experiments. The aim of this research was to study their growth capacities in liquid media containing different boric acid concentrations, with two objectives: to elucidate the fate of boron during growth using spectrophotometric techniques and also to preliminary determine using proteomics, if there are proteins involved in the tolerance mechanisms.

2. Materials and Methods

2.1 Liquid media

Two liquid media were used. One of them was Minimal Medium (MM) with composition, in g/L: glucose, 10.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.20; $FeSO_4.7H_2O$, 0.01; pH 7.0 ± 0.5. A stock solution with the double concentration was prepared (i.e; in g/L: glucose, 20.0; L-asparagine, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.40; $FeSO_4.7H_2O$, 0.02) and 10 mL of it were added to 100 mL flasks that were sterilized by autoclave. Then, 0.84 mL or 1.67 mL of a concentrated sterile boric acid solution (440 mM) was added to each flask to a reach a final concentration of 20 mM and 40 mM, respectively, after completing 20 mL with distilled sterile water. These final concentrations were selected after the results obtained in previous studies [24], where tolerance to boron (from 20 to 440 mM) was analyzed for eight actinobacteria initially isolated. The pHs measured after the addition of the sterile concentrate boric acid were 5.58 and 5.44 for 20 mM and 40 mM, respectively.

MM was used to prevent boric acid forming a complex with some components of the medium, leaving them less available for cells, fairly common situation in rich media [25]. The other liquid medium used was Soil Extract (SE). This was prepared using 300 g of soil

taken at a depth of 0–15 cm from surface, which were placed in 600 mL of tap water and heated to boiling for 1 h with constant stirring. After 24 h it was centrifuged at 11,700 rpm for 15 min, filtered, adjusted to pH 7.0 (± 0.5) and partitioned in 100 mL flasks, which were autoclaved [26]. Then, 0.84 mL or 1.67 mL of a concentrated sterile boric acid solution (440 mM) was added to each flask to a final concentration of 20 mM (pH 5.60) and 40 mM (pH 5.46) respectively, after adding water to reach 20 mL as final volume in all cases. The boric acid solution was always sterilized separately from the liquid media because heat induces borate esters formation between glucose and boric acid, preventing availability of the carbon source for microorganism growth.

2.2 Microorganisms, inocula preparation and culture

The growth of two selected actinobacteria (*Streptomyces* sp. 053 and *Streptomyces* sp. 128) in MM and SE media, with and without the addition of boric acid (H_3BO_3), was studied. Those strains were chosen because of promising results regarding boron tolerance obtained in previous work [24].

Since boron inhibits spores germination [27], fresh inocula were prepared from spore suspensions (4×10^6 CFU/mL) cultured in MM without the addition of boric acid (8 – 12 h depending on the strain) at 30 °C and 250 rpm. In all cases, 650 μL of the fresh inocula were added into 100 mL flasks containing 20 mL of sterile liquid medium with the addition of boric acid solutions to a final concentration of 20 or 40 mM. Also, the same procedure was followed for each media without boric acid added which were used as growth controls. They were incubated from 5 to 7 days (according to the strain) at 30 °C, 250 rpm in orbital shakers, in order to assess growth.

Samples were taken periodically by sacrificing a total of six flasks in MM and a similar amount in SE, including duplicates for each media and condition as follows: without boric acid (as control) and with the two different concentrations (20 and 40 mM). Biomass and the remaining boric acid concentrations were determined in all cases in duplicates.

2.3 Biomass concentration

As *Streptomyces* are filamentous bacteria, it is not possible to measure growth by optical density [2]. Therefore, the biomass concentration was determined by dry weight, from its recovery by filtration of the production of a complete flask (20 mL) at different incubation times. For this, a manifold system with three stainless steel sterile funnels with cellulose nitrate membranes of 47 mm diameter and 0.45 um pore size (Sartorius) were used, through which the supernatants were filtered using a vacuum pump (Microsart® e.jet, Sartorius) and bacteria were retained. The biomass was determined by carrying membrane dry weight (24 h at 105 °C), with bacteria retained on the filter. The supernatants were used to analyse the residual boric acid concentrations.

Specific growth rate (μ) was calculated according to:

$$dX/dt=\mu X \quad (1)$$

where X (g/L) is the biomass concentration and t (h) is the time.

2.4 Residual boron concentration

The concentration of the residual boron in the culture supernatants obtained before was analysed using the Azomethine H spectrophotometric method [28]. This was selected because of the high quality of results, sensitivity (concentrations in the order of mg/L), low costs, simplicity, and accessibility to perform routine tests.

To prepare the calibration curve, 2.5 mL of 1000 mg/L boric acid standard solution (Merck) were brought to 100 mL in a volumetric flask with distilled water, resulting in a concentration of 25 mg/L. From this, solutions with the following concentrations were prepared: 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/L (recommended linearity level). All of them and one without boric acid (just distilled water) were used to measure Absorbance by UV-visible single beam spectrophotometer (Spectrum SP-1103) at 414 nm. These measurements were performed after 60 to 120 minutes of prepared the solutions, to avoid variations in the values [29].

The specific boric acid consumption rate (q_A) was calculated according to:

$$q_A = (1/X)[(dC_A)/dt] \quad (2)$$

where C_A (mg/L) indicates de boric acid concentration.

2.5 Scanning electron microscopy and Energy dispersive spectroscopy X-ray

In order to perform the morphological, structural, and compositional characterization of solid surfaces, pure cultures of the selected strains were observed through a scanning electron microscope (SEM) vacuum under JEOL JSM - 6480 LV (Thermo Electron), capable of accelerating voltage between 0.2 and 30 kv. For that the bacteria were grown at 30 °C for 72 h in MM, without and with boric acid in concentrations of 20 and 40 mM. After that, cultures were centrifuged at 5,400 rpm for 10 min (the supernatant was discarded) and 500 μL of 2% glutaraldehyde were added (binder) and left for 48 h at 4 °C. Then, cells were dehydrated by successive washes with 500 μL of ethanol at 30%, 50%, 70%, 80%, 96%, and absolute ethanol. After each addition, the samples were centrifuged at 5,400 rpm for 10 min, supernatant was discarded and the next alcohol concentration was added. Strains in absolute ethanol were delivered to LASEM (Electron Microscopy Laboratory of INIQUI, CONICET-UNSa) where the critical points and a conductive coating of gold/palladium through a metallizer (DESK-IV) were done. This step was necessary since bacteria are non-conductors. The metallization also plays a protective role by making material more resistant to prevent burning or alteration by the action of the high voltage used [30]. The chemical analysis of the observed accumulation was performed by an Energy Dispersive X-ray (EDS) analyser (Thermo Electron, SIX System Model NORAN NSS-100), equipped with secondary electron sensors and backscattered held high and low vacuum.

2.6 Protein extraction and quantification

Cultures of strain 053 grown in MM for 72 h with and without boric acid concentrations (20 and 40 mM), were centrifuged at 5,400 rpm for 5 min and washed twice consecutively with 25 mM Tris-HCl buffer pH 8.0. Then the cells were resuspended in 120 μL of 8 M urea 25 mM Tris-HCl buffer pH 8.0 with protease inhibitors (PI) to avoid lysis of the proteins released during extraction. To obtain the quantity and quality of protein required, the cell was disrupted by mechanical means, after addition of 0.1 mL of glass beads doing approximately 30 pulses of 1 min mixing in a vortex with one minute intervals between them in an ice bath. Next, a hole in the bottom of the tube was done with a needle and another tube was placed below and centrifuged at 1,000 rpm for two minutes. The upper tube was discarded and 120 μL of 8 M urea solution 4% 3-[(3-cholamidopropyl)-dimethylammonium]-1-propane sulfonate (CHAPS) and dithiothreitol (DTT) 50 mM were added to the lower tube. It was homogenized with vortex, centrifuged for 10 min at 12,000 rpm and 4 °C, and proteins obtained were quantified. Total protein concentration of the samples was determined by Lowry method [31]. Standard curve with bovine serum albumin (BSA) was built using solutions of known concentrations prepared from a 1 mg/mL BSA solution. When proteins were resuspended in rehydration buffer (RHB), the standard curve was performed with BSA dissolved in RHB.

2.7 Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE)

Proteins present in the samples were denatured with 5% sodium dodecyl sulphate (SDS) for 3 min and then one-dimensional electrophoresis was performed using a Mini Protean 3 (Bio-Rad) and a buffer with the following composition: 0.1% (w/v) SDS, 25 mMTris-HCl pH 8.8, and 192 mM glycine. While the proteins were in the stacking gel, the intensity was 15 mA and the composition was: 0.125 M Tris-HCl pH 6.8, 5% (v/v) acrylamide/bisacrylamide (30:0.8), 130 mM ammonium persulphate, 0.025% (v/v) tetramethylethylenediamine (TEMED), and 2% (w/v) SDS. Instead, intensity was 20–25 mA when proteins were located in the separating gel, which composition was: 7–15% (v/v) acrylamide/bisacrylamide (30: 0.8), 2% (w/v) SDS, 130 mM ammonium persulphate, 0.025% TEMED (v/v), and 0.375 M HCl pH 8.8.

2.8 Two-dimensional electrophoresis (2DE)

The proteomic approach consisted in protein separation by two-dimensional gel electrophoresis (2DE): in a first dimension IPG strips were used (GE Healthcare Life Science) and then in a second dimension SDS-PAGE gels of 13×13 cm 2DE, two strips (control gel and with boron) 7 cm non-linear 3/10 were used.

Isoelectric focusing (IEF) was performed in the first dimension. For this, dissolved proteins were loaded in a rehydration RHB buffer (composition: 8 M urea, 4% (w/v) CHAPS, 0.5% (v/v) ampholytes, 0.00125% (w/v) bromophenol blue, 50 mM DTT) in the rehydration tray (IPBox) and then the strips were placed above. Rehydration cuvette was incubated at 20 °C for 16 h. Once hydrated, the strips were placed in the bowl of IEF (EttanTM IPGphor3, GE Healthcare), covered with mineral oil and the following program (EttanTM IPGphor3, GE Healthcare) was set to run the first dimension: 1) 250 V, 15 min; 2) 6000 V, 2 h; 3) 6,000 –

24,000 V h; 4) 500 V, 2 h. Once the IEF was complete, the strips were removed from the cuvette; excessive mineral oil was removed and stored at −20 °C.

For SDS-PAGE electrophoresis (second dimension), the strips were thawed for 15 min and then equilibrated for 15 min with balancer buffer I (composition: 0.375 M Tris-HCl pH 8.8; 6 M urea, 2 % (w/v) SDS, 20% (v/v) glycerol, 130 mM DTT) and 15 min with the equilibrating buffer II (composition: 0.375 M Tris-HCl pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 135 mM iodoacetamide). Then, the strips were placed in a SDS-PAGE gel and fixed with 1 mL agarose solution (composition: 0.5% (w/v) agarose, 0.002% (w/v) bromophenol blue, 1X buffer electrophoresis), previously melted and tempered. The electrophoresis cuvette was filled with electrophoresis buffer and 6 – 9 mA intensity was applied overnight.

2.9 Gel staining and analysis of protein synthesis

Two different stains, one with CoomassieBrillant Blue (CBB) for one-dimensional gels, and the second silver nitrate for two-dimensional gels, were used (this was prepared on the same gel after the first staining with CBB). In the case of CBB staining gels, they were incubated for 30 min in a dye solution containing 0.1% (w/v) Coomassie Blue - G250, 10% (v/v) acetic acid, and 25% (v/v) isopropanol and finally the gels were transferred to a bleaching solution of 10% (v/v) acetic acid and 10% (v/v) isopropanol. For silver nitrate staining, the suggested kit protocol was followed (ProteoSilver™ Plus, Sigma).

The image acquisition was performed by gel scanning III Image Scanner (GE Healthcare Life Sciences). Image Master 2D Platinum v 7.0 (GE Healthcare Life Sciences) software for comparative analysis of two-dimensional gels were used, which allowed to define differentially spots expressed between two or more situations analysed using the complimentary statistical analysis.

3. Results and Discussion

3.1 Growth in liquid media

The growth of both strains, *Streptomyces* spp. 053 and *Streptomyces* spp. 128, was negatively affected by the presence of boric acid in the liquid media studied. The biomass concentrations obtained for strain 053 in MM with 20 mM and 40 mM of boric acid were 20% and 12% lower, respectively, than the control without boric acid (MM) and 8% and 12% lower in SE with 20 mM and 40 mM of boric acid, respectively, respect to the corresponding control (SE without boric acid).

Strain 053 grew better (producing higher concentration of biomass) in both media with 20 mM boric acid, compared to 40 mM (Figure 1.a and Figure 1.b).

Growth of strain 128 in MM was similar with 20 and 40 mM boric acid. However, the biomass concentration was 20% lower than the control (without boric acid) (Figure 1. c) [32]. The decrease in growth was more remarkable (36%) in SE medium with 40 mM of boric acid (Figure 1.d).

Regarding the removal capacity of boric acid, strain 128 was better than strain 053 in all the conditions assessed (Table 1). The removal capacity of the strains was similar for both media used with the same boron concentration but it was negatively affected when the concentration increased, being more remarkable for *Streptomyces* spp. 053.

Conversely to our results showing that the liquid media tested (MM and SE) did not affect the boron removal capacity, Polti et al. [33] studying the chromium removal capacity of *Streptomyces* sp. MC1 found that the liquid media had a great impact in it. Indeed, after 48 hours' incubation in MM supplemented with 50 mg/L of Cr (VI) the bacteria reduced 75% of Cr (VI), while the reduction was only of 30% when cultured in SE for 96 hours being 10 mg/L the initial concentration.

The specific growth rate (μ) was similar for strain 053 in all the conditions, with the exception of the control in SE (without boric acid, in which μ was around 3.5 higher), and for strain 128 in SE control and with 20 mM of boric acid. In all the other conditions for *Streptomyces* sp. 128, the specific growth rate was one order of magnitude lower (Figure 2). It is interesting to note though that the highest values of the boron removal specific rate (q_A) were found for the highest initial boron concentration (40 mM) despite the fact that the growth was negatively affected.

The μ values obtained in this study for both strains in SE with 40 mM of boric acid, were one order of magnitude lower than those obtained by De Jager et al. [34], who worked with *Streptomyces coelicolor* A3(2) in a pressurized continuum reactor using glucose as carbon source (*medium* International *Streptomyces* Project, medium 2; ISPM 2). Coisne et al. [35] and Shahab et al. [36, 37], who worked with the same strain and medium (ISPM 2) but in discontinued cultures without pressurization, obtained similar μ values than De Jager et al. [34]. Since in none of the above mentioned cases there was a stressing agent such as boric acid in our study, the growth rate of the target strains in its presence is noteworthy. Taştan et al. [38] studying boron removal from water by microalgae *Chlorella* sp., obtained superior q_A values superior (0.175 mg A/(g biomass. h) using a concentration of 0.134 mM boric acid) than those presented here, although the conditions were different and the assays lasted 15 days.

3.2 Bio-precipitates

Pure cultures of the two selected actinobacteria were grown during 72 h at 30 °C and 250 rpm in MM without and with boric acid (20 and 40 mM initial concentrations) and were watched with the scanning electronic microscope. Numerous structures (accretions) were observed in both strains grown in the presence of boric acid that did not appear in the control without acid (Figure 3, black circles). The different magnifications used enabled the observation of these abundant forms in the medium with 20 mM of boric acid (Figure 3b and g). In the medium with 40 mM boric acid they were also present although were scarce and with different sizes, which might suggest different stages of development (Figure 3d and h). Also, at the higher boric acid concentration, the cells were closer together, as if they were adhered to each other, quite contrary to what happens in the other cases.

In order to determine the composition of the structures observed, EDS microanalysis of some of them was conducted revealing similar results. To illustrate (Figure 4) the quantitative analysis performed in the accretion found in 053 culture with 20 mM boric acid (Figure 3c, black ellipsis) revealed that this structure contains a high boron percentage and small amounts of magnesium and iron, the latter in lower quantities than those in the error margin (Table 2). Both elements were added as part of the micronutrient solution to the culture medium. The sodium found therein is likely to have come from the water.

Also the quantitative analysis performed in the accretion found in the culture of strain 128 with 40 mM boric acid (Figure 3h, black ellipsis) revealed that this structure contains only boron and oxygen in high percentages (Table 2).

Given the morphological similarities in the structures found, even when they were from different strains, it was thought they might be boric acid crystals remaining without dissolving in the liquid medium. However, acid concentrations used in the culture media are far below the solubility limit of this compound in the given conditions (0.124 mg $B(OH)_3$/100 mg H_2O (20 mM) and 0.247 mg $B(OH)_3$/100 mg H_2O (40 mM) at 30 °C, constant stirring at 250 rpm), since boric acid solubility at 30 °C is 6.23 g $B(OH)_3$/100 g H_2O. This, considering similar density between water (1 g/mL) and the solutions as they are diluted aqueous solutions (Figure 5).

Similarly and in order to establish comparisons, the boric acid (in solid state) used in preparing the culture media was analysed in the electronic microscope (Figure 6) as well as with EDS (Figure 7). As expected, the percentage of atomic composition obtained through the EDS corresponded to that of boric acid (data not shown) and it was different from the structures obtained in the culture.

All the results obtained suggest that the accretion found may be a bio-precipitate obtained through a Biologically Induced Mineralization (BIM), formation type which would justify its being outside the cells, as observed. Minerals formed by BIM are often characterized by poor crystallinity, lack of specific crystal morphologies, and broad particle-size distributions [39], as we could observe here. Both the fine crystal size and the spheroidal shapes are sometimes used as indicators of bacterial induction [40].

Furthermore, the capability of both strains to form these bio-precipitates is probably the strategy that they developed to tolerate high boron concentrations modifying the chemical composition of the external fluid through metabolic activities. The chemical equilibria between soluble and insoluble phases are influenced by physico-chemical components of the environmental matrix as well as other abiotic components, including dead biota and their decomposition products.

3.3 Protein Synthesis

Streptomyces spp. 053 protein profiles with (20 and 40 mM) and without boric acid after 72 h of culture in MM at 30 °C and 250 rpm were obtained through mono dimensional electrophoresis with SDS PAGE (Figure 8). Important differences were observed in some protein bands when the strain was grown in the presence of boric acid (Figure 8, see the

arrows). Clearly, the presence of the acid brings out some protein oversynthesis. The differential synthesis of these proteins may be the result of some of the strain's mechanisms to resist the toxicity brought about by boric acid (Figure 9). Most of the bands differentially expressed from the strains exposed to boron are between 20 and 90 kDa. Parsell and Lindquist [41] reported that heavy metals may induce synthesis of stress proteins (known as *Heat shock protein*, Hsp), depending on the microorganism type.. These proteins act in normal conditions but they may be over synthetized under stress. Their molecular weights vary between 15 and 30 kDa (family of small Hsp) and up to 110 kDa [42]. Thus, the protein over synthesis in this work may be an adaptive response of the cells to acid exposure. Twenty synthetised membrane proteins were isolated by Wimmer et al. [43] by means of chromatography and bidimensional electrophoresis and identified by MALDI-TOF microsomal solutions of roots of *A. thaliana* in the presence of boron. The proteomic analysis showed that many membrane isolated proteins are typical components of the microdomains and that their capacity to bind boron increases together with the concentration.

On the basis of the preceding information for the proteins synthesized in the presence of boron both in *A. thaliana* and *Zea mays*, the ones over synthesized in this work could be homologues, given that their molecular weights are between 10 and 90 kDa and their pIs are between 4 and 6, but there is not enough evidence regarding bacteria to support this hypothesis.

Three membrane proteins, BOR1, DUR3, and FPS1 related to boron in *Saccharomyces cerevisiae*, appear to be involved in boric acid tolerance and in keeping the concentration of protoplasmic boron [44].

As to the presence of this type of proteins in bacteria, Ahmed and Fujiwara [45] proposed the existence of a bearing protein in the membrane of *Bacillus boroniphilus*, similar to that of ATR1 and BOR1, which allows boron extra low intracellular concentration even though it may be high outside (450 mM maximum). These authors justified this behaviour through the existence of some mechanism which enables pumping boric acid out of the cell to keep the internal concentration lower than the external one and proposed that the mentioned outward flow in boron tolerant bacteria may be due to a protein.

On the other hand Miwa and Fujiwara [46] when conducting studies on boron accumulating bacteria isolated from activated sludge (none of them *Streptomyces*), reported a great variability in the intracellular boron content among species. They justified that proposing different uses of boron in bacteria, such as membrane composition, transport mechanisms, and in proteins and/or organic acids formation of which may bond to or use boron. They also suggested that there may exists an intracellular boron regulation mechanism in bacteria, not only to acquire it when it is scarce but also to avoid the toxic effects of excess.

Despite the advances commented so far there has been no information as to a boron transporter in bacteria in general nor in *Streptomyces* in particular, or about ATR1 or BOR1 homologues in the databases of the bacterial genome sequences. Even though in this research neither synthesized proteins nor their role have been identified, the results obtained

in strain 053's differential protein synthesis in the presence of boric acid suggest there may be a bearing protein similar to that proposed by Ahmed and Fujiwara [45] for *Bacillus boroniphilus*.

4. Conclusions

The selected strains, *Streptomyces* spp. 053 and *Streptomyces* spp. 128, grew in Minimal Medium and Soil Extract, without and with boric acid at 20 and 40 mM. Increasing boric acid concentrations in the media affected negatively the specific growth rate at the time that increased the specific boric acid removal rate. Both strains showed great capacity to remove boron from the liquid media.

Scanning electronic microscopy revealed that strains 053 and 128, when grown in boric acid, produced accretions containing boron, by means of a likely boron-cell interaction mechanism conducive to the formation of a boron bio-precipitate. The conditions of culture, the crystal sizes, and their spheroidal shapes let us conclude about a biologically induced mineralization mechanism. Although there is abundant information about almost all groups of minerals (arsenates, chlorides, fluorides, carbonates, phosphates, sulfates and sulfides, silica, oxides, and hydroxides) produced by biomineralization, this is the first time, to the best of our knowledge, that this is described for boron.

The formation of bio-precipitate could be understood as an adaptive response developed by the cells to boric acid exposure, for which many proteins are differentially synthetized. This strategy for resisting the potentially toxic effect of boric acid by removing and immobilizing the boron in the bio-precipitate, may have a great potential application in the remediation of soils contaminated with boron compounds.

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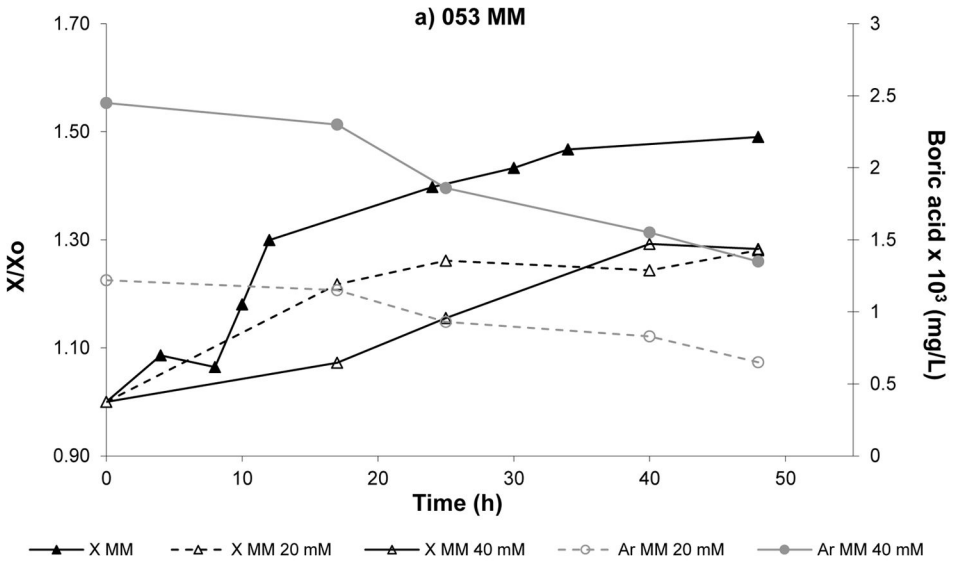
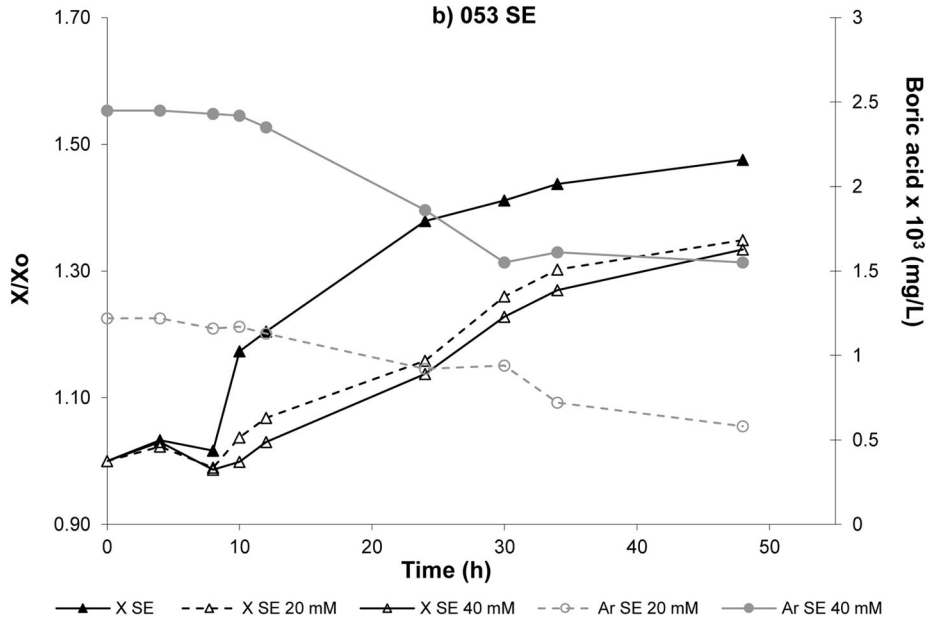
HIGHLIGHTS

- Two *Streptomyces* strains showed great capacity to remove boric acid in liquid media
- Specific boric acid removal rate increased with boric acid concentrations
- A boron bio-precipitate produced in the presence of boric acid was detected by SEM
- Different proteins were synthetized as an adaptive response to boric acid exposure
- Bio-precipitate formation strategy in boric acid presence, may be useful in remediation





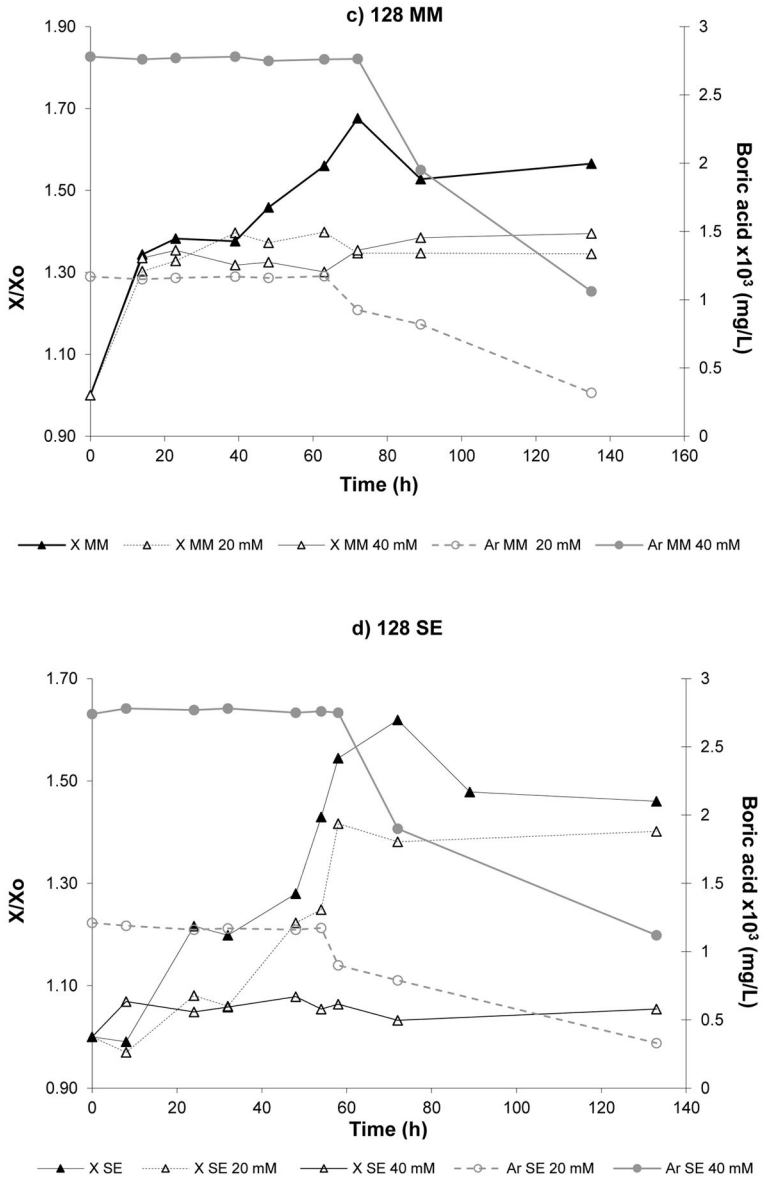


Figure 1.

Growth curves of *Streptomyces* sp. (X: biomass concentration) normalized with the initial biomass concentration (X_o) and residual boric acid measurements (Ar) for both strains: a) 053 in Minimal Medium (MM), b) 053 in Soil Extract medium (SE), c) 128 in MM, d) 128 in SE without boric acid added (controls) and with initial boric acid concentrations of 20 mM (1.22 mg/L) and 40 mM (2.78 mg/L).

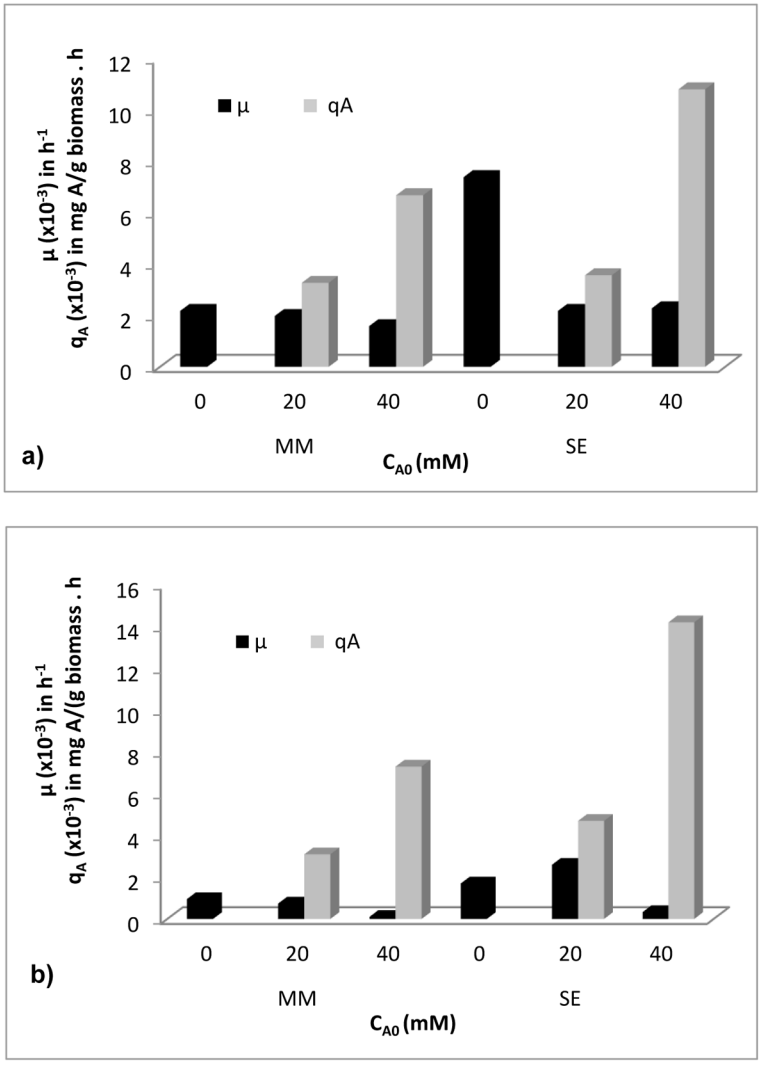


Figure 2.

Specific growth rate (μ, in h^{-1}) and boric acid specific consumption rate (q_A,, in mg A/(g biomass. h)) for the selected strains: a) 053 and b) 128, cultured in Minimal Medium (MM) and Soil Extract (SE) without boric acid and with initial concentrations (C_{A0}) of 20 and 40 mM.

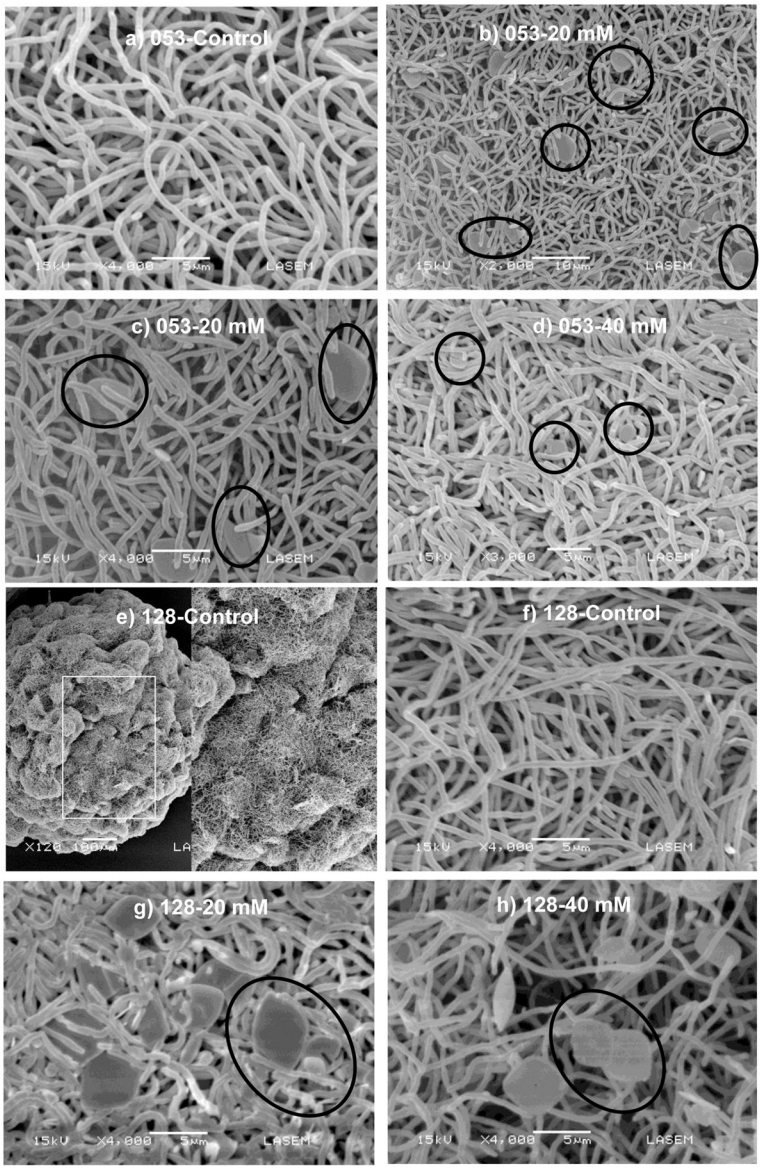


Figure 3.

Electron micrographs of two *Streptomyces* grown in Minimal Medium for 72 h at 30 °C and 250 rpm. a) Control strain 053 without boric acid (x4000), b) Strain 053 in 20 mM boric acid (x2000), c) Strain 053 in 20 mM boric acid (x4000), d) Strain 053 in 40 mM boric acid (x3000), e) Strain 128 without boric acid (Control, x120), f) Strain 128 without boric acid (Control, x4000), g) Strain 128 in 20 mM boric acid (x4000), h) Strain 128 in 40 mM boric acid (x4000). Black circles show bio-precipitates formed at different concentrations of boric acid.

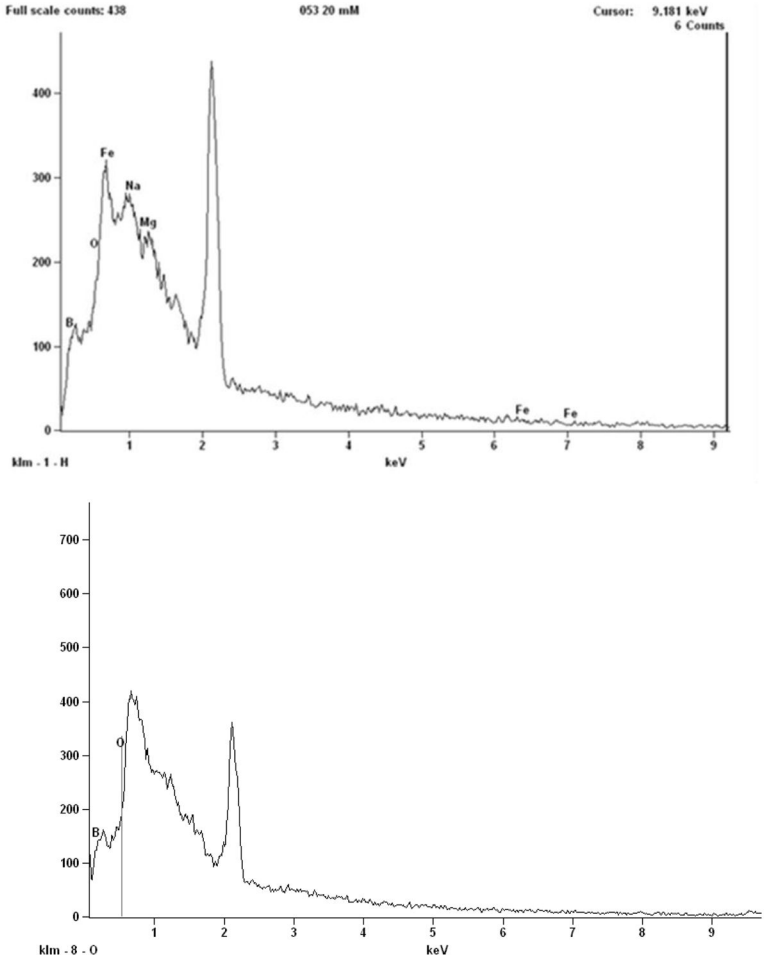


Figure 4.

EDS spectrum of one of bio-precipitate present in *Streptomyces* grown in Minimal Medium for 72 h at 30 °C and 250 rpm: a) *Streptomyces* sp. 053 with 20 mM boric acid, b) *Streptomyces* sp. 128 with 40 mM boric acid.

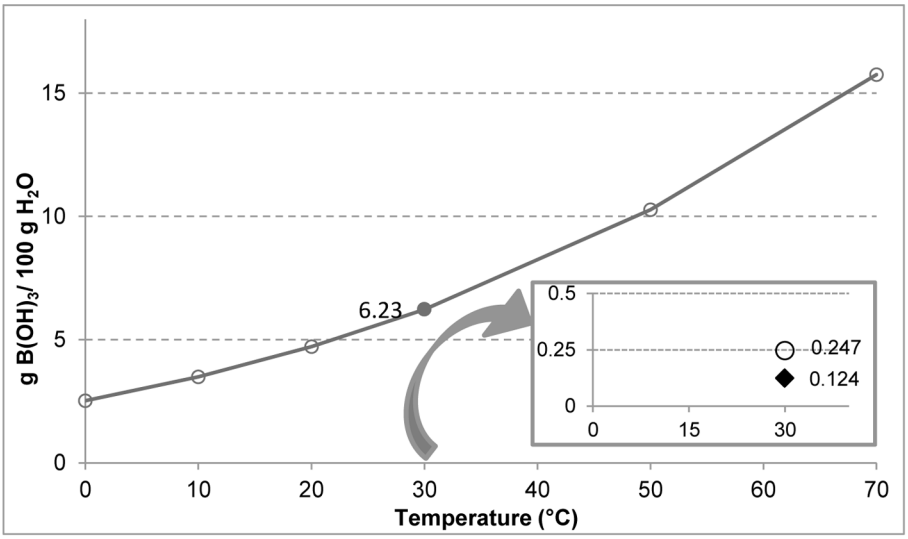


Figure 5.

Boric acid solubility, in g $B(OH)_3$/100 g H_20, as function of temperature. The black point represents the solubility at 30 °C. The arrow points out a zoom in to show that boric acid concentrations in the media at 30 °C, 0.124 mg $B(OH)_3$/100 mg H_2O (20 mM) and 0.247 mg $B(OH)_3$/100 mg H_2O (40 mM), were below the saturation curve.

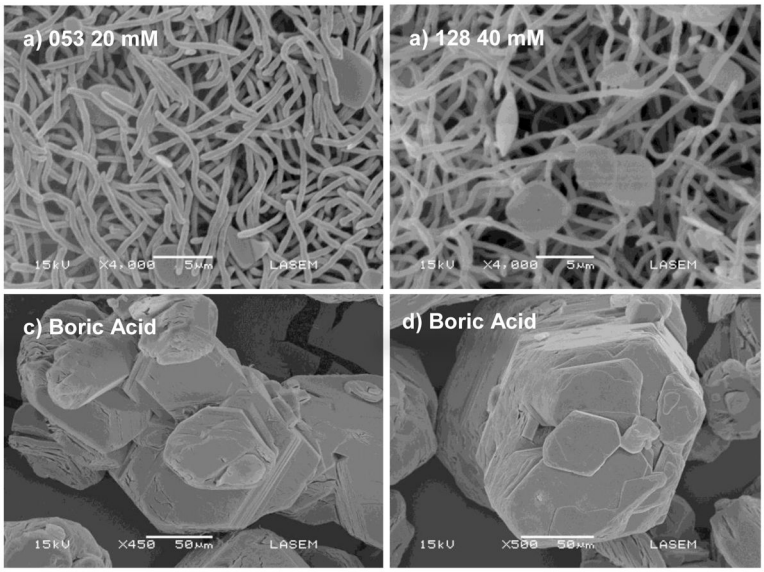


Figure 6.

Electron micrographs. *Streptomyces* sp. grown in Minimal Medium for 72 h at 30 °C and 250 rpm: a) strain 053 grown with 20 mM boric acid (×4000), b) Strain 128 grown with 40 mM boric acid (×4000), c) boric acid in solid state (×450), d) boric acid in solid state (×500).

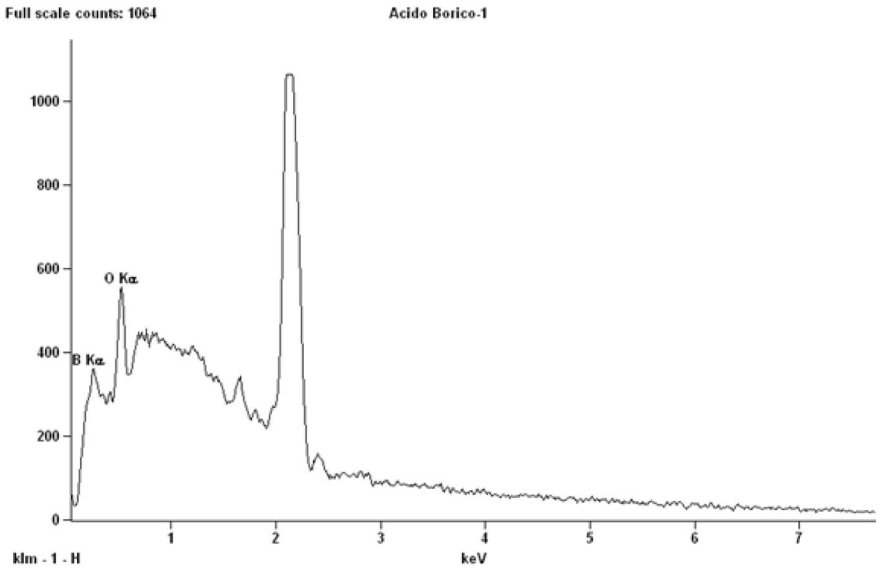


Figure 7.
EDS spectrum of the boric acid in solid state used for the preparation of culture media.

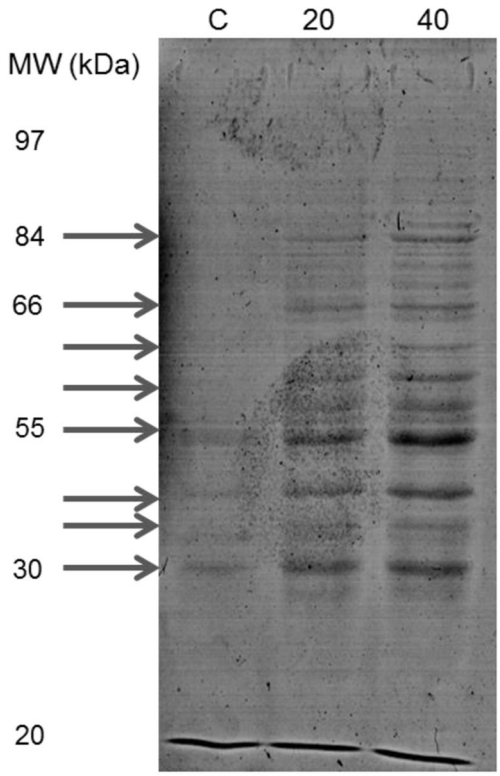


Figure 8.

SDS-PAGE. Protein profiles of *Streptomyces* sp. 053 grown in Minimal Medium for 72 h at 30 °C and 250 rpm. Without boric acid (C) and in the presence of 20 and 40 mM. The gels were stained with Coomassie Brilliant Blue - G250. The arrows indicate molecular weights (MW) in kDa marker.

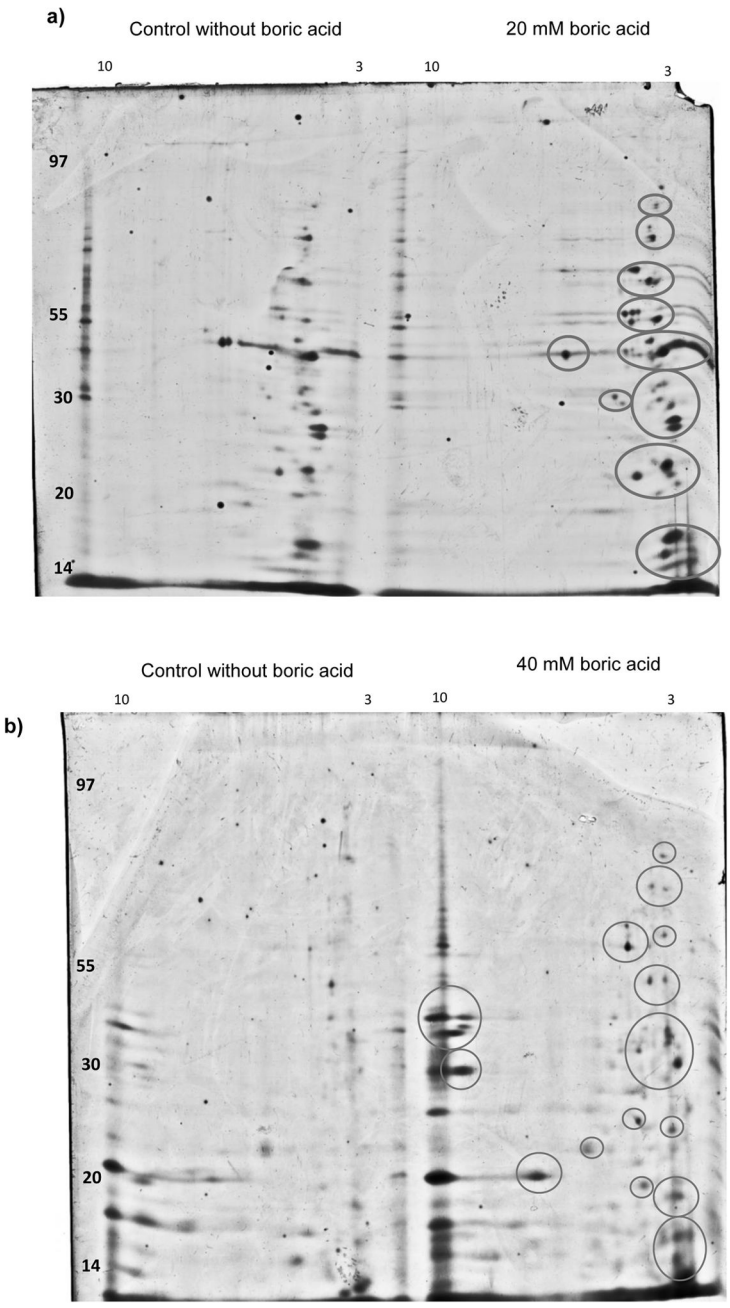


Figure 9.

Differential protein synthesis of *Streptomyces* sp. 053 grown in Minimal Medium at 30 °C for 72 h and 250 rpm in the presence of boric acid: a) 20 mM b) 40 mM. The protein profile was visualized after staining with Coomassie Brilliant Blue G-250 and with silver nitrate. Differentially synthesized proteins compared to controls are indicated by black circles. The horizontal numbers indicate the pH range; the vertical molecular weight marker is in kDa.

Table 1

Boric acid removed from the liquid media after growing *Streptomyces* sp. strains 053 and 128 in minimal medium (MM) and soil extract medium (SE) with 20 and 40 mM of boric acid at 30 °C and 250 rpm.

Medium	Boric acid initial concentration (mM)	Boric acid removed (%)	
		***Streptomyces* spp. 053**	***Streptomyces* spp. 128**
MM	20	59.02	72.65
MM	40	36.73	61.87
SE	20	52.46	72.73
SE	40	36.73	59.12

Table 2

Atomic percentage composition obtained through the EDS analysis of a structure observed in *Streptomyces* sp. 053 and *Streptomyces* sp. 128 grown in Minimal Medium with 20 mM and 40 mM boric acid, respectively, at 30 °C for 72 h and 250 rpm.

Strain	Element	Weight (%)	Error (%)	Formula	Composition (%)
Streptomyces sp. 053	B	28.63	+/− 3.82	B_2O_3	92.18
	O	66.16	--	--	--
	Na	2.38	+/− 0.92	Na_2O	3.21
	Mg	2.46	+/− 0.67	MgO	4.07
	Fe	0.37	+/− 1.26	Fe_2O_3	0.53
	Total	**100.00**			**100.00**
Streptomyces sp. 128	B	31.06	+/− 4.04	B_2O_3	100.00
	O	68.94	--	--	--
	Total	**100.00**			**100.00**

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