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Characterization of Cultured Rod-shaped Magnetotactic*Betaproteobacteria*from Skudai River, Malaysia

Laila Muftah Zargoun[1], Nor AzimahMohd Zain[2*] and Shafinaz Shahir[2]

[1]Department of Microbiology, Faculty of Medical Technology, Ben Walid University, Libya
[2]Department of Biosciences and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti-TeknologiMalaysia, 81310 Skudai, Johor Bahru, Malaysia; Azimah@fbb.utm.my

Abstract

Objectives: Magnetotactic bacteria (MTB) represent a widespread group of phylogenetically, physiologically and morphologically aquatic prokaryotes with a distinct ability to migrate along the earth's geomagnetic field using intracellular magnetic organelles called magnetosomes (MSs). **Methods:** In this study, a new MTB, *Alcaligenessp*SUM 123 was successfully isolated from Skudai river in Johor Bahru, Malaysia. The bacterium was cultivated in enrichment growth media (GM) at room temperature 25 °C after isolation using a neodymium magnet. **Findings:** Phylogenetic analysis using 16S rRNAgenoe sequence revealed that the new strain SUM 123 of *Alcaligenessp*belongs to *Betaproteobacteria* class. The strain SUM 123 contained three or more round shaped, random distributed magnetosomes (MSs) of 80-120 nm in size observed under Scanning Transmission Electron Microscope (STEM). The minerals in MTB were studied by X-ray Energy Diffraction (EDX) analysis. Furthermore, the peaks that are attributed to the 220, 311, and 210 of MS were confirmed using X-ray Diffraction (XRD). **Application/Improvement:** The band around 580 cm^{-1} was related to Fe-O functional group of magnetite confirmed by Fourier Transform Infrared (FTIR) Spectroscopy.

Keywords: 16S rRNA, *Alcaligenes sp., Betaproteobacteria,*MagnetotacticBacteria,Magnetosomes

1. Introduction

MTBs are wide range of gram-negative prokaryotes that varied in morphologically from spirillum, rodid, vibriod, and coccoid[1–3]. They can generate intracellular magnetic minerals such as MSs that are aligned typically in their chain structure[4]. Characteristically, MTB are strongly geomagnetically sensitive. This property causes an increase in migration and alignment alongside the geomagnetic field lines[5]. Generally, MTBs are dispersed within the aquatic systems, in which an oxygen gradient exists, like stratified water columns or lacustrine sediments[6,7]. In these systems, they are capable of representing nearly 30% of natural bacterial communities[8]. The magnetotaxis model is formed based on an idea indicating that MTB use their MSs for the navigation of the oxic–anoxic interface in such a way that the ideal oxygen concentration can be found[9]. Moreover, their existence has been proved in a number of natural environments[3]. MTB characteristically exist in environments such as lakes[10], rivers[11], and in polluted water bodies and waste water produced through dyeing and printing processes[12].

Some investigations carried out by scholars[13], and[3], on the orientation and morphology of MSs, they stated that their structures have bullet, cuboid, cubooctahedron,

*Author for correspondence

elongated hexagonal prism shapes. Furthermore, these shapes are normally varied by two or more MSs per chain[14]. Note that in MTB, some other intracellular structures have been identified consisting of some elements like iron–phosphorus[15], phosphorus[16], poly-hydroxybutyrate[17], or sulphur[18]. In[19,20] maintain that due to their association with such different ions, it is possible that MTB have important effects in geochemical cycling.

For bacterial MSs, a number of commercial uses have been recommended, for example magnetic targeting of pharmaceuticals, hyperthermia, cell separation and contrast-enhancement agents in MRI[20]. Magnetotaxisrefers to the navigation of magnetotactic bacteria along the magnetic field; a phenomenon that can be usefully applicable to robotics. Hence, the detection, isolation, and culture of MTBs from various ecosystems require further investigation to better understand and increase their potential for future applications.

Most of the identified MTB generally correspond to the *Alphaproteobacteria* class, however, MSs-like inclusions and magnetic orientation have been identified in some representatives of the *Gammaproteobacteria* class[21], the *Deltaproteobacteria* class[19,20], *Nitrospira* phylum[22], and *Betaproteobacteria* class[23]. Numerous kinds of *Alcaligenessp*that belong to *Betaproteobacteria* class were reported with diverse applications, e.g., *Alcaligenessp* AM4 was capable of phenol degrading[24]. The strain H 16 of *Alcaligenessp*has generated poly-beta-hydroxy-butyrate (PHB)[25]. The *Alcaligenesfaecalis* GPA-1 was used for the production of thermostable extracellular α-amylase[26].

In this study, we isolated and characterized the new strain 123 of *Alcaligenessp* as magnetotactic bacterium. The bacterium was isolated from freshwater sediment of Skudai river and was found to be *Betaproteobacteria*. The characterization of the new strain of MTB and its MSs were carried out using Scanning Transmission Electron Microscope (STEM), X-ray Energy Diffraction (EDX), X-ray Diffraction (XRD), and Fourier Transform Infrared (FTIR) Spectroscopy. This will potentially serve as a new interest area for the detection, isolation and characterization of freshwater MTBs. In addition, the discovery of new MTB will improve the understanding, applications and utilization of MTB in diverse applications such as waste water treatment.

2. Methodologies

2.1 Magnetotactic Bacteria Collection and Isolation

The sediments and water samples were collected from the shallow edge of Skudai river (Johor Bahru, Malaysia), and then placed in tightly capped 0.5 litter glass bottle. The sample bottle was subsequently stored in a darkroom temperature (25 °C) for 2 weeks. This was followed by placing a neodymium magnet at the surface of the water for 2 hours to enhance the migration of the bacteria towards magnetic field. After that, a sterile pipette was carefully used to collect 1 mL of water sample from the container near the position of the magnet. Thus, the collected water sample was transferred into a 1.5 mL plastic tube. Later, a neodymium magnet placed near the end of tube for 3 hours. Approximately, 0.25 mL water containing MTBs was collected from the end of tube for further study[27]. The laboratory set-up for the isolation of MTB is presented in Figure 1.

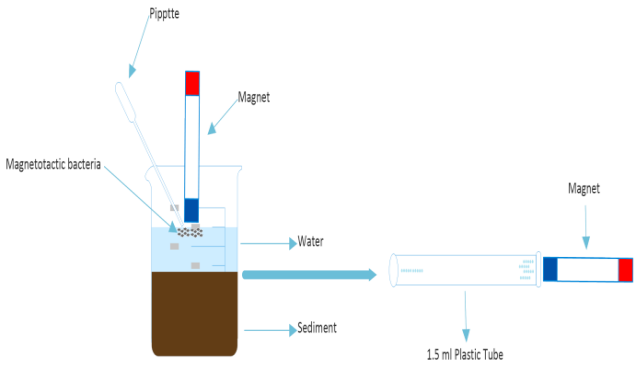

Figure 1. Set-up for isolating magnetotactic bacteria (MTB) using a neodymium magnet.

The concentration of iron and other metals of 0.5 g sediment sample was analysed using Atomic Absorption Spectroscopy (AAS) whereas temperature and pH were determined during the sample collection.

2.2 Purification of MTB

The bacteria isolated from the water sample was grown in a liquid growth media (GM)in the proportion of 1: 3 (v/v) containing;0.68 g of KH_2PO_4; 0.12 g of $NaNO_3$, 10 mL of Wolfe's vitamin solution, 5.0 mL of Wolfe's mineral solution, 2.0 mL of 0.01 M ferric quinate solution, 2 mg of resazurin,

0.05 g of sodium acetate, 0.05 g of sodium thioglycollate, 0.37 g of tartaric acid, 0.37 g of succinic acid per litter of distilled water[28]. The pH of the medium was adjusted to 7.0 with 5M NaOH before supplementing with 1.2% (w/v) agar in the growth media. After 2 weeks, the grown bacteria were transferred into the sterile agar media in Petri dishes and cultivated in the dark for 2 days at room temperature. Next, the colonies were isolated from the agar plates and streaked repeatedly to obtain pure colonies before inoculation in the growth media (GM)[29]. Lastly, the isolated magnetotactic bacterium colony was observed through microscope to confirm a pure bacterium that was subsequently maintained at 30 °C in screw-capped culture tubes containing sterile GM.

2.3 Identification of MTB

2.3.1 16S rRNA Gene Sequence Analysis

Genomic DNA was extracted from 2.5 mL of bacterium culture of strain SUM 123 with Promega Wizard® DNA Extraction Kit. Almost full-length of 16S rRNA gene was amplified from the DNA extract with specific primers 27F –AGAGTTTGATCMTGGCTCAG-3' and 1429R primer 5'-TACGGYTACCTTGTTACGACTT-3'[30]. The polymerase chain reaction (PCR) was run at (95 °C for 3 min) as initial denaturation; 30 cycles of denaturing (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 2 min) with final extension at (72 °C for 5 min). The PCR product was purified using *Qiagen* PCR purification kit. The total DNA was quantified at OD_{260} and OD_{280} with a Nano drop ND-1000 spectrophotometer. Next, the purified PCR was sent to Medi Gene Laboratories SdnBhd Malaysia for DNA sequencing before being analysed using the NCBI-BLAST program (https://www.ncbi.nml.nih.gov).

2.3.2 Biochemical Test

Several biochemical tests were carried out to further characterise the bacterium. These tests included catalase test, oxidase test, nitrate test, citrate test, urease test, motility test, and starch test[31].

2.4 Characterization of Magnetotactic Bacteria

2.4.1 MTB Iron Analysis

Cell pellets of the isolated magnetotactic bacteria and non magnetotactic bacteria (*Escherichia coli*) were harvested from media containing ferric quinate as iron source. Subsequently, about 1 mg of wet biomass was added to the mixture of sulphuric acid, perchloric acid and nitric acid solution in the ratio (19: 12: 1) and boiled to digest the cell. This was added to 1 mL of 1N hydrochloric (HCl) acid diluted with deionised distilled water (ratio 10: 90) before Atomic Absorption Spectroscopy (AAS) analysis to determine the iron content of the cell. Similar procedure was followed using *E. coli* as a control[32].

2.4.2 Scanning Transmission Electron Microscopy (STEM-EDX)

The whole cells for STEM were prepared by placing a drop of suspension onto a form var-carbon coated copper grid after dilution with sterilized phosphate buffer solution. This was accomplished without adding any negatively stains and before air drying. The grids were examined using a high resolution Scanning Transmission Electron Microscope (Model: Hitachi SU8020) operating at 20 keV. The elemental and chemical species present in the isolated MTB were detected using an Energy Dispersive X-ray (EDX).

2.4.3 X-ray Diffraction Analysis (XRD)

Dried sample of the isolated MTB was subjected to XRD analysis using the BrukerD8 Advanced X-Ray Diffractometer with Graphite-monochromatized Cu Kα radiation. The resulting diffractograms were recorded at 2θ from 20° to 80° with 10 s per 0.03 stepas described by[33].

2.4.4 Transmission Electron Microscope (TEM)

The TEM and HRTEM observation of single magnetite crystal was carried out using BIO-TEM HITACHI HT7700 at 120 KeV. The d-spacing were determined from the patterns of magnetosome crystal[34].

2.4.5 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The chemical structures of magnetosomes synthesized by SUM 123 was investigated using FTIR analysis in the range of 400-4000 cm^{-1}, by using Perkin Elmer model 2000[3,24,35].

3. Experimental Results

3.1 Isolation and Purification of the MTB

A new strain of *Alcaligenes sp.* was isolated and cultivated successfully in growth medium. The isolated SUM 123 grew fast at microaerobic condition. The purity of the strain was confirmed by microscopy after repeated streak on agar Growth Medium (GM). The motility of SUM 123 strain was also observed under light microscope. The bacterium appeared as rod shapes with 100x magnification.

In general, it is a difficult task to isolate and cultivate MTB in pure culture in laboratory. This is mainly because of the fact that they are fussy and redox sensitive[3, 24, 35]. The replication of isolation has been shown to be difficult; though, a number of MTB strains are accessible currently in pure culture. To some extent, this problem is due to lack of a detailed specification of isolation techniques and also unavailability of a reliable method in literature. This project is focused on conducting the magnetotactic bacteria isolation using growth medium containing ferric quinate as iron source that can facilitate the isolation of magentotactic bacterium strain SUM 123. The number of particular morphotypes of MTB is normally increased in those natural samples (microcosms) that are kept back under a dim light, in such a way that the photosynthetic organism overgrowth can be prevented and also they can be devoid of being mixed. This way, oxygen and possibly other chemical gradients can be stabilized in the sample[3, 24, 35]. Majority of known MTB like organisms require gradient, which reasonably grow within culture medium where there is an oxygen concentration gradient and a low concentration of nutrient. These types of cultures are designed principally for chemolithoautotrophs that have been shown applicable to the isolation of novel MTB strains. This is because fast growing heterotrophs surpass the fussy MTB in a richer medium that is comprised of organic carbon sources. Even in cases where MTB were the prevailing fraction in the inoculums[36], the method that was based on magnetically collecting the cells directly from the environmental samples with no additional purification mostly caused contamination. Therefore, the isolation of cultured strains of MTB can be done through formation of colony, occasionally in shake tubes; magnetic enrichment; and reiterated rounds of serial dilution to extinction[36].

3.2 Phylogenetic Analysis

The newly obtained 16S rRNA gene sequence was deposited in NCBI Gene Bank database (www.ncbi.nlm.nih.gov) under the accession number KR107950 for AlcaligenesspSUM 123. Figure 2 presents the phylogenetic tree of the SUM 123 strain of the isolated MTB constructed using the neighbour-joining method.

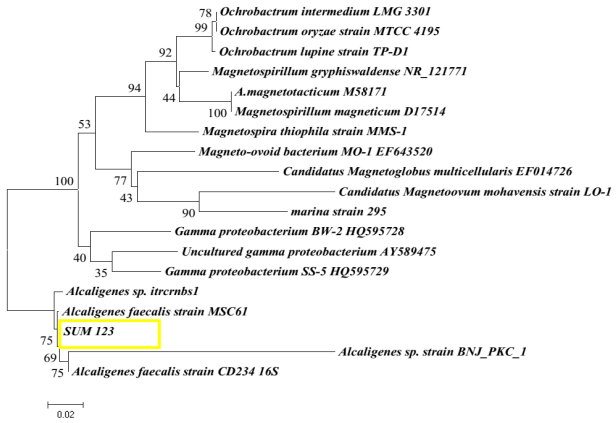

Figure 2. Phylogenetic tree of strain SUM 123 examined by 16S rRNA gene sequences.

The phylogenetic tree presented in Figure 2 was constructed using BLAST-Webpage and MEGA6 Software. The result of multiple alignments for phylogenetic tree showed SUM 123 strain was clustered with a bootstrap value of 500 and scale bar 0.02 substitutions per site. This was observed to be 99% similar to *Alcaligenessp,* which is related to the class *Betaproteobacteria*, phylum *proteobacteria*. The *Alcaligenessp* strain SUM 123 is a typical aerobic, short-rod to rod shape, Gram-negative bacteria that can grow fast in micro-aerobic condition. This study reported the first strain SUM 123 of *Alcaligenessp* that characterized as magnetotactic bacteria. In previous studies, most of magnetotactic bacteria belong to *Alphaproteobacteria*as marine vibrio strains MV-1[37], *Gammaproteobacteria*eg strains BW-2[38], *Deltaproteobacteria* such as *Desulfovibriomagneticus* strain RS-1[39], *Nitrospira* like strain MHB-1[22]. However, to date, the isolation and identified of MTB that related to *Betaproteobacteria* are so limited. Various *Alcaligenes* species were isolated from different sources and were applied in various application such as producing polyhydroxyalkonates (PHA)[25], as well as producing of siderophore[40].

According to the results of biochemical tests, the strain is an actively motile and capable of reducing nitrates, and positive for oxidase, catalase, and citrate yet negative for urease and starch activities.

3.3 Sediment Sample Analysis

The sediment separated from Skudai river Johor was analysed using AAS. The metals found include Fe, Mg, Al, Zn, Ni, As, and Na at various concentrations. The order of the concentrations of metals is Al> Na> As> Mg> Zn> Ni, 263, 45.63, 14, 4.148, 1.74, 0.337, 0.006 ppm, respectively. Therefore, the sediments serve as either carrier or tracers for heavy metal elements in aquatic environments. Riverine sediments play a significant role as pollutants stocks and reflect the history of river pollution[41]. A study of heavy metals in Skudai river using reed grass Phragmiteskarka revealed that the Skudai river system contained Zn, Cu, Ni, Cr, As, Pb, Hg, Cd, while sediment contained Zn, Cr, Cu, Pb, Ni, As, Hg (increasing magnitude of concentration)[42]. The findings by [42] are in good agreement with our findings especially As and Ni. However, our study reports additional metals (Na, Fe, Mg, Al) but the disparity arises from the presence of toxic heavy metals like Hg, Cd and Pb. The presence of the metallic elements indicates the distinctive ability of MTB to grow and survive in metals-contained environments.

3.4 Characterization of MTB

3.4.1 Iron Content in Strain SUM 123

The AAS results revealed that the *Alcaligenes sp.* SUM 123 contained more iron than the non-magnetotactic*E-scherichia Coli* (control) bacteria cell. The iron content of isolated bacteria was 8.270 ppm compared to 0.012 ppm for the *E. coli*. This is an indication that MTBs accumulate more iron than non-magnetic cells. The results further confirm the existence of a definite correlation between magnetic response and iron content of the cells.

3.4.2 Morphology of Magnetotactic Bacteria and Magnetosomes

The structure of MTB and its magnetosomes were observed by STEM. As shown in Figure 3, the rod shaped of MTB has dimensions of 1.50 × 2.09 μm while the round shaped MSs are 80-100 nm in size. In most cases, MSs of MTBs synthesized in aquatic environments are arranged in a chain or chains which form typical crystals. However, in this study the MSs appeared as single or randomly dispersed magnetosomes in the MTBs. This may be due to the absence of a gene capable of producing magnetosomes in chain. Recently, inspection of *Magnetospirillummagneticum*revealed that specific genes in bacteria play a significant role in the formation of MSs and regulation of the shapes and size of MTB crystals[43, 44].

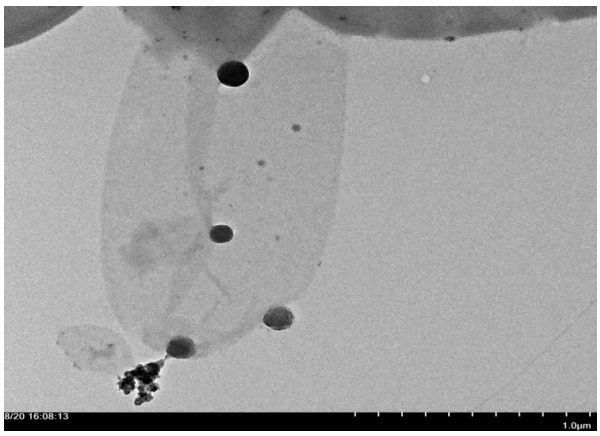

Figure 3. Scanning Transmission Electron Microscope (STEM) of isolated MTB that appeared with rod shape (Scale bar: 1μm).

Previous studies stated that the chain configuration of MSs are significantly influenced by the cytoskeletal acidic proteins filaments;[45]. The interaction of mamJ of a cytoskeleton like structure directs the assembly and location of the magnetosome vesicles. The occurrence of magnetosomes randomness in MTB is similar to those obtained from the uncultured coccus *Bilophococcusmagnetotacticus*.

3.4.3 Chemical Properties of MTBs

The use of STEM imaging showed that in the isolated cells, there were broad shaped, dark granule structures occupying a great fraction of the cell volume (see Figure 4(a)). EDX spot analysis was carried out upon various parts of the granule in the cell and findings showed that the granule comprised elements such as oxygen (O), phosphorus (P), aluminium (Al), carbon (C), sulphur (S), and zinc (Zn) in addition to very in significant quantity of yttrium (Y),iron (Fe), and chromium (Cr). Though, normally, MTB can be comprised of phosphorus and sulphur internal granules[16, 18], the phosphorus peaks were assumed to be originating from the cell background. Findings of the present research are confirmed by[46]. Both MSs and P-rich granules allowed MTB to separate the elements into various compartments. In addition, the

P-rich granules caused an increase in the storage of phosphates and toxic metals in MTBs[47]. On the other side, the collection and accumulation of other ions are also enhanced by the granules' unstructured nature[48]. Note that any function of aluminium in bacteria has not been found; although, a number of scholars have suggested that a function of p-granules can be the detoxification of metals, including aluminium[49]. More research should be carried out to explain the reason these bacteria accumulate metals ions in inclusion of cells. In a laboratory environment, cadmium, zinc, aluminium, strontium, and manganese have been created in the bacteria granules that have been exposed artificially to these elements[50]. The present study demonstrates that the dark granules can be naturally comprised of some amount of zinc, iron, aluminium, and arsenic, which is consistent with findings indicating that those heavy metals can occur in sediment samples from Skudai river sediment.

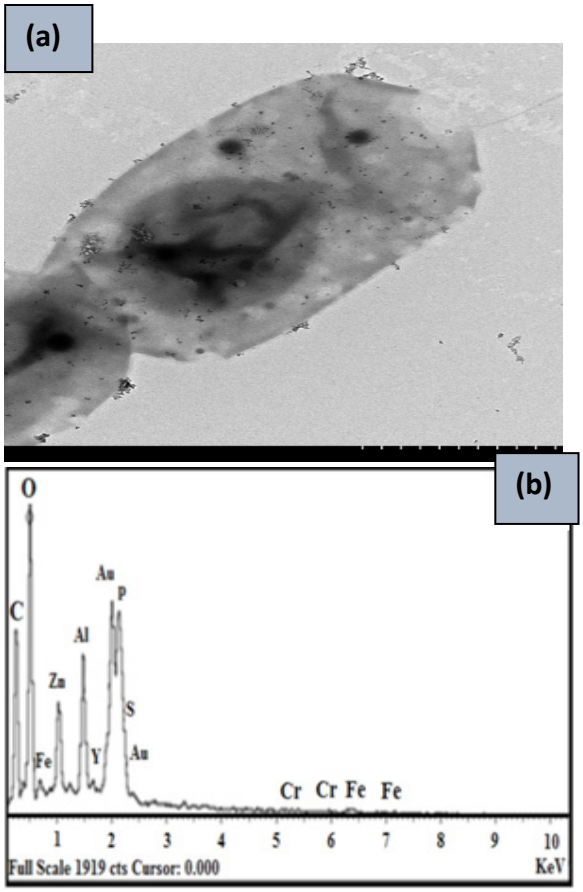

Figure 4. (a) STEM image indicating dark granules in MTB and (b) EDX spectra with detected elements; iron, aluminium, phosphorus, oxygen, carbon, zinc, chromium, silver, and yttrium.

3.4.4 Crystalline Structure Analysis

The HRTEM result verified the presence of the magnetite phase as the magnetosomes mineral in *Alcaligenessp* SUM 123. The high magnification of TEM image is presented in Figure 5(a). Figures5 (b) and 5(c) showed the crystalline character with lattice spacing of 0.24 nm and 0.31 nm which can be indexed to the (311) and (220) planes of magnetite, respectively. The results are in agreement with those reported elsewhere[41].

The phase purity and composition of MTB and magnetosome was evaluated by XRD and compared with the spectra data from previous studies. Figure 6 presents the XRD diffraction spectra showing the characteristic peaks of magnetite.

The main peaks observed at 2θ = 25.65°, 28.92°, 37.25°, 45.23°, 55.57°, 62.83° and 70.0° were attributed to the (210), (220), (311), (400), (422), (440), and (533) crystal planes of magnetite (Fe_3O_4). The XRD pattern of *Alcaligenessp* SUM 123 magnetosome was similar to those obtained elsewhere. The crystalline structure of magnetite phase is confirmed by XRD which is shown in the (311), and (220). The (311) indexes correspond to lattice spacing with 0.31 nm, whereas the (220) indexes to lattice 0.24 nm which were confirmed by the XRD analysis.

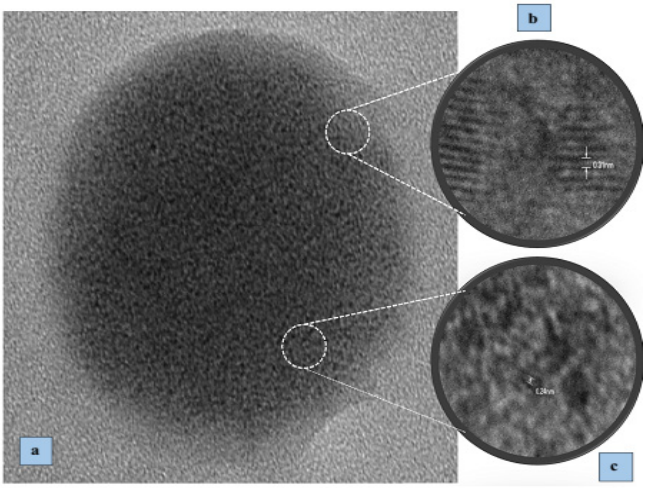

Figure 5. (a) transmission electron microscope of single magnetosome, (b-c) high resolution transmission electron microscope (HRTEM) of magnetosome.

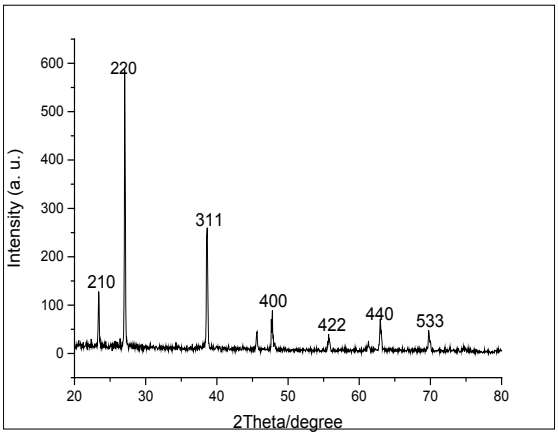

Figure 6. X-ray diffraction patterns of magnetosome.

3.4.5 Fourier Transform Infrared (FTIR) Spectroscopy

The characterization of magnetite within magnetosomes was done through displaying the characteristic peak that was centred at 580 cm^{-1} in Figure 7. As can be seen from Figure 7, the peak at 3280 cm^{-1} correspond to O-H stretching, the band at 2926 cm^{-1} is related to the CH group, the peak at 1634 cm^{-1} and 1530 cm^{-1} correspond to amide I and amide II bands of the protein peptide bonds, and finally, the peak around 1375 cm^{-1} is related to the COOH group, whereas the characteristic bands around 1054 cm^{-1} are correspond to the C-O stretching. Comparing FTIR spectra between[33] and MSs of the present research, it was revealed that the surface of the magnetosome exist carboxyl, hydroxyl and amino groups, which are the components of the magnetosome membrane[33].

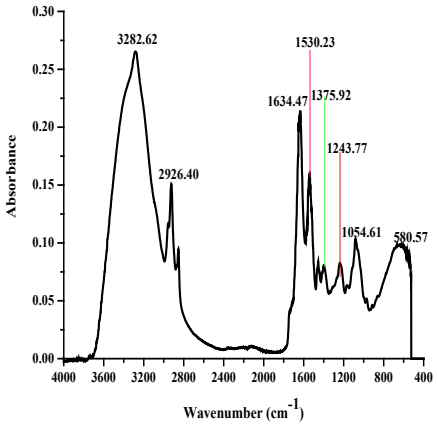

Figure 7. FTIR peaks are revealed to the appearance of functional groups of magnetosome membrane.

4. Conclusion

The study successfully isolated and characterized an *Alcaligenes sp.,* strain SUM 123 MTB, class *Betaproteobacteria,* from Skudai River in Johor Bahru, Malaysia. The morphology of the MTB revealed single rod structured and randomly dispersed magnetosomes devoid of the characteristic chains observed in other isolated MTBs. Elemental analysis showed presence of P-rich intracellular granules indicating the existence of P, K, Fe, Al, Cr, Y, Zn and oxygen in MTB. The mechanism of magnetic and other minerals accumulation together with intracellular crystal structures of MSs was also highlighted.

5. Acknowledgement

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