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Ilic, Dora S.; Dimkic, Ivica Z.; Waisi, Hadi K.; GKOREZIS, Panos; Hamidovic, Saud R.; Raicevic, Vera B. & Lalevic, Blazo T. (2019) Reduction of hexavalent chromium by Bacillus spp. isolated from heavy metals-polluted soil. In: CHEMICAL INDUSTRY & CHEMICAL ENGINEERING QUARTERLY, 25(3), p. 247-258.

DOI: 10.2298/CICEQ180607003I
Handle: http://hdl.handle.net/1942/29959

REDUCTION OF HEXAVALENT CHROMIUM BY *BACILLUS* spp. ISOLATED FROM HEAVY METALS-POLLUTED SOIL

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Abstract

Hexavalent chromium, Cr(VI), one of the major pollutants from industrial facilities, is very toxic and harmful for human health and environmental quality. Due to the lack of conventional methods, bioremediation was recommended as an environmentally friendly and effective technique. The aim of this paper was the isolation, identification and selection of the microorganisms which are capable of Cr(VI) reduction *in vitro*. Heavy metal concentration, detected in four soil samples, within and around the former bicycle factory "Rog" (Republic of Slovenia), was measured using the ICP-OES method. Bacteria were isolated and tested for chromium tolerance using LB agar supplemented with various Cr(VI) concentrations, whilst Cr(VI) reduction and bacterial growth was determined using the LB liquid medium. From 53 bacterial isolates, five of them showed a tolerance of 1000 mg/L of Cr(VI). Those five isolates showed the capability of growth under various Cr(VI) concentrations (50-1000 mg/L). Initial Cr(VI) concentrations ranging from 50 to 100 mg/L were completely reduced by four bacterial isolates, whilst 500 to 1000 mg/L by *Bacillus safensis* 342-9. Using 16S rDNA and *tuf* gene sequence analyses, isolates 270-9R and 342-9 were identified as *Bacillus safensis*, isolates 351-9 and 270-9C as *Bacillus subtilis* subsp. *subtilis*, and 212-9 as *Bacillus thuringiensis*. These results indicated that these bacteria may be a promising tools for remediation of metal-polluted sites.

Keywords: *Bacillus* spp., bioremediation, heavy metal pollution, hexavalent chromium reduction, indigenous bacteria

INTRODUCTION

Chromium is one of the most hazardous heavy metals and it is broadly dispersed in environment because of anthropogenic (industrial) activities [1] and geogenous processes [2]. Although chromium is an important component for the utilization of carbohydrates [3] and the metabolism [4], its hexavalent form, Cr(VI), is responsible for the environmental pollution and that form has carcinogenic effects on living systems [5]. Cr(VI) represents one of the most important soil and groundwater contaminants [6] and is more soluble and toxic compared to the trivalent form [7]. Hexavalent chromium diffuse away easily from the pollution sites [8] to other sites. Similar problems are present at the factory "Rog" in Ljubljana (Republic of Slovenia), where a galvanization process and the chroming of bicycle frames have been performed since 1951. However, decreasing market demand, caused by the breakdown of the former Yugoslavia, as well as the high-cost of production, resulted in closing the factory in 1994. Unfortunately, leaking of liquid from cracks within the galvanization baths has been ongoing for many years since production activities stopped. This liquid, which contains large concentrations of heavy metals [9] leaks out of the objects and flows down into the Ljubljanica River to this day, which represents a serious environmental problem.

Conventional methods, such as chemical reduction, adsorption or ion exchange are available for Cr(VI) removal, but these techniques are less-efficient [10] and often followed by production of toxic byproducts [11]. Thus, it is necessary to introduce environmental friendly techniques for the removal of Cr(VI) [12]. One possible method which could be applied for this purpose is bioremediation [13], which has been considered as a possible strategy for Cr(VI) removal using indigenous microbial populations [14]. Removing the Cr(VI) using bacteria received significant attention, especially because of the cost of chemicals which are used in large-scale clean up technologies [15]. The capability of Cr(VI) reduction was achieved in bacterial genera such are *Pseudomonas* [16], *Escherichia* [17], *Arthrobacter* [18] etc. under the presence [19] or absence [20] of oxygen. Indigenous microbial populations have a key role in removal of Cr(VI) from polluted sites [21] and their efficiency depends on bacterial species and various environmental factors [22].The purpose of this study was to determine the reduction capability of indigenous bacteria, isolated from heavy metal-polluted soil in Cr(VI) reduction under laboratory conditions.

EXPERIMENTAL SETUP

Sampling

The samples of heavy metal-polluted soil for this research were taken in July 2010 at the location of the former bicycle production facility "Rog" (Ljubljana, Republic of Slovenia) located at N 46°3'7.44" E 14°30'53.43". The samples were taken aseptically in sterile plastic bags from the soil surface layer (0-20 cm) at four sites: under the central galvanization facility (site 1), from the subsurface layer in the same location (site 2), from the surface layer at the other side of the facility, toward Ljubljanica River (site 3), and between the facility and Ljubljanica River (site 4). The control sample was taken near the park in the city center of Ljubljana (46°08'16.27" E 14°51'9.313"). Samples from all sites were used for the preparation of composite samples and stored at 4°C for further analyses.

The chemical analyses of samples

Determination of pH value was performed using potentiometric method with a combined glass electrode in soil/water and soil/1M KCl mixture (ratio 1:2.5). $CaCO_3$ content was measured volumetrically using a Scheibler calcimeter, humus content was determined using the Kotzmann method, total N using the Kjeldahl method, and available P and K using the Al method [23]. Metal extraction from the soil and acid mineralization, was performed using a microwave assay Berghof Speed wave 4 (Germany). Digestion of 0.5 g of dry sample during 16h at 105°C was performed using HNO_3, HCl and H_2O_2 [24]. Measurement was carried out using Spectro Genesis ICP-OES method coupled with Smart Analyzer Vision software (SPECTRO Analytical Instruments GmbH, Boschstr. 10. 47533 Kleve, Germany). Curve formation was performed based on individual standards of 1g/L concentration (Ultra Scientific U.S.A.) and multi-element standards (SPS-SW2, LGC, UK) for Fe, Mn, Na, Zn, Cu, Cr, Ni, Pb, Cd, As, Hg, Mo, S, Ca, Mg, Co, Sb, Sn, B, Sr, Li, Al, Ba, Se, and V. The obtained results of chemical analyses were compared to the standards [25,26].

Prevalence and isolation of chromium resistant bacteria

The abundance of aerobic heterotrophic chromium resistant bacteria of overburden samples (20 g) were determined using Luria Bertani (LB) agar medium (pH maintained to 5.0; 7.0; and 9.0). The medium was enriched with sterile $K_2Cr_2O_7$ dilution up to a final concentration of 500; 1000; 1500 and 3000 mg/L Cr(VI). After incubation, which was performed in

incubator (Binder, Germany) at 30°C for 5 days, the number of bacteria was expressed as CFU/g. Morphologically different colonies were counted and transferred on fresh LB agar plates amended with 2 mM of Cr(VI) and stored until used for further research.

Bacterial tolerance on Cr(VI)

Tolerance of all bacterial isolates (53 isolates) to Cr(VI) was tested using modified Sayel et al. method [3] on a LB agar medium, supplemented with 100; 500; 750; 1000; and 1500 mg/L of Cr(VI). This medium was inoculated with 20h-bacterial cultures and after an incubation period of 4 days at 30°C, Cr(VI) tolerance was determined visually. The Cr(VI)-free agar LB medium inoculated with bacterial cultures was used as a control treatment. The most tolerant bacterial strains were used for a Cr(VI) reduction assay.

Screening of Cr(VI) reduction and bacterial growth

Cr(VI) reduction ability of bacterial isolates was tested using the previously described LB liquid medium enriched with 10 g/L glucose. Erlenmeyer flasks (250 mL) containing 50 mL of LB medium supplemented with $K_2Cr_2O_7$ dilution up to final Cr(VI) concentration of 50; 100; 200; 300; 500; and 1000 mg/L were inoculated with 10% (*v*/*v*) 20h-old bacterial inoculum (1×10^8 CFU/mL) under aseptic conditions. Incubation was performed at 30°C at 120 rpm in an orbital shaker (IKA, IK-3692500, Germany) for 48 h. The supernatant was analysed for residual chromium following the DPC method [27] by measuring the absorbance at 540 nm using a DR 3800 sc VIS Spectrophotometer (Denmark). The Cr(VI) reduction was observed in different intervals of incubation (time zero, and after 2; 4; 6; 18; 24; and 48 h). A control treatment (without bacterial cells) was also used in this experiment for the purpose of measurement of abiotic Cr(VI) reduction. All measurements were performed in triplicates. The growth of bacteria capable of Cr(VI) reduction in a liquid medium was measured spectrophotometrically at 600 nm using a DR 3800sc VIS Spectrophotometer (Denmark) under the same conditions and at the same intervals of incubation as in the Cr(VI) reduction screening.

Morphological characterization and molecular identification of bacterial isolates

Morphological characterization of five selected (212-9, 342-9, 270-9R, 351-9, and 270-9C) Cr(VI) reducing bacteria (shape, colour, and size) was carried out by growing them on an agar medium. The cell morphology of chromium resistant bacteria was examined using a

Leica DMSL (Germany). The performed tests were pertaining to Gram's staining, shape, and spore formation. Further identification of bacterial isolates was performed using molecular characterization. *Bacillus* spp. genomic DNA was isolated according to the protocol from Dimkić et al. [28]. First, the 16S rDNA gene, using universal primers $UN1_{16s}F$ (5′-GAGAGTTTGATCCTGGC-3′) and $UN1_{16s}R$ (5′-AGGAGGTGATCCAGCCG-3′) was amplified. To improve identification, *tuf* gene (encoding for elongation factor TU) was amplified using a previously described methods [29,30]. For the amplification of *tuf* gene, tufGPF (5′-ACGTTGACTGCCCAGGACAC-3′) and tufGPR (5′-GATACCAGTTACGTCAGTTGTACGGA-3′) primers were used. The PCR amplification was carried out in a 25 µL volume of a mixture containing 2.5 µL of 10×KAPA Taq buffer; 1.75 and 2 µL of 25 mM $MgCl_2$ (KAPA Biosystems, USA) for 16S and *tuf*, respectively;
0.5 µL (10 mM) of dNTPs (KAPA Biosystems, USA);
1 µL and 2.5 µL of each primer (10 µM) for 16S and *tuf*, respectively;
17.15 and 13.9 µL of DNase/RNase free water (Gibco, UK) for 16S and *tuf*, respectively;
0.1 µL (5U/µL) of KAPA Taq polymerase (KAPA Biosystems, USA); and 1 µL of template DNA.

16S rDNA PCR reaction was performed in 30 cycles, 50°C was primer annealing temperature. PCR *tuf* amplification included 35 cycles, with primer annealing temperature at 55°C. The PCR products (1500 bp for 16S rDNA and 791 for *tuf*) were purified with a QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany) and sent for sequencing to the commercial service (Macrogen, Netherlands).

The obtained sequences were checked with the 16S and GenBank database using the BLAST search program 2.5.0 (http://www.ncbi.nlm.nih.gov/). ClustalW was used for multiple sequence alignment, and evolution analyses were conducted in MEGA 7. The evolution history was inferred using the Neighbor-Joining method and evolution distances were computed using the Kimura 2-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown and *Clostridium botulinum* was included as an out-group.

Statistical analysis

The Kolmogorov–Smirnov test was used for analysis of variance, as well as Levene's test for homogeneity of variance. The variance analysis (ANOVA) was performed, whilst for the means separation of Cr(VI) reduction and bacterial growth in different concentrations of

selected isolates Tukey's HSD (honest significant difference) test was applied. Level of significance was $P<0.05$. All experiments were performed twice and for ensuring minimal error margin in three repetitions for each sample. STATISTICA v.7 (StatSoft, Inc.) and IBM SPSS Statistics v.20 (SPSS, Inc.) were used for processing data.

RESULTS AND DISCUSSION

Chemical characterization of the soil samples

The soil samples were characterized as neutral to slightly alkaline, with a low to medium $CaCO_3$ content, medium organic C and total N content, as well as a medium to high content of P and K (Table 1). According to the standards [25,26], content of Ba, Cr, Cu, Pb, Ni, and B and S, respectively, were above the remediation value. It is evident that long-term galvanization and other industrial processes in the former bicycle factory have had environmental consequences in this area. Chromium and nickel content was higher compared to other heavy metals (Table 1). On the other hand, the control soil sample was considered unpolluted. From data it is obvious that the general order of metal abundance in the soil was Fe>Ni>Cr>Al. A higher presence of Fe compared to other metals was previously reported [31].

Table 1

Microbiological diversity and Cr(VI) tolerance

The microbial diversity of soil is linked to environmental quality; an environment under stress may maintain microbial diversity [32]. According to the morphological characterization and Gram reactions a total of 53 isolates were described. The majority of them formed small spherical white to creamy colonies. These isolates belong to Gram positive spore-forming rods indicating their belonging to the *Bacillus* genera or similar genera. Previously published studies also suggest the domination of Gram positive bacteria in metal-polluted locations [33,34].

Table 2

However, the obtained results (Table 2) suggest that the prevalence of Cr(VI) resistant bacteria depends on initial Cr(VI) content in the medium, pH value, and sampling location.

A majority of bacteria were capable to grow in a pH 7 to pH 9 range. The highest bacterial activity was found at sample site 2. Addition of Cr(VI) resulted in a reduction of the bacterial number at sample sites 1 and 4. The presented data shows that with an increase in the Cr(VI) content in the LB medium, decrease of the number of Cr(VI) tolerating bacterial isolates was observed (Table 3). At concentration of 100 mg/L of Cr(VI), depending on the sample, 53.8 to 78.6% of isolates were capable of growth, whilst at 500 mg/L of Cr(VI), the tolerance rate of bacteria was 29.4% to 38.5%. Only a few isolates were capable of tolerating concentrations above 1000 mg/L Cr(VI): isolates 270-9C; 270-9R; and 342-9 from sample site 2, isolate 351-9 from sample site 3, and 212-9 from sample site 4. Rahman et al. [35] reported that only 12% of bacteria were capable of growth at 1000 ppm Cr(VI) were also detected at 500 ppm. However, Dey et al. [36] found that the rate of bacterial growth reduction was about 50% at a Cr(VI) concentration of 2 mM compared to the control, which is in agreement with this study.

Table 3

Molecular characterization of Cr(VI) reducing bacteria

Because of their tolerance on high Cr(VI) concentrations, five isolates were selected for further identification and Cr(VI) reduction assay. Isolates 270-9C, 270-9R, 212-9, 351-9, and 342-9, which showed an ability to reduce various initial Cr(VI) concentrations, were Gram positive, spore-forming short rods, creamy colonies on LB plates. Phylogenetic reconstructions based on 16S rDNA and *tuf* nucleotide sequences of the tested isolates are shown in Figure 1. According to the results after 16S rDNA sequencing it becomes clear that identification at this level is insufficiently discriminatory (Figure 1A). Two isolates 270-9R and 342-9 linked to *Bacillus safensis*, *Bacillus pumilus* and *Bacillus zhangzhouensis* where BLAST results revealed 99.87% identity. Isolates 270-9C and 351-9 were linked to the *Bacillus subtilis* strain with 99.89% identity as well as to *B. subtilis* subsp. *inaquosorum* with a slightly lower identity 99.78%. Also, a similar observation was noticed for the 212-9 isolate which was supported by high bootstrap values and indicated its belonging to a species of *Bacillus thuringiensis* and *Bacillus toyonensis* with 99.34%. Although the 16S rDNA has been a powerful tool for identification at the genus level, its use at the species level was doubtful, particularly in distinguishing certain *Bacillus* spp. [37]. On the other hand, gene targeted tufGPF and tufGPR universal primers, previously designed and proven for better

identification of all *Bacillus* spp. [29] were used in this study (Figure 1B). However, sequencing of *tuf* gene unequivocally determined the isolates even below the species level, indicating its higher discriminatory ability. The isolates 270-9R and 342-9 was supported by high *tuf* bootstrap values and linked unequivocally to *B. safensis* (CP018100) while isolates 270-9C and 351-9 were confirmed as *B. subtilis* subsp. *subtilis* (CP021921). On the other hand, the 212-9 isolate was identified as *B. thuringiensis* (CP003687), as shown in Figure 1B. The sequences generated after *tuf* PCR amplification for 212-9, 342-9, 270-9R, 351-9, and 270-9C were submitted to the NCBI GenBank database under accession numbers MH122621, MH122622, MH122623, MH122624, and MH122625, respectively. The *tuf* gene can be a powerful tool for the differential characterisation of different *Bacillus* groups (*B. subtilis* and *B. cereus*), because such separation cannot be achieved by 16S rDNA sequencing [29].

Figure 1

Bacterial isolates and their growth kinetics and Cr(VI) reduction potential

The results presented in Table 4 show that the Cr(VI) reduction rate was influenced by Cr(VI) content, bacterial isolates, and time of incubation. Screening of Cr(VI) reduction indicates that all bacterial isolates were capable of reducing Cr(VI). In most of the samples Cr(VI) reduction was finished by discoloration of the liquid medium. Out of five bacterial isolates, four were capable for total reduction of 50 mg/L Cr(VI): isolate 342-9 after 6h, isolate 270-9R in 18h, isolate 212-9 after 24h of incubation, and isolate 351-9 after 48h of incubation. The isolate 270-9C which is the same species as 351-9 (*B. subtilis* subsp. *subtilis*), also showed a statistically significant reduction rate ($P<0.05$), higher than 99%. Bacterial isolates were also able to reduce 100 mg/L Cr(VI). In the previous concentration rate, the isolate 342-9 was the fastest in reduction capability. The isolates 342-9 and 270-9R showed maximal potential of reduction 200 mg/L Cr(VI) after 24h. At higher initial Cr(VI) concentrations, the reduction rate was the lowest compared to previous experiments. However, isolate 342-9 showed maximal efficiency in reduction of all examined Cr(VI) concentrations. At the same incubation times, the un-inoculated liquid medium did not show any Cr(VI) reduction (Table 4). On the other hand, all bacterial isolates were capable of growth in the liquid LB medium containing various Cr(VI) concentrations. In most of the samples the growth rate decreased with an increase of Cr(VI) content in the medium (Figure

2, 3, 4). The growth histograms of bacterial isolates 342-9, 270-9R (Figure 2) and 270-9C (Figure 3A) showed a moderate decrease in OD values after 24 and 48h of incubation for a concentration range from 300 to 1000 mg/L Cr(VI), whilst a statistically significant change in growth was not noted for isolate 351-9 (Figure 3B) after 24h and 48h incubation periods and a concentration range of 300-1000 mg/L Cr(VI). Contrary to that, for the isolate 212-9, statistically significant growth ($P<0.05$) rapidly decreased after 48h at the highest tested concentration with an evident stationary phase (Figure 4).

Figure 2

Figure 3

Figure 4

Table 4

Since the determination of *Pseudomonas dechromaticans* as the first described Cr(VI) reduction microbe [38], research regarding Cr(VI) reducing bacteria has continued and a lot of information has been collected about Cr(VI) reduction in laboratory and field conditions [39]. Thus, there is various evidence showing the efficiency of numerous bacteria in Cr(VI) reduction [40]. The isolates SUK 1201 and SUK 1205 showed a total reduction of 0.1 mM Cr(VI) in 48h [36]. Furthermore, *Nesterenkonia* sp. MF2 was able to reduce 0.2 mM Cr(VI) in 24h [41]. Likewise, a similar Cr(VI) reduction capability was found in *Exiguobacterium* sp. ZM-2, which reduced 0.5 mM Cr(VI) in 56h [42], and in *Arthrobacter* sp. SUK 1201, with a reduction rate of about 75% of 800 μM Cr(VI) within 48h [36]. However, it is evident that the reduction strongly depends on the initial Cr(VI) concentration, which has been previously demonstrated [14,43]. Incomplete reduction of the highest Cr(VI) concentration is similar with previous findings [18]. Several studies have addressed the ability of Cr(VI) reduction by *Bacillus* strains. *Bacillus subtilis* BYCr-1 was able to completely reduce 0.2 mM Cr(VI) after 48 h of incubation [44]. Cr(VI) concentration of 50 mg/L was partially reduced using *Bacillus subtilis* MNU16 to 13.23 mg/L after 72 h of incubation [45]. Significant decrease from 75 to 25 mg/L Cr(VI) by *Bacillus thuringiensis* BRC-ZYR2 was observed previously [46]. *Bacillus thuringiensis* SUCR186 showed the ability to carry out the total

reduction of 0.2 mM Cr(VI) and partial reduction of 0.4 to 1.0 mM Cr(VI) [47], whilst this study showed the ability of *Bacillus thuringiensis* 212-9 to carry out the complete reduction of 1mM Cr(VI). Several reports indicate the role of *Bacillus safensis* in Cr(VI) toleration and resistance [48,49]. To author`s knowledge, only one report [50] has addressed the reduction capability of *B. safensis* MX-3, with maximum reduction rate of 150 μg Cr(VI). Similar capability of *B. safensis* was confirmed by our study using strain 270-9R, with maximal reduction rate up to 200 mg Cr(VI). However, the strain MX-3 has a lower reduction ability compared to our isolate 342-9, which was able to completely reduce 1000 mg Cr(VI) and may be considered as encouraging strain for improving our knowledge about chromate bioremediation. All examined bacterial isolates were capable of reducing different Cr(VI) concentrations in liquid medium, which might indicate that $Cr(OH)_3$ accumulates close to the bacterial cells and prevents the deleterious influence of chromium [35]. As reported previously [51], it is speculated that Cr(VI) reduction in *Bacillus* spp. is localized on the chromosomal DNA. In several *Bacillus cereus* and *Bacillus thuringiensis* strains, promoter *chrI* are responsible for regulation of Cr(VI) reduction genes [52]. Mangaiyarkarasi et al. [53] suggest that expression of reductase, which is localized on cell membrane, mediate in Cr(VI) reduction. However, properties and reduction potential of chromate reductase in *Bacillus* spp. have not been determined completely [54].

CONCLUSIONS

The presented results clearly demonstrate that indigenous *Bacillus* spp. strains from heavy metal-polluted overburden soil were capable of Cr(VI) reduction. These bacterial isolates might be a promising candidates for bioremediation of Cr(VI)-contaminated soils and the improvement of environmental quality in heavy metal-polluted industrial sites. Further projects will be focused on mechanisms of bacterial Cr(VI) reduction and application of molecular tools for enhancement in Cr(VI) reduction efficiency.

Acknowledgments

The authors confirm the partial support of Ministry of Education, Science and Technological Development of Republic of Serbia (Grant Nos. TR31080 and 173026).

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Figure Captions

Figure 1. Neighbor-joining phylogenetic tree based on 16S rDNA (A) and *tuf* gene sequences (B) (1500 and 791 bp, respectively) showing the relationship of the tested isolates (212-9, 342-9, 270-9R, 351-9, and 270-9C) and related reference strains of the genus *Bacillus*. *Clostridium botulinum* (NR_036786 and NC_010674) was used as an out-group. Bootstrap values (expressed as a percentage of 1000 repetitions) are displayed at points of branching. The horizontal bar indicates a genetic distance of 0.02 for 16S rDNA and 0.05 for *tuf* gene.

Figure 2. The growth kinetics of the *Bacillus safensis* isolates 270-9R (A) and 342-9 (B) in liquid medium supplemented with different initial Cr(VI) concentrations. Values followed by the same letter within columns, within each time point of sampling, are not significantly different ($P < 0.05$), according to Tukey's HSD test.

Figure 3. The growth kinetics of the *Bacillus subtilis* subsp. *subtilis* isolates 270-9C (A) and 351-9 (B) in liquid medium supplemented with different initial Cr(VI) concentrations. Values followed by the same letter within columns, within each time point of sampling, are not significantly different ($P < 0.05$), according to Tukey's HSD test.

Figure 4. The growth kinetics of the *Bacillus thuringiensis* isolate 212-9 in liquid medium supplemented with different initial Cr(VI) concentrations. Values followed by the same letter within columns, within each time point of sampling, are not significantly different ($P < 0.05$), according to Tukey's HSD test.

Table 1. Chemical characterization and heavy metal content of the soil samples

Parameter	Unit	Sample soil sites				
		1	**2**	**3**	**4**	**Control**
pH in KCl	-	6.88	7.41	7.60	7.73	7.03
pH in H_2O	-	7.52	7.97	8.03	8.00	7.53
$CaCO_3$	%	0.99	1.83	2.39	2.25	1.83
Organic C	%	1.84	2.22	2.67	2.61	4.87
Total N	%	0.16	0.19	0.23	0.23	0.42
P_2O_5	mg/100 g	15.93	38.75	47.78	48.10	23.15
K_2O	mg/100 g	26.10	24.00	25.60	28.40	30.50
Fe	g/kg	142.07	143.90	135.02	168.67	26.93
Mn	mg/kg	501.20	458.90	407.80	571.10	1343.40
Na	mg/kg	5177.00	5454.00	5319.00	5348.00	55.30
Zn	mg/kg	535.30	453.30	644.70	497.70	128.80
Cu	mg/kg	1956.00	1470.60	1828.80	1650.90	20.80
Cr	mg/kg	9457.80	9519.00	9796.80	12144.20	28.70
Pb	mg/kg	1317.90	1218.70	1249.50	1277.00	76.00
Ni	mg/kg	21690.30	19536.30	19886.50	25219.10	32.40
Cd	mg/kg	1.66	1.15	1.00	1.21	0.00
As	mg/kg	11.22	7.34	7.54	9.10	15.60
Hg	mg/kg	0.00	0.00	0.00	0.00	0.00
Be	mg/kg	0.00	0.00	0.00	0.00	0.00
Mo	mg/kg	4.45	4.45	4.44	5.86	0.00
S	mg/kg	884.40	805.80	871.90	981.20	86.10
Ca	mg/kg	16584.60	16291.40	16516.70	16031.80	31508.80
Mg	mg/kg	2515.70	3183.60	2737.00	2334.20	9189.60
Co	mg/kg	274.02	236.65	233.26	291.84	7.30
Sb	mg/kg	2.50	3.30	6.30	3.00	3.42
Sn	mg/kg	147.42	137.37	141.65	179.62	0.00
B	mg/kg	559.58	960.19	949.23	1182.78	0.60
Sr	mg/kg	183.50	187.60	190.80	216.30	0.00

Li	mg/kg	4.20	4.90	4.50	4.30	18.60
Al	mg/kg	2062.70	1738.90	1758.20	1880.40	22399.10
Ba	mg/kg	1445.14	1522.78	1523.61	1343.75	124.90
Se	mg/kg	0.32	0.33	0.27	0.33	0.00
V	mg/kg	19.86	19.86	21.37	27.51	46.80

Table 2. Prevalence of Cr(VI) resistant bacteria (1×10^4 CFU/g)

Sample	**Cr (VI) concentration (mg/L)**					
soil sites	**500**			**1000**		
	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9
1	0.0±0.00	0.4±0.10	2.9±0.30	0.0±0.00	0.7±0.10	2.6±0.44
2	0.0±0.00	53.2±7.68	0.4±0.16	1.1±0.40	127.1±16.33	16.5±3.44
3	0.1±0.07	1.9±0.38	2.1±0.38	0.2±0.12	1.0±0.41	1.9±0.46
4	0.0±0.00	0.5±0.12	27.5±3.12	0.0±0.00	0.0±0.00	23.5±2.65
Sample	**Cr (VI) concentration (mg/L)**					
soil sites	**1500**			**3000**		
	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9
1	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
2	0.0±0.00	0.0±0.00	126.3±9.64	0.0±0.00	0.4±0.07	0.1±0.07
3	12.3±1.92	37.1±2.75	13.6±1.93	0.0±0.00	0.3±0.12	0.1±0.00
4	0.0±0.00	0.9±0.21	0.0±0.00	0.0±0.00	0.4±0.17	0.2±0.17

Table 3. Tolerance on Cr(VI) within bacterial population

Sample	**Cr(VI) concentration (mg/L)**											
soil sites	**0**		**100**		**500**		**750**		**1000**		**1500**	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1	17	100.0	8	47.1	5	29.4	1	5.9	0	0.0	0	0.0
2	13	100.0	7	53.8	5	38.5	4	30.8	3	23.1	3	23.1
3	9	100.0	6	66.7	3	33.3	2	22.2	1	11.1	1	11.1
4	14	100.0	11	78.6	5	35.7	2	14.3	1	7.1	1	7.1

Table 4. The capacity of Cr(VI) reduction in different concentrations by indigenous bacteria

Conc. of Cr (mg/L)	Isolate	Time (h)						
		0	2	4	6	18	24	48
50	270-9C	$3.31^{a*} \pm 0.02$	$1.23^{a} \pm 0.02$	$0.70^{a} \pm 0.01$	**$0.22^{b} \pm 0.01$**	**$0.13^{d} \pm 0.01$**	**$0.09^{b} \pm 0.01$**	**$0.01^{b} \pm 0.00$**
	342-9	$0.33^{d} \pm 0.01$	**$0.07^{e} \pm 0.01$**	**$0.05^{f} \pm 0.01$**	**$0.00^{e} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	212-9	$1.35^{b} \pm 0.01$	$0.35^{b} \pm 0.01$	**$0.20^{c} \pm 0.01$**	**$0.07^{d} \pm 0.01$**	**$0.01^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	270-9R	$0.41^{c} \pm 0.01$	**$0.21^{cd} \pm 0.01$**	**$0.10^{e} \pm 0.01$**	**$0.01^{e} \pm 0.00$**	**$0.00^{b} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	351-9	$0.28^{d} \pm 0.01$	**$0.18^{d} \pm 0.01$**	**$0.14^{d} \pm 0.01$**	**$0.11^{c} \pm 0.01$**	**$0.09^{c} \pm 0.01$**	**$0.04^{c} \pm 0.01$**	**$0.00^{b} \pm 0.00$**
	Control	$0.28^{d} \pm 0.01$	$0.26^{c} \pm 0.00$	$0.26^{b} \pm 0.00$	$0.29^{a} \pm 0.00$	$0.27^{a} \pm 0.00$	$0.30^{a} \pm 0.00$	$0.28^{a} \pm 0.01$
100	270-9C	$2.81^{a} \pm 0.01$	$1.60^{a} \pm 0.02$	$1.04^{a} \pm 0.01$	$0.67^{a} \pm 0.01$	**$0.12^{b} \pm 0.01$**	**$0.08^{b} \pm 0.01$**	**$0.01^{b} \pm 0.00$**
	342-9	$0.28^{e} \pm 0.01$	**$0.10^{f} \pm 0.01$**	**$0.04^{e} \pm 0.00$**	**$0.00^{f} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	212-9	$1.51^{b} \pm 0.02$	$0.88^{b} \pm 0.01$	$0.61^{b} \pm 0.01$	**$0.15^{d} \pm 0.01$**	**$0.00^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	270-9R	$0.84^{c} \pm 0.01$	$0.58^{c} \pm 0.02$	$0.30^{cd} \pm 0.01$	**$0.04^{e} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{b} \pm 0.00$**
	351-9	$0.55^{d} \pm 0.01$	$0.45^{d} \pm 0.01$	$0.34^{c} \pm 0.02$	**$0.23^{c} \pm 0.01$**	**$0.08^{c} \pm 0.01$**	**$0.05^{c} \pm 0.01$**	**$0.00^{b} \pm 0.00$**
	Control	$0.27^{e} \pm 0.00$	$0.26^{e} \pm 0.00$	$0.27^{d} \pm 0.00$	$0.28^{b} \pm 0.00$	$0.28^{a} \pm 0.00$	$0.29^{a} \pm 0.00$	$0.27^{a} \pm 0.00$
200	270-9C	$3.26^{a} \pm 0.01$	$3.03^{a} \pm 0.01$	$2.56^{a} \pm 0.03$	$1.71^{a} \pm 0.01$	$0.46^{a} \pm 0.02$	**$0.20^{b} \pm 0.01$**	**$0.02^{b} \pm 0.00$**
	342-9	$0.85^{e} \pm 0.00$	$0.43^{e} \pm 0.01$	**$0.20^{d} \pm 0.01$**	**$0.17^{e} \pm 0.00$**	**$0.06^{cd} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{c} \pm 0.00$**
	212-9	$2.90^{b} \pm 0.01$	$1.77^{b} \pm 0.02$	$1.00^{b} \pm 0.01$	**$0.26^{d} \pm 0.01$**	**$0.07^{c} \pm 0.00$**	**$0.01^{d} \pm 0.00$**	**$0.00^{c} \pm 0.00$**
	270-9R	$1.82^{c} \pm 0.01$	$1.23^{c} \pm 0.01$	$0.98^{b} \pm 0.01$	**$0.12^{f} \pm 0.01$**	**$0.01^{d} \pm 0.00$**	**$0.00^{d} \pm 0.00$**	**$0.00^{c} \pm 0.00$**
	351-9	$1.10^{d} \pm 0.02$	$1.05^{d} \pm 0.02$	$0.97^{b} \pm 0.04$	$0.71^{b} \pm 0.01$	$0.47^{a} \pm 0.02$	**$0.14^{c} \pm 0.01$**	**$0.02^{b} \pm 0.00$**
	Control	$0.35^{f} \pm 0.01$	$0.37^{f} \pm 0.00$	$0.36^{c} \pm 0.00$	$0.38^{c} \pm 0.00$	$0.35^{b} \pm 0.00$	$0.35^{a} \pm 0.01$	$0.33^{a} \pm 0.00$
300	270-9C	$3.46^{a} \pm 0.03$	$3.34^{a} \pm 0.01$	$2.37^{a} \pm 0.02$	$2.92^{a} \pm 0.03$	$0.73^{b} \pm 0.02$	$0.43^{b} \pm 0.01$	**$0.15^{c} \pm 0.02$**
	342-9	$1.70^{d} \pm 0.02$	$0.87^{e} \pm 0.01$	**$0.15^{e} \pm 0.01$**	**$0.07^{f} \pm 0.01$**	**$0.00^{e} \pm 0.00$**	**$0.00^{f} \pm 0.00$**	**$0.00^{d} \pm 0.00$**
	212-9	$3.31^{b} \pm 0.02$	$2.95^{b} \pm 0.01$	$1.70^{c} \pm 0.01$	$1.22^{c} \pm 0.01$	$0.55^{c} \pm 0.02$	**$0.20^{e} \pm 0.01$**	**$0.00^{d} \pm 0.00$**
	270-9R	$2.69^{c} \pm 0.01$	$2.28^{c} \pm 0.01$	$2.00^{b} \pm 0.01$	$1.62^{b} \pm 0.01$	$1.30^{a} \pm 0.01$	$1.00^{a} \pm 0.01$	$0.57^{a} \pm 0.01$
	351-9	$1.70^{d} \pm 0.02$	$1.70^{d} \pm 0.01$	$1.67^{c} \pm 0.01$	$1.11^{d} \pm 0.01$	$0.62^{c} \pm 0.02$	**$0.24^{d} \pm 0.01$**	**$0.13^{c} \pm 0.01$**
	Control	$0.41^{e} \pm 0.00$	$0.40^{f} \pm 0.00$	$0.39^{d} \pm 0.00$	$0.40^{e} \pm 0.00$	$0.40^{d} \pm 0.00$	$0.39^{c} \pm 0.00$	$0.38^{b} \pm 0.00$
500	270-9C	$3.21^{b} \pm 0.01$	$3.11^{c} \pm 0.01$	$2.16^{c} \pm 0.02$	$1.50^{d} \pm 0.02$	$0.55^{d} \pm 0.01$	**$0.42^{c} \pm 0.00$**	**$0.26^{d} \pm 0.01$**
	342-9	$1.80^{d} \pm 0.01$	$0.75^{e} \pm 0.01$	$0.68^{e} \pm 0.01$	**$0.21^{f} \pm 0.00$**	**$0.15^{f} \pm 0.02$**	**$0.00^{d} \pm 0.00$**	**$0.00^{e} \pm 0.00$**
	212-9	$3.49^{a} \pm 0.01$	$3.48^{a} \pm 0.01$	$3.49^{a} \pm 0.01$	$2.43^{b} \pm 0.01$	$1.98^{b} \pm 0.01$	$1.10^{b} \pm 0.06$	$0.50^{c} \pm 0.02$
	270-9R	$3.47^{a} \pm 0.00$	$3.34^{b} \pm 0.01$	$3.30^{b} \pm 0.01$	$3.15^{a} \pm 0.02$	$2.70^{a} \pm 0.02$	$2.51^{a} \pm 0.01$	$2.12^{a} \pm 0.01$
	351-9	$2.89^{c} \pm 0.02$	$2.61^{d} \pm 0.02$	$1.81^{d} \pm 0.01$	$1.59^{c} \pm 0.01$	$1.30^{c} \pm 0.01$	$1.02^{b} \pm 0.02$	$0.76^{b} \pm 0.02$
	Control	$0.45^{e} \pm 0.00$	$0.47^{f} \pm 0.00$	$0.46^{f} \pm 0.00$	$0.44^{e} \pm 0.00$	$0.45^{e} \pm 0.01$	$0.44^{c} \pm 0.01$	$0.44^{c} \pm 0.01$
1000	270-9C	$3.12^{c} \pm 0.01$	$2.98^{c} \pm 0.02$	$2.60^{c} \pm 0.01$	$3.34^{b} \pm 0.03$	$1.59^{d} \pm 0.02$	$1.20^{c} \pm 0.01$	$0.92^{c} \pm 0.01$

342-9	$1.93^{d} \pm 0.01$	$1.50^{d} \pm 0.02$	$0.89^{d} \pm 0.01$	**$0.20^{e} \pm 0.01$**	**$0.16^{f} \pm 0.01$**	**$0.10^{f} \pm 0.00$**	**$0.00^{f} \pm 0.00$**
212-9	$3.41^{b} \pm 0.01$	$3.50^{a} \pm 0.00$	$3.47^{a} \pm 0.01$	$3.49^{a} \pm 0.01$	$3.48^{a} \pm 0.00$	$3.43^{a} \pm 0.02$	$3.10^{a} \pm 0.02$
270-9R	$3.48^{a} \pm 0.01$	$3.47^{a} \pm 0.01$	$3.49^{a} \pm 0.01$	$3.33^{b} \pm 0.01$	$3.20^{b} \pm 0.01$	$3.02^{b} \pm 0.01$	$2.92^{b} \pm 0.01$
351-9	$3.50^{a} \pm 0.00$	$3.39^{b} \pm 0.01$	$3.10^{b} \pm 0.01$	$2.98^{c} \pm 0.02$	$2.80^{c} \pm 0.02$	$0.83^{d} \pm 0.02$	**$0.30^{e} \pm 0.01$**
Control	$0.50^{e} \pm 0.00$	$0.52^{e} \pm 0.01$	$0.49^{e} \pm 0.00$	$0.48^{d} \pm 0.00$	$0.47^{e} \pm 0.01$	$0.49^{e} \pm 0.01$	$0.49^{d} \pm 0.01$

[a] Mean values of dihexavalent chromium reduction (n = 3) by indigenous bacteria with standard error are shown.

* Values followed by the same letter within columns, per each concentration of Cr(VI), are not significantly different ($P < 0.05$), according to Tukey's HSD test.

The values in bold represent the statistically significant Cr(VI) reduction for a particular isolate, compared to the control, per each concentration of chromium traced in time.

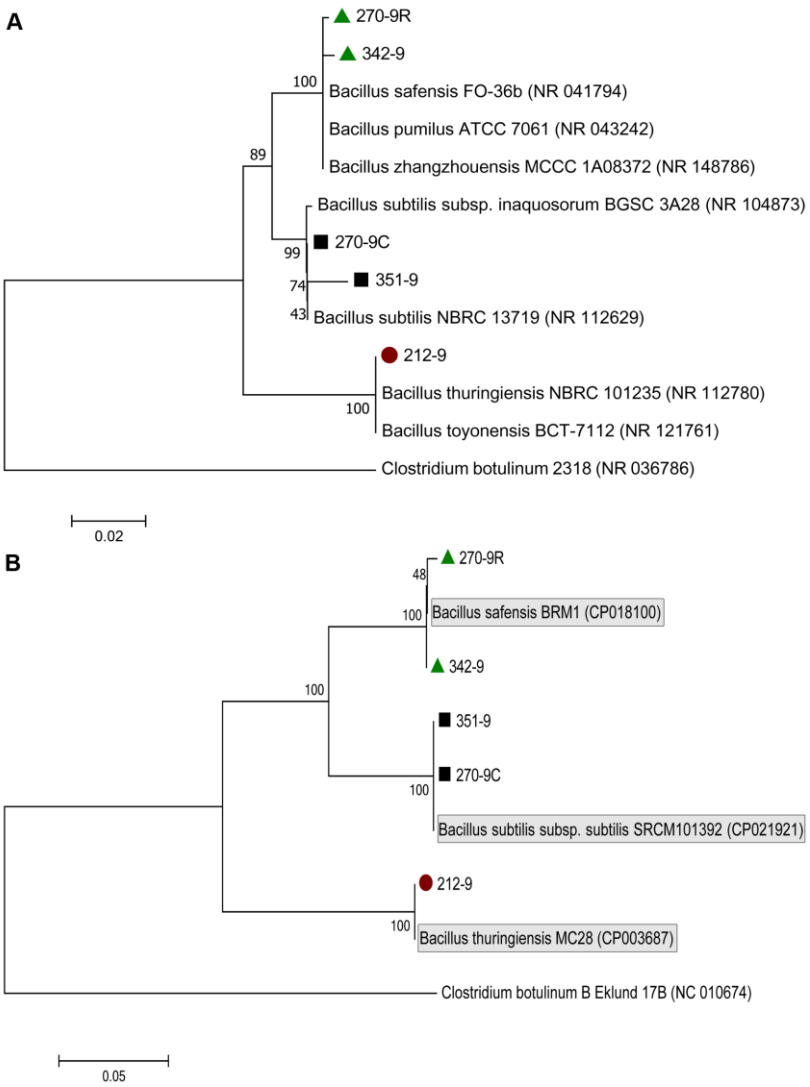
A
270-9R
342-9
100
Bacillus safensis FO-36b (NR 041794)
Bacillus pumilus ATCC 7061 (NR 043242)
89
Bacillus zhangzhouensis MCCC 1A08372 (NR 148786)
Bacillus subtilis subsp. inaquosorum BGSC 3A28 (NR 104873)
270-9C
99
351-9
74
43
Bacillus subtilis NBRC 13719 (NR 112629)
212-9
Bacillus thuringiensis NBRC 101235 (NR 112780)
100
Bacillus toyonensis BCT-7112 (NR 121761)
Clostridium botulinum 2318 (NR 036786)
0.02
B
270-9R
48
100
Bacillus safensis BRM1 (CP018100)
342-9
100
351-9
270-9C
100
Bacillus subtilis subsp. subtilis SRCM101392 (CP021921)
212-9
100
Bacillus thuringiensis MC28 (CP003687)
Clostridium botulinum B Eklund 17B (NC 010674)
0.05

Figure 1

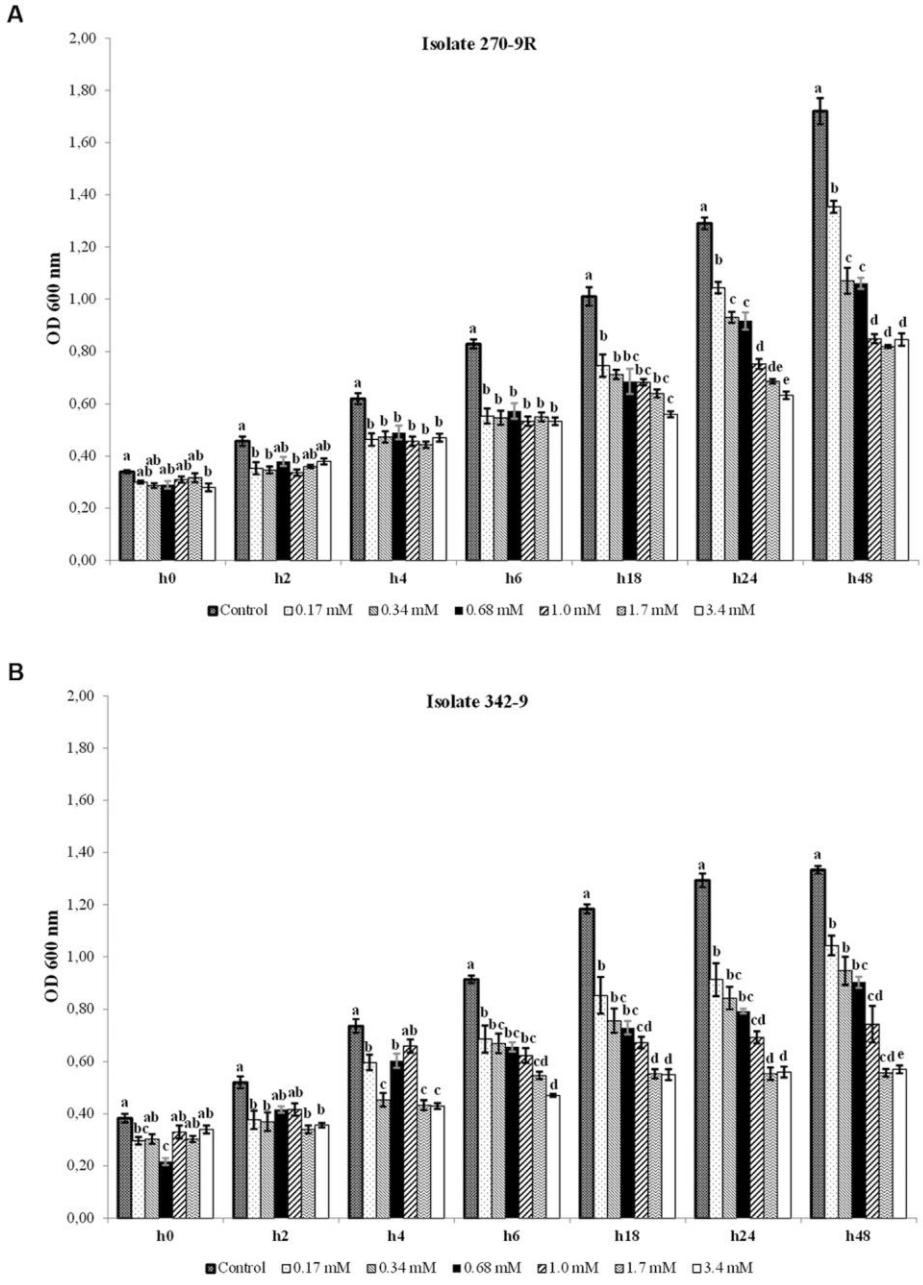
A
Isolate 270-9R
OD 600 nm
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
h0
h2
h4
h6
h18
h24
h48
Control
0.17 mM
0.34 mM
0.68 mM
1.0 mM
1.7 mM
3.4 mM
B
Isolate 342-9
OD 600 nm
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
h0
h2
h4
h6
h18
h24
h48
Control
0.17 mM
0.34 mM
0.68 mM
1.0 mM
1.7 mM
3.4 mM

Figure 2

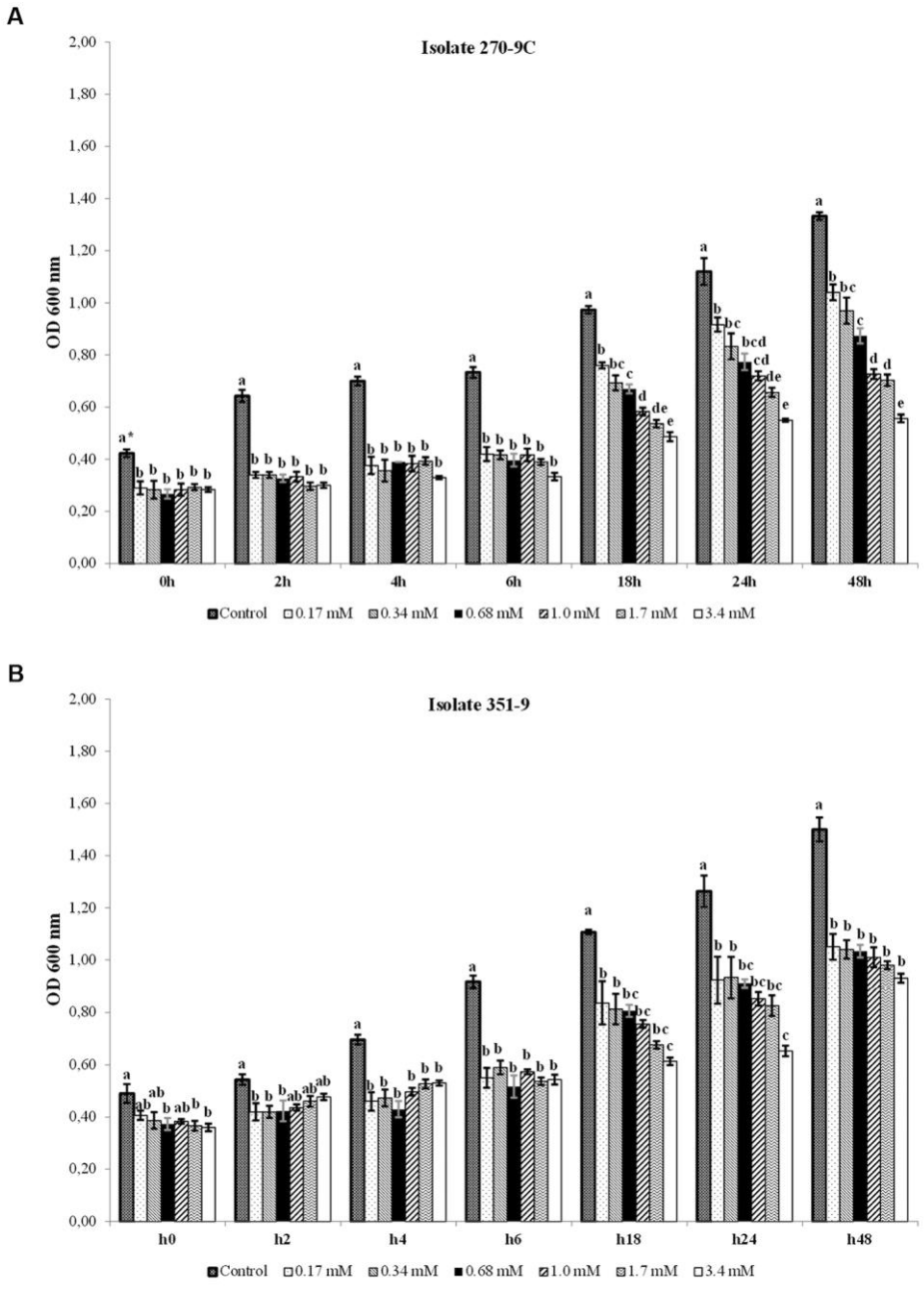
A
Isolate 270-9C
OD 600 nm
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
4h
6h
18h
24h
48h
Control
0.17 mM
0.34 mM
0.68 mM
1.0 mM
1.7 mM
3.4 mM
B
Isolate 351-9
OD 600 nm
h0
h2
h4
h6
h18
h24
h48
Control
0.17 mM
0.34 mM
0.68 mM
1.0 mM
1.7 mM
3.4 mM

Figure 3

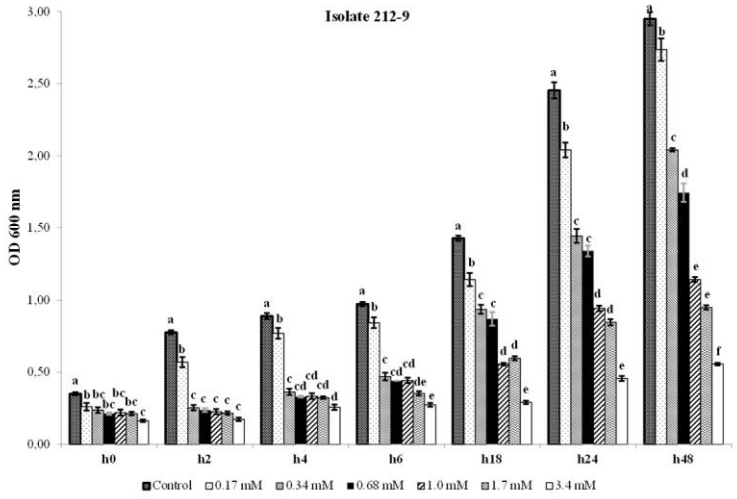
Isolate 212-9
OD 600 nm
3,00
2,50
2,00
1,50
1,00
0,50
0,00
h0
h2
h4
h6
h18
h24
h48
Control
0.17 mM
0.34 mM
0.68 mM
1.0 mM
1.7 mM
3.4 mM

Figure 4