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Investigation of the Siderophore Production and Associated Heavy Metal Accumulation Potential of *Brevibacillus laterosporus* 301/İK3-2

Brevibacillus laterosporus 301/İK3-2'nin Siderofor Üretimi ve İlişkili Ağır Metal Biriktirme Potansiyelinin Araştırılması

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ABSTRACT

Siderophores are secondary metabolites released into the environment by various microorganisms, fungi and plants to chelate iron from the surrounding environment. It is known that siderophores bind to other metals besides iron. Today, heavy metals, which are released as an undesirable result of industrial development, accumulate at high rates and pose a significant threat to biological living things. In this sense, remediation of heavy metal contaminated sites is an urgent requirement. Siderophores are promising agents for the removal of heavy metals from natural habitats with the role of bioremediation. In this study, the effect of heavy metals on the growth and siderophore production of *Brevibacillus laterosporus* was investigated. Maximum siderophore production was determined as 50 % at 48 h in the metal free growth media. In addition, maximum siderophore production was determined in the presence of various heavy metals including 5 µM Hg^{2+}, 0.5 mM Ni^{2+}, 0.1 mM Co^{2+} and 2.5 µM Fe^{2+}. Intracellular uptake of the mercury was also measured using optical emission spectroscopy and compared with siderophore production values of the *B. laterosporus*. The maximum biosorption of mercury was measured to be 40% in 5 µM Hg^{2+} containing media at 48 h of incubation. The results show that siderophore production is affected by uptake of various metals, and are usable for removing of heavy metals from environmental habitats.

Key Words
Brevibacillus laterosporus, siderophore, heavy metal, secondary metabolite, CAS assay.

ÖZ

Sideroforlar, çevrelendikleri ortamdan demiri şelatlamak için çeşitli mikroorganizmalar, mantarlar ve bitkiler tarafından çevreye salınan ikincil metabolitlerdir. Sideroforların demir dışında diğer metallere de bağlandığı bilinmektedir. Günümüzde endüstriyel gelişmenin istenmeyen bir sonucu olarak açığa çıkan ağır metaller yüksek oranlarda birikmekte ve biyolojik canlılar için önemli bir tehdit oluşturmaktadır. Bu anlamda, ağır metalle kontamine olan alanların iyileştirilmesi acil bir gerekliliktir. Sideroforlar, biyoremediasyon rolü ile ağır metallerin doğal yaşam alanlarından uzaklaştırılması için umut vaat eden ajanlardır. Bu çalışmada ağır metallerin *Brevibacillus laterosporus*'un büyümesi ve siderofor üretimi üzerindeki etkisi araştırılmıştır. Maksimum siderofor üretimi metal içermeyen büyüme ortamında 48 saatte %50 olarak belirlendi. Ayrıca, maksimum siderofor üretimi 5 µM Hg^{2+}, 0,5 mM Ni^{2+}, 0,1 mM Co^{2+} ve 2,5 µM Fe^{2+} gibi çeşitli ağır metallerin varlığında belirlendi. Civanın hücre içi alımı, optik emisyon spektroskopisi kullanılarak da ölçüldü ve *B. laterosporus*'un siderofor üretim değerleri ile karşılaştırıldı. Civanın maksimum biyosorpsiyonu, 48 saatlik inkübasyon sonunda 5 µM Hg^{2+} içeren ortamda %40 olarak ölçüldü. Sonuçlar, siderofor üretiminin çeşitli metallerin alımından etkilendiğini ve ağır metallerin çevresel habitatlardan uzaklaştırılması için kullanılabileceğini göstermektedir.

Anahtar Kelimeler

Brevibacillus laterosporus, siderofor, ağır metal, sekonder metabolit, CAS testi.

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INTRODUCTION

Iron is one of the main limiting factors and an essential nutrient for microbial life, as it is an essential cofactor for various cellular processes, including respiration, amino acid metabolism, biosynthesis of sterols and DNA in all eukaryotes and most of the prokaryotes. Although it is abundant in nature, it is not easily found in the preferred form. Iron undergoes rapid oxidation from the Fe^{2+} form to Fe^{3+} in the presence of oxygen and neutral pH, eventually forming ferric oxyhydroxide, which has extremely low bioavailability by microorganisms, almost impossible to obtain, and insoluble. Therefore, bacteria and fungi produce high affinity chelating, low molecular weight and water-soluble compounds called siderophores (400-1000 kDa). There are also cyanobacterial, mammalian and plant siderophores. A wide variety of siderophore types are produced by different organisms whose production processes are controlled by different genes at the molecular level for accumulation, mobilization and transportation of iron for metabolism [1,2]. In contrast to their structurally different configurations and properties, all siderophores exhibit a conserved structure consisting of non-functional units bound by molecules such as transferrin and lactoferrin. These compounds often have a peptide backbone that interacts with outer membrane receptors located on the cell surface. These molecules contain the most effective types of iron-binding ligands in nature, consisting of hydroxamate, catecholate or α-hydroxycarboxylate ligands [3]. Siderophores, produced by bacteria for iron uptake can also chelate metals other than iron [4]. It has been shown that metals other than iron can regulate the production of siderophores. This process depends on the concentration of metals in the growth media; the excess of the metals can affect the siderophore production negatively.

Siderophores have great biotechnological potential due to their structural and functional properties. With the emergence of novel molecular methods and technologies, further research and development is required to utilize the beneficial properties of these molecules in both medical and environmental microbiology. In recent studies, it has been shown that siderophores can be used for reducing environmental pollution [5], and in some studies, the use of microbial siderophores can be a solution for some diseases caused by iron deficiency in some plants [6]. In metal competition tests with siderophores, it has been shown that siderophores bind iron with the highest affinity, but also bind various heavy metals with a certain affinity.

In addition to some ions such as Na^{2+}, Mg^{2+}, and K^{+}, which are the basic metabolic elements in cells, there are also metals that participate in the structure of cellular processes. Some of these elements are Fe^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Mo^{2+}, and it has been shown that these metals can exist from µM levels to mM levels in bacterial cells [7]. Heavy metal groups are generally known with their toxic effects on living organisms. In addition, it has been reported that some metals such as Hg^{2+}, Pb^{2+}, Ag^{2+}, Al^{2+} have no biological functions. On the other hand, some exceptional heavy metals such as; vanadium, tungsten, and cadmium, were detected with their vital functions for some organisms [8].

Hg^{2+} is an element that is found in very low concentrations in environment and has some toxic effects on cells. Generally, the combustion of coal and petroleum products and industrial by-products generates mercury emissions and Hg^{2+} spreads to the atmosphere, water and terrestrial systems. Additionally, environmental Hg^{2+} participates in the environmental cycle as a result of geological and biological processes. Mercury fumes are absorbed by natural and anthropogenic sources and then dispersed into the atmosphere. The Hg^{2+} can also accumulate in food chain. Mercury exists in the atmosphere as vapor and volatile forms. In nature, mercury can be found in Hg^{+2}, HgO or methyl/ethyl forms. All of these forms are toxic, water-soluble and dispersible [9]. In addition, mercury ions can be found in the form of HgS by adhering to sulfur ions in the soil.

In this context, it was aimed to determine the siderophore production of *B. laterosporus* and investigate the effect of siderophore production on heavy metal uptake. In the study, the type of siderophore produced by the bacteria was determined. The effects of heavy metals such as Fe^{2+}, Co^{2+} and Ni^{2+} and Hg^{2+} on siderophore production were analyzed at various concentrations. Mercury was selected among the metals for the metal uptake experiments in this study, taking into account the effects of this metal on the environmental habitats. Correspondingly, siderophore production ratios were also measured to compare with the rate of uptake of mercury into the cell from the media.

MATERIALS AND METHODS

Chemicals and bacterial strains

Geobacillus kaustophilus DSM 7263^T was obtained from the *Bacillus* Genetic Stock Center at the Ohio State University. *Brevibacillus laterosporus* 301/İK3-2 strain isolated from the Marmara Sea and identified by the VITEK system was obtained from Dr. Yosun Mater (Gebze Technical University). *Pseudomonas aeruginosa* PO1 strain was obtained from Yavuz Sezen's laboratory (Gebze Technical University). Unless indicated, all chemicals and buffers were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Determination of siderophore production in the culture supernatant

Siderophore production was determined according to the CAS agar and CAS liquid method of Payne et al. [10]. Bacterial cultures were grown in an iron-depleted minimal medium (MM9) for all experiments. All used glass materials were treated with 10% HCl in order to remove the metal ions in the environment and then passed through distilled water. Glass materials were finally made ready for use by autoclaving at 121 °C for 20 min and sterilizing. The CAS agar assay is a universal test for the detection of siderophores. Test based on the affinity of the siderophore for ferric iron (Fe^{+3}). CAS agar plates contain hexadecyltrimethylammonium (HDTMA) which is blue in color due to the complex formation of Fe^{+3} and CAS dye. HDTMA, as a detergent, prevents the precipitation of colour in the bacterial medium and promotes bacterial growth. In the presence of siderophore, the following reaction occurs, free dye is released and the blue color turns into orange.

$$Fe^{3+} - Cas\ \ complex + Siderophore \rightarrow\rightarrow Fe^{3+} - Siderophore + Cas\ (orange)$$

B. laterosporus 301/İK3-2 and *P. aeruginosa* PO1 (selected as positive control organism) were transferred to LB agar medium and incubated at 37 °C. Bacterial colonies were transferred to CAS agar medium and incubated at 37 ºC for 168 h. Orange colored regions just outside the growth rings indicate siderophore formation and show positive results for the CAS agar test [11]. Orange rings proving the presence of siderophore production were measured at 24-h intervals and these formations were photographed after 168 h. In order to determine the production of siderophores with the CAS liquid assay, pre-cultured bacteria in MM9 broth, which does not contain iron, were transferred to fresh MM9 broth at a ratio of 1/100 and was incubated at 37 ºC at 225 rpm. The supernatant was collected after centrifugation at 10.000 rpm for 5 minutes at 20 °C. For the detection of siderophore concentration by CAS liquid assay, 0.5 ml of supernatant was mixed with 0.5 ml of CAS solution and then 10 µl of shuttle solution (sulfosalicylic acid, 0.2 M) was added. A culture-free MM9 medium was used as a blank. Color change in references (0.5 mL MM9 medium 0.5 mL CAS solution + 10 µM shuttle solution) and for samples were determined by measuring at A630 nm wavelength. All experiments were performed 3 times for both cultures. The secreted siderophores bind and release the dye and change the color from blue to orange inside the media.

Calculation of siderophore content was performed using the following method: Ar is the reference value (CAS reagent) and As is the sample value (630 nm) for the equation.

$$\%\ Siderophore = \frac{(Ar - As)}{Ar} \times 100$$

Determination of Siderophore Type

The type of siderophore was determined using Arnow's and Csaky's assays [12,13]. In both assays, first *B. laterosporus* 301/İK3-2 was pre-cultured in MM9 broth medium, then, it was transferred to MM9 broth medium in a 1/100 ratio and incubated at 225 rpm, at 37 ºC. At the end of the 48 h growth phase, 1 mL of the culture was taken and centrifuged at 10.000 rpm for 5 min at 20 ºC. For the Arnow test, which was used for identifying the catechol type siderophore, 1 mL 0.5 N HCl and 1 mL nitrite-molybdate reagent were added into 1 mL supernatant. If bacteria produce catechol-type siderophores, the mixture is expected to turn yellow in this step. Finally, 1 mL of NaOH solution was added to the mixture. In this step, the mixture should turn red, if it is producing catechol-type siderophores. The color change solution due to siderophore formation was measured at 510 nm in the spectrophotometer. For the Csaky test, which was used to identify hydroxamate-type siderophores, 1 mL of H_2SO_4 (6N) was added to the 1 mL

obtained supernatant, and the mixture was kept at 130 °C for 30 min., after this step 3 mL of sodium acetate was added. Then, 1 mL of sulfanilic acid solution and 0.5 mL of iodine solution were added and the mixture was incubated for 5 min, and 1 mL of sodium thiosulfate solution was added. Finally, after adding α-naphthylamine to the solution, 10 mL of distilled water was added to the mixture and incubated for 30 min at room temperature. The samples were measured at 526 nm by the spectrophotometer.

Optimization of Siderophore Production Time

The maximum siderophore production time was detected using a CAS liquid assay as mentioned above. Siderophore measurement was performed by taking samples from the medium at 0, 4, 8, 12, 16, 20, 24, 48, 72, 96, 120, 144 and 168 h of incubation, and OD_{600} of the samples were also measured to determine cell growth.

Effect of heavy metals on siderophore production

The effect of heavy metals on siderophore production was determined using different heavy metal concentrations for the bacterial growth mediums. Siderophore production was detected with CAS liquid assay as described above. The growth mediums for *B. laterosporus* were prepared by adding different concentrations of $HgCl_2$ (0, 5, 10, 15 µM), $NiSO_4$ (0, 0.5, 1.0, 1.5 µM), $CoCl_2$ (0, 0.1, 0.3, 0.5 mM), and $FeCl_2$ (0, 2.5, 5, 10 µM) [14]. The percentage of siderophore production as well as cell growth was determined by taking samples from cultures at 0, 24, 48, and 72 h.

Detection of cellular Hg^{2+} uptake with ICP-OES

The Hg^{2+} uptake from the medium was quantitatively determined by ICP-OES (Inductively coupled plasma-optical emission spectrometry). In order to detect cellular mercury uptake, *B. laterosporus* preculture was inoculated 1/100 on MM9 medium containing $HgCl_2$ at different concentrations given above and incubated at 37 °C, 225 rpm for 72 h. Samples (10 mL) taken from growing bacterial cultures at 24 h intervals were centrifuged at 10.000 rpm for 5 min. mercury in obtained supernatants were measured at 194.168 nm, which is the predicted emission line value for Hg^{2+} in ICP-OES, and the absorption rate for Hg^{2+} in the medium of bacterial cultures was calculated. In addition, cell density and siderophore production at different Hg^{2+} concentrations were measured as previously described. Studies in optical emission spectrometry were carried out in Gebze Technical University Environmental Engineering Department Inorganic Instrumental Analysis Laboratory.

Results and Discussion

The ability of *B. laterosporus* to produce a siderophore was first detected with the CAS agar plate test. After 168 h incubation, the orange zone formed around *B. laterosporus*, as well as *P. aeruginosa*, which is known to be a good siderophore producer used as a positive control. The other microorganism G. kaustophilus showed a negative result for siderophore production (Fig. 1). The diameters of the orange zones formed by the bacterial colonies growing on the CAS agar plates were measured; 14 mm for *P. aeruginosa* and 10 mm for *B. laterosporus* were detected.

Produced siderophores remove iron which results in the change of the blue CAS solution to orange in *P. aeruginosa* PO1 as well as *B. laterosporus* cultures. The highest percentage of siderophore production was determined as 87.1% for *P. aeruginosa* PO1 and 28.6% for *B. laterosporus*, respectively. Siderophore production by *B. laterosporus* started in the early phase of growth and increased gradually until the start of the death phase. The maximum production of siderophore was determined as 50% at 48th h (Fig. 2). It has been previously reported that the concentration of siderophores decreases as the bacteria consume the nutrient and the cells begin to break down with time [15].

B. laterosporus exhibited positive results with the Arnow method for catecholate-type siderophores and the negative result with the Csaky method that was used for the determination of hydroxamate type siderophores.

Concentrations of metals used in the study were determined on the basis of previous studies [14,16]. Iron is a trace element necessary for the growth of all organisms. Contrary to Hg^{2+}, Co^{2+} and Ni^{2+}, the concentrations of Fe^{3+} were used in much lower amounts in this study. It is known that siderophore secretion is suppressed in the presence of highly available iron (Fe^{3+}) concentration, but a low amount of iron is required for the growth of organisms [17]. In order to identify the relationship of metal ions with growth and siderophore production, simultaneous growth values and siderophore secretion level were determined for *B. laterosporus*, MM9 media is used for all experiments with different metal ion concentrations.

According to the results with iron, the highest production of siderophore was determined as 32.57% and 31.98% in the medium containing 2.5 µM and 5 µM Fe^{3+}

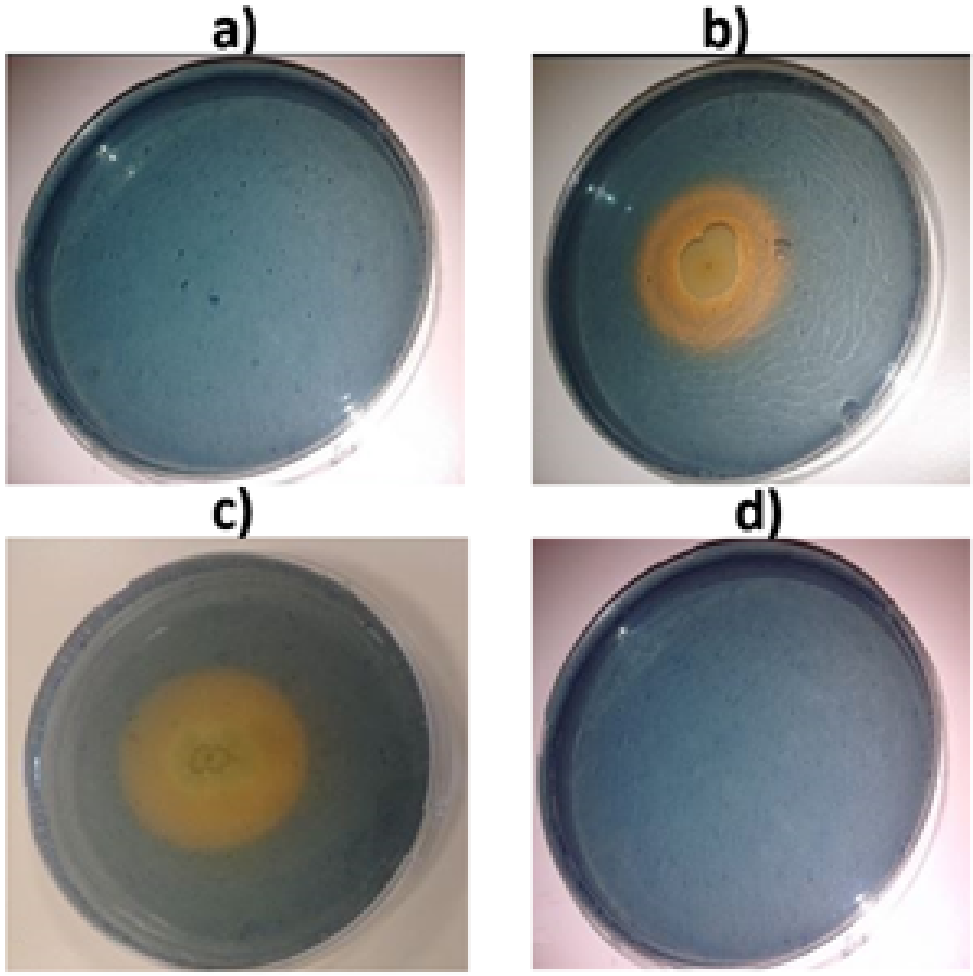

Figure 1. CAS agar test results. a) Control, b) *B. laterosporus*, c) *P. aeruginosa* PO1 as positive control, d) G. kaustophilus DSM 7263T as negative control.

at 48th h, respectively (Fig. 3a). Besides, the cell growth was stimulated with the increasing amount of iron concentrations. Remarkable cell growth was observed at 24 h in the presence of 10 µM Fe^{3+}. The results showed consistency with previous studies in the literature. In the study of Rachid et al. [18], siderophore production was suppressed when the ferric iron content was above 200 µg/L in the media, and siderophore production was positively affected when the ferric iron content was below 160 µg/L in the media.

Cobalt is an important metal for organisms which is found in the structure of many organic compounds such as cobalamin, nitrile hydratase and methionyl aminopeptidase [19]. In this study, the highest siderophore production was observed in the cobalt-free control group until the 48th h. In spite of this, siderophore production exceeded the control group (28%) at 0.1 mM (35.7%) and 0.3 mM (32.9%) Co^{2+} concentrations at 72 h (Fig. 3b). Considering the effect of Co^{2+} on cell growth, the highest value was observed in the cobalt-free group at the 24th h. Cell growth was at low levels for the first 24 h in the mediums containing various levels of cobalt concentrations. However, cell growth decreased after the 24th h in the control group. In the following hours, cultures containing cobalt showed adaptation to this metal and cell growth increased at 0.1- and 0.3-mM cobalt concentrations containing mediums. But at 0.5 mM cobalt concentration, cell growth was suppressed after 48 h. Similarly, in a study with a desferrioxamine B-type siderophore, cobalt formed a strong complex with the siderophore and increased cobalt mobilization [20]. The results show that there is a correlation between cobalt levels and siderophore production rates, low cobalt concentration stimulates the secretion of siderophore. Ni^{2+} is an element that is found as a cofactor in many enzyme structures and plays a major role for diverse biological metabolic processes. Nickel also plays a role

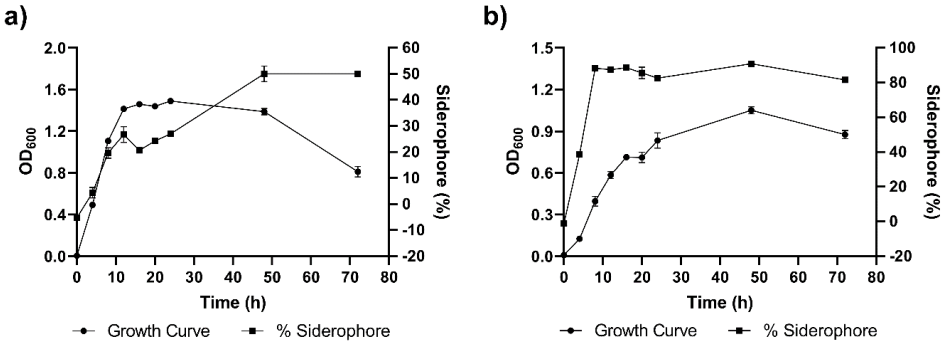

Figure 2. Time dependent siderophore production for *B. laterosporus* (a) and *P. aeruginosa* (b).
B. laterosporus exhibited positive results with the Arnow method for catecholate-type siderophores and the negative result with the Csaky method that was used for the determination of hydroxamate type siderophores.

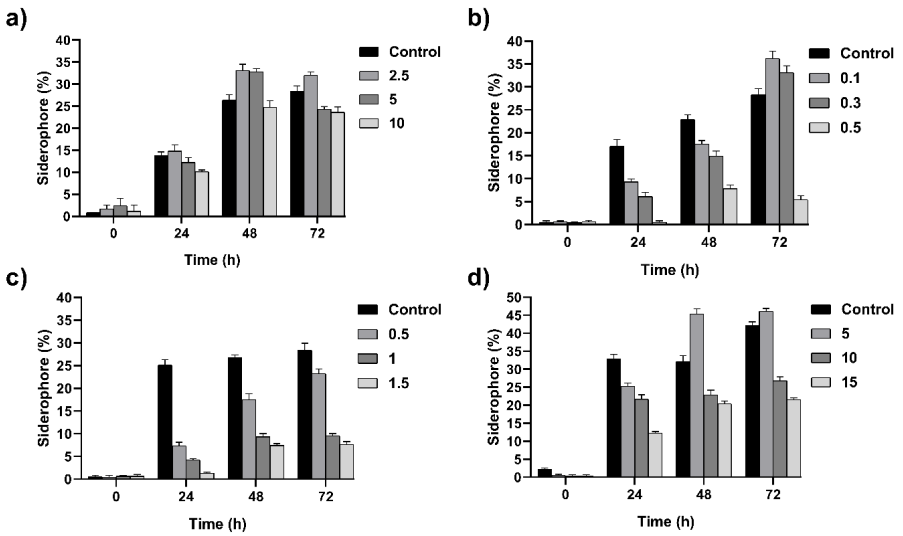

Figure 3. The effect of different concentrations (µM) of Fe^{3+} (a), Co^{2+} (b), Ni^{2+} (c) and Hg^{2+} (d) on time-dependent siderophore production in *B. laterosporus* 301/iK3-2.

in energy production [21]. Unlike other metals, nickel did not contribute positively to the production of siderophores. Recent studies show that the excess of nickel can repress siderophore production [22]. Cell growth remained lower than the control group at all nickel concentrations until the 72nd h (Fig. 3c).

Hg^{2+} has no essential biological function and causes toxic effects at very low concentrations. The effects of mercury on *B. laterosporus* siderophore production and cell growth are shown in Figure 3d and 4d, respectively. At the 24th h, the highest siderophore production was observed in the Hg2+ free control group (33.9%). However, siderophore production increased in the cultures containing 5 µM Hg at 48 and 72 h. In this concentration, siderophore levels reached 47.1% and 46.6%, respectively. As expected, mercury suppressed cell growth as its concentration increased.

With the increasing production of siderophores, it is expected that the mercury will form a complex with the siderophore and as a result, the free Hg^{2+} presence inside the media will be decreased. For this purpose, the removal of free mercury in the medium by siderophore was determined at different time intervals and concentrations using ICP-OES. The highest mercury uptake was observed at

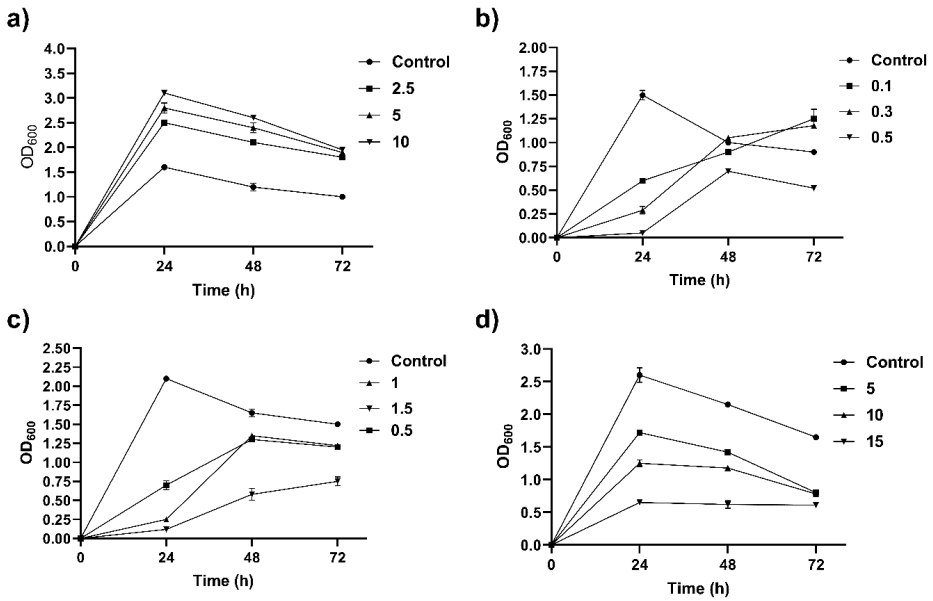

Figure 4. The effect of different concentrations (µM) of Fe^{3+} (a), Co^{2+} (b), Ni^{2+} (c) and Hg^{2+} (d), on the growth of *B. laterosporus* 301/İK3-2.

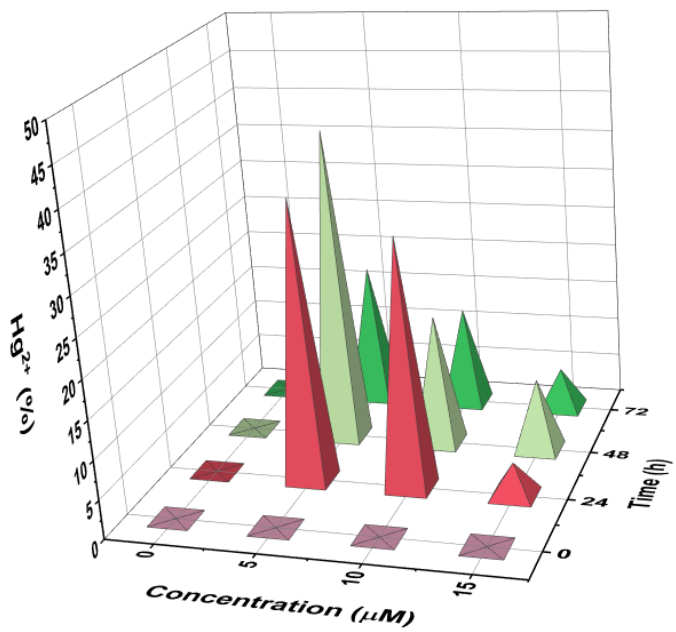

Figure 5. Mercury uptake by *B. laterosporus* with respect to Hg^{2+} concentrations and incubation time.

the 48th h, on the contrary, the least metal uptake was observed at the 72nd h (Fig. 5). The highest Hg^{2+} uptake occurred at the 48th h and the results showed consistency with the findings of siderophore production. It was determined that the cellular uptake percentage of Hg^{2+} was 16.62% in the medium containing 10 µM Hg^{2+}. The lowest cellular uptake was determined as 9.1% at 15 µM Hg^{2+} concentration.

Conclusions

Siderophores bind certain metals with different levels of affinity and form complexes. Revealing the relationship of siderophores with various metals beside iron is an important phenomenon in terms of contributing to the understanding of natural tolerance mechanisms against toxic metals that have adverse environmental effects. Therefore, metals such as Fe3+, Ni2+, Co2+ and Hg2+ are selected to reveal their effects on *B. laterosporus* siderophore production. All the metals tested in this study except mercury have essential roles for living organisms.

It was determined that heavy metals at high concentrations negatively affected the production of siderophores. It is known that 3 heavy metals (Co2+, Ni2+, Hg2+) whose effects on siderophore production percentages have been evaluated, can create toxic effects at their high concentrations. All three heavy metals suppressed bacterial growth. The percentages of siderophore production were very close at 72nd h. There was no significant change in the percentage of siderophore production after this period.

In parallel with the production of siderophores in *B. laterosporus*, it is expected that the mercury ratio in the medium will decrease as a result of the siderophore complex with mercury. In order to support the removal of mercury in the medium by the siderophore, the amount of mercury in the medium was measured at different time intervals and at different concentrations using optical emission spectroscopy. According to the results we obtained, the highest cellular mercury uptake of the bacterial cultures was at the 48th h when the siderophore production was also at maximum level. at the 48th h, the highest mercury absorption was observed at the 5 µM Hg2+ concentration (40%) where the maximum siderophore production was observed. Although it is known that mercury can be taken into the cell with the help of other types of mechanisms [23], it was shown in the present study that as the siderophore production increased concentration of mercury in the supernatant decreased.

Siderophores have become the subject of many scientific studies in terms of their high potential usage areas. For other metals used other than Hg2+, the metal ratios taken into the bacterial cells were not specifically determined. In our study, the interaction of mercury and siderophore was specifically investigated. Our aim was to observe whether the type of siderophore produced by *B. laterosporus* is effective for the removal of mercury pollution. In this context, we tried to reveal the benefits of the removal of mercury pollution of catecholate siderophore type. The data obtained in this study contains parallel results with many previous studies and show promising results for the removal of mercury pollution.

Authors' contributions

FİÖ designed the study. BA and FİÖ performed the study. FİÖ, AT and BA analyzed the data. AT and FİÖ wrote the paper. All authors read and approved the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

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