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Native Bacteria Isolated from Phosphate Deposits Reveal Efficient Metal Biosorption and Adhesion to Ore Particles

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Abstract: Mining and processing phosphate ore are among the essential branches of the economy in some developing countries, including Algeria. Conventional ore beneficiation methods can harm the environment by consuming tremendous amounts of water resources (during washing and flotation), potentially hazardous chemicals, and thermal energy. Mine water contains toxic metals that, when released, interfere with environmental functioning. Therefore, in line with environmental needs, conventional methods should be gradually replaced with safe biotechnological processes. This study aimed to investigate the biosorption and adhesion abilities of native microorganisms isolated from Djebel Onk ore (Algeria). The examined bacterial strains differed in their metal accumulation efficiency. The incubation of phosphate ore with the native strain *Bacillus* HK4 significantly increased the recovery of Mg and Cd (at pH 7, 8147.00 and 100.89 $\mu g/g^{-1}$, respectively). The HK4 strain also revealed better adhesion to the ore particles than the reference strain of *Bacillus subtilis*. Thus, biosorption could be more effective when using the native HK4 strain, which can remove Cd and/or Mg over a pH 4–10 range. Moreover, concerning the unique adhesion capacity of HK4, the strain can be considered in the design of bioflotation methods, as well as in the development of an eco-friendly method of ore and post-flotation waste beneficiation.

Keywords: microorganisms; phosphate ore; Cd; Mg; biosorption; adhesion; metal impurities

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1. Introduction

Mining, including the extraction and processing of phosphate ores, is one of the most important branches of the economy in developing countries, including Algeria. Although it is associated with producing a considerable amount of waste, which is dangerous to humans and the environment (due to the accumulation of pollutants released during processing), at least part of the contaminants (for example, metals) can be potentially reused, as long as more efficient and cheaper methods are developed. Thus, one of the challenges of the modern mining industry is developing environmentally friendly ore processing and waste reuse technology based on natural processes. During phosphate beneficiation processes, remarkable amounts of water polluted with metals are released into the environment, posing a risk to ecosystems' balance, stability, and sustainability. Since metals are highly persistent as pollutants, they can accumulate in water and soil ecosystems, potentially posing an enduring threat to all organisms [1].

According to EU policy, cadmium content in soil, phosphate fertilizers, and raw material must be limited. Consequently, countries with phosphorene deposits rich in

cadmium and other metal impurities must limit sales, as demand for their raw material will gradually decrease [2]. Such consequences may impact Nauru, Senegal, the USA (North Carolina deposits), Tunisia, Morocco, Israel, Egypt, Syria, and Algeria. According to Mar and Okazaki [3], cadmium content in phosphate ores from these countries can reach up to 243 mg Cd per kg of phosphate rocks. A reasonable and profitable solution for countries possessing raw material rich in Cd is further ore beneficiation, including decadmiation. Conventional ore beneficiation methods should be gradually replaced with safe biotechnological methods in line with environmental needs. The most promising of these methods—bioflotation, bioleaching of metals, or biosorption of metals—engage microorganisms [4–7]. Microorganisms might also be successfully used to treat post-flotation wastes, wastewater after ore processing, and metal recovery after appropriate biomass processing [1,8–15]. Therefore, it is necessary to select and characterize bacterial strains with high biosorption capabilities.

Biosorption is the ability of biomolecules or biomass to bind specific ions, removing them from aqueous solutions. Biosorption must be distinguished from bioaccumulation, which involves active transport through cell membranes and the storage of pollutants inside cells [1,8,9]. This phenomenon is studied intensively due to its vast potential to purify water, sewage, industrial waste, and by-products of ore processing. Its advantages include rapid kinetics and high efficiency in a wide range of physiochemical conditions; the renewability of biomaterials; the possibility of removing a few metals simultaneously; good recovery of metals from biomass; reduced use of potentially dangerous reagents; and the opportunity to recover and reuse metals; and its relatively low cost [1,10–15]. The aforementioned biosorption process involves chemical and physical mechanisms that act separately or, more often, simultaneously. Physical processes primarily involve Van der Waals' attraction forces, while chemical processes create stronger chemical bonds between the functional groups of a biosorbent and the adsorbed substance. In general, the coordination of complex formation, chelation, and ion exchange must be considered when describing the mechanism of biosorption [1,16]. The most effective method of biomass recovery after heavy metal biosorption in the liquid environment relies on the immobilization of bacteria or the biosorbents they produce on a polymeric column bed. Heavy metals accumulated by the biomass can be readily recovered by chemical desorption, e.g., with 0.1 HCl, precipitation, and electrowinning. The bed with a biosorbent can be reused in the next biosorption cycle [14,17].

One of the most effective mechanisms of heavy metal biosorption is immobilization in the mucous matrix of extracellular polymeric substances (EPSs). The main components of EPSs are polysaccharides and proteins. Lipids, nucleic acids, and other organic and inorganic components can also be found there [18–22]. EPSs protect microorganisms against mechanical damage, dehydration, physicochemical stressors (pH, temperature, pressure, salinity, radiation, heavy metals) and biological ones (antibiotics, bacteriocins, bacteriophages, and phagocytic and secretory activity of the immune system). EPSs improve the adhesive properties of microorganisms, which are crucial to the formation of three-dimensional biofilms [18]. The EPSs' barrier is characterized by a significant sorption capacity and binds heavy metal ions at a safe distance from the cell, reducing their toxicity and bioavailability. Additionally, EPS-mediated binding of metal ions does not require metabolically valuable energy, as it is based on the differences between electric charges of the reacting particles. Negatively charged functional groups, such as amino, ester, hydroxyl, carboxyl, carbonyl, and sulfonic groups present in EPSs and on the cell surface, adsorb metal cations as a result of electrostatic forces. Therefore, protons and/or cations of light metals (sodium, potassium, calcium), naturally associated with functional groups on the surface of bacterial cells, are displaced by heavy metal ions present in an aqueous solution [20]. Literature data indicate that using both living and dead biomass as a sorption bed can lead to the removal of heavy metal ions from the solution [20].

Due to a very beneficial surface-to-mass ratio and various functional groups on the surface of cells, microorganisms appear to be perfect biosorbents. Therefore, it is unsurpris-

ing that they have attracted the attention of scientists working on ecological methods of environmental remediation. Due to the different structures of their cell wall, bacteria are characterized as Gram-positive or Gram-negative. Gram-positive bacteria have a thick layer of peptidoglycan with teichoic acids taking on a chelating role. Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane rich in lipopolysaccharides [23]. The composition of these layers can be wildly different in various species, giving them different biosorption properties. Bacteria have been studied for their biosorption capabilities. Among the commonly studied species with a high application potential, some of the most promising are Gram-positive bacteria from the genus *Bacillus*. Most of these species, which exhibit outstanding biosorption capabilities, are isolated from specific, highly contaminated environments. Long-term exposure to severe stress factors stimulates those bacteria to develop and reinforce their unique properties, allowing them to bind substantial amounts of metals [1,24–36].

This study is a continuation of our earlier work, in which we analyzed the metal biosorption abilities of five bacterial species from the collection of the University of Silesia in Katowice after incubation with phosphate ore from Djebel Onk [35]. These organisms did not originate from heavy metal-contaminated sites and did not exhibit any exceptional biosorption capabilities. Therefore, in this study, we have focused on native species isolated from phosphate ores in Djebel Onk. We isolated over 160 species of microorganisms and selected four Gram-positive bacterial strains of different morphology, characterized by rapid growth in basic/cheap media and good biomass production, to test their biosorption-related properties further. The main aim of our study was to search for heavy metal-resistant species capable of efficient biosorption of Cd and Mg. The choice of metals was dictated by their critical importance for industry related to the production of phosphoric acid, fertilizers, and, thus, the potential contamination of soil ecosystems. A low MgO product is desirable for producing phosphoric acid, as well as for use in the fertilizers industry. Magnesium is the most undesirable impurity in phosphate ore. Fertilizers produced using metal-contaminated ores are of a lower standard due to the extensive contamination of soils during their application. Therefore, the authors emphasize the necessity of removing Mg during ore beneficiation [37–39]. For the same reasons, removing the Cd impurity is highly desirable. Moreover, we assessed the relationship between the pH of the solution during incubation and the scale of microbial biosorption. After biosorption tests, we assessed the adhesion capacity of the most promising strain, previously labeled with quantum dots. We noted significant differences in biosorption capabilities, which depended on the strain and pH of the solution. We also determined the two most promising bacterial strains belonging to the genus *Bacillus*.

2. Materials and Methods

The phosphate ore originated from Djebel Onk (Kef Essnoun region) and was acquired courtesy of the National Company of Iron and Phosphate FERPHOS. The material we studied was a loose, yellowish-gray sedimentary rock. Ore samples were collected from the site without excavation or other human activity. The sampling site was recommended by the employees of FERPHOS Company and was rich in ore. However, it had not been exploited for several years for safety reasons. Samples (loose sedimentary rock) were collected using sterile spatulas and placed in sterile plastic containers of 100 mL each. The material in the plastic containers was stored in air-tight conditions and sealed in zipped plastic bags. After collection, the samples were transported to the laboratory to isolate native microorganisms. After appropriate mechanical treatment, the remaining parts of the samples were used for physicochemical analyses and biosorption tests.

2.1. Characteristics of the Raw Ore (before Mechanical Preparation)

A granulometric study was conducted first after transporting ore samples to the laboratory. The particle size analysis of the sample revealed that 3% of the particles were

less than 80 µm in size (Table 1). Seventeen granulometric classes were distinguished and characterized by their metal contents and mineralogical composition.

Table 1. Particle size distribution of phosphate ore from Djebel Onk. R—mass retained; CR—cumulative mass retained; CP—cumulative passing.

Fractions	R (g)	R (%)	CR (g)	CR (%)	CP (g)	CP (%)
≥8 mm	34	1.7	34	1.7	1998	99.9
5–8 mm	42	2.1	76	3.8	1924	96.2
4–5 mm	17	0.9	93	4.7	1907	95.4
2.5–4 mm	35	1.8	128	6.4	1872	93.6
2–2.5 mm	20	1.0	148	7.4	1852	92.6
1.6–2 mm	27	1.4	175	8.8	1825	91.3
1–1.6 mm	55	2.8	230	11.5	1770	88.5
0.8–1 mm	10	0.5	240	12.0	1760	88.0
500–800 µm	105	5.3	345	17.3	1655	82.8
315–500 µm	212	10.6	557	27.9	1443	72.2
250–315 µm	264	13.2	821	41.1	1179	59.0
160–250 µm	861	43.1	1682	84.1	318	15.9
125–160 µm	162	8.1	1844	92.2	156	7.8
80–125 µm	60	3.0	1904	95.2	96	4.8
63–80 µm	47	2.4	1951	97.6	49	2.5
40–63 µm	35	1.8	1986	99.3	14	0.7
≤40 µm	9	0.5	1995	99.8		
Total	1995	99.8				

2.2. Measurements of Metal Concentration in Phosphate Ore

To measure metal concentration, ore samples of each granulometric class were mineralized in wet conditions using the ETHOS UP Microwave Digestion System (Milestone Inc., Shelton, CT, USA). After the material was dried (temp 30 °C, 0.5 h), 0.200 g samples were placed directly into Teflon vessels and covered with concentrated acids in the following volumes: 6 mL HNO3 (65%), 2 mL HCl (35%), and 4 mL HF (40%). The samples in the vessels were placed in a microwave oven and mineralized according to the program: the first step (raising the temperature to 220 °C)—power: 1800 W, time: 25 min, temperature: 220 °C; the second step (maintaining the temperature of 220 °C)—power: 1800 W, time 20 min. After this step, the samples were cooled, and 24 mL of 4% H_3BO_3 was added to each dish to neutralize the remaining HF. Then, the vessels were placed in the microwave oven once again according to the program: the first step (raising the temperature to 120 °C)—power 1200 W, time: 10 min; the second step (maintaining the temperature of 120 °C)—power 1200 W, time 5 min. After mineralization, the samples were cooled and filled to 50 mL with deionized water. Metal contents in the samples were measured using iCE™3500 Atomic Absorption Spectrometer (AAS, ThermoFisher Scientific, Cambridge, UK) [35,40]. Each sample was measured in two repetitions. The results of these analyses were collected in Table 2. The mineralogical composition was assessed in each fraction using the XRD method.

2.3. Mineralogical Composition Assessment: X-ray Diffraction (XRD)

XRD analyses were performed on powdered samples using a PANalytical X'Pert Pro MPD (PANalytical, Almelo, The Netherlands) powered by a Philips PW3040/60 X-ray generator and fitted with a 1D silicon strip detector (X'Celerator) with a 2.122° 2θ active length. The measurements were performed using Cu Kα-radiation with a wavelength of 0.1540598 nm, an acceleration voltage of 40 kV, a current of 40 mA, and 0.02° 2θ step sizes between the angles of 5° and 70° 2θ and a 200 s measurement time per step. Powder diffraction analysis parameters are gathered in Table 3.

Table 2. Metals concentrations (μg g^{-1}) in different fractions of phosphate ore from Djebel Onk.

Fractions	Metals						
	Cd	**Cu**	**Mn**	**Fe**	**Mg**	**Ni**	**Zn**
≥5 mm	23.7	7.9	17.3	1210.0	3893.9	1.5	166.6
4–5 mm	23.6	8.8	20.2	1577.2	4681.8	1.7	144.5
2.5–4 mm	34.7	9.9	23.1	1527.3	4102.6	1.7	163.6
2–2.5 mm	42.0	10.2	22.6	1626.0	4336.5	1.6	167.2
1.6–2 mm	44.3	9.8	23.9	1692.3	4280.8	1.7	172.1
1–1.6 mm	45.8	10.6	21.9	1627.4	5213.4	1.7	174.4
800 μm–1 mm	42.7	9.1	20.6	1533.4	3635.7	1.8	164.1
500–800 μm	30.2	10.7	14.8	1533.3	4395.9	1.5	161.8
315–500 μm	19.9	11.5	12.6	1516.6	3350.0	1.5	146.0
250–315 μm	19.8	10.5	10.8	1423.6	2747.0	1.5	147.7
160–250 μm	25.3	9.9	14.5	1296.0	4172.2	1.7	179.8
125–160 μm	32.4	13.4	18.5	1530.5	3969.6	2.0	206.3
80–125 μm	36.8	13.1	23.4	1629.0	5369.1	1.8	217.5
63–80 μm	-	-	-	-	-	-	-
40–63 μm	49.1	9.5	30.5	2072.9	5217.8	17.4	234.3
≤40 μm	49.6	10.9	29.3	2144.5	4391.0	6.4	256.6

Table 3. Powder diffraction analysis parameters.

Radiation	CuKα1 (λ = 1.540598 Å) Ni filter for Kβ elimination
Tension	40 kV
Current	40 mA
Range	5°–70° 2Θ
Step size	0.02° 2Θ
Time limit	200 s
Detector type	X'Celerator—strip detector

The data obtained were processed using HighScore+ software (version 4.1, PANalytical, Almelo, The Netherlands), linked to the ICSD database (2015) and the PDF4+ ICDD database (2018). For the standardless, quantitative phase analysis, the Rietveld method was used. The Rietveld structure fit module is a part of the HighScore Plus program suite [41]. Quantitative phase analysis can be performed on multi-phase samples using the formalism described by Hill and Howard [42]. The Rietveld method is a full pattern fit method. The measured profile and a profile calculated from crystal structure data were compared. Through variation of many parameters, the difference between the two profiles is minimized.

To obtain quantitative calculations, the semi-automatic Rietveld mode in HS+ was used. The refinement was carried out until good statistical parameters were obtained: R expected = 4.30; R profile = 6.50; Weighted R profile= 8.61; Goodness of Fit =4.01. The results of XRD analyses for each ore fraction are presented in Table 4.

2.4. Mechanical Preparation of the Ore for Testing

In the next stage, ore was prepared for biosorption tests. To achieve a homogenous fraction, mechanical procedures (crushing, grinding, and sifting) were conducted according to the algorithm presented in Figure 1.

Table 4. Mineralogical composition of different fractions of phosphate ore from Djebel Onk.

Fractions	Mineralogical Composition (%)				
	Calcite	**CFA**	**Clinoptilolite**	**Dolomite**	**Quartz**
≥5 mm	5	64	2	29	1
4–5 mm	5	66	1	27	1
2.5–4 mm	7	58	1	33	1
2–2.5 mm	4	69	2	23	2
1.6–2 mm	9	69	1	18	2
1–1.6 mm	4	69	1	25	2
800 µm–1 mm	4	73	0	18	4
500–800 µm	2	85	0	13	1
315–500 µm	0	97	0	2	0
250–315 µm	0	93	2	5	0
160–250 µm	1	89	0	6	4
125–160 µm	3	73	1	21	2
80–125 µm	1	62	2	29	6
63–80 µm	3	60	2	35	0
40–63 µm	4	59	7	30	0
≤40 µm	5	54	13	28	0

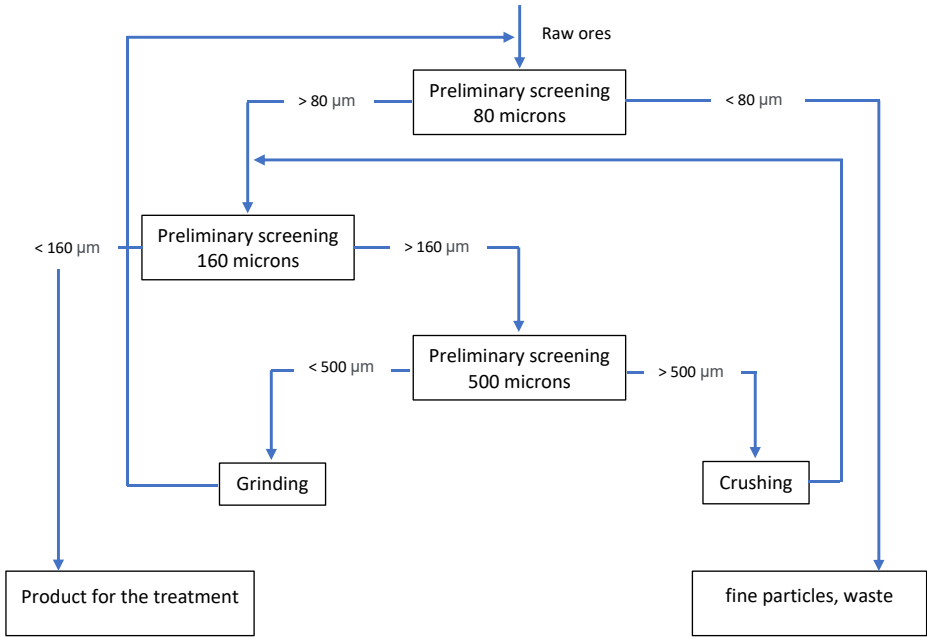

Figure 1. Technological procedures (mechanical preparation) conducted for preparation of 80–160 µm fraction.

A homogenous fraction (80–160 µm) was subjected to assessment of its mineralogical composition using the XRD method. For this fraction, the results were as follows: carbonate fluoroapatite (CFA; $Ca_5(PO_4,CO_3)_3F$) 87%, dolomite ($CaMg(CO_3)_2$) 10%, calcite ($CaCO_3$) 1.5%, clinoptilolite (Ca_2–$3[Al_3(Al,Si)_2Si_{13}O_{36}]\cdot 12H_2O$) 1.0%, and quartz ($SiO_2$) 0.5%. (Figure 2). Before biosorption tests, the ore was sterilized to prevent any microorganisms from interfering with the experiment. Samples of 1 g of processed ore were weighed, autoclaved (121 °C, 2 h), and stored in sterile conditions for further use.

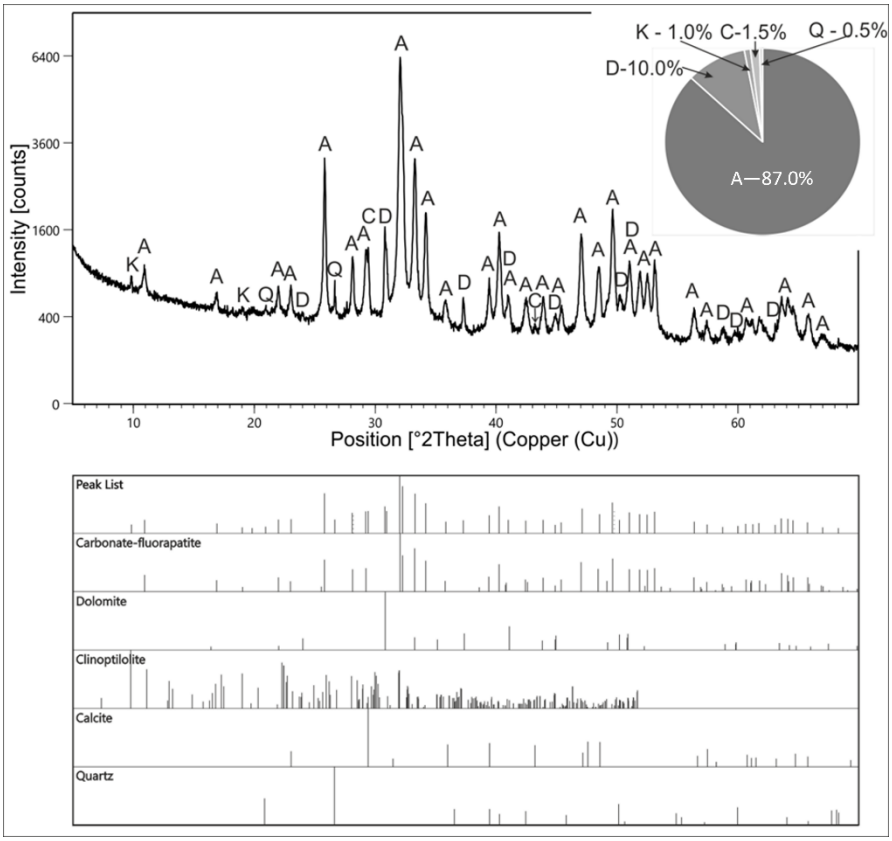

Figure 2. XRD pattern of a Djebel Onk sample after mechanical preparation (fraction 80–160 μm), with the results of qualitative analysis (table showing the matched patterns) and semi-quantitative calculations (pie chart, all values are weight). Mineral symbols: A—apatite, C—calcite, D—dolomite, K—clinoptilolite, Q—quartz.

2.5. Isolation and Identification of Microorganisms from Raw Ore

To isolate native bacteria from the phosphate ore from the Djebel Onk mine (Algeria), 10 g of ore was suspended in 90 mL of sterile 0.98% normal saline (NaCl), shaken for 1 h at 120 rpm, and serially diluted. An aliquot of 0.1 mL of each ore dilution (from 10–1 to 10–6) was spread on the solid culture media LB and R2A (BTL, Łódź, Polska). The cultures were incubated for 96 h at 28 °C. Single bacterial colonies were passaged on fresh media to obtain pure cultures. Four morphologically different strains were selected for identification and biosorption testing among the isolated bacteria. Molecular identification of bacterial strains was based on the 16S rDNA gene fragment sequence. Polymerase chain reaction (PCR) with primers 8F and 1492R was performed as described by [43]. Next, 1484 bp PCR products were cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and sequenced at Genomed S.A. (Warsaw, Poland). The edition of sequences was conducted manually using Chromas Lite 2.01 (Technelysium Pty Ltd., Brisbane, Queensland, Australia). Chimera detection was performed using Decipher 2.19.2 [44]. Obtained sequences were aligned to the reference sequences of the 16S rRNA gene available in the GenBank database (National Centre for Biotechnological Information) using BlastN. The strain HK1 was indicated as *Lysinibacillus* sp. based on a 16S rDNA sequence that was 99.70% identical to the sequences of NCBI accession numbers: CP026120.1, KF228905.1 and CP104728.1, representing two strains of *Lysinibacillus sphaericus* and *Lysinibacillus fusiformis*, respectively. The strain HK2 was indicated as *Pseudarthrobacter* sp. based on a 16S rDNA sequence that was >99% identical to the sequences of NCBI accession numbers: KR085945.1, KR085776.1, KR085778.1, CP047898.1 representing two strains of *Pseudarthrobacter oxydans*, *Pseudarthrobacter scleromae*, and *Pseudarthrobacter psychrotolerans*, respectively. The strain HK3 was indicated as *Bacillus mycoides* based on a 16S rDNA

sequence that was 99.93% identical to the sequences of *B. mycoides* under NCBI accession numbers: MT827167.1, CP031071.1 The strain HK4 was indicated as *Bacillus* sp. Based on a 16S rDNA sequence that was 99.93% identical to the sequences of NCBI accession numbers: KX281166.1, KU551251.1, KU551122.1, and CP053764.1 representing two strains of *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus velezensis*, respectively. The sequences were deposited in GenBank with the following accession numbers: MZ046078 (USK1), MZ046079 (HK1), MZ046080 (HK2), MZ046081 (HK3), MZ046082 (HK4).

2.6. Biosorption Testing

Native microorganism strains chosen for tests and *Bacillus subtilis* USK1 from the University of Silesia in the Katowice collection (as a reference strain) were cultured in LB medium for 24 h. Then, bacterial cultures were washed and suspended in 0.98% NaCl. Shortly before incubation with ore, the bacteria were centrifuged, and biomass was used for the next steps of the experiment. Portions of 1 g sterilized ore samples were incubated (RT with constant stirring; 24 h) in 50 mL of NaCl (0.168 mol/L) and $CaCl_2$ (0.5 mol/L) solution to support metal extraction [45,46]. The process was conducted under different pH conditions (4, 7, and 10). Next, biomass was added to each vial and the suspension was incubated (28 °C with constant shaking) for 20 minutes. After incubation, samples were set aside for 5 min to facilitate the sedimentation of ore particles. Next, the suspension of microorganisms was gently transferred to sterile 50 mL centrifuge tubes, and the procedure was applied as previously described [35]. Briefly, the suspensions of microorganisms were centrifuged (Ultracentrifuge Beckman Optima LE-80K) at 8000 rpm (4 °C; 15 min). Biomass was frozen at −70 °C and lyophilized (Freeze dryer Alpha 1–4; Christ, Germany) at −35 °C and 0.2 mBar for 24 h. Portions of ~0.02 g of dried microorganisms were mineralized with 0.5 mL of ~65% HNO_3 at 110 °C for 48 h. After mineralization, the samples were diluted with deionized water to a total volume of 5 mL. Cd and Mg contents were measured by AAS methods with an iCE™ 3500 Atomic Absorption Spectrometer (AAS, Thermo Fisher Scientific). The quality of the analytical procedure was confirmed using standard solutions from Merck at the initial concentration of 1 g of metal L^{-1} of water. The metal content was expressed as μg g^{-1} of biomass.

2.7. Adhesion Tests

Selected microorganisms were incubated on a rotary shaker (120 rpm) for 20 min with phosphate ore. Incubation was conducted at three different pH: 4, 7, and 10. The level of adhesion of the microorganisms was measured spectrophotometrically after 10 min (CECIL 9000) by assessing the optical density (OD) before and after induction at 600 nm. The test was also conducted under the same conditions, but without ore, to evaluate the degree of microbial sedimentation (control group).

An additional test was conducted for the reference strain (USK1) and the strain with the highest Cd and Mg sorption properties (HK4) to assess the level of bacteria adhesion to ore particles. Firstly, both strains were marked with quantum dots (CdTe QD; PlasmaChem GmbH). The bacteria were incubated with QD for about 20 minutes (red for the USK1 strain and yellow for the HK4 strain), gently washed twice, centrifuged and resuspended in water. Next, QD-labeled bacteria were incubated with the ore for 20 min on a rotary shaker (120 rpm) at room temperature. In the first attempt, we incubated the ore with each of the bacteria separately. In the second trial, we incubated the ore with both strains simultaneously. The ore particles were then gently washed twice and microscopic slides were prepared. The slides were analyzed using an Olympus FV1000 confocal laser scanning microscope at a 405 nm laser line (diode laser). Z-stacks were acquired with a step size of 2 μm.

2.8. Statistical Analysis

Tests to determine the metal concentration in bacteria, as well as the adhesion, were performed (in triplicate for metal analysis and five replicates for the adhesion tests). Moreover, each measurement was taken in two technical replications. The results were expressed

as mean ± SD. The analysis of variance, followed by the Fisher's Least Significant Difference test (LSD, ANOVA; $p < 0.05$), was performed to assess the significance of differences in metal biosorption, as well as adhesion among the studied bacterial strains and different pH. Statistical analysis was conducted using Statistica 13.1 software.

3. Results

3.1. Cadmium Biosorption by Native Bacteria

ANOVA analysis revealed a significant influence of strain (F = 28.48; $p < 0.00001$) and pH (F = 26.63; $p < 0.00001$) on Cd biosorption level. The interaction between both factors was also significant (F = 4.70; $p = 0.00013$). *Pseudarthrobacter* sp. HK2, *Bacillus mycoides* HK3, and *Bacillus* sp. HK4 efficiently accumulated cadmium at pH 4. The average concentration of Cd in the biomass of these strains after incubation at pH 4 was 43.83, 60.84, and 85.01 μg g^{-1}, respectively. At pH 7, a significantly higher accumulation than the reference USK1 strain showed strains HK1, HK3, and HK4. Increasing the pH to 10 created the least beneficial biosorption conditions for the studied bacterial strains. In these conditions, high Cd accumulation was achieved by two *Bacillus* strains: *B. mycoides* HK3 and *Bacillus* sp. HK4. These strains' average Cd accumulation at pH 10 reached 38.11 and 84.99 μg g^{-1}, respectively (Figure 3). Statistical analysis revealed the ability of Cd accumulation by *Bacillus* sp. HK4 and the reference strain *Bacillus subtilis* USK1 were independent of pH; however, a high difference in Cd accumulation between these strains was reported. The Cd concentration in the biomass of *B. subtilis* USK1 was higher than in the control group (bacteria incubated without ore), but average values never exceeded 20 μg Cd g^{-1}. The same trend was observed for *Bacillus* sp. HK4, but the average Cd accumulation was above 80 μg g^{-1}, indicating this strain is a very good biosorbent of Cd, which is active in a wide range of pH. Post hoc analysis showed that *Bacillus* sp. HK4 had the highest mean Cd accumulation at pH 4 compared to other strains. Meanwhile, Cd biosorption by the other studied strains, *Pseudarthrobacter* sp. HK2 and *B. mycoides* HK3, was highly dependent on the pH, reaching the lowest values at pH 10 and the highest at pH 4 (Figure 3). In summation, Cd accumulation by native strains, especially *Bacillus* sp. HK4, was higher than the reference strain, *B. subtilis* USK 1, which originated from the uncontaminated environment.

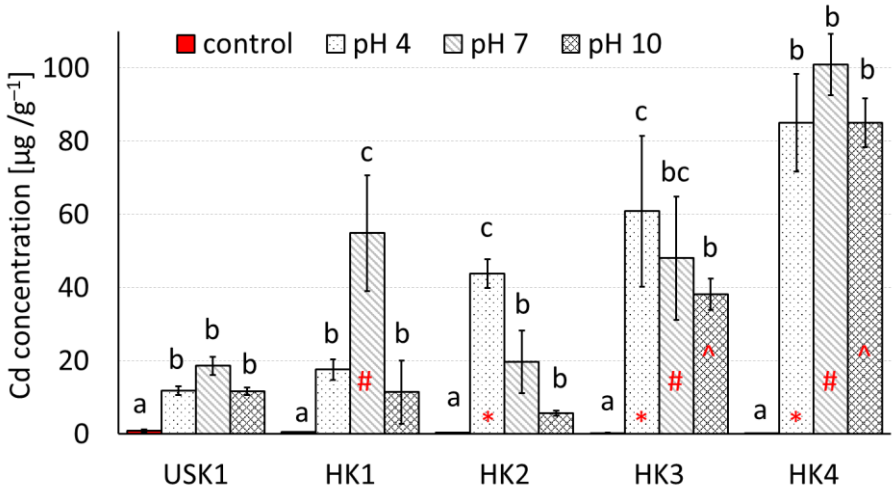

Figure 3. Cd accumulation (mean ± SD; μg g^{-1}) in the biomass of bacterial strains isolated from the phosphate ore (HK1–HK4 strains) and *B. subtilis* USK1 reference strain after incubation (20 min; 28 °C; pH 4 or 7 or 10) with the ore from Djebel Onk. The same letter (a, b, c) in a particular strain denotes no significant differences among pH groups. The red marks mean the differences between each native strain and the reference strain USK1 at pH4 (*), pH7 (#), and pH10 (^) analyzed separately (ANOVA, LSD; $p < 0.05$).

3.2. Magnesium Biosorption by Native Bacteria

Statistical analysis highlighted the significant influence of strain (F = 16.84; $p < 0.00001$) and pH (F = 26.12; $p < 0.00001$) on Mg biosorption. The interaction between both factors was also significant (F = 4.70; $p = 0.00049$). *Bacillus* sp. HK4 also revealed very high efficiency in Mg accumulation. Mg content in its biomass at pH 7 (mean: 8147 µg Mg g^{-1}) was 272 times higher than in the control, where bacteria were incubated without ore (Figure 4).

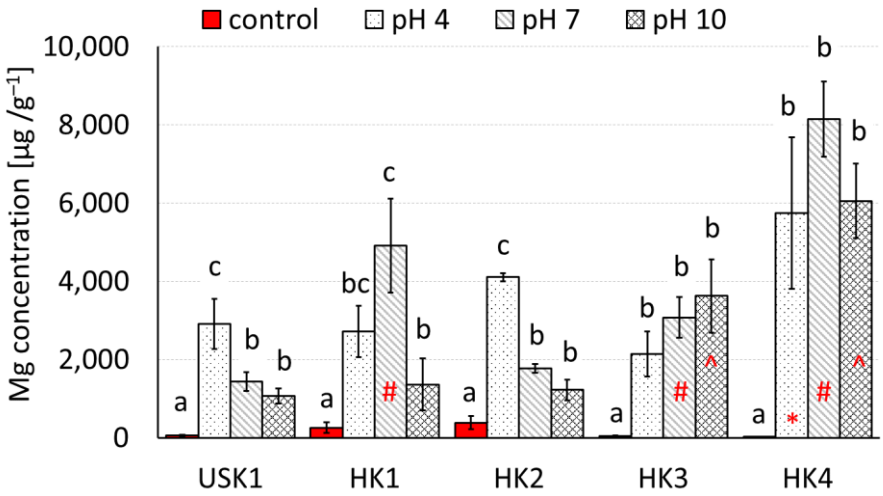

Figure 4. Mg accumulation (mean ± SD; µg g^{-1} in the biomass of bacterial strains isolated from the phosphate ore (HK1–HK4 strains) and *B. subtilis* USK1 reference strain, after incubation (20 min; 28 °C; pH 4 or 7 or 10) with the ore from Djebel Onk. The same letter (a, b, c) in a particular strain denotes no significant differences among pH groups. The red marks indicate the differences between each native strain and the reference strain USK1 at pH4 (*), pH7 (#), and pH10 (^) analyzed separately (ANOVA, LSD; $p < 0.05$).

At pH 7, relatively good Mg biosorption was also shown by *Lysinibacillus* sp. HK1 and *B. mycoides* HK3. The HK3 strain also demonstrated high Mg biosorption at pH 10. *Bacillus* sp. HK4 and *Pseudarthrobacter* HK2 are very efficient in Mg accumulation at pH 4 (5741 and 4103 µg Mg g^{-1}) (Figure 4). As in the case of Cd, *Bacillus* sp. HK4 revealed a high Mg biosorption capacity over a wide pH range.

Besides that strain, *B. mycoides* HK3 has not changed its capability for Mg accumulation at different pH. For the reference strain, *B. subtilis* USK1, and *Pseudarthrobacter* sp. HK2, a negative relationship between Mg accumulation and pH (accumulation was the highest at low pH) was reported. At pH 10, high Mg accumulation was found for *Bacillus* sp. HK4 and *B. mycoides* HK3. At pH 7, the highest was found in the HK4 strain and then HK1 and HK3, which had significantly higher Mg in biomass than in the reference strain USK1 (Figure 4).

3.3. Adhesion of Native Bacteria to Ore Particles

Incubation of microorganisms without ore (control group) revealed a sedimentation level between 4 and 11%. The highest adhesion of bacteria onto particle surfaces was found at pH 7 for HK4 (~80%) and HK2 (~45%). *B. subtilis* USK1 adhesion onto the ore at pH7 was ~20%. At pH 4, the strains HK2 and HK4 revealed similarly high adhesion, while at pH 10, strain HK4 was, once again, the most efficient (Figure 5A–D). Thus, the HK4 strain perceived the best adhesion in a broad pH range. In the next step, this strain was analyzed using confocal microscopy.

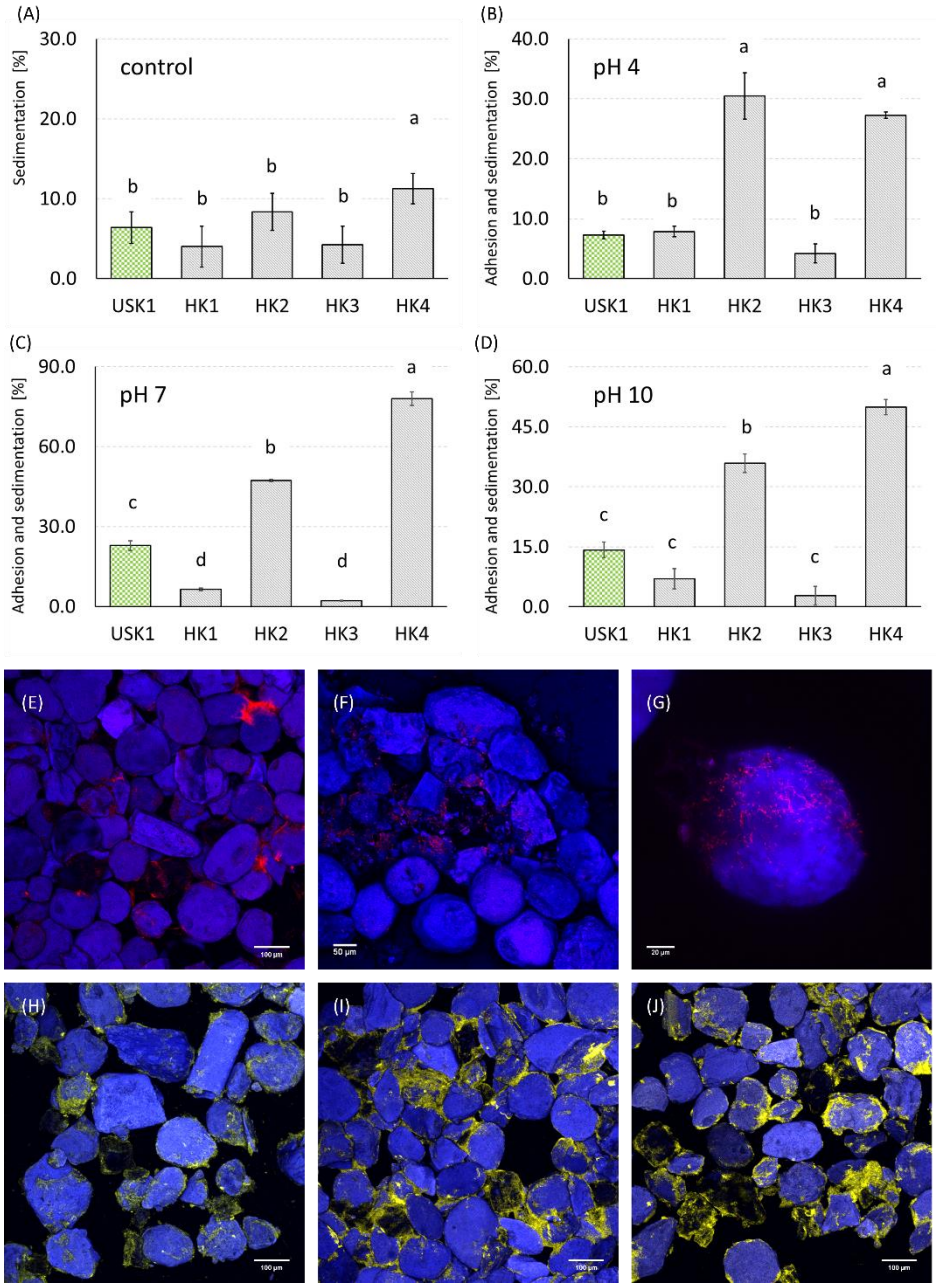

Figure 5. Adhesion or/and sedimentation (**A**–**D**) of selected native bacteria and *Bacillus subtilis* USK1 (served as a reference strain) onto ore from Djebel Onk after incubation at various pH, and adhesion of reference USK1 strain (**E**–**G**) or native strain (**H**–**J**) onto the ore particle surface. Microorganisms were labeled with quantum dots: red—USK1 and yellow—HK4. The same letter (a, b, c, d) in a particular pH denotes no significant differences among strains.

After labeling the USK1 (reference) and HK4 strains with quantum dots, incubation with ore was carried out again, followed by observations of particles conducted using a confocal microscope. Slide analysis confirmed the excellent adhesion of the native HK4 strain to the surface of ore particles (marked in yellow). The control strain also showed adhesion (marked in red), but to a much lesser extent than the native strain (Figure 5E–J). When the ore was incubated with both strains simultaneously, much better adhesion of HK4 than USK1 was observed. Notably, for ore particles on which adhesion of the reference USK1 strain was observed, the native HK4 strain was also present (Figure 6). Thus, both

strains have similar adhesion properties. However, the native HK4 strain is much better developed in this regard.

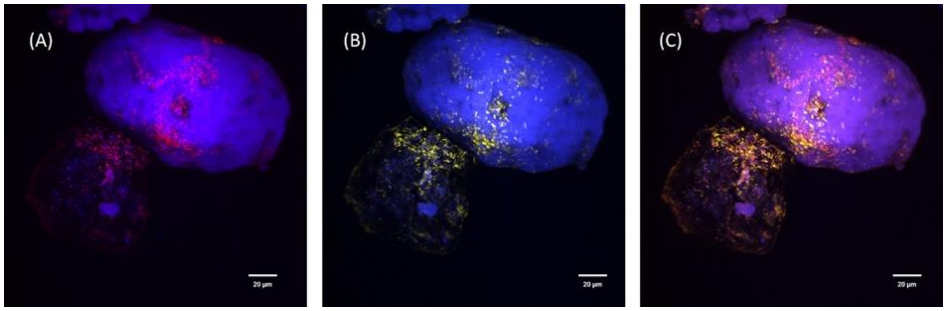

Figure 6. Adhesion of reference USK1 strain (**A**) or native strain (**B**) and merged imagines (**C**) onto the ore particle surface. Bacteria were labeled with quantum dots: red USK1 and yellow HK4.

4. Discussion

In 2019, we attempted to use microorganisms to purify phosphate ores from Djebel Onk for the first time [35]. We selected species from the collection of the University of Silesia in Katowice, and the Cd accumulation in the biomass of most of the studied microorganisms did not exceed 3 μg g^{-1}. In two cases, it reached values close to or slightly above 7 μg Cd g^{-1}, and in one case, it reached a value of 13.6 μg Cd g^{-1}. This result was disappointing, as efficient biosorption was not observed, despite previous encouraging reports on the capabilities of *B. subtilis* in this area [1,23,25,30,34]. Even a strain isolated from heavily contaminated soil that displayed siderophores [47] did not accumulate large amounts of Cd. Recent studies conducted with native bacteria shed new light on the design of contemporary, environmentally friendly treatment methods for phosphate ores.

Compared to the bacteria used in our previous study [35], Cd (and Mg) concentrations in native bacteria were much higher. The most effective strain, *Bacillus* sp. HK4, isolated from raw ore, accumulated over 80 μg Cd g^{-1}, irrespective of the pH of the incubation solution. The latest paper by Fathollahi et al. [48] presents a meta-analysis of metal biosorption by bacteria. Biosorption efficiency in the context of physiochemical parameters, including pH, was discussed. Changing the pH may affect the solubility of all components involved in the process, and quantity and quality of active binding sites of the biomass. At lower pH, the amount of H^+ ions increases, competing with metal ions for the binding site on the surface of biosorbents. In turn, a higher pH reduces the solubility of metal ions, but also reduces the amount of H^+ ions that could compete for the binding site on the bacterial surface. Notably, the change in pH also affects bacteria, changing the properties (composition) of the cell wall, ESPs, their metabolism, and thus the ability and efficiency of biosorption. Considering all these components, the biosorbent-heavy metal system works best in a pH range of 6–7.5, confirmed in the meta-analysis [48]. The result we obtained is consistent with this. However, certain strains evolving under particular/extreme conditions may reveal some unique/specific properties (e.g., due to unique polysaccharides composition), which shift the optimum of biosorption to a higher/lower pH. Our studies also exhibited good biosorption results at lower and higher pH, especially for HK4. Due to the evolution in extreme conditions created by phosphate ore, it can be assumed that these bacteria have developed a unique cell wall surface and/or EPSs, ensuring effective biosorption in a wide range of pH. Evidence shows that EPSs produced by native strains can change their properties and sorption efficiency. For example, Zeng et al. [49] showed that EPSs produced by *Bacillus* sp. S3, a new hyper antimony-oxidizing bacterium previously isolated from contaminated mine soils, was effective in the removal of different heavy metals. Additionally, Arce-Inga et al. [50] emphasize the significance of EPSs (produced by native *Bacillus* sp.) in cadmium removal from the environment. Feria-Cáceres et al. [51] report a native *Staphylococcus* sp. (2–3). The strain produces sticky EPSs with anionic properties, supporting the formation of EPS–metal complexes. Our HK4 strain may possess a unique

cell wall construction and EPSs. However, this concept should be confirmed in further studies focused on the structure and properties of the cell wall and EPSs (e.g., assessing polysaccharides and proteins composition and their variety) of the HK4 strain.

In a study by Roşca et al. [34], a detailed analysis of the removal of Cd ions from liquid effluents using two bacterial species was conducted. *Bacillus megaterium* isolated from food products was one of them. Biosorption measurements were conducted on pH range, incubation time, temperature, initial metal concentration, and the amount of biosorbent. The authors reported the highest Cd accumulation by *B. megaterium* (15.1 µg Cd g^{-1}) at pH 4, 35 °C, biosorbent dose of 3 g L^{-1}, and 20 min of incubation. Although we used a lower amount of biosorbent (about 0.2–0.3 g·L^{-1}), these findings are comparable to our results for *B. subtilis* USK1 from the collection of the University of Silesia in Katowice. Nevertheless, the Cd accumulation for *B. megaterium* and *B. subtilis* USK1 was much lower than that of the native strain, *Bacillus* sp. HK4 (Figure 3). In this context, Yilmaz and Ensari [26] have demonstrated the interesting biosorption capabilities of the native, HM-resistant strain of *Bacillus circulans* EB1. In a solution containing 28.1 mg Cd L^{-1}, accumulation as high as 5.8 mg Cd g^{-1} of dry biomass was observed after the first 8 h of growing cell incubation. However, Cd biosorption by the resting cells (biomass produced in the absence of Cd, dried overnight at 80 °C and then incubated like growing cells in a solution containing 28.1 mg Cd L^{-1}) for fresh and dry biomass was significantly higher and reached 9.8 and 26.5 mg Cd g^{-1}, respectively. The authors claimed that higher Cd accumulation by resting cells compared to growing ones could be an effect of the metabolic activity of growing cells that could influence their less efficient biosorption properties. Another study on strains isolated from industrial waste-activated sludge included 37 native strains [30]. Two of them, *Bacillus* sp. C13 and *Bacillus* sp. C16, had high resistance and high capability to accumulate metals such as Cd, Cr, Mn, and Pb. In that study, biosorption in the alkaline environment was higher than in the acidic environment. Once again, the authors pointed to competition between H^+ and metal ions for adsorption sites as a contributing mechanism. Namely, in the presence of H^+ (lower pH), the active groups of polymers are protonated, preventing metal ions from binding. These groups are negatively charged at lower H^+ concentrations (higher pH), which favors the binding of metal ions [30].

The phenomenon of biosorption is extraordinarily complex and depends on many variables [22,48]. Therefore, Ahmad et al. [28] used an artificial neural network (ANN) to predict the biosorption capabilities of *B. subtilis* to remove Cd ions from an aqueous solution. Their investigation concluded that the biosorption capability of *B. subtilis* can be as high as 251.91 mg Cd g^{-1} in the following optimal conditions: pH 5.91, temperature 45 °C, time of contact 3 h, initial Cd concentration of 496.23 mg per L. In that case, the optimal conditions for biosorption shifted more towards acidic pH, which can be related to interactions between the negatively charged surface of the biosorbent and a positive charge of Cd ions [1,28]. Boyanov et al. [24] conducted X-ray absorption fine structure (XAFS) spectroscopy, where they tested the ability of *B. subtilis* to bind Cd ions to its cell walls. The research was conducted in the pH range of 3.4–7.8 and proved that various functional groups might be engaged in binding Cd ions, depending on the pH. When pH was below 4.4, Cd was mainly bound by phosphoryl ligands, and when pH reached higher values, a significant role of carboxyl groups in binding Cd was described. Activation of an additional binding site (ascribed by the authors to a phosphoryl site with a smaller Cd-P distance than that observed at a pH below 4.4) was also observed at pH 7.8 [24]. In the context of the results quoted above, our strain, *Bacillus* sp. HK4, will be investigated further in future studies, as it demonstrates the capability of binding Cd in a wide pH range. Moreover, it might be applicable in high phosphate concentrations (such as in soil and communal waste), as it was isolated from phosphate ore.

Native bacteria isolated from phosphate ore from Djebel Onk have also exhibited high capabilities of magnesium sorption. Isolated strains accumulated Mg in concentrations exceeding 2000 µg g^{-1}, and for *Bacillus* sp. HK4, the biosorption exceeded 8000 µg Mg g^{-1} at pH 7 (Figure 4). These are much higher than the values obtained for *B. subtilis* USK1

from the University of Silesia in Katowice [35]. In that study, Mg accumulation in the biomass of microorganisms ranged from 324 to 2698 μg Mg g^{-1} and was higher at pH 7 than at pH 3 [35], emphasizing the potential biotechnological applications of native species. Accumulation of large amounts of Mg is significant because it helps remove unwanted Mg from phosphate ores, and it could help reclaim this element from waste and recycle it.

In this study, we also confirmed the remarkable adhesion capacity of the native HK4 strain (Figures 5 and 6), especially at neutral pH. Again, this can be attributed to the unique cell wall and EPSs construction and the need for certain minerals. Growing microorganisms usually have a high demand for minerals. Calcium is found in large amounts of phosphate ore (in apatite and dolomite). Meanwhile, magnesium is found only in dolomite [52]. For our ore, carbonate fluoroapatite $Ca_5(PO_4,CO_3)_3F$) and dolomite ($CaMg(CO_3)_2$) were present (Table 4). Interestingly, Oknin et al. [53] discovered that Mg can affect biofilm formation by *Bacillus* species. Additionally, 50 mM or higher concentration of Mg ions can inhibit biofilm formation, but not the growth of bacterial cells. The authors concluded that Mg ions specifically decrease the expression of genes involved in biofilm formation, inhibiting extracellular matrix synthesis [53]. Notably, the authors mentioned that other divalent ions (such as Ca^{2+}) did not inhibit biofilm synthesis by *Bacillus* species [53–56]. However, this problem requires a deeper analysis, as not all studies are consistent in this regard. For example, Mhatre et al. [56] showed that Ca^{2+} ions, under specific conditions, can inhibit the expansion of *B. subtilis* colonies and biofilm development.

However, focusing only on the properties of Mg and considering that dolomite contains magnesium (which can slow down biofilm formation), it is safe to assume that after a sufficiently long incubation of *Bacillus* sp. with phosphate ore, such biofilm can develop in different dynamic/time on the surface of dolomite and apatite. Its development on the surface of dolomites might be slowed (or stopped altogether) by Mg ions. At the same time, because apatite does not contain Mg, bacterial biofilm may develop more easily on its surface. This quality may be valuable when designing further procedures to create an ecological method of ore treatment, as it is a potential factor that differentiates both minerals. However, the selectivity of native bacteria isolated from Djebel Onk ores is in need of detailed study. Subsequent tests may be performed using QD-labeled bacteria and a simplified model ore. To prepare such a sample, pure apatite and dolomite (particles of different sizes/shapes) could be mixed in predetermined proportions.

5. Conclusions

Incubation of phosphate ore with the HK4 native bacterial strain (for 20 min at different pH) can significantly increase the recovery of metals such as Mg, Cd, and possibly others. Native *Bacillus* species are especially promising and worthy of recommendation. They can be potentially helpful in designing eco-friendly ways to clean ore and post-flotation wastes and recycle metals. Undoubtedly, further studies are required to determine the selectivity of adhesion and mechanisms that enable certain species to survive and develop in the extreme conditions of phosphate deposits in Djebel Onk.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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