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Microbacterium sp. MRS-1, a potential bacterium for cobalt reduction and synthesis of less/non-toxic cobalt oxide nanoparticles (Co_3O_4)

Sathyavathi Sundararaju[1,2*], Manjula Arumugam[2] and Prakash Bhuyar[3]

Abstract

Background: Detoxification of heavy metal pollutants in wastewater has become a serious problem to surrounding environment. This research was conducted to utilize a potential heavy metal-resistant bacterium for the remediation of cobalt metal and simultaneous synthesis of cobalt oxide nanoparticles in the form of powder for various industrial applications. Metal oxide nanoparticles have great applications in electrochemical devices such as supercapacitors, biosensors, and batteries.

Method: A heavy metal-resistant bacterium *Microbacterium* sp. MRS-1 isolated from electroplating industrial effluent reduced cobalt ions from an initial concentration of 200 mg/L to 26 mg/L were analyzed by atomic absorption spectroscopy. Instrumental analysis of bacterially synthesized Co_3O_4 has been characterized. Cytotoxicity of synthesized nanoparticles was assessed by MTT assay.

Results: *Microbacterium* sp. MRS-1 isolated from electroplating industrial effluent was found to be suitable for cobalt oxide nanoparticles as it showed tolerance towards high concentration of metal. The nutrient broth containing metal solution and *Microbacterium* sp. MRS-1 showed color change from light pink to dark pink indicated the formation of extracellular nanoparticles. It also converted soluble cobalt salts into less soluble cobalt oxide nanoparticles outside the cell which allows easy recovery of nanoparticles without the destruction of cells and simultaneous detoxification of toxic metal ions. Electron microscopic imaging verified that nanoparticles were predominantly surrounding the bacterial cells and SEM imaging revealed that the produced particles were in the range of 10–100 nm in size. XRD spectrum exhibited 2θ values were corresponding to cubic face-centered cobalt oxide (Co_3O_4) nanoparticles.

Conclusion: The present study investigated new prospective for eco-friendly detoxification of toxic heavy metal Co from metal-polluted sites and the production of cobalt oxide nanoparticles in powder form for clinical and other industrial applications.

Keywords: *Microbacterium* sp. MRS-1, Cobalt reduction, Cobalt oxide nanoparticles (Co_3O_4), Cytotoxicity assay

* Correspondence: sathyasundararaju@gmail.com
[1]Department of Pathology, Sidra Medicine, Doha, Qatar
[2]Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India
Full list of author information is available at the end of the article

1 Introduction

Metal oxides have interesting physico-chemical, electronic, and optical properties [1]. Therefore, metal oxides such as copper oxide, iron oxides, and cobalt oxides have been extensively synthesized and used in industries such as electroplating, textiles, steelmaking, drug delivery, and as biomedicine [2]. For the multifunctional, antiferromagnetic p-type semiconductor properties, cobalt oxides have been widely used in electrochromic sensors, energy storage, heterogeneous catalysis, pigments, dyes, and in lithium-ion rechargeable batteries as an anode material [3–5]. Like many other heavy metals, cobalt ions present in the wastewater discharged from these industries are of a major concern for the environment. Abatement strategies such as bioremediation showed a promising environmentally friendly approach to address heavy metal contamination. Various chemical and physical methods have been proposed for the synthesis of cobalt oxide nanoparticles, but the conventional methods generate toxic wastes and create secondary pollutants to the environment [6]. In order to overcome the problems of toxic waste generation, plant extracts and microbes can be used in an economical and ecofriendly method for the production of nanoparticles for various applications [7]. Green synthesis of magnetic behavior CO_3O_4 nanoparticles by an *Aspalathus linearis'* leaves natural extract has been reported [5]. Recently, CO_3O_4 have been chemically synthesized to develop magnetic semiconductor spintronic devices [8]. Also, cobalt oxide (CoO, Co_3O_4, or Co_2O_3) is a common component in several pigments [9].

Environments polluted with metals lead to the selection of indigenous metal-resistant microorganism, which can be exploited for the production of useful metal/metal oxide nanoparticles and also to minimize metal toxicity in the environment [10]. However, the production of cobalt oxide nano powder is considered as a promising technology for a wide range of applications including magnetic resonance imaging contrast agent, anticancer drug delivery system for cancer diagnosis and chemotherapy [11], biosensor properties for DNA genotyping [12], and amino acid detection [13]. The utilization of microorganism for the synthesis of metal/metal oxide nanoparticles is a natural and eco-friendly process, which represents a promising alternative to the usage of expensive chemical reactants, oxidizing compounds, and its harmful effects [14, 15]. Heavy metal-resistant microorganisms are of great significance in detoxification of soluble toxic inorganic metal ions to insoluble less toxic metal/metal oxide nanoparticles can be made either by extracellular biomineralization, biosorption, complexation or precipitation, or intracellular bioaccumulation [16]. Isolation and screening of heavy metal-resistant bacterial strain resistant to cobalt such as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* have been reported previously [17, 18]. Various applications of *Microbacterium* sp. have been reported in different fields such as bio-emulsifier production and extracellular synthesis of gold, silver, and nickel oxide nanoparticles [16, 19, 20]. Synthesis of hollow cobalt oxide nanoparticles by yeast cells [21] and magnetic cobalt oxide by *Brevibacterium casei* have been reported recently [22]. As it is well demonstrated that cobalt-based nanoparticles are promising and significant candidates for enormous biomedical applications due to their unique characteristics [23]. In the present study, we report the heavy metal-resistant bacterial strain *Microbacterium* sp. MRS-1 as a potential candidate for the detoxification of cobalt oxide nanoparticles in the form of CO_3O_4 and bioremediation of toxic heavy metals. This study is the first report on the biosynthesis of cobalt oxide nanoparticles (CO_3O_4) in powder form using *Microbacterium* sp. MRS-1.

2 Materials and methods

2.1 Bacterial cultivation

The metal-resistant bacterium, *Microbacterium* sp. MRS-1 isolated from electroplating industrial effluent showed resistant to cobalt on nutrient agar was taken for this study based on its MIC [16]. It was grown in nutrient broth supplemented with 200 mg/l of cobalt chloride and incubated at 30 °C for 120 h at 180 rpm on the rotary shaker. The culture was centrifuged at 3000 rpm 4 °C for 10 min. Biomass monitored at every 24-h interval was recorded by measuring the absorbance at 600 nm and heavy metal concentration was analyzed using AAS. Each experiment was carried out in triplicate.

2.2 Biomass characterization

The extracellular aggregate was collected and washed twice with deionized water by repeated centrifugation steps. UV-DRS (diffused reflection spectra) of the cobalt oxide nanoparticles were recorded using a JASCO-V-550, double beam spectrophotometer with PMT detector. The presence of functional group was identified by FT-IR spectroscopy and morphological studies were carried out using SEM, Hitachi S-4500 microscope operating at 120 kV accelerating voltage attached with EDAX. AFM experiments were carried out by A100SGS atomic force microscope using a Si tip. AFM imaging was done in tapping mode to study the surface morphology. XRD analysis carried out on a PAN analytical-X'pert PRO (PAN analytical, Netherland) and the measurement carried out at 40 kV and 30 mA in Cu KV radiation.

2.3 Characterization of nanoparticles

To evaluate the morphology of the cobalt oxide nanoparticles, freshly harvested cobalt treated bacteria were

washed with d H_2O. It was suspended in sterile d H_2O and placed on carbon grid which was then pre-treated with 1% phosphotungstic acid for 1 h and pre-treated grid was placed on electron microscope. TEM (model Philips-TACHNAI10, Netherland) equipped with tungsten filament electron source, operated at and 80 kV and Olympus camera has been used for imaging the morphological structure and bacterial interaction of cobalt oxide nanoparticles were imaged. The TGA was carried out using Universal V4.7A thermogravimetric analyser TGA Q50 V20.10 Build 36.

2.4 MTT assay

5-diphenyl tetrazolium bromide (MTT) assay was carried out for cytotoxicity, and L929 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate, and antibiotic/antimycotic solution (100×, 1 mL/100 mL of medium). The cells were maintained in 5% CO_2–95% atmosphere under high humidity at 37 °C. Cells were assessed for cell viability by trypan blue dye exclusion assay as described earlier [24], and batches showing more than 98% cell viability were used in this study. L929 cells were treated with different concentrations of Co_3O_4-NPs (50, 75, and 100 mg/mL) for 24 h. After 24 h of incubation, 10 μL of MTT reagent was added to each well and was further incubated for 4 h. Formazan crystals formed after 4 h in each well were dissolved in 150 μL of detergent and the plates were read immediately in a microplate reader (BIO-RAD microplate reader-550) at 570 nm. Wells with complete medium, nanoparticles, and MTT reagent, without cells, were used as blanks. Untreated L929 cells as well as the cell treated with (50, 75, and 100 mg/mL) concentration of Co_3O_4 for 24 h were subjected to the MTT assay for cell viability determination. The stock solution was then diluted in culture medium to reach the desired concentrations for cell treatment. Approximately 1×10^5 mL^{-1} cells (L929) in their exponential growth phase were seeded in a flat-bottomed 96-well polystyrene coated plate and were incubated for 24 h at 37 °C in a 5% CO_2 incubator.

3 Results

3.1 Spectroscopy analysis

In this study, investigation of the bioremediation potential of the *Microbacterium* sp. MRS-1 to reduce Co (II) ions was carried out. The isolated metal-resistant bacterium *Microbacterium* sp. reduced 174 mg/L of cobalt ions from an initial concentration of 200 mg/L (Fig. 1) and shown here the conversion of soluble cobalt ions into less soluble cobalt oxide nanoparticles outside the cell. The nanoparticles produced extracellularly were deposited on the bottom of the flask and change in medium color to pink was observed. The spectrum obtained from UV-vis spectroscopy (Fig. 2) showed no absorption peak in the control, whereas, the culture exposed to cobalt ions showed distinct broad absorption

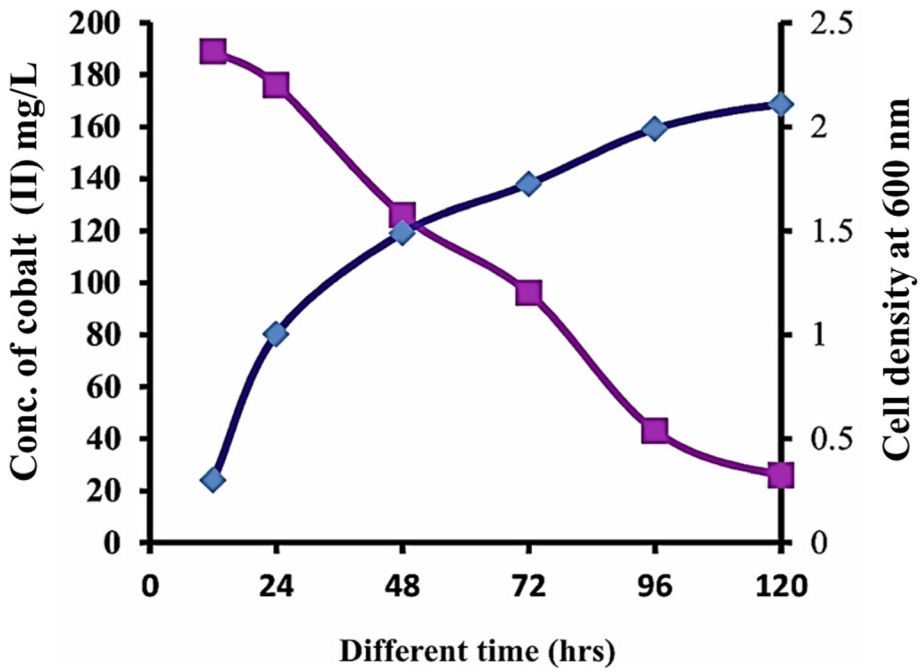

Fig. 1 Growth kinetics and Co removal by *Microbacterium* sp. MRS-1 at pH 7 and 30 °C. The initial concentration of Co (II) was kept at 200 mg/L

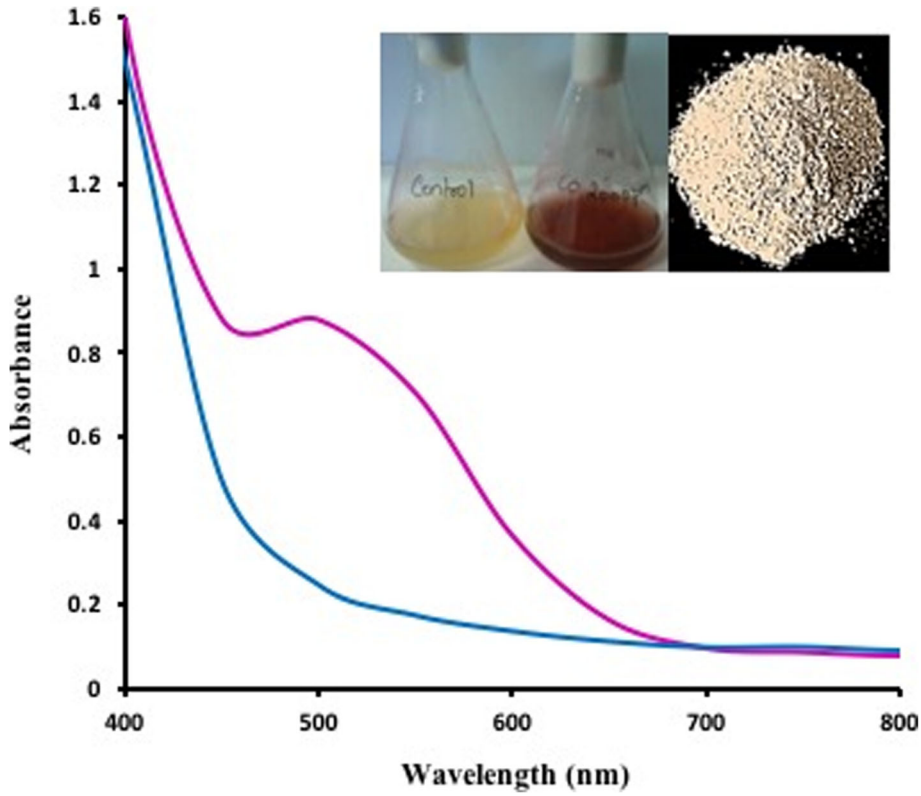

Fig. 2. UV–DRS absorption spectra of extracellular cobalt oxide nanoparticles (Co_3O_4) synthesized by *Microbacterium* sp. MRS-1 incubated with 200 mg/L of $CoCl_2$ at 37 °C at 180 rpm for 120 h

peak around 450–600 nm which can be attributed to the formation of nanoparticles [25]. The presence of broad resonance indicated the evidence of aggregation of nanoparticles. Extracellularly synthesized cobalt oxide nanoparticles have been characterized by FT-IR, which is a very useful tool to investigate the interactions between bacterial cell wall components and cobalt ions.

3.2 Microscopy

Scanning electron microscopy showed uniform distribution of cobalt oxide nanoparticles. During elemental analysis of the synthesized nanoparticles, a high intense peak of oxygen was detected. The majority of the particles were uniformly spherical within the size range of 10–70 nm. The elemental ratio of Co to O in the cobalt

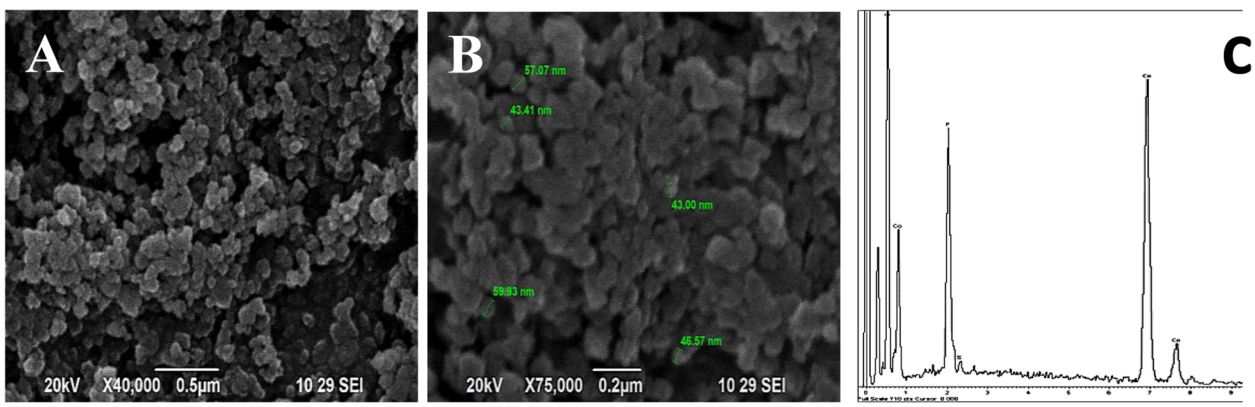

Fig. 3 Scanning electron microscopy (**a**, **b**) and (**c**) EDAX analysis of cobalt oxide nanoparticles (Co_3O_4) synthesized by *Microbacterium* sp. MRS-1 treated with 200 mg/L $CoCl_2$. EDS spectrum recorded for the cobalt oxide nanoparticles with different X-ray emission peaks labeled. (Scale bar **a** 0.5 µm, **b** 0.2 µm)

oxide nanoparticles is 54:34 (Fig. 3). In atomic force microscopic analysis, both spherical and pentagon-like cobalt oxide nanoparticles were observed in the range of 20–90 nm in size (Fig. 4). The XRD pattern of the cobalt oxide nanoparticles is shown in Fig. 5. All the diffraction peaks in the XRD can be indexed to cubic face centered Co_2O_3 and Co_3O_4. The peaks correspond to diffraction from (440), (622) planes showed good agreement with the JCPDS-pattern (JCPDS file no.: 02-1079) of Co_2O_3 and diffractions from (220), (311), (222), (400), (422), (511) planes showed good agreement with the JCPDS-pattern (JCPDS file no.: 78-1969) of Co_3O_4. Comparison of JCPDS data confirms the formation of Co_3O_4 as the major fractions together with Co_2O_3 (Fig. 5).

3.3 TGA

The thermal gravimetric analysis of the synthesized cobalt nanoparticle showed gradual decomposition steps. Decomposition of cobalt nanoparticle was initiated at 100 °C at which, only about 7% of the decomposition was observed. The next stage of decomposition was observed at 225 °C where nearly about 15% of weight loss was observed, mass loss started at about 250 °C to 400 °C corresponding to ~ 50% of the total mass. The reaction terminated in the range of 500–700 °C (Fig. 6). The FTIR spectra of the synthesized samples showed the evidence for the presence of an organic coating. The decomposition was observed in the TG analysis of cobalt nanoparticles could be due to the breakdown of the protein ligands.

3.4 Tem

Different TEM images revealed an average particle size and clearly observed the spherical and pentagon morphology of the synthesized cobalt oxide nanoparticles. Surface interactions between the synthesized cobalt oxide nanoparticles and bacterial cell wall were clearly observed and evidenced the aggregation of the nanoparticles based on TEM analysis. The aggregates were observed in the nutrient medium, and the TEM micrographs revealed the presence of particles in the range of 10–100 nm in size and the outer surface of the bacterial cell could be clearly observed (Fig. 7a–c). Cobalt oxide nanoparticles were bound on the cell wall at one pole of the bacteria under cobalt containing environment for 24 h (Fig. 7a). The accumulation of cobalt oxide nanoparticles all over the bacterial cell at 48 h can be clearly observed (Fig. 7b), and it is clearly seen that the cobalt nanoparticles were deposited as thick layer on the cell wall of the bacteria due to the prolonged exposure for about 72 h (Fig. 7c). Therefore, the isolated bacterial cells at the early stage of nanoparticles synthesis are rod shaped with smooth surfaces, but roughness of the surface developed during prolonged incubation.

3.5 MTT assay

Various metal oxides cytotoxicity by using 5-diphenyl tetrazolium bromide (MTT) assay has been widely demonstrated. The percentage cell viability of L929 cells were recorded at 24 h exposure of Co_3O_4 -NPs. Cell viability at 50, 75, and 100 mg/mL of Co_3O_4-NPs exposed for 24 h were recorded as 100%, 84.5%, and 47.83%, respectively (Fig. 8). The increase in exposure of high concentration cobalt oxide nanoparticles at 50 mg/ml concentrations did not cause any decrease in cell viability, as the concentration of nanoparticle increased up to 100 mg/mL showed significant decrease in cell viability. The decrease in percentage of cell viability was 15% at

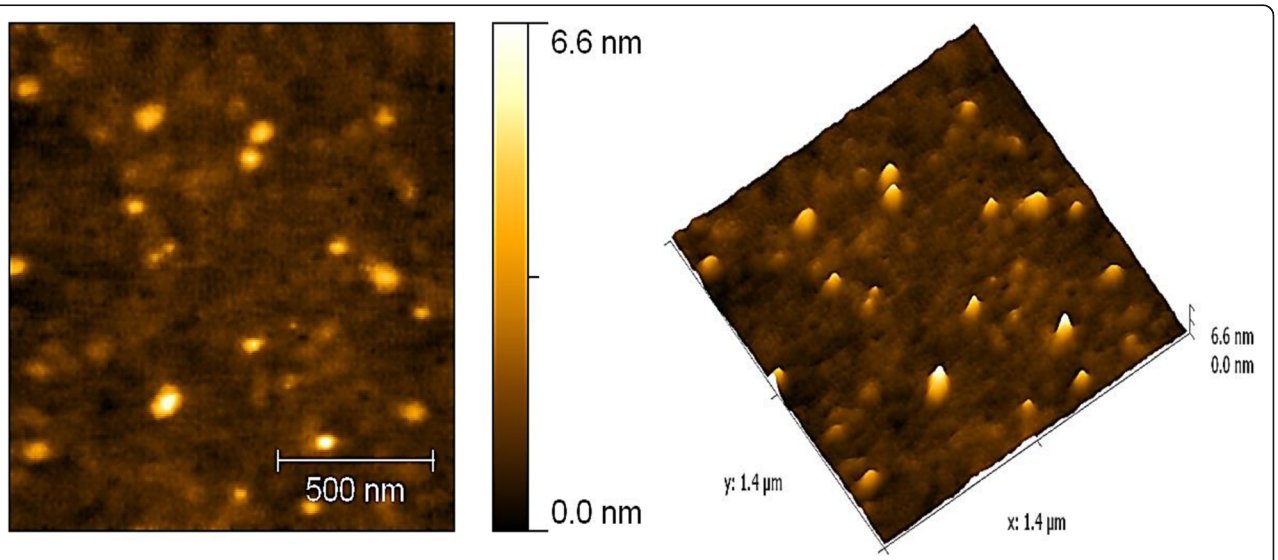

Fig. 4 Cobalt oxide nanoparticles (Co_3O_4) synthesized by *Microbacterium* sp. MRS-1 treated with $CoCl_2$ of 1 µm field size. 1.4 × 1.4 µm

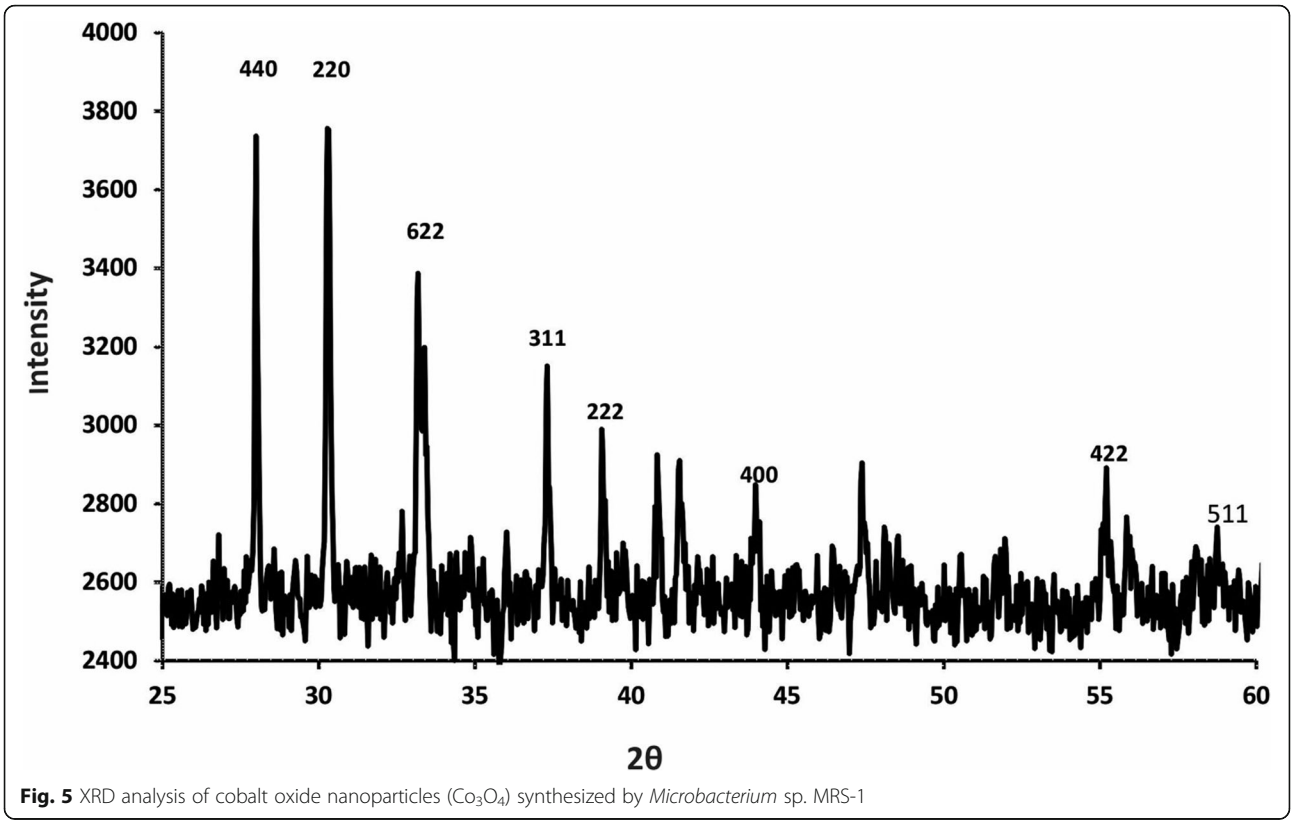

Fig. 5 XRD analysis of cobalt oxide nanoparticles (Co_3O_4) synthesized by *Microbacterium* sp. MRS-1

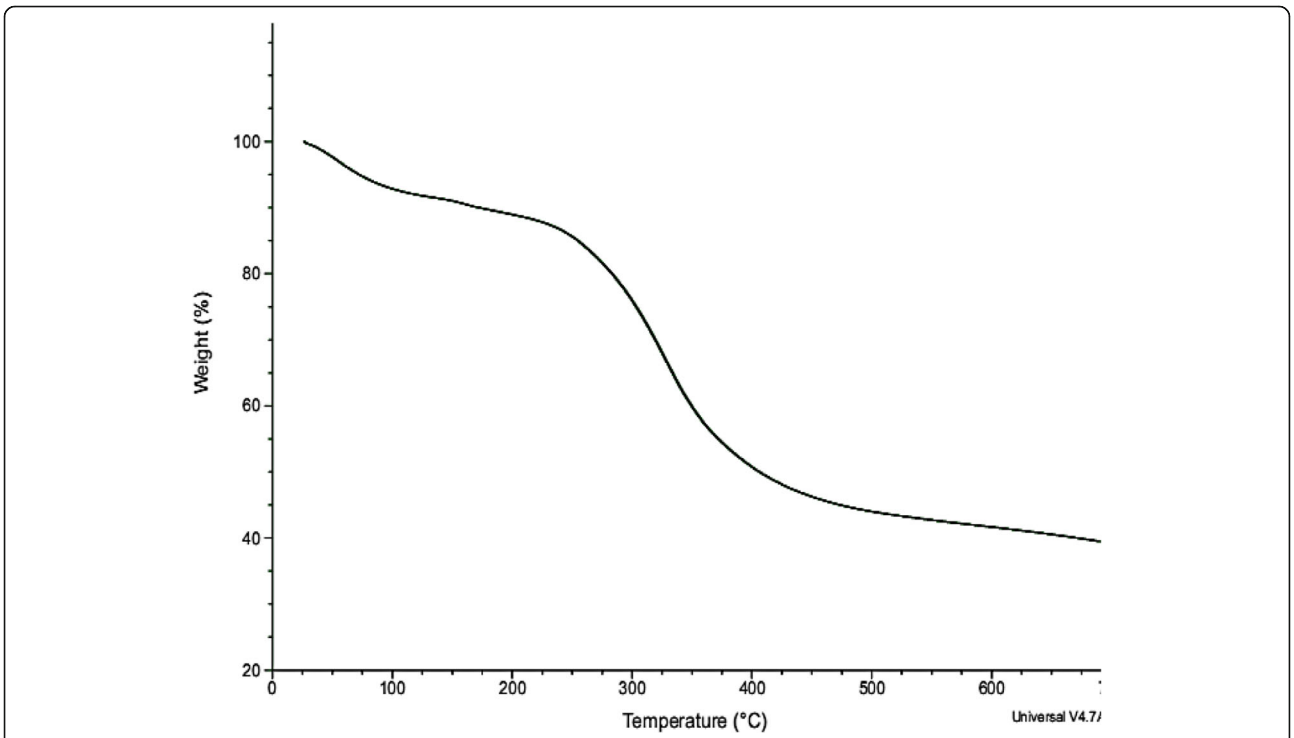

Fig. 6 Thermal gravimetric (TG) curve of bacterially (*Microbacterium* sp. MRS-1) synthesized cobalt oxide nanoparticles (Co_3O_4)

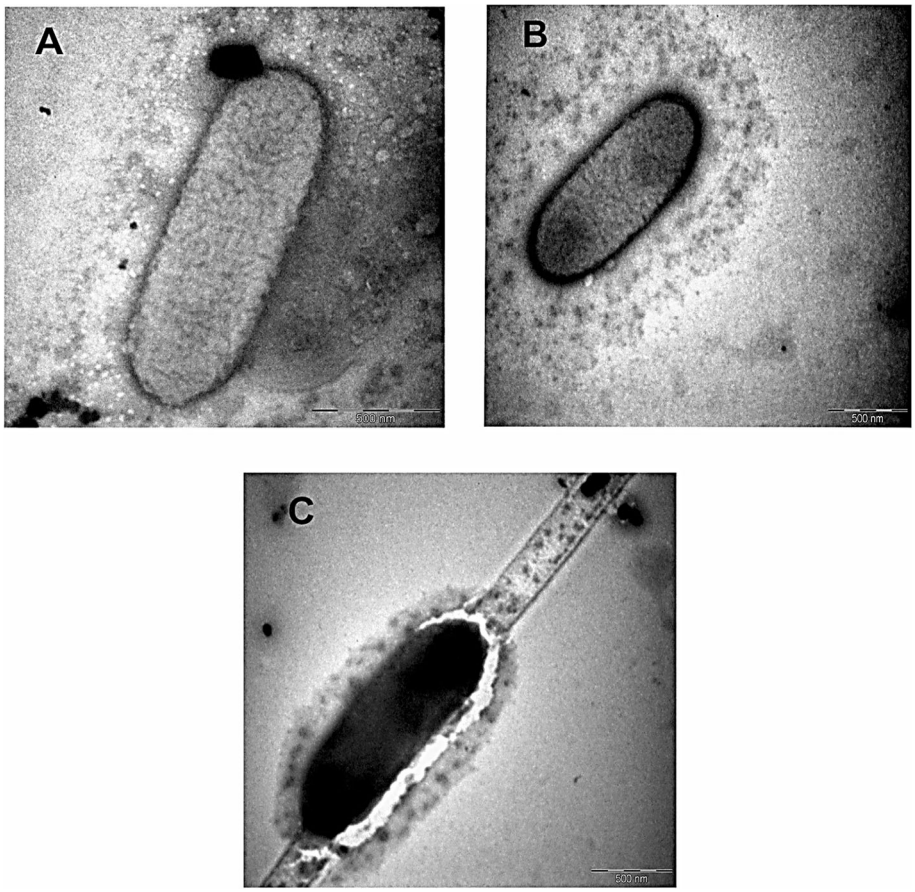

Fig. 7 Transmission electron micrograph of cobalt oxide nanoparticles (Co_3O_4) synthesized using *Microbacterium* sp. MRS-1. **a** Large pentagon-like crystalline cobalt oxide nanoparticles were bound on the cell wall at one pole of the bacteria under cobalt containing environment for 24 h. **b** Cobalt oxide nanoparticles (Co_3O_4) were synthesized and accumulated all over the bacterial cell. Arrow indicates the synthesized cobalt nanoparticles around the bacterial cell, and it can be clearly seen that the cobalt nanoparticles were deposited as thick layer on the cell wall of the bacteria for 48 h to the metal environment. **c** Fully mineralized bacterium encrusted with cobalt oxide nanoparticles observed at 72 h of exposure to the metal environment

75 mg/ml and approximately 50% decrease in cell viability at 100 mg/mL which showed concentration-based cytotoxicity at very minimum level when compared with previous cytotoxicity studies on nanoparticles.

4 Discussion

Extracellular production of less soluble cobalt oxide nanoparticles in the form of pale pink color powder has been observed in agreement with the previous reports for nanoparticle production using *Geobacter sulfurreducens* and *Brevibacterium casei* [26, 27]. FT-IR experiment was performed to study the functional groups of the cobalt nanoparticles. It showed absorption peak at 846 cm^{-1} which indicates the presence of C–H bending vibration (data not shown). Also, it showed a sharp single peak at 694 cm^{-1}, indicates the presence of optical vibrations of cobalt oxide which has been reported in the previous study [28]. From the surface topography of the cells incubated with cobalt chloride during 24 to 72 h, we observe deformation in the cellular structure due to increasing exposure time to cobalt ions. It is reported that the deformation of the cells is a kind of stress response to protect themselves against high concentrations of metal ions [24]. Previous study showed that cobalt oxide (Co_3O_4) nanoparticles exposure did produce cytotoxicity at the concentration of 25 μg/mL after 6 h in human small airway epithelial cells [29]. Obtained results showed that high exposure of biologically produced Co_3O_4 nanoparticles affects the cell viability at a very minimum level as revealed by cell viability assay.

5 Conclusion

The present study demonstrated the feasibility of *Microbacterium* sp. MRS-1 cells to synthesize extracellular cobalt oxide nanoparticles in powder form, while remediating cobalt in culture media. The extracellularly

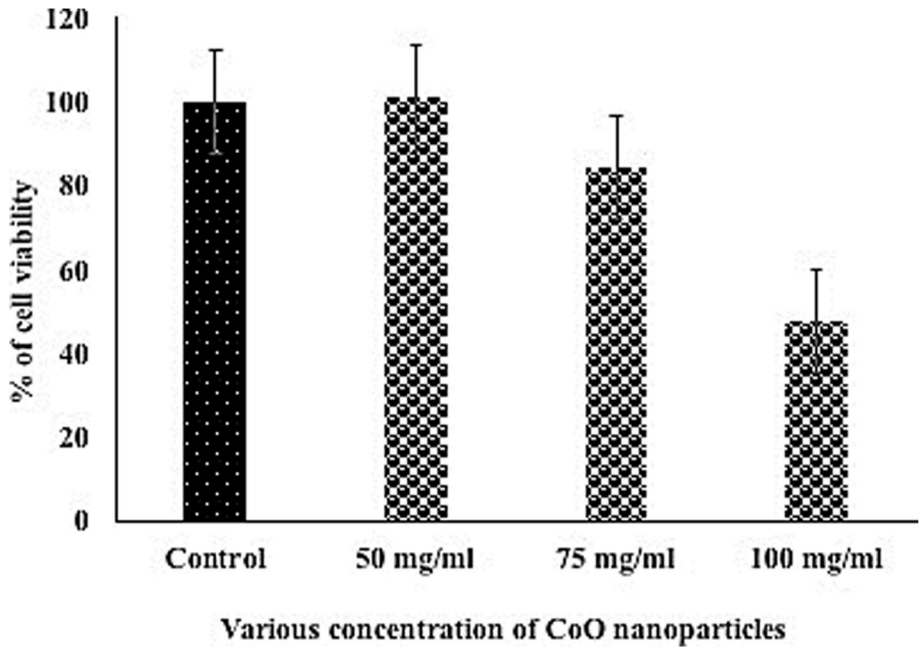

Fig. 8 MTT Assay-Percentage of cell viability L929 cells were recorded at 24 h exposure of Co_3O_4-NPs. Cell viability at 50, 75, and 100 mg/mL of Co_3O_4-NPs exposed for 24 h with control cells without bacterially synthesized Co_3O_4 nanoparticles

synthesized cobalt oxide nanoparticles were easily recoverable and can be easily utilized for industrial applications as it has low level of cytotoxicity. This study provides the simple eco-friendly way to remediate environmental contamination of cobalt and the production of cobalt oxide nanoparticles for clinical and other industrial applications.

Abbreviations

Co (II): Cobalt ions; $CoCl_2$: Cobalt chloride; Co_3O_4: Cubic face-centered; NPs: Nanoparticles; UV-DRS: Ultraviolet-diffused reflection spectra; FTIR: Fourier transform infrared (FTIR) spectroscopy; XRD: X-ray diffraction; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy; AAS: Atomic absorption spectroscopy; AFM: Atomic force microscopy; EDAX: Energy-dispersive X-ray spectroscopy; TGA: Thermogravimetric analysis; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; JCPDS: Joint Committee on Powder Diffraction Standards; C-H: *Carbon-hydrogen bond*; CO_2: Carbon-dioxide; d H_2O: Distilled water; H: Hydrogen; DMEM: Dulbecco's modified Eagle's medium; FBS: *Fetal bovine serum*

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Authors' contributions

Sathyavathi Sundararaju (SS): planning, experiment performance, writing the manuscript. Manjula Arumugam (MA): performed few experiments, major corrections. Prakash Bhuyar (PB): provided valuable suggestions to improve the quality. All authors read and approved the final manuscript.

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Author details

[1]Department of Pathology, Sidra Medicine, Doha, Qatar. [2]Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India. [3]Algae Culture Collection Center & Laboratory, Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia.

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