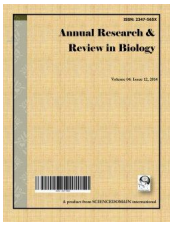

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Magnetotactic Characterization and Environmental Application *P. aeruginosa* kb1 Isolate

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Authors' contributions

This work was carried out in collaboration between all authors. Author HK managed the analyses of the study. Authors MFE and MMA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors NA and SME managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The interest in magnetic nanoparticles (MNP) is rising in the recent years. *Pseudomonas aeruginosa* Kb1 isolated from Egyptian habitat found to synthesis intracellular magnetosomes. This work aimed at characterization of produces magnetosomes and prospective application of the magnetotactic *P. aeruginosa.*
Methodology: The Kb1 isolate was grow in a batch experiment for three days. The synthesis of magnetosomes was confirmed by TEM microscope. The produced MNP were extracted, purified and characterized. The ability of *P. aeruginosa* Kb1 to remove heavy metals from its solutions was examined as well.
Results: The biosynthesis of mature magnetosomes, quasi-spherical or cubical in shape and 30- 70 nm in size, was affirmed after 72 h. The produced particles were crystals and displayed a super paramagnetic behaviour. Elemental analysis of the extracted MNP produced by *P. aeruginosa* revealed the presence of carbon, oxygen, iron and chloride. This result indicates that the

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composition of the produced magnetosomes is Fe_3O_4 mainly while the finding of high carbon level could be refer the protein coat surrounding the magnetosomes. The FTIR spectrum analysis reveled the detection of Fe-O group which point to magnetite as the main MNP. The existence of H-O group, N-H group, C-H, C=C and C-C groups prove presence of the protein coat. Furthermore, magnetotactic *P. aeruginosa* was able to remove 99.4% and 70% of Cd^2 and Pb^2 at initial concentration of 4 ppm although it survived at high concentrations (up to 8 ppm) of each.
Conclusion: *Pseudomonas aeruginosa* Kb1 is a promising candidate for magnetic nanoparticles production and heavy metal removal.

Keywords: Heavy metals removal; magnetosome characterization; MTB; Pseudomonas aeruginosa.

1. INTRODUCTION

Prokaryotic microorganisms are key players in depositing and weathering of the Earth's crust mineral contain [1,2]. The biomineralization of intercellular inorganic minerals is not often exist among prokaryotes and can be categorized to biologically induced and biologically controlled method [3]. The biosynthesis of intercellular MNP by Prokaryotic genera is an obvious example for the biomineralization process. The distinct group of prokaryotes that create particular organelles, so-called magnetosomes, are known as magnetotactic bacteria (MTB). MTB are Gram-negative, motile, aquatic and microaerophilic/anaerobic bacteria, inhabiting both freshwater and marine ecosystems [4]. Magnetosomes are membrane-coated MNP of magnetite (Fe_3O_4) or greigite (Fe_3S_4) that are aligned in regular chains in the bacterial cells and function as a geomagnetic field sensor [5].

Magnetotactic bacteria abundantly inhabit diverse environments (especially aquatic) such as lakes, rivers, ponds, estuaries and salt marshes, lagoons, mangrove swamps, intertidal zones, deep-sea sediments and soils as well as some extreme ecosystems. MTB discovered internationally in all continents [6]. Although the abovementioned diversity of MTB, a few species were isolated and maintained in pure culture [7]. Among the most well-known MTB, the obligate microaerobic *Magnetospirillum magnetotacticum* strain MS-1 was described [8]. On the other hand, *Magnetospirillum* sp. strain AMB-1 and *M. gryphiswaldense* MSR-1 were able to grow aerobically [9,10].

The applications of MTB in the medical and environmental application are commonly focused, in spite of the rare investigations on their application in environmental remediation [11]. In this respect, the bioremoval of heavy metals was further studied [12-15]. However, the selective biosorption for Au (III) and Ag (I) in Au–Cu and Cu–Ag in binary ion systems by MTB was reported [14,15].

Investigation of unexplored ecosystems could resulted in isolation and characterization of novel MTB. In previous study several magnetotactic bacteria have been isolated from Egyptian habitats and their magnetic behavior was confirmed [16]. One isolate was identified as *P. aeruginosa* reporting the first indication of magnetosome biosynthesis by this bacterial type according to our knowledge. While the formation of nanomagnetic crystals by *P. aeruginosa* have never been studied, the current study aims at production and characterization of magnetosomes by *P. aeruginosa* kb1 isolate. Prospective environmental applications of this isolate were investigated for heavy metals removal.

2. MATERIALS AND METHODS

The previously isolated *P. aeruginosa* kb1 (KT962901) was grown in a 5 litres tightly closed bottle approximately fully-filled with M3 medium in a batch experiment. M3 medium contains (g/l): KH_2PO_4: 0.15, $MgSO_4.7H_2O$: 0.1g, NH_4Cl: 0.40g, Hepes: 2.38 g, yeast extract: 0.1g, peptone: 3.0 g, sodium pyruvate: 3.0 g, and a 1ml EDTA trace element solution according to Widdel and Bak [17]; then the pH was adjusted to 6.8-7 and autoclaved at 121°C for 30 minute. After cooling and before inoculation filter-sterilized 400 µl/l of 1M ferric citrate, 1 ml/l of 1 M DTT; and sodium thioglycolate, 0.5 g/l were added and the bottles were shacked well till homogenization occurs. The bottle was inoculated and incubated at 30°C at static incubator for three days. After the incubation period the bacterial cultures were centrifuged at 12000 rpm for 15 minute and the pellets were washed three times with distilled water then suspended in 100 ml of a buffer solution contains 20 mM hepes and 4 mM EDTA.

2.1 Magnetosomes Extraction and Purification

The magnetosomes were extracted from the bacterial cells to evaluate their characteristics. A physical method using ultrasonication was followed for cells dispersion and magnetosome extraction. The bacteria cells were suspended in the PBS buffer flowed by three cycles of freezing and thawing before being crashed using an ultrasonicator (600 W/cm^2, 2.5 min, and 30 pluses) in ice bath [18]. The magnetosomes released from the crashed bacterial cells were collected by strong permanent magnet. The cell debris and other impurities were removed through several washes with PBS buffer.

2.2 Morphological and Analytical Characterization of Magnetosomes

The magnetosomes synthesis by *P. aeruginosa* kb1 was confirmed by TEM imaging of bacterial cells during batch experiment. The extracted magnetosomes were further characterized using TEM as well. The pelleted cells as well as extracted magnetosomes were washed three times PSB, suspended in distilled water, fixed onto a 300-mesh carbon-coated copper grid, the grids were air dried for 20 min subsequently observed by transmission electron microscope (JEM-2100, JEOL, Japan) at 200 kV with magnification range from 1000x to 50000x. The Image program was used to measure the diameters of one hundred nanoparticles at the different functionalization steps, which were analyzed using a lognormal distribution. Selected-area electron diffraction analysis was conducted with TEM (JEM-2100, JEOL, Japan) at 200 kV.

The surface morphology of extracted air dried magnetosomes (for 48 h at 70 °C) was characterized utilizing a scanning electron microscope (SEM) on a FEI Quanta FEG 250 instrument operated at 20 kV. Infrared reflectance spectra were carried out on oven dried (for 2 days at 72°C) magnetosomes using a spectrometer (Jasco FT/ IR 6100 – Japan) with instrument resolution of about (1 cm^{-1}), in the wavenumber region (400–4000 cm^{-1}) at room temperature. Magnetization was measured using a vibrating sample magnetometer (VSM). About 0.5 g of the extracted bacterial magnetosomes were lyophilized and subjected to Vibrating Sample Magnetometer (Lake Shore Model 7410 USA) which provides field strength up to 31 kOe.

2.3 Potential Removal of Heavy Metals (Cd and Pb) by *P. aeruginosa* kb1

To examine the ability of *P. aeruginosa* kb1 isolate to remove Cd^2 and Pb^2, 1.0 x 10^{10} cells from overnight cultures harboring the magnetosomes as indicated by TEM was utilized. The cells were collected by centrifugation (10,000 rpm for 10 min at 4°C), washed twice with 10 mM hepes buffer (pH 7.4) and EDTA (25 mM, pH 7.0) to chelate and remove the attached metal ion residues from the growth medium. The collected cells were resuspended in the same buffer containing Cd and Pb individually with concentrations (1, 4, 6, 8 mgL^{-1}). The solutions containing cells and heavy metals (Cd^2 and Pb^2) were gently agitated for 3 h. The uncultured medium was running as a negative control. After the agitation, the cells were removed by centrifugation then Cd^2 and Pb^2 were removed from the cell surfaces. The supernatant after centrifugation was collected for analysis of remaining Cd and Pb by atomic absorption spectrophotometry [19].

3. RESULTS AND DISCUSSION

3.1 Morphological and Analytical Characterization of Magnetosomes

The bacterial cells of *P. aeruginosa* kb1 were confirmed to contain intracellular magnetosome during the growth period in batch culture for 72 hours.

The TEM images of the bacterial cells illustrated the existence of 5-20 electron-dense particles that are readily observed (Fig. 1). The largest particles were located in the center or bottom of the cell. These pictures affirmed the biosynthesis of mature magnetite nanoparticles through cultivation of *P. aeruginosa* kb1 72 h. A number of quasi-spherical or cubical particles were observed with 30- 70 nm size range.

The imaging of the constructed magnetosomes indicated the changes in their shape and size with increasing the incubation period of *P. aeruginosa* from spherical nanoparticles into cubic superstructures and from 5-30 nm to 30-70 nm, respectively (Fig. 1a-1d). The magnetite nanoparticles were intracellular as they were located inside the cytoplasmic membrane. The electron-dense particles in the cells of *P. aeruginosa* were not arranged in a single chain although it were arranged at the center of the bacterial cytoplasm.

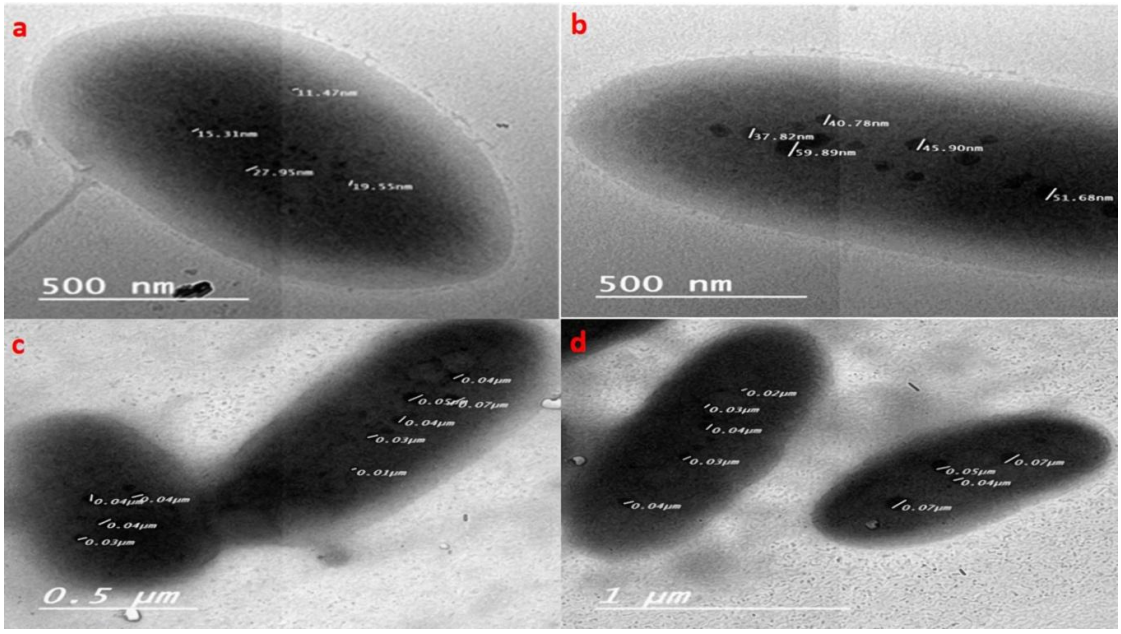

Fig. 1. TEM images of bacterial cells of *P. aeruginosa* after 24 and 72 h of incubation in batch experiment . The figure shows the constracted magnitosomes and their size. image after 24h (a) represent the immature magnetosomes while the mature magenetosomes (after 72 h) are presented at different magnification in images b, c and d)

The intracellular magnetosomes produced by *P. aeruginosa* were extracted by ultrasonication. The TEM images of extracted magnetic nanoparticles showed that the size of extracted magnetosomes ranged from 40-50 nm (Fig. 2a). The size of magnetosome isolated from *P. aeruginosa* was amongst the sizes recorded for intracellular magnetosomes biosynthesized by numerous bacteria in previous studies. The magnetosome crystals in this size found to be stable individual nanoparticles as previously reported that crystals ranged from 35 to 120 nm were stable [20]. Several sizes of magnetosomes have been documented for different bacteria. The magnetosomes size were 20 nm for the recombinant AMB-1 [21], 81 x 58 nm for a marine magnetotactic *spirillum axenic* QH-2 isolate [22], 54 x 43 nm for *Magnetospirillum* strain WM-1 [23], 30-43 nm for *Shewanella oneidensis* MR-1 [24] and 113 x 40 nm for a thermophilic magnetotactic bacteria HSMV-1 [25]. These nanoparticles appeared to be well separated from one another since they were stabilized by the proteins present on the surface (Fig. 2a and 2b). Nemours studies elucidated the formation of proteins membrane surrounding the formed magnetosomes [26,27]. High magnification image of the cubes reveal the existence of voids which may refer to the aggregation of smaller spherical nanoparticles (Fig. 2a and 2b). Although that aggregation, the magnetite nanoparticles produced by *P. aeruginosa* kb1 were extremely stable in solution for weeks as a result of the biosynthesis of a protein coat around the magnetosomes. In this respect, Gorby et al. [28] explained the well separation of the intracellular magnetite nanoparticles as a result of the membranous vesicles enveloped the magnetosomes. This protein membrane coting the magnetosomes resulted in increasing the biocompatibility and dispersibility of the nanoparticles without further treatments. The preference of the biologically produced MNP regarding the advantages of membrane cotes have been frequently reported formerly [27,29,30].

The crystallinity of MNP synthesized by *P. aeruginosa* kb1 was confirmed by selected area electron diffraction analysis (SAED) (Fig.3). The SAED showed the presence of well defined diffraction spots indicating that nanoparticles are crystalline in nature. Although the crystalline characteristics of magnetosomes was reported previously for several magnetotactic bacteria by many researchers including Pósfai et al. [31] and Li et al. [32], there was no record for SAED study of *P. aeruginosa.*

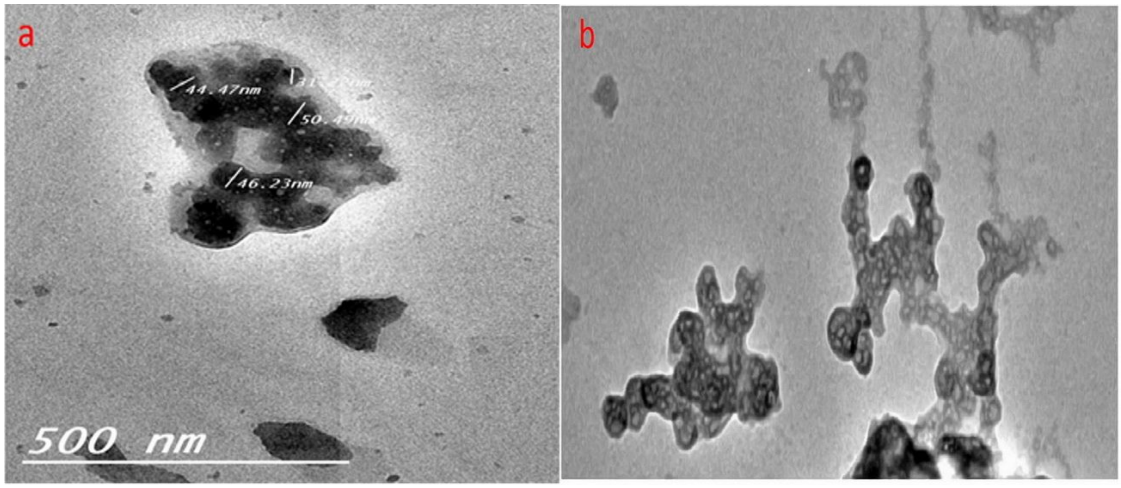

Fig. 2. Magnetosomes size (A) and shape (B) after partial physical extraction with ultrasonic waves (note the protein coat surrounding each particle)

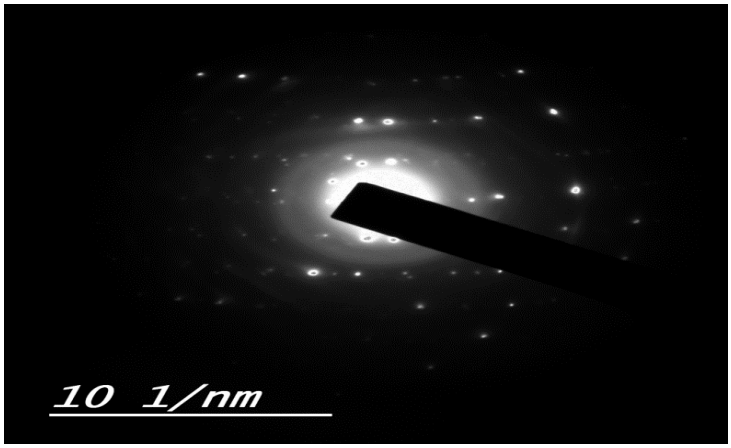

Fig. 3. Selected area electron diffraction analysis of magnetosome isolated from *P. aeruginosa* kb1.

3.2 SEM Analysis

Scanning electron microscope (Fig. 3) indicates that the shape of mature magnetosome is cuboidal to octahedral shape and the average size is about 44-64 nm (average 54.25 nm). Elemental analysis of the extracted magnetosomes produced by *P. aeruginosa* particles revealed the presence of carbon, oxygen, iron and chloride. The percentage of the elements were oxygen 39.29%, carbon 28.7%, iron 17%, chloride 11.24% and sodium 4.49% (Figs. 4 and 5). The absence of sulfur in the elemental composition revealed that magnetic components of magnetosomes are consisted of two kinds of elements, iron and oxygen. It could be deduced that the composition of the produced magnetosomes is Fe_3O_4 mainly. The detection of high carbon percentage could be refer the electron dense cover around MNP. In accordance with this work, several previous studies reported the existence of iron oxide in magnetosomes, probably magnetite (Fe_3O_4), whereas greigite (Fe_3S_4) was never found [33-35].

3.3 FTIR Spectral Analysis

The surface nature of extracted magnetosomes was studied by the FTIR spectroscopy to characterize the functional groups of magnetosome membrane. The obtained FTIR spectrum presented in figure (6) revealed the existence of H-O group and N-H group at

3787.51cm^{-1} and 3431.71 cm^{-1}, respectively. The bands at 2922.59 cm^{-1}, 1619.91 cm^{-1}and 1047.16 cm^{-1} can be assigned to the presence of C-H, C=C and C-C groups, correspondingly. Additionally the strong peak observed at 745.35 cm^{-1} was attributable to Fe-O group, while vibration band at around 610 cm^{-1} along with a small band at 547 cm^{-1} is due to the Fe-O bending mode. In this respect, Fe-O group increase the probability of magnetite as the main magnetic nanoparticles accumulated by *P. aeruginosa* Kb1 while the presence of organic groups confirm the synthesis of a protein membrane coting the magnetosomes. In agreement of these results, earlier reports indicated the existence of amino and carboxyl groups on the surface of the magnetosome [36,37].

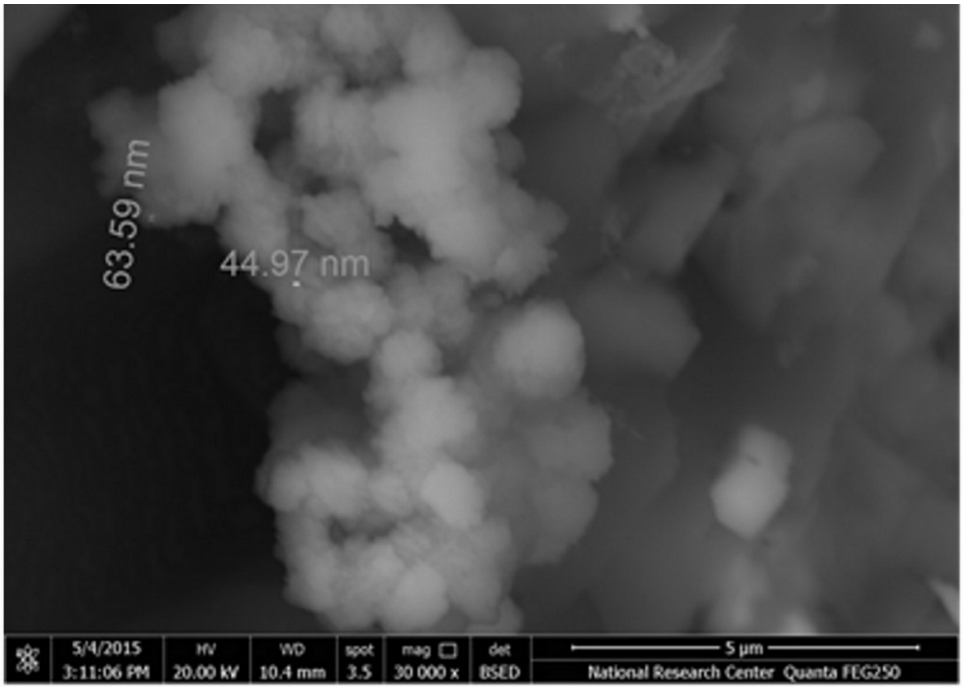

Fig. 4. Analysis and characterization of magnetosomes after extraction by SEM (Scanning Electron Microscope)

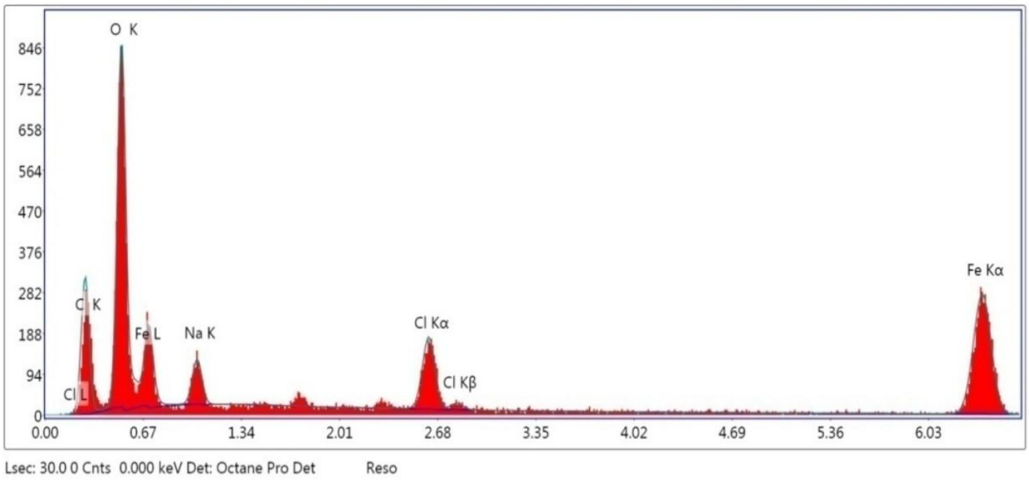

Fig. 5. Energy dispersive X-ray microanalysis spectrum of strain *P. aeruginosa* Kb1, Fe and O were found present in the bacteria

3.4 VSM Analysis

Magnetization measurements of the magnetosomes produced by *P. aeruginosa* Kb1 isolate were carried out using vibrating sample magnetometer (VSM). The curves of field dependence of magnetization are presented in figure (7). The curve of hysteresis and magnetization are completely reversible at room temperature demonstrating a super paramagnetic behaviour of magnetosomes prepared at normal condition. The recorded saturation magnetization (Ms) values were about 600 memu/g while the retentivity was calculated to be 235 memu/g approximately.

3.5 Removal Efficacy of Cd and Pb by *P. aeruginosa* kb1

In order to demonstrate the prospective environmental applications of MTB *P. aeruginosa* Kb1, the the capability of viable cells to eliminate divalent heavy metal ions, Cd^2 and Pb^2, from their solutions was investigated. Cells of *P. aeruginosa* kb1 were applied to solutions contain different concentrations of either Cd^2 or Pb^2 (1, 4, 6, 8 ppm) and then the remaining of each was determined to estimate its removal. The obtained data reveled that the highest Cd^2 removal percentage (99.4%) was recorded at initial concentration of 4 ppm while the highest removal of Pb^2 was 70% at 1ppm and 4 ppm (Fig. 8). The removal capability of *P. aeruginosa* kb1 toward both elements was declined when applied to solutions contains higher concentrations (over 4 ppm) of either Cd or Pb. This decrease in the removal aptitude of the bacterial cells could refer to the toxicity of high concentrations Cd and Pd. The growing MTB could remove the metals through taking them up inside the cells or accumulate them on the cell wall. The MTB found to remove plutonium and heavy metals

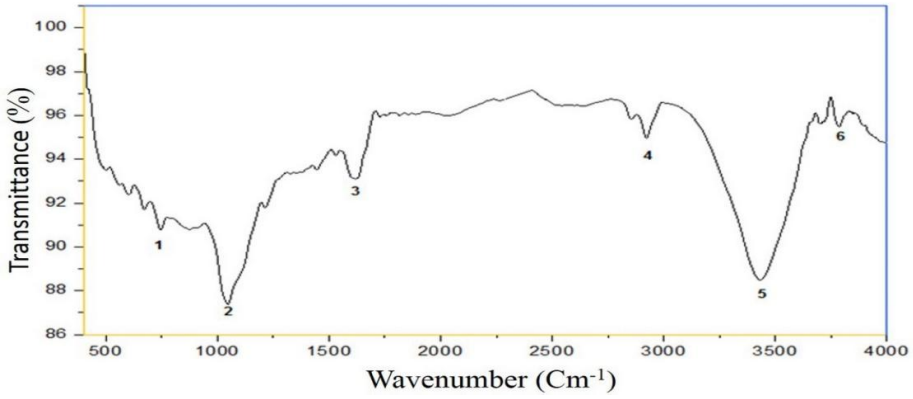

Fig. 6. FTIR spectra of extracted magnetosomes from *P. aeruginosa* Kb1. The adsorption peaks (No.1-6) magnetosomes were at 745.35, 1047.16, 1619.91, 2922.59, 3431.71 and 3787.51 cm^{-1}

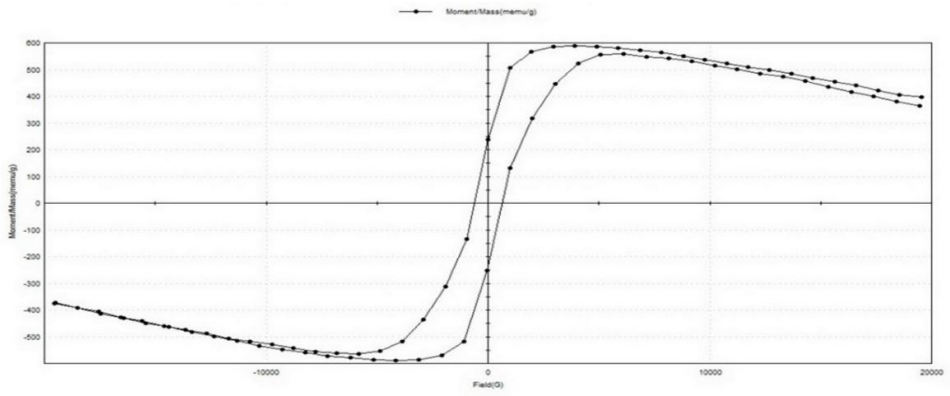

Fig. 7. Magnetization properties and hysteresis loop of the extracted magnetosomes

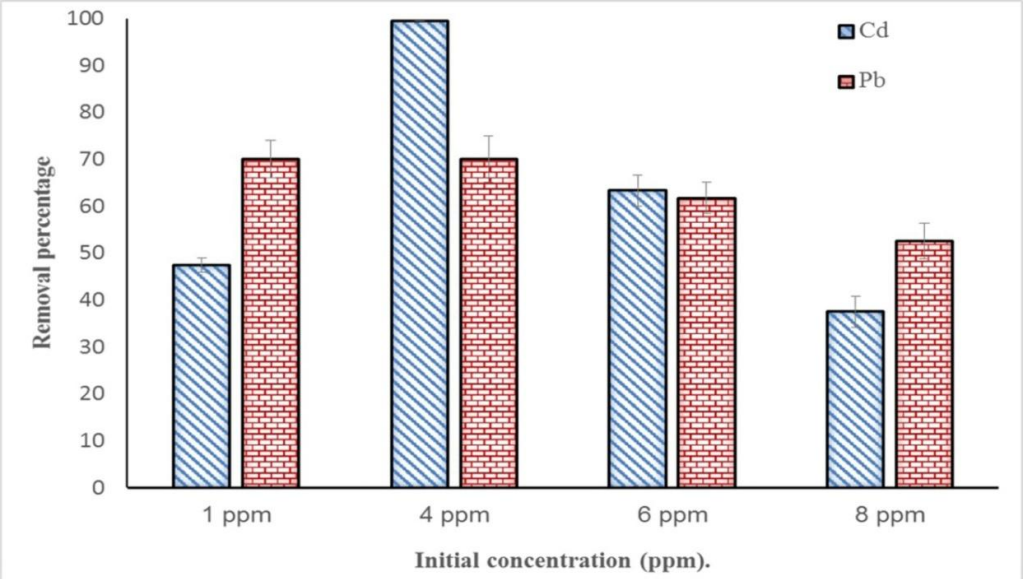

Fig. 8. Heavy metal (Cd and Pb) removal percentage (± sd) from different initial concentrations after the application of growing culture of *P. aeruginosa* kb1 in contrast to control untreated samples

including cadmium (more than 95% of its initial concentration) by *Desulfovibrio magneticus* [38,39]. Although the above mentioned decline, *P. aeruginosa* kb1 remained able to remove 37.5% and 53.5% of Cd and Pb, respectively from their solutions at initial concentration of 8 ppm. As reported by Lang and Schüler [40] resistance of MTB to toxic concentrations divalent heavy metal including zinc, cadmium, cobalt and others based on the existence of specific proteins (such as MamB and MamM) in the composition of CDF (cation diffusion facilitator) family of metal transporters that function as efflux pumps for heavy metal ions. Not only cell surface proteins but also granules and capsules could be essential in the bioremoval of heavy metals and different ultrastructure resulted in binding of different metals by MTB [41,42]. Otherwise, metallic elements firmly fixed to the membrane as inorganic precipitates [12,39-41].

4. CONCLUSION

MTB is the key agents of biogeochemical cycling of iron and other elements. The isolated *P. aeruginosa* kb1 was confirmed to produce MNP. The intercellular magnetosomes size was ranged from 30-70 nm after 72 h of growth. The extracted magnetosomes were 40-50 nm, crystalline in nature and coated with protein membrane. The paramagnetic behaviour of magnetosomes produced by *P. aeruginosa* Kb1 was confirmed by VSM analysis. MTB *P. aeruginosa* kb1 revealed a notable potential in removing heavy. *P. aeruginosa* kb1 removed up to 99.4% and 70% of Cd and Pb from their initial concentrations, respectively and it resists high concentrations (up to 8 ppm) of each. The environmental applications of MTB *P. aeruginosa* kb1 is auspicious as it could be collected from contaminated solution by super magnets and required further studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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