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Research paper

Burkholderia Fungorum, A promoter biological tool for heavy metals bioresorption

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ABSTRACT

In this work, we studied the effect of *Burkholderia fungorum* strain Bf01 bacterium on three heavy metals bioresorption: cadmium, copper and zinc.

The heavy metals bacterium resistance was studied in liquid minimum standard medium, added with increasing metals concentrations. Furthermore, the *Burkholderia fungorum* strain Bf01 was monitored during its growth for its capacity to reduce high metals. The strain Bf01 showed high Minimal Inhibitory Concentrations about (1500 mg/L, 400 mg/L and 50 mg/L) for Cadmium, Zinc and Copper, respectively. Therefore, it was assumed that *Burkholderia fungorum* strain Bf01 had a high metals resistance degree especially for cadmium and it exhibited a high adsorption affinity and removal metals from bacterial suspensions. As a result, *Burkholderia fungorum* strain Bf01 presents an excellent biological tool for heavy metals bioresorption for its efficiency, reliability and low cost.

1. INTRODUCTION

Water pollution is now a major scourge of modern world. With the rapid industries development such as metal plating facilities, mining operations, fertilizer industries, tanneries, batteries, paper industries and pesticides, etc., heavy metals wastewaters are increasingly discharged into the environment.

The ever increasing demand for food products has led to the use of a large number of chemical products (chemical fertilizers, pesticides, etc.), often as inputs. These cultural practices have as consequences a weakening of the soils (vulnerability of the soils), as well as an important water pollution, in particular that caused by heavy metals which represents a danger for health and the environment (Ganesan, et al. 2020, Zaynab, et al. 2022). Unlike organic contaminants, heavy metals are not biodegradable and tend

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to accumulate in living organisms and many heavy metal ions are known to be toxic or carcinogenic and pose a menace to environment (Fu et Xi 2020, Zaynab, et al. 2022). Several inorganic metals, such as zinc (Zn), chromium (Cr3+), manganese (Mn), copper (Cu), calcium (Ca), nickel (Ni), magnesium (Mg), and sodium (Na), are essential for metabolic and redox processes in trace amounts. Mercury (Hg), silver (Ag), cadmium (Cd), lead (Pb), aluminum (Al), and gold (Au) are heavy metals that have no role in biochemical functions and are very harmful to the living organisms (Sharma, Tripathi, et al. 2021, Sharma and Kumar 2021).

Faced with more and more stringent regulations, nowadays heavy metals are the environmental priority pollutants and are becoming one of the most serious environmental problems. In fact, in order to protect living organisms and the environment, the heavy metals should be removed or immobilized using useful technologies. To provide such treatment, many conventional chemical methods are used, including chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and the recovery Evaporative (Camargo, et al. 2016, Chaemiso and Nefo 2019, Qin, et al. 2020, Shrestha, et al. 2021). These methods can be inefficient, sometimes extremely expensive, produce a lot of toxic chemical sludge (Medfu Tarekegn, Zewdu Salilih and Ishetu 2020, Riseh, et al. 2022).

The choice of heavy metals removal methods requires critical approach since it must take into account the eco-efficiency of such action. In recent years, the attention of researchers focused on biological treatment using microorganisms to reduce toxic metals amount to acceptable environment limits (Rizvi, et al. 2020, Filote, Rosca and Hlihor 2020, Blaga, Zaharia and Suteu 2021, Priya, et al. 2022). There are three main benefits of using biotechnology for the pollutants elimination; First, biological processes can be performed in situ on the contaminated site; they are generally harmless to the environment (no secondary pollution) and are profitable. Different biomass types have been investigated for heavy metals biosorption properties. Using microorganisms as a heavy metal biosorbent is an effective, ecofriendly and economical alternative to existing treatments (Filote, Rosca and Hlihor 2020, Alwaleed, Latef and Mostafa 2021)

Microorganisms used in bioremediation are indigenous or non-indigenous, which they can be introduced to contaminated sites in different ways. Using indigenous microorganisms in contaminated environments is the most important approach that challenges solving problems related to pollutants biodegradation and bioremediation (Verma and Jaiswal 2016, Oziegbe, et al. 2021).

Flavobacterium, Pseudomonas, Bacillus, Arthrobacter, Corynebacterium, Methosinus, Rhodococcus, Mycobacterium, Stereum hirsutum, Nocardia, Methanogens, Aspergilus niger, Pleurotus ostreatus, Rhizopus arrhizus, Azotobacter, Alcaligenes, Phormidium valderium, Ganoderma applantus are some microbial species that help in bioremediation of heavy metals (Andreazza, et al. 2011, Oubohssaine, Sbabou and Aurag 2022, Priya, et al. 2022)

In this work, we study the effect of a rhizospheric bacterial strain *Burkholderia fungorum Bf01* on the reduction of three metals: cadmium, zinc and copper. *B. fungorum* is widely distributed in nature and its basic habitat is soils.

2. MATERIAL AND METHODS

2.1 Bacterial strain

The bacterium *Burkholderia fungorum* strain Bf01 was isolated from peanut (*Arachis hypogea*) endophytes from Algerian oasis (Sebseb-Ghardaia, Algeria), at the Soil Biology Laboratory of Université des sciences et Technologie Houari Boumedienne- Algeria (USTHB). The strain was characterized by phylogenetic method and was stored in glycerol (20%) at -70°C.

2.2 Culture medium

The Sucrose minimal salts Low Phosphates (SLP) medium (1% sucrose, 0.1% (NH_4) SO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.01% NaCl, 0.05% Yeast extract, pH 7.4), was used for bacterial cultures (Jiang, et al. 2008). The bacterial suspensions were used to follow bacterial growth with and without heavy metals, as well as to study the metals bioresorption.

The bacterial cultures were carried out using flasks (Schott) of 500 ml, filled to $1/5^{th}$ with SLP liquid medium. The pH was adjusted to 6-6.5 for Cd and Cu respectively and to 7 for Zn, corresponding to optimum retention pH of each metal (Vijayaraghavan and Yun 2008). The flasks were incubated at 28°C, under agitation at 200 rpm. Bacterial growth was carried out by reading the OD_{600} at different times (0, 15, 24, 48, 72, 96, 120 hours).

The bacterial cells of the exponential growth phase (between 12 and 15 hours of incubation) were collected to test their heavy metals resistance and to determine the Minimal Inhibitory Concentrations (MIC) (Jiang, et al. 2008).

2.3 Heavy metal solutions

The effect of different concentrations of three metals cadmium, zinc and copper, on bacterial growth was studied in the present work. To do this, a concentration range for each metal stock solution of 10 g/L was prepared from 50 to 10000 mg/L (0-50-100-200-300-400-500-1000-2000-3000-4000-5000-10000 mg/L) and filtered at 45µm.

2.4 Determination of Minimal Inibitiory Concentrations

The MIC is commonly used to study a strain sensitivity to antibiotics. Its application for other toxic elements allows the determination of bacteria resistance threshold to these molecules. The MIC of a toxicant is defined as the lowest concentration of this molecule capable of preventing the visible development of the bacteria under standardized conditions (Untereiner 2008).

To determine MIC of the Bf01 strain to the metal elements (Cd, Zn and Cu), the OD_{600} was followed using different metals concentrations (Cd: 500-2500 mg/L, Zn: 300-600 mg/L, Cu: 20-200 mg/L). The kinetics were compared to a bacterial growth kinetic in a SLP medium without metal, which represents the experiment control.

2.5 Heavy metal resistance of B. fungorum strain Bf01

The heavy metal resistance evaluation of Bf01 strain was determined by monitoring reduction rate of bacterial growth in the presence of different metals concentrations, at two different times (24 hours and 50 hours) and has been calculated using the Equation 1:

$$\text{Growth reduction rate (\%)} = \left(1 - \frac{Y}{Yt}\right) \times 100\ \% \quad (1)$$

Y : OD_{600} at 24h or 50h in the presence of metal concentration $\boldsymbol{x}$,

Y_t : OD_{600} at 24h or 50h of the control

2.6 Heavy metals adsorption by B. fungorum strain Bf01

The metal dosage was carried out using "Atomic Absorption Spectrophotometry-AAS" Flame mode (Chang, Law and Chang 1997), followed simultaneously with Bf01 strain growth.

The metal biosorption was estimated by analyzing the residual metal of the supernatant after centrifugation at 15000 rpm for 15min. The metal removed from the aqueous solution was calculated

by determining the difference between the initial and the final metals concentrations (Sinha and Mukherjee 2009) following Equation 2:

$$V_e = V_i - V_r \quad (2)$$

V_e *:* eliminated volume

V_i : Initial volume (at t_0)

V_r : residual volume

3. RESULTS AND DISCUSSION

3.1 Determination of MIC and evaluation of bacterial resistance

The growth kinetics showed in Figure 1, revealed different responses at different metal concentrations.

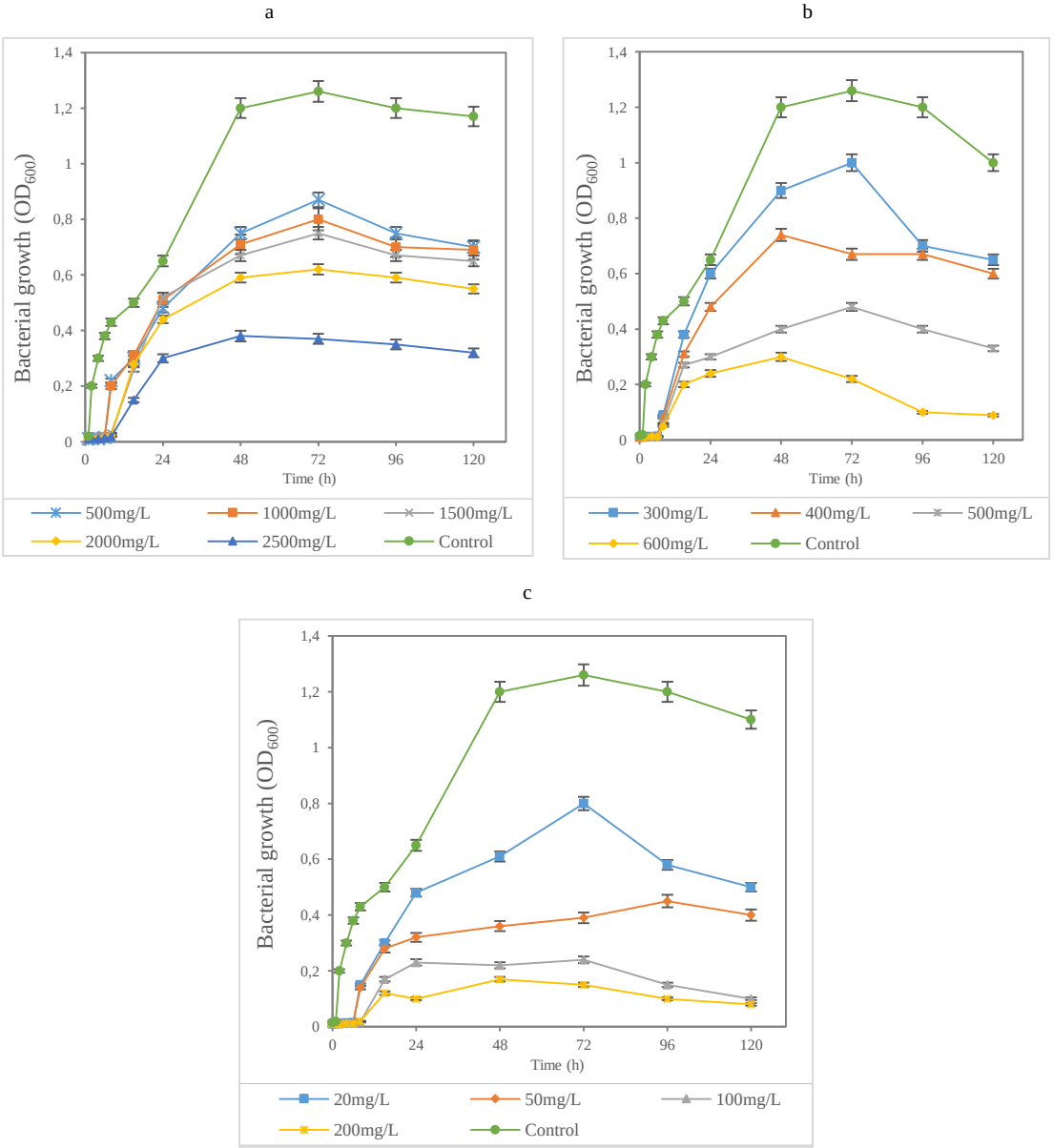

Fig.1. Increasing concentrations effect of **(a)** Cadmium (500 – 1000 - 1500 – 2000 – 2500 mg/L), **(b)** Zinc (300 – 400 - 500 – 600 mg/L) and **(c)** Copper (20 – 50 - 100 – 200 mg/L), on *B. fungorum* strain Bf01 growth in SLP liquid medium

The effect of Cd, Zn and Cu on cell growth of strain *B. fungorum Bf01* cultured in the SLP medium was investigated. From the bacterial growth curves with and without metals, the kinetics followed the same appearance, except for the long lag phase of growth with metals kinetics. For the control, rapid growth could be observed and the lag phase does not exceed 1-2h, however it was approximately 8h for growth with metals. In contrast, growth inhibition was observed for the growth curves of Cd2000, Zn500 and Cu100, indicating that strain *B. fungorum* Bf01 was able to tolerate very high concentrations of metals Cd, Zn and Cu, when cells were cultured over 96h. Furthermore, the growth of *Bf01* was inversely proportional to the metals concentrations. This could be due to a significant bacteria toxicity, probably caused by a cell morphology alteration (Zhang and Min 2010, Chatterjee, Ghosh and Mukherjea 2011). Indeed, it has been shown previously for other *Burkholderia* species (Xu, et al. 2009) that such behavior is due to heavy metal ion deposition, precipitation of organic functional groups or metal sequestration, considering that adsorption and/or complexation could be occurring between heavy metals and microbially secreted EPS (Vishan, Sivaprakasam and Kalamdhad 2017, Naveed, et al. 2019, You, et al. 2021).

To evaluate the metal tolerance of *strain Bf01*, growth reduction rates in the presence of different concentrations of Cd, Zn and Cu were established at two different times (24h and 50h), and were represented in the following histograms (Figure 2).

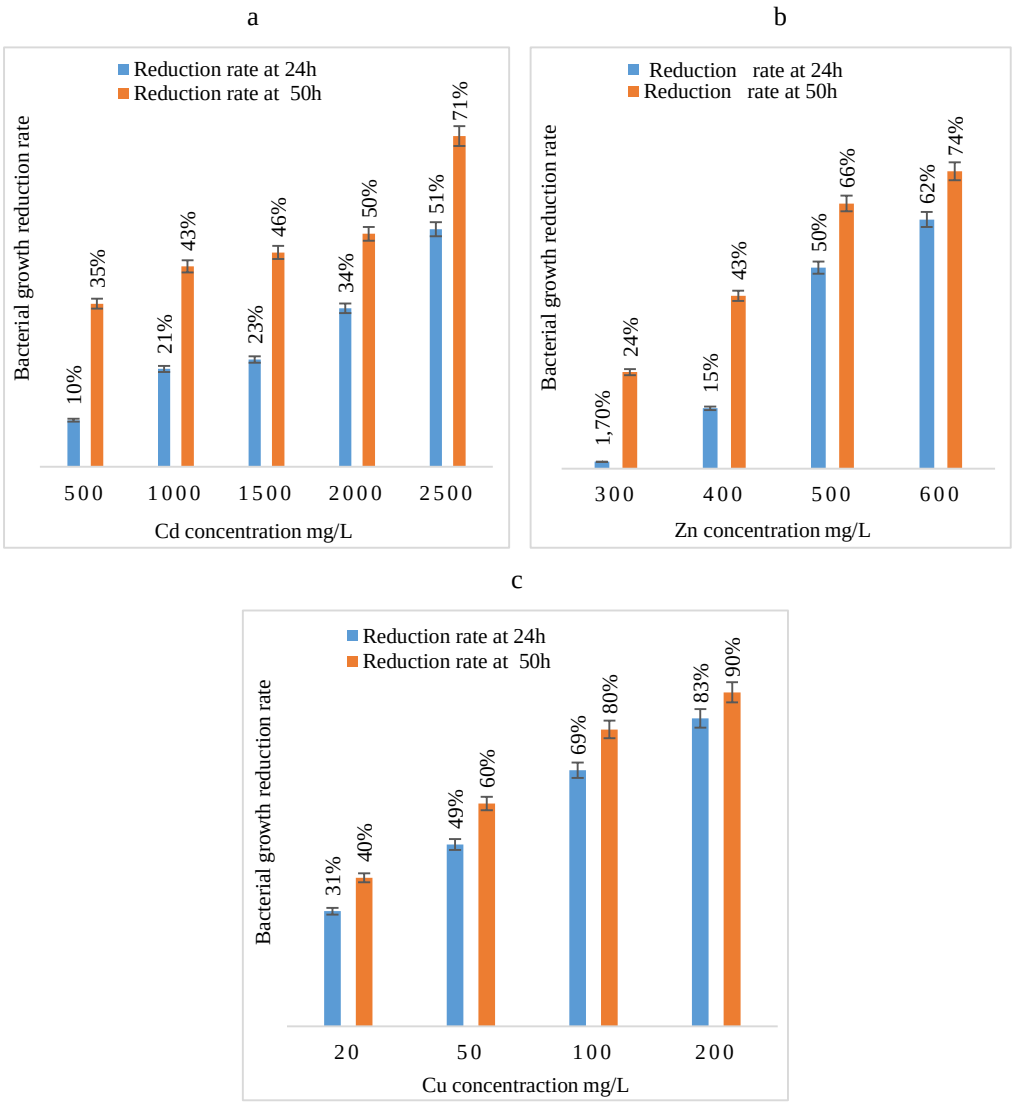

Fig 2. Growth reduction rate of *Burkholderia fungorum* Bf01strain in the presence of different concentrations of (a) Cd, (b) Zn et (c) Cu

The analysis of the growth reduction rate of *B. fungorum* strain Bf01 in liquid SLP medium, allowed to note that at 24h of incubation, growth was reduced to approximately half (51, 50 and 49%) for (Cd, Zn and Cu) and high concentrations up to (2000, 500 and 50 mg/L) were respectively tolerate by the bacterium. However, at 50h of incubation, the growth was inhibited with reduction rates of (71, 66 and 60%) for the concentrations (2000, 500 and 50 mg/L) of Cd, Zn and Cu respectively.

According to the results in figures 1 and 2, *B. fungorum strain Bf01* shows significant tolerance to the three metals studied. In fact, the Minimal Inhibitory Concentrations (MIC) for cadmium, zinc and copper could be included between 1500- 2000 mg/L, 400- 500 mg/L and 50- 100 mg/L respectively.

To identify the toxicity order of *B. fungorum* strain Bf01 to Cd, Zn and Cu, we had compared the growth kinetics at heavy metals MICs (Cd-1500 mg/L, Zn-400 mg/L and Cu-50 mg/L) (Fig 3).

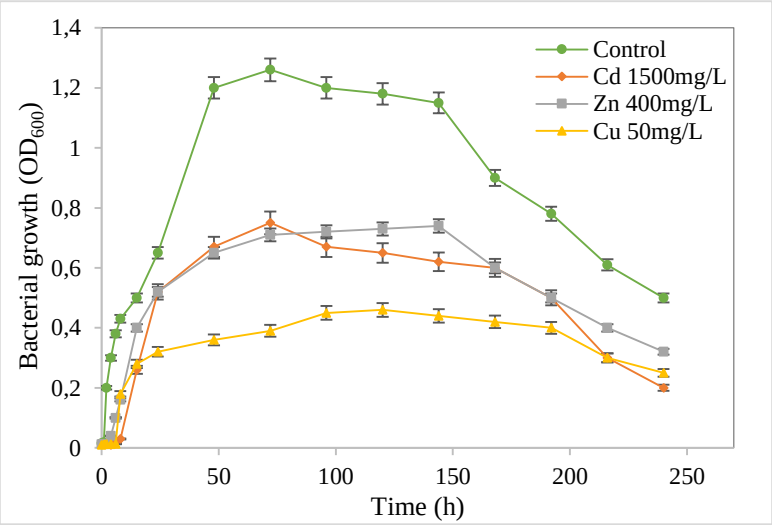

Fig 3**.** Comparison of the growth kinetics of *Burkholderia fungorum* strain Bf01 in the presence of the three heavy metals (Cd, Zn and Cu) at tolerance levels.

The growth kinetics with metals showed the same pattern as the control (without metal), therefore, a significant growth reduction was observed. After 30 hours, cell biomass production was reduced almost four times in the presence of Cu50 mg/L, 2.4 times for Cd1500 mg/L and two times in the presence of Zn400 mg/L. We can also note that despite this significant *B. fungorum* growth reduction in the presence of the three heavy metals; the bacterial growth after 30 hours remained appreciable (Fig. 3). Therefore, it has been highlighted that the toxicity order of Bf01 strain for these three metals, was: Copper > Zinc > Cadmium.

Comparing the heavy metals MICs of Bf01 strain with those obtained in previous works, it was suggested that Bf01 shows a significant tolerance to the three metals studied. Indeed, the Cd MIC for B*urkholderia fungorum* was 400mg/L (Liu, et al. 2019), 0.89 mM for *Burkholderia cepacia* GYP1 (Zhang, et al. 2019), 3.56 mM for *Planococcus rifietoensis* (Bhakta, et al. 2014), 2.22 mM for *Halomonas* BVR 1 (Rajesh, et al. 2014), 2.67 mM for *Citrobacter* sp. JH 11-2 (Shim, et al. 2015), 4.45 mM for *Enterobacter* sp. OCPSB1 (Shim, et al. 2015) and *Pseudomonas* sp.M3 (Shim, et al. 2015), 3.65 mM for *Salmonella enteric* (Shim, et al. 2015), and others in previous reports (Bhakta, et al. 2014). In another work, a strain *Paraburkholderia fungorum BRRh-4*. was isolated from a bacterial community used as *Plant Growth Promising Rhizobacteria* (PGPR) and exposed at high heavy metals concentrations of Pb+, Cd+, Cr+, Ni+, Au+ and Ag+ (Banach, et al. 2020, Raihan, et al. 2022). Furthermore, five isolated strains belonged to the genera *Sphingomonas, Stenotrophomonas and*

Arthrobacter, showed high resistance to copper (MIC ranged from 3.1 to 4.7 mM), Zn^{2+} ranged from 0.8 to 17 mM and Cd^{2+} ranged from 0.4 to 3.6 mM (Altimira, et al. 2012). In other research, bacteria isolated from different industrial locations were tested for metal resistance against CdP^{2+}, NiP^{2+}, HgP^{2+}, CuP^{2+} and PbP^{2+} by determining the minimal inhibitory concentration ranging from 10 to 250 mg/L. The strain Bf01, has showed a high resistance for Cd but less important for Zn compared to that revealed in other works with strains of the same species, that has revealed Zn 1200 mg/L (Liu, et al. 2019), or for its potential of Cadmium sequestration (Zhang and Min 2010).

3.2 Cd, Cu and Zn adsorption kinetics by B. fungorum strain Bf01

The concentration of the three metals was followed during the bacteria growth for 10 days.

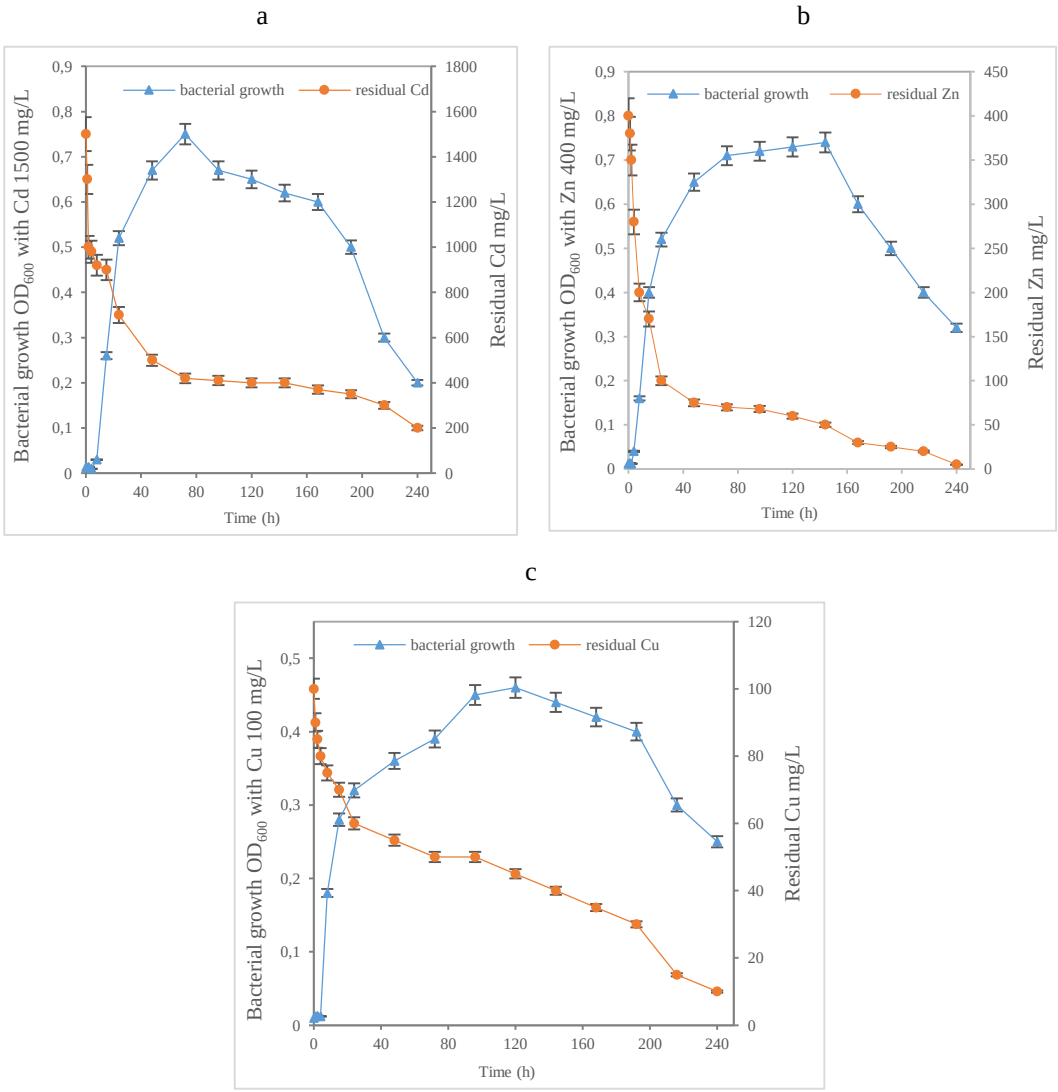

Fig.4. Metals reduction monitoring of **(a)** Cd (1500 mg/L), **(b)** Zn (400 mg/L) and **(c)** Cu (100 mg/L) during bacterial growth

Figure 4 shows that the concentration of heavy metals was inversely proportional to the bacterial growth. the metals decrease started from the first hour of contact with the bacteria. in fact, during the first 48 hours, a considerable and rapid metals decrease was recorded. During this phase the metal decrease rates were (11.84 mg $L^{-1}.h^{-1}$, 6.74 mg $L^{-1}.h^{-1}$ and 0.98 mg $L^{-1}.h^{-1}$) for Cd, Zn and Cu respectively (Phase 1). Between 48 and 192 hours, the rates of metal reduction and bacterial growth have decreased

and the kinetics are slowed down and form a plateau (0.6, 0.36 and 0.1 mg $L^{-1}.h^{-1}$) for Cd, Zn and Cu respectively (Phase 2). Between 192 and 240 hours, a slight rates increase resumes (3.03 and 05.1 mg $L^{-1}.h^{-1}$) for Cd and Cu, however the elimination of Zn remained slightly constant. This reduction was about 80, 98 and 97% for Cd, Zn and Cu respectively, compared to the initial metal concentrations, approaching a complete metal removal after 10 days of inoculation (phase 3). The elimination of heavy metals by *B. fungorum strain Bf01* starts during the growth lag and exponential phases and even from the first hours of bacteria contact with the metal. Most of the metal elimination was achieved either during (case of Zn and Cu), or just after the growth exponential phase (case of Cd).

The reduction of Cd, Zn and Cu in the presence of Bf01 *strain*, suggests that the metals elimination was achieved through the bacteria cell that could have effective mechanisms to detoxifying the three metals (Choińska-Pulit, Sobolczyk-Bednarek and Łaba 2018, You, et al. 2021). This was probably achieved according to two complementary processes: bioaccumulation, that would occur mainly during the growth active phase (Congeevaram, et al. 2007, Sinha and Mukherjee 2009, Fan, Okyay and Rodrigues 2014), and a bio-adsorption could happen during the stationary and decline phases (Wang, et al. 1997). Indeed, several works carried out in the bioremediation field, using live bacteria, have shown that a large part of the metal is eliminated during the active growth phase (exponential phase). On the other hand, a dependence of cell density on metal reduction is noted during this phase (Sinha and Mukherjee 2009), during which, most of the electrochemical and ionic exchanges take place, including the metal ions transfer and their biosorption (Beisl, et al. 2019). However, a regular and constant removal during the stationary phase has been noted and considered independent of cell growth (Chang, Law and Chang 1997, Damodaran, Suresh and Mohan 2011, Pratama 2020). During 2nd phase, the bacterial metabolism and metals elimination are slowed down, characterized by the depletion of nutrients and elements necessary for cell multiplication. During this phase, the exopolysaccharide (EPS) would be produced, that is favored by the stress culture conditions (Sinha and Mukherjee 2009, Maier and Pepper 2015). During the 3rd phase, the resumption of the metals elimination could be related to the presence of secondary metabolites in the medium like the EPS which play an eminent role in the heavy metals biosorptions (Blaga, Zaharia and Suteu 2021, Chug, et al. 2021). Indeed *B. fungorum* is considered as a good producer of exopolysaccharides, which may be responsible for heavy metals bioresorption in the present study (Zhang and Min 2010). The production of such polymers by bacteria, in these conditions hostile to growth, gives them a protective role (Fu et Wang 2011, Damodaran, Suresh and Mohan 2011, Liu, et al. 2019).

4. CONCLUSION

In the present study we have demonstrated the ability of *B. fungorum* strain Bf01to reduce heavy metals in aqueous solution. The Bf01 has showed a very good resistance and a high tolerance towards the studied metals, where high Minimal Inhibitory Concentrations (MIC) (1500, 400 and 50 mg/L) for Cd, Zn and Cu were carried out respectively. The metal removal begins from the first contact hour with speeds fast enough reduction. *B. fungorum* strain Bf01 was able to eliminate almost all of the three metals up to 80, 98 and 97% for Cd, Zn and Cu, respectively, after just 10 days of incubation.

The results show that, given its effectiveness, availability and low cost, *B. fungorum* strain Bf01 can be a great tool for seeing promising biological bioremediation of heavy metals. Bioremediation techniques seem to have a bright future. Their cost compared to the physico-chemical techniques is favorable. They are proven to be a useful alternative to conventional systems for the removal of toxic metals in industrial effluents. Bioremediation is still far from being fully controlled and requires investigation and further research in the direction of modeling and regeneration of biosorbent mass.

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