Isolation and Characterization of Magnetite Producing Bacteria from Fresh and Marine Water Sediments of Pakistan

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ABSTRACT

Objective: It is reported that only 5% of all the microorganisms that existed have been cultured and 95% have not been discovered yet or remain uncultivable. In recent times, it is an emerging field to isolate different types of bacteria that have the property to accumulate biominerals intracellularly as iron nanoparticles in the form of ferric oxide (magnetite) that can be used instead of artificially producing iron nanoparticles. Most of them belong to the alpha-Proteobacteria, beta-Proteobacteria, and gamma-Proteobacteria classes.

Methods: In the current study, the samples of fresh and marine water sediments were analysed for the presence of magnetite producing bacteria. The modified iron-containing medium was used to isolate and culture the bacterial strains. The attraction of culture present in the medium towards the magnet showed the presence of iron particles in bacterial strains while in another experiment, the turning of the medium colour from brown to black is also an indication of iron particles in the medium. The hanging drop technique also confirmed the presence of magnetite-producing bacteria. The extracted magnetite from isolated strains has unique crystal morphology. The extracted magnetite was characterized by Fourier transform infrared spectroscopy, X-ray powder diffraction and Scanning Electron Microscopy.

Results: The results of the study showed that the isolated produced magnetite and screening was confirmed by hanging drop method applied under magnetic field. The analytical technique like FTIR showed Fe-O stretching and bending vibrations between 550 and 650 cm^{-1}. The XRD peaks at 101 at 19°, 311 at 25°, 400 at 30° and 511 at 37° representing the presence of magnetite (Fe_2O_3) in the sample and SEM analysis showed the morphology of the magnetites.

Keywords: Magnetite producing bacteria, Biomineralization, Fourier transform infrared spectroscopy, Scanning Electron Microscopy.

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1. Introduction

Different microelements like iron, sulfur, magnesium, etc. are used by bacteria that act as important cofactors for different processes in their life cycle. Iron is an important microelement that influences the wide range of different cellular processes in the bacterial cell. These processes include photosynthesis, DNA biosynthesis, membrane energetics, H_2 production, nitrogen fixation, consumption, and oxygen transport. The presence of iron oxide molecules in the bacterial cell makes them unique and important as they behave like little magnets [1, 2].

Many microorganisms can produce different types of minerals intracellularly and this ability helped the scientists in understanding the evolutionary trends of bacterial phylogeny [3]. There is a well-understood example of magnetite producing bacteria that can sense and align along with the geomagnetic field [4–6]. Magnetosomes are biologically produced magnetite nanoparticles. These iron nanoparticles can be used instead of artificially or chemically produced magnetite nanoparticles and this attribute make them ideal for use in biotechnological purpose [7–9]. The process of biomineralization helps bacterial cells to use the iron present in the environment and convert it into magnetite nanoparticles and accumulated them in cells. Different types of mechanisms are used for the assimilation and utilization of iron present in the bacterial cell [8, 10, 11].

Different techniques and conditions are used in the process that helps in culturing these microbes. Different conditions provided to the bacterial cell cultures such as iron and sulphur contents, oxygen availability, nitrogen and carbon sources in medium, pH, and temperature could help in accumulating the iron oxide nanoparticles in cells [12]. The resemblance of the characteristics of magnetite producing bacteria with other bacterial strains of Magnetotactic bacterial groups of different classes shows that they have a wide range of diversity in the classification of microbiology [13–15].

In this study, the samples of fresh and marine water sediments were analysed for the presence of magnetite producing bacteria. The morphological and magnetic behaviour of the selected bacterial strains were characterized and studied. The biochemical and Physico-chemical characteristics of magnetite producing bacteria were also studied. The Magnetite producing bacteria were analysed

for the qualitative enzyme assays, which can provide insight for further study. The extracted magnetite was characterised by Fourier transform infrared spectroscopy and Scanning electron microscopy.

2. Materials and Methodology

2.1. Sample collection and Growth medium preparation

About 200 ml of samples of water sediments were collected from the freshwater stream of village Rumli (Islamabad) and River Swat (Saidu Sharif), while marine sediments were from the Arabian Sea (Karachi). The sample jars with tight lids were incubated for 15 days in dim light at room temperature with a strong magnet attached to the sample jar. This method is known as the magnet enrichment technique.

A modified iron growth medium was used for the growth of selected magnetite producing isolates. The composition of modified iron growth medium was tryptone (5 $g.L^{-1}$), beef extract (3 $g.L^{-1}$), sodium chloride (5 $g.L^{-1}$), ferric citrate (20 $ml.L^{-1}$), and Agar 2% in 100 ml of distilled water [16–18].

2.2. Identification of Magnetite producing Bacteria

The hanging drop technique was used to observe the movement of bacterial culture under the light microscope by using a magnet. According to Lefèvre and Bazylinski in 2013, the hanging drop method was applied to confirm the magnetite producing bacteria in water sample and they will show movement to the magnet direction. The modified iron broth medium was inoculated with the selected bacterial strains and incubated for 15 days, the colour of the medium changed from light brown to dark brown or blackish that indicated the presence of iron in the medium [2].

2.3. Molecular identification of selected strains

1. DNA was partially sequenced for 16S rRNA gene by Macrogen, Inc., Seoul, South Korea after the extraction of DNA by the Phenol/chloroform DNA extraction method. The 16S rRNA primer of selected bacterial strains were analysed for the similarity index in NCBI using BLAST software. The Phylogenetic trees of selected bacterial strains were constructed using MEGA 7 software [18, 19].

2.4. Assessment of Physico-chemical and Enzymes activities

The physical properties such as temperature and pH with incubation time were optimized for the cultures. The growth was analysed at five different temperatures (25, 30, 37, 45, and 50°C) and pH values (3, 5, 7, 9, and 11) for 5 days [20]. The biochemical parameters such as oxidase, catalase, citrate, indole, MR/VP and triple sugar iron were estimated for the growth of isolates. The isolates were analysed for the enzyme's activity qualitatively. Four enzyme assays (amylase, protease, lipase and cellulase) were performed for the presence and absence of various enzymatic activities by isolates [21].

2.5. Magnetite Extraction

The selected bacterial culture of 4 strains (RY-MS-3, RY-1b-4 and RY) were suspended in modified iron growth medium broth in separate flasks and were incubated at 37°C in a shaking incubator

for 20 days. After this, the broth containing the bacterial culture was centrifuged at 8000 rpm for 15 minutes at 4°C. Then sterilized buffer solution was added to each sample of bacterial culture. 2 mL of phosphate buffer solution was added to all the samples of centrifuged suspension bacterial culture and sonicated it for 120 minutes at 30 W. Now the suspension was left for about 7 days to settle down the magnetite crystals. The samples were re-suspended in phosphate buffer solution and were stored at room temperature [22, 23].

2.6. Characterization of Magnetite

Fourier Transform Infrared (FTIR) spectroscopy

The extracted magnetite nanoparticles were then characterized by using the ATR-FTIR (Parkin Elmer spectrum 65 FTIR) spectrometer. This analysis was used for the structural analysis of the purified compound. The extracted magnetite after drying out overnight was analysed by FTIR. The samples were then scanned from 4000-400 cm^{-1}. The spectrum was recorded using an attenuated reflectance technique involving diamond crystals [20, 24].

X-ray powder diffraction (XRD)

X-ray powder diffraction (Xpert Pro. PANalytical) was used to identify the phase of the crystalline object and provide details about the size of the cell unit. The phase and purity of the extruded magnetosomes were tested by X-ray diffraction (XRD) at a distance of 2θ [18, 25, 26].

Scanning electron microscopy (SEM)

The surface topology of extracted magnetite nanoparticles was examined by scanning electron microscopy (JSM5910, JEOL, Japan). Magnetite nanoparticles were washed with distilled water thoroughly and mounted on copper stubs with gold paint to view the topology of the sample [18, 27].

3. Results

3.1. Isolation of Magnetite producing Bacteria

Ten isolates were isolated from freshwater samples from Rumli village (Islamabad) and Saidu Sharif Swat (Swat River). The isolated strains were separately inoculated on a modified iron growth medium. Three out of ten strains showed magnetic behaviour in the magnetic enrichment technique and hanging drop technique.

3.2. Identification and characterization of Magnetite producing Bacteria

The morphological characteristics of Magnetite producing bacteria were analysed by Gram staining technique (Table 1).

Table 1. Gram staining and Magnetic assessment test of selected isolates.

Strains	Gram Nature	Cells Shape	Magnetic assessment test	Colour of medium
RY-1b4	Negative	Cocci	Positive	Changed to dark brown
RY-MS3	Positive	Rods	Positive	Changed to dark brown
RY	Negative	Cocci	Positive	Changed to dark brown

3.3. Magnetic assessment of Magnetite producing Bacteria

The strains showed a movement towards the magnet in all following three ways:

The cultures inoculated into the modified iron growth medium broth showed a positive response toward the magnets and moved in the direction of magnets (Fig. 1). The hanging drop method showed the positive response of cultures towards the attached magnets to the slide. It showed the movement of the cells in the direction of magnets. The colour of the medium was converted from light brown to dark brown that indicated the presence of iron in the medium (Table 1).

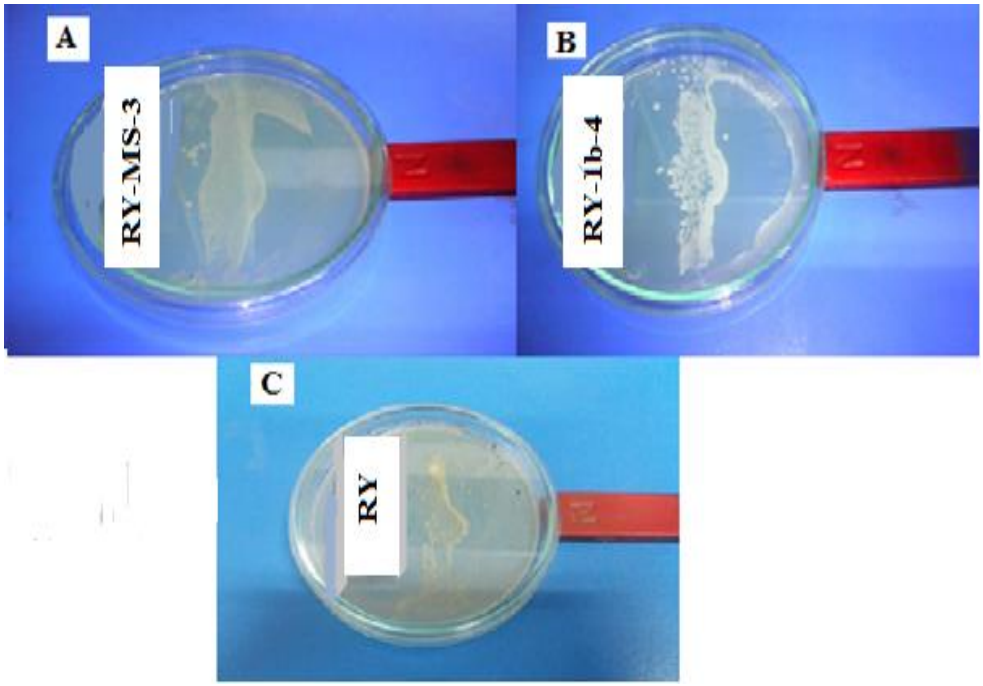


Fig.1. Magnetic assessment of Magnetite producing Bacteria on Petri plates. (A) RY-MS-3, (B) RY-1b-4, (C) RY.

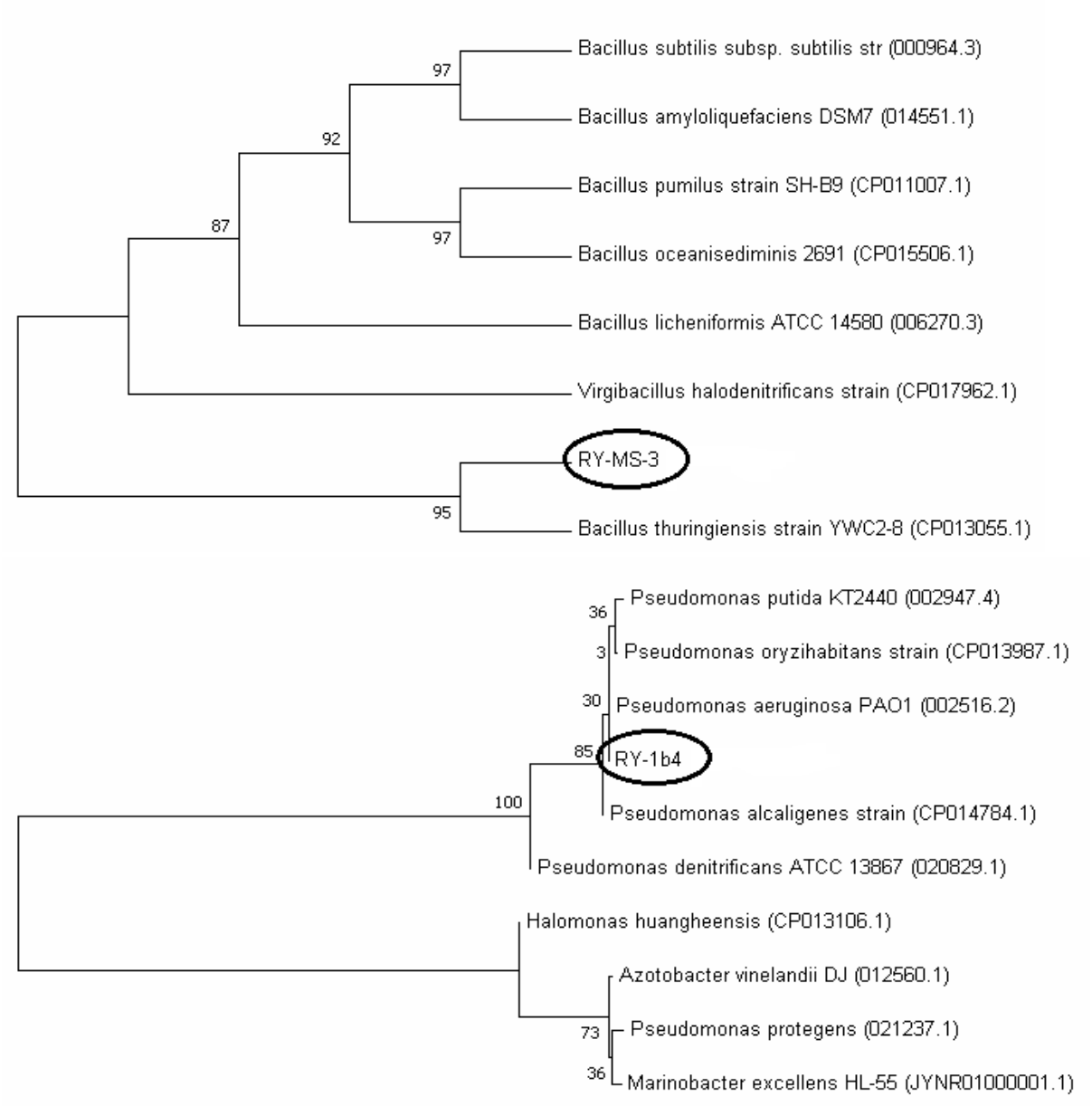
3.4. Molecular identification through 16S rRNA gene sequencing

The 16S rRNA sequences of selected strains were compared in the GenBank databases, using the NCBI BLAST software with other bacterial sequences. The obtained aligned bacterial sequences were then used to construct a phylogenetic tree by the neighbour-joining method using MEGA 7 software.

- RY-MS3 showed 97% identity with *Bacillus sp.* (*Bacillus subtilis*)
- RY-1b4 showed 100% identity with *Pseudomonas aeruginosa*
- RY showed 99% identity with *Escherichia coli*

Table. 2. Identification and Accession numbers of selected strain.

Strain names	Identity	Accession numbers	Locations	Isolation methods
RY-MS-3	*Bacillus thuringiensis*	MK332439	Rumli village	Magnetic enrichment method
RY-1b-4	*Pseudomonas aeruginosa*	MK332438	Sawat River	Magnetic enrichment method
RY	*Escherichia coli*	ON661873	Arabian seawater	Magnetic enrichment method



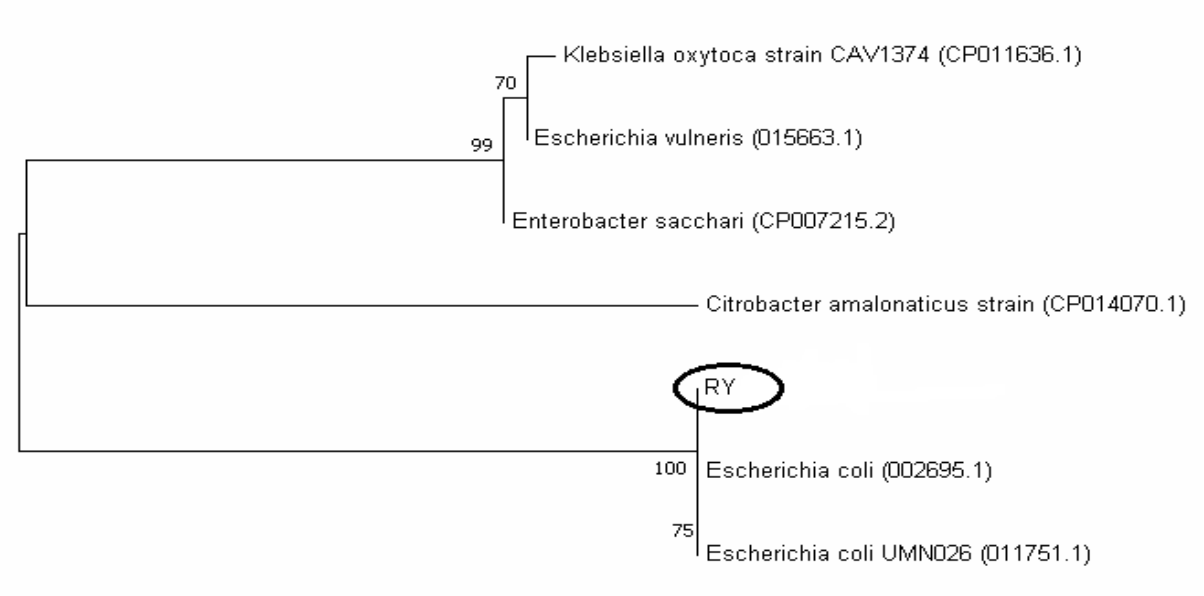


Fig. 2. Phylogenetic trees of selected magnetite producing bacterial strains.

3.5. Biochemical and Physico-chemical characteristics of isolates

The biochemical characterization and enzymatic study of the isolated strains were shown in tables 3 and 4.

Table. 3. Biochemical and Physico-chemical characterization of selected strain.

Characteristics	RY-1b4	RY-MS3	RY
Catalase	+	+	+
Oxidase	_	_	+
Citrate	+	_	+
Indole	_	+	_
MR	+	+	+
VP	_	_	_
Sugar fermentation	_	+	+
Temperature (°C)			
25	+++	+++	++
30	++	+++	+++
37	++	++	+++
45	_	_	+
50	_	_	_
pH			
3	_	_	_
5	++	++	++
7	+++	+++	+++
9	+	+	+

11	_	_	_

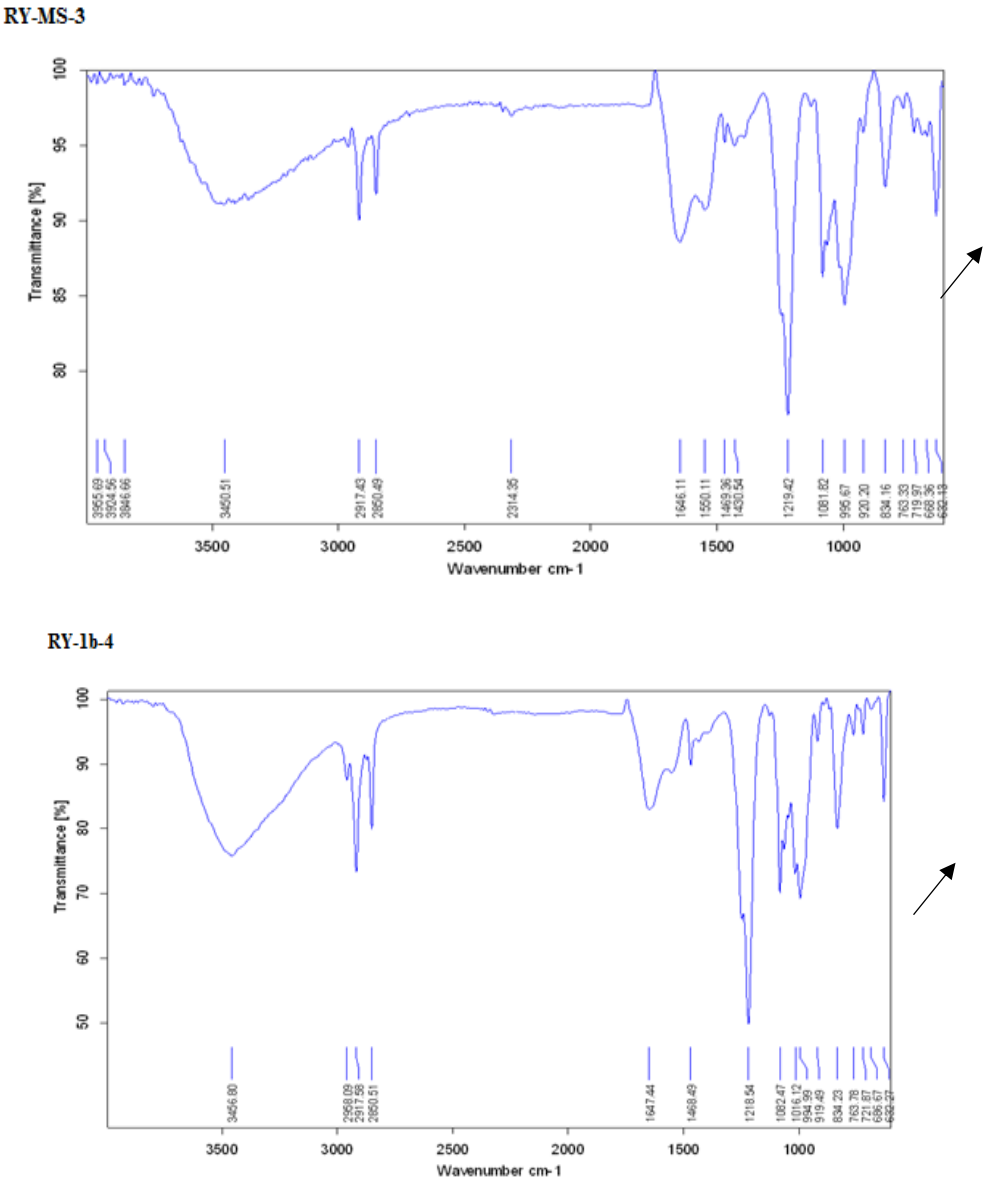
Table. 4. Qualitative screening for enzymatic activities of isolated strains.

Strains	Amylolytic	Proteolytic	Lipolytic	Cellulytic
RY-1b4	+++	+++	_	+++
RY-MS3	_	+++	+	+++
RY	_	_	+	+++

3.6. Characterization of Magnetite

Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectra showed the transmission bands for the magnetite (Fe_3O_4) groups. Fe-O stretching and bending vibrations between 550 and 650 cm^{-1} were observed for the samples as represented in Fig. 3.



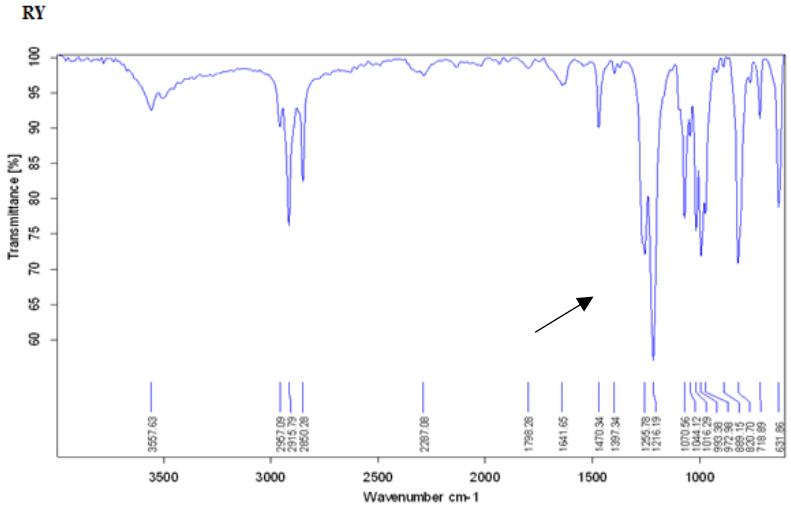
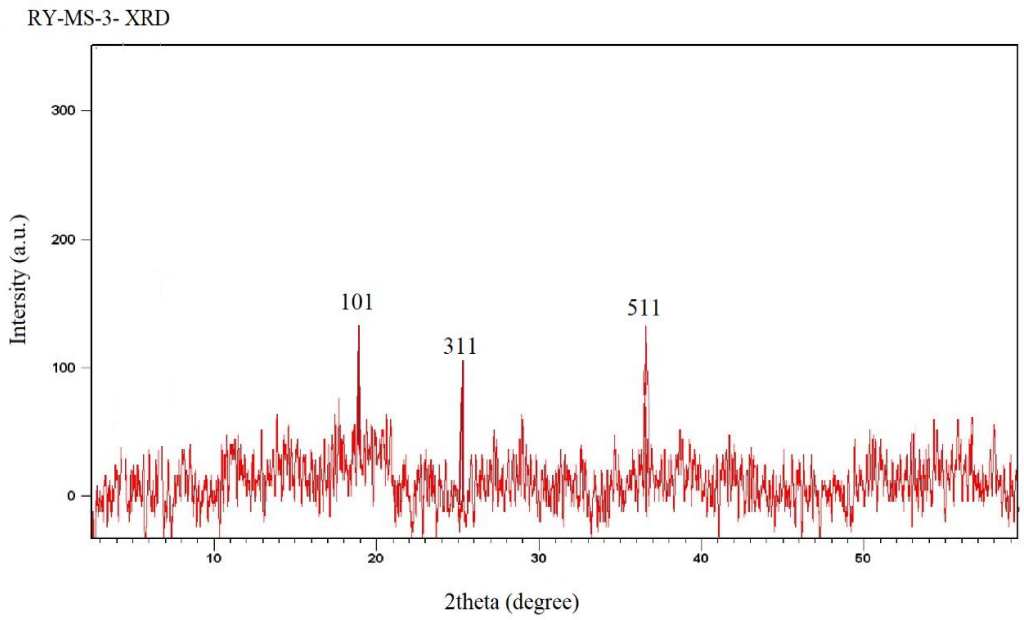


Fig. 3. FTIR spectrum of Magnetite sample produced in Modified iron-containing medium extracted from bacterial strains (1) RY-MS-3 (2) RY-1b-4 (3) RY

X-ray powder diffraction (XRD)

The diffraction pattern in Fig. 4 showed the entire indication of magnetite present in samples. The peaks at 101 at 19°, 311 at 25°, 400 at 30° and 511 at 37° representing the presence of magnetite (Fe_2O_3) in the sample. The presence of sharp, deep and wide peaks ensured the crystal formation present in the samples.



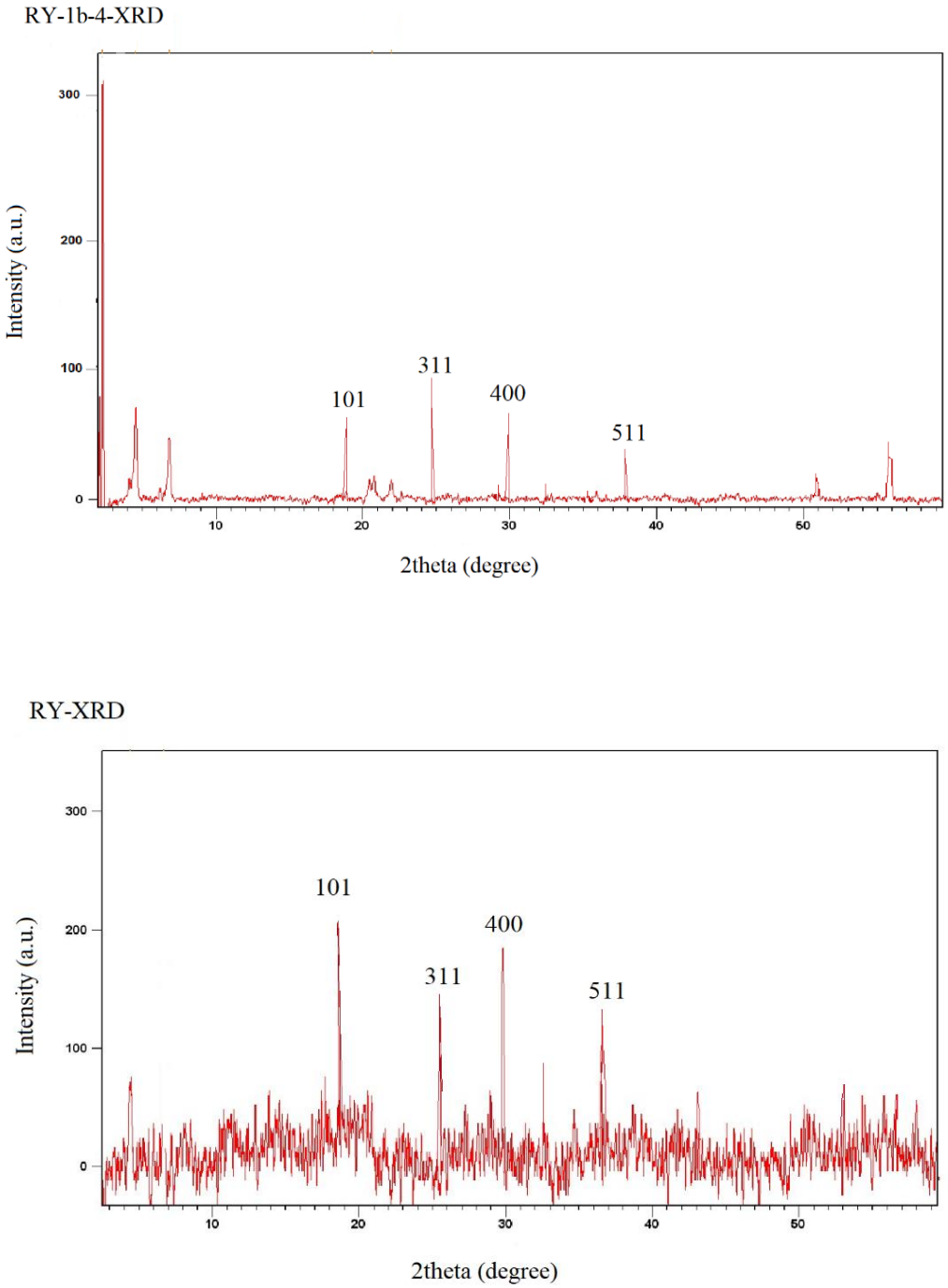


Fig.4. *X-ray powder diffraction patterns of extracted magnetosomes from* RY-MS-3, RY-1b-4 and RY strains.

Scanning electron microscopy (SEM)

The scanning electron microscopy showed the topology of magnetite produced by RY-MS-3, RY-1b-4 and RY (Fig. 5).

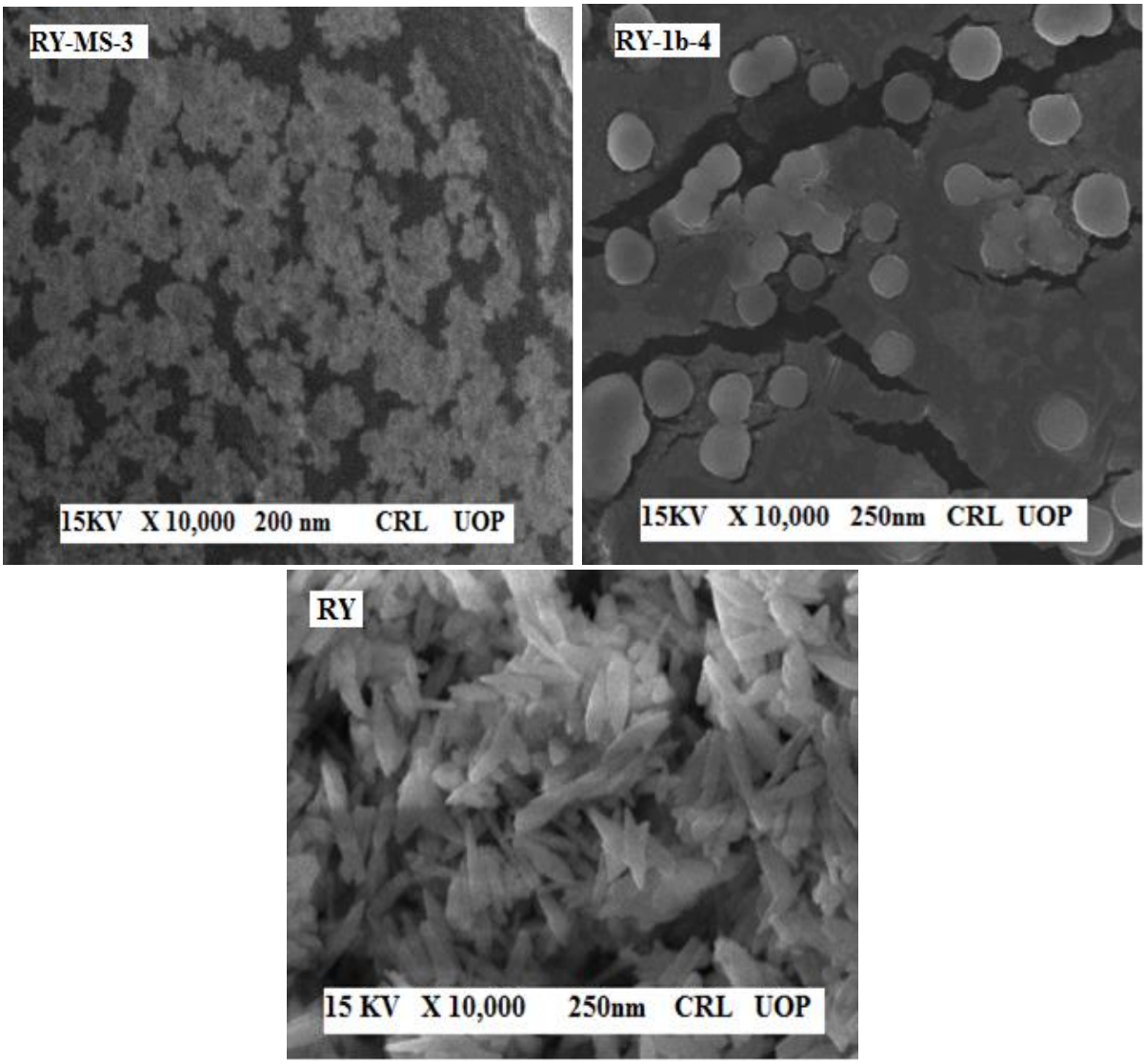


Fig. 5. Scanning electron micrographs of magnetite nanoparticles from RY-MS-3, RY-1b-4 and RY strains.

4. Discussion

The strains RY-1b-4, RY-MS-3, and RY showed positive response toward the magnet and a clear movement of cultural strain towards the magnet was observed and the growth was confirmed towards the walls of the test tubes that were set with bar magnets aside after about 1 week of incubation time (Fig. 1). It was reported that a significant change in the colour from light brown to blackish colour of broth culture occurred due to the precipitation of Fe_3S_4 compounds by sulphate reduction [28, 29]. In this study, during the magnetic assessment, a change in the colour of broth from light brown to blackish (dark brown) was observed and confirmed (Fig. 1). By using the hanging drop method with the magnetic field, the MTB isolates were observed microscopically and confirmed their movement in the direction of the magnet. It was reported about the bacterial strain *Acidthiobacillus ferrooxidans* has the property of producing magnetosomes inside its cell. *Acidthiobacillus ferrooxidans* is an autotrophic and aerobic bacterium and it was easy to culture [22,

30]. It was observed first time that *Acidthiobacillus ferrooxidans* was a weak Magnetotactic bacterium and detection of the intracellular magnetosomes showed its magnetic response. In one report, it was stated that *B. subtilis* strains have the potential for the extracellular biosynthesis of Fe_3O_4 nanoparticles [31, 32]. While in another report, that *Pseudomonas* sp., has also developed the property of producing the smallest nanoparticles of iron or its oxide. *Pseudomonas* sp. belongs to the order Gamma-Proteobacteria and recently a novel species of MTB was identified in this order [33, 34]. It was reported in 2012, that *Pseudomonas* sp., formed the iron nanoparticles of Fe_2O_3 of an average size of 200 nm [35, 36]. In the current study, the isolation of *Bacillus subtilis* and *Pseudomonas* sp. showed the same responses toward the magnet as having the magnetosomes [37, 38]. In the current study, the production of iron in culture media could be due to *Bacillus* sp. But as very few species of magnetosomes producing bacteria were identified up till now, there was a possibility that these species like *Bacillus* can produce magnetosomes intracellularly like MTB. While bacteria have *fur* genes that were responsible for regulating the protein named Fur protein that induced the production of magnetite nanoparticles (Magnetosomes) in bacterial cells [11, 39, 40]. These proteins were also present in γ-Proteobacteria such as *E. coli* and *Pseudomonas aeruginosa*. The storage and utilization of iron uptake from the environment were directly or indirectly regulated by Fur protein. A Magnetotactic bacterial strain named *Magnetospirillum gryphiswaldense* also has the same *fur* genes and Fur protein [36]. There was also a mechanism for producing siderophores. Siderophores were relatively low-molecular-weight Fe (III) specific chelating compounds that are mostly produced by prokaryotes under low concentrations of iron in medium conditions. *Escherichia coli* was an example of siderophores producing microbes [36, 41].

In the current study, the analysis of biochemical characteristics of isolates was performed to check their resemblance to MTB. The results matched with the previously reported studies on MTB. The microorganisms that existed in the oxygenated settings, produce catalase enzymes to neutralize the toxic forms of oxygen metabolite as H_2O_2 [42, 43]. It was reported in 1991, *Magnetospirillum gryphiswaldense* MTB was catalase and oxidase-positive and motile. Catalase-positive bacteria included facultative anaerobes also, which confirmed the results of the catalase test in previous studies [44]. In another report, the MTB does not produce cytochrome oxidase and are oxidase negative. Three isolates produced negative results for oxidase in the current study, which were supported by the findings of the previous study [45, 46]. In the current study, nine isolates confirmed the negative results for indole production. This finding was supported by Blakemore and Maratea (1981), who reported that *Magnetospirillum magnetotacticum* showed negative results for indole. It was also reported that *Aquaspirillum magnetotacticum* was observed negative for the triple sugar iron test which perfectly resembled the current study in which five isolates out of ten confirmed the previous study and had negative results for the triple sugar iron test [47]. The positive results differentiate bacteria from other bacteria based on the ability to reduce sulphur and ferment carbohydrates (Table. 3). In the current study, the enzymatic activity assays were also performed for the isolated strains. In amylolytic activity, five isolates did not hydrolyse the starch

and showed negative results, which showed that these isolates did not possess an amylase enzyme to degrade the starch. Three isolates (RY-1b2, RY-1b4 and RY-MS2) showed great activity for starch degradation. In the present study, nine isolates showed positive results for the cellulose activity assay. Cellulase enzyme was an industrially important degradative enzyme that plays an important role in detergents (Table. 4).

Temperature and pH were the important parameters that greatly affect the growth of microorganisms. All the isolates showed good growth at temperatures of 25°C, 30°C, 37°C, and 45°C. Five isolates were observed with maximum growth at 30°C and the log phase remained up to 96h of incubation, after which a clear decline was observed in the growth due to the depletion of nutrients in the growth medium and the production of secondary metabolites by bacteria. While from these findings, it can be concluded that the isolate can tolerate the high temperatures and can be thermophilic and the result can be related to the findings [48, 49]. It was reported in 2007 that magnetite producing bacteria grow best at 25-37°C. Magnetite producing bacteria present wide geographic distribution that's why the temperature range for these can be wide (Fig. 3, Table. 3) [25, 50]. It was reported that magnetite producing bacteria grow best at neutral pH. In the current study, the isolates were observed with a wide range of pH values, but most of the isolates showed a good growth rate at neutral pH 7. Neutral pH values support the biomineralization of iron in magnetite producing bacteria, as acidic and alkaline pH values interfere with the process (Fig. 3, Table. 3).

The FTIR analysis also showed a similar resemblance of peaks of iron oxide that appeared 550-650 cm^{-1}. All the spectra contained strong peaks at 633, 632, and 631 cm^{-1} that showed Fe-O vibrations of iron oxide. The strong peaks on 1016, 889, 834, and 833 cm^{-1} represented the Fe-OH group. It was reported that the Fe-O and Fe-OH group's peaks appeared at 570 and 890-1094 cm^{-1} respectively that showing a good resemblance with the current study results (Fig. 4) [47, 51]. In another study, it was also confirmed the FTIR peaks appeared for iron oxide at 629 and 631 cm^{-1} for *Iron sulfide magnetotactic bacteria* CT-K1 and *Bacillus cereus* TP6-4 respectively [24].

X-ray diffraction analysis showed the presence of iron oxide. 2θ corresponds to the scattered crystal angle, where on the sharp edge of the sample the clarity will be much higher. Elevations of 101 and 311 indicated the presence of iron oxide in the sample and the broad spectrum is due to the crystallinity of iron oxide. According to Bini et al. (2012), wavelengths of 311 showed iron oxide crystallinity. While according to Revathy et al. (2017) *Magnetospirillum gryphiswaldense* (MSR-1) produced magnetosomes and peaks appeared at 27.05°, 30.30°, 35.71°, 43.31°, 55°, corresponding to Fe_3O_4. In the present study, peaks showed similarities to 27° (311), 30° (400), and 38.5° (511). These results revealed that XRD analysis showed the presence of Fe_3O_4 in the sample. The magnetite particles produced by selected bacteria have provided good evidence for immediate use in future studies.

The SEM micrographs showed the shape and size of the biologically produced magnetite nanoparticles. It was reported the SEM analysis of the magnetite nanoparticles showed resemblance

to the topology of the magnetite produced by RY-MS-3. The bacterial strain *C. sinuosa* and *P. capillacea* synthesized iron oxide nanoparticles of different sizes between 20 nm to 500nm while the SEM images of these nanoparticles also showed the topology that resembled the iron oxide nanoparticles of RY-MS-3 and RY and also the sizes of 200 and 250 nm respectively [24, 34, 52].

Conclusion

We investigate that three bacterial strains *Bacillus* sp.*, Pseudomonas* sp.*, and E. coli* can produce magnetite particles in the presence of iron in the medium. The parameters temperature and pH were optimized to get a better yield of magnetite production. The analytical analysis like FTIR, XRD, and SEM also confirmed the production of magnetite in the bacterial cell.

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This work has been done in the Microbiology Lab, Department of Microbiology, Quaid-i-Azam University, Islamabad.

Conflict of Interest Statement

Authors report no conflict of interest.

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