International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Isolation of Magnetotactic Bacteria from Freshwater Sources of Goa

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Abstract

This study focused on the isolation and characterization of magnetotactic bacteria (MTB) from freshwater sources in Goa. Samples were collected in sterile 1L plastic containers, pre-treated with 70% alcohol and sterile distilled water, and transported in biohazard bags. Water and sediment were collected in a 1:3 ratio and incubated undisturbed for two months at 25°C with a slightly loosened lid to promote microaerophilic conditions. The formation of a blackish layer indicated low oxygen levels.

Enrichment involved placing the containers under the influence of magnets. Aliquots from both poles were collected and subjected to CRT purification using a Pasteur pipette and a bar magnet to isolate MTB by attracting them to the pipette tip. This process lasted 30 minutes, after which the pipette tip was broken off, and the aliquot was collected with a sterile needle and syringe for inoculation onto Flies Media.

Isolates grown on the media exhibited varied morphologies and characteristics. Their motility and magnetotaxis were confirmed using the hanging drop method with a magnet placed at the edge of the drop. Gram staining and the KOH string test determined the Gram-positive/negative nature of the bacteria. The Hugh-Leifson test and Thioglycollate Broth Test identified their microaerophilic respiratory nature. All isolates showed nitrate reduction.

Further analysis revealed that 4 out of 6 tested isolates formed capsules. Siderophore production was observed in 5 isolates, indicated by a yellow halo. Exopolysaccharides (EPS) were produced by 4 isolates. Scanning Electron Microscopy confirmed rod-shaped morphology and revealed internal magnetosomes. XRD analysis identified perovskite as the elemental structure of the magnetosome.

The study highlights the largely unexplored potential of magnetotactic bacteria beyond their known bioremediation capabilities for sequestering organic pollutants, suggesting numerous avenues for future research and exploitation.

Chapter 1 Introduction

1.1 WHAT ARE MAGNETOTACTIC BACTERIA?

Magnetotactic bacteria (MTB) are gram negative and gram -positive bacteria that reside in fresh and marine waters. Magnetosomes, which are responsible for the cells' magnetotactic behaviour, also known as magnetotaxis, are biomineralized by these bacteria. They are morphologically, phylogenetically, and physiologically varied in nature. Coccus (spheres), bacillus (short or long rods), vibrios (curved rod), spiral (twisted), and even multicellular magnetotactic prokaryotes are the most common morphologies of MTB. Just one magnetosome mineral is biomineralized by the majority of MTB strains (Blakemore et al, 1975).

There are numerous cases where geology and biology intersect directly. Magnetotactic bacteria are maybe the greatest illustration of this. These are live organisms with the capacity to make small magnetite crystals in their cells. They do so by transporting iron from the outside world into their cells, where proteins biomineralize it into nano-sized magnets made of magnetite (Fe3O4) or greigite (Fe3S4). These aquatic critters employ magnets to align themselves with the Earth's magnetic field in a passive manner a compass, similar to how a compass could be used by an adventurer to find his bearings. Once oriented, bacteria can swim up or down in the water column to find a suitable environment using their polar flagellum (Dusenbury, David B, 1996).

1.2 HISTORY

It was first described in 1963, Salvatore Bellini of the University of Pavia. While analysing bog sediments under his microscope, he discovered a colony of bacteria that displayed a distinct pattern of movement. He named the microbes Magneto sensitive Bacteria because they travelled in the direction of the North Pole.. Further on, Richard P. Blakemore rediscovered MTB in 1974 having magnetosomes within cells of MTB. (Blakemore et al, 1975).

In his 1963 publication, Salvatore Bellini was the first to describe MTB (Bellini, 1963). Because his research was published in Italian, he is rarely recognised with their discovery. Richard Blakemore is most commonly credited with discovering MTB since he published an English-language article describing the disease in the magazine Science in 1975. (Blakemore, 1975). In Woods Hole, Massachusetts, these were found MTB in sediments near Eel Pond in a salt marsh (Blakemore, 1975). The ability of these species to manufacture magnetic particles comprised of magnetite (Fe3O4), greigite (Fe3S4), or both, within their cells, is their defining feature (Bazylinski et al., 1995).

These bacteria have the ability to respond positively to external magnetic fields and swim in their direction. The intrinsic mechanism of production of nano-sized magnetic particles inside the cells is responsible for this response. The microorganisms are made to function or behave like microscopic, self-propelled compass needles, aligning them with the magnetic field outside. An organic membrane surrounds each nanocrystal., which is referred to as a 'magnetosome.' (Lefèvre et al, 2011).

1.3 MECHANISM

The affectability of magnetotactic microscopic organisms by the fact that these microscopic organisms speed chains of precious stones of appealing minerals inside their cells contributes to the Earth's attractive field. To date, all magnetotactic systems have failed infinitesimal life forms are point by point to quicken either magnetite or greigite. MTB magnetotaxis can be classified into two categories: polar and axial. An axially oriented MTB travels back and forth in every direction, whereas a polar MTB moves in a preferred direction related to the local field. (Barber-Zucker et al, 2016).

Based here on magnetic crystal formations inside, polar MTB either are south- or north-seeking. North-seeking MTBs are found primarily in the northern hemisphere and travel towards to southern polar region of a bar magnet, while south-seeking MTBs are found primarily in the south pole and travel towards to the northern polar region of a bar magnet. The north- and south-seeking MTBs are commonly seen near the geographic equator, moving south and north respectively (Kopp et al, 2007).

The magnetosomes are present in the cell in the form of chains which align whenever there is a trigger of magnetic field in the vicinity. Present in these magnetosomes are mineral crystals which are magnetic in nature, covered by a phospholipid layer along with proteins that particularly present in Magnetotactic Bacteria. MTB are fastidious in nature and require specialized conditions to isolate and grow (Wang et.al, 2020).

1.4 CHARACTERISTICS of MTB

Magnetotactic bacteria are divided into 2 divisions based on their particle size such are: magnetite (Fe3O4), greigite (Fe3S4). The response of MTB depending on the magnetic field of the earth is due to intracellular enveloped magnetic particles which are of the microphilic environment and are membrane bound nanoparticles. The bacteria can either have one or both of the molecules depending on the environment and conditions they are exposed to. MTB cells possess upto – 3% iron in dry weight, ranging from 10–13 to 10–15 grams per cell (Vargas et. al, 2018).

Magnetotactic bacteria are classified into three categories based on the sort of magnetic mineral nanocrystal they produce: 1. greigite (Fe3S4) or 2. a combination of greigite and pyrite (FeS2), 3. a combination of magnetite and greigite.

These bacteria preferentially live (or settle) in the aerobic- non-aerobic transition zone (OATZ) where oxygen-rich water (or deposits) comes into contact with oxygen-poor water. Inside, bacteria produce a series of magnetic nanoparticles that act as nano-sized magnets, allowing them to settle and survive along the Earth's geomagnetic lines in both the Northern and Southern Hemispheres. Bacteria with higher than optimal oxygen levels rotate the flagella counterclockwise and move to OATZ. Bacteria in environments below ideal oxygen levels, on the other hand, rotate the flagella clockwise and swim backwards towards OATZ (Chen et. al, 2010).

1.5 HABITAT FOR GROWTH of MTB

The majority of MTBs currently known were obtained either saline or still waters (or sediment), and their genomes have helped researchers better understand and cultivate these microbes. Furthermore, genome sequencing has allowed researchers to investigate genes involved in magnetosome formation in order to develop magnetic particles for economic and clinical purposes in the future.

When trying to separate MTB from samples collected mostly MTB are often drawn to the southern end of a bar magnet in the Northern Hemisphere, but to the north end of a magnet in the Southern Hemisphere. Using a transparent plastic container for collecting sediment and water from such a natural source, such as a freshwater reservoir, is one of the most frequent ways to enrich MTB. At the sediment-water interface, the south end of a bar magnet is positioned against the outside of the container just above the silt.. (Flies et al, 2005).

1.6 DIVERSITY of MTB

MTB are classified in the following phyla:

Proteobacteria, *Nitrospirota*, *Omnitrophota*, *Latescibacterota*, *Planctomycetota*, *Nitrospinota*, *Hydrogenedentota*, *Elusimicrobiota*, *Fibrobacterota*, *Riflebacteria*, *Bdellovibrionota,* UBA10199, *Desulfobacterota*.

From the above mentioned phyla those that are known to be cultured and uncultured MTB are *Alpha*-, *Gamma*-, and *Deltaproteobacteria* classes of the *Proteobacteria* phylum and with the *Nitrospirae* phylum. All cultured species are either microaerophiles, anaerobes and the capability of exhibiting nitrogease activity thus fixing and denitrifying atmospheric nitrogen (Flies et al, 2005a).

The cultured species *Alpha*- and *Gammaproteobacteria* classes are microaerophilic that can grow chemolithoautotrophically by making use of reduced sulfur compounds as electron sources and chemoorganoheterotrophically using organic acids required as electron and carbon sources. The *Deltaproteobacteria* are sulfate-reducing anaerobes that are capable of growing chemoorganoheterotrophically. Thus, it does showing great importance in the natural environment by iron, nitrogen, sulfur, and carbon cycling (Amann et al, 2007).

1.7 CULTIVATION AND ISOLATION OF MAGNETOTACTIC BACTERIA

For many years, future and present researchers in this field have been disappointed by the inability to isolate new MTB strains because of their extended cell-dividing cycles and the lack of appropriate enrichment and isolation media. This frustration stems in part from MTB's widespread presence in aquatic settings and the relative simplicity with which it can be collected and separated for study (Flies et al, 2005).

According to Bazylinski et al,2013, because all known magnetite-producing MTB are microaerophiles, anaerobes, or facultatively anaerobic microaerophiles, semisolid oxygen concentration gradients or anaerobic liquid environments are commonly used for their growth. In general, richer media with greater concentrations of carbon and nitrogen sources tend to be better richer media with lower quantities of carbon and nitrogen sources are better for initial enrichment and isolation of MTB. Despite the fact that certain species have been domesticated, such as all magnetotactic Deltaproteobacteria, are obligate anaerobes, most MTB accept brief oxygen exposures during magnetic purification and inoculation, obviating the need for strict oxygen exclusion during cell manipulations (Lefèvre et al, 2013).

The bacteria was incubated for a period of 2months in a plastic container. After some time has elapsed, the bacteria can be taken with a pipette from the inside of the container close to magnet, and then enhanced with a capillary racetrack and a magnet. The bacteria can be deposited onto a copper grid and viewed using transmission electron microscopy or placed on a glass slide using a hanging drop method and examined in a light microscope (TEM)/ STEM.

A Pasteur pipette sealed at one end is utilised in a more commonly used 'Capillary racetrack' (CRT) approach for MTB isolation and purification. The pipette is sanitised and an absorbent cotton plug is inserted very loosely into the narrow neck of the capillary tube. Using a syringe, sterile water is injected via the pipette's broad mouth until the capillary is full. Excess water is drained from above the cotton plug, and the cotton is firmly packed into the capillary's neck.

The silt containing MTB is then deposited in the pipette's reservoir. The capillary's sealed end is inserted into the slide holder and set on the microscope stage. The south pole of a stirring bar magnet is then exposed to it. A phase contrast microscope is used to track the movement of an actively swimming MTB. After a few minutes, the motile MTB collecting at the capillary tip is harvested by breaking off the tip. Finally, a sterilePasteur pipette is used to extract the contents. This is used as an MTB inoculum for purification and enrichment investigations (Prabhu and Kowshik, 2016).

1.8 USES AND EXTRACTION OF MAGNETOSOMES

Magnetotactic bacteria are members of a genus of bacteria that produce magnetosomes, which are iron oxide nanoparticles wrapped with biological material. The magnetosomes are used as a compass by these bacteria to navigate in the direction of the earth's magnetic field. This compass aids bacteria in determining the best conditions for growth and survival. We look at how magnetosomes are used in medicine, including magnetic resonance imaging (MRI), magnetic hyperthermia, and medication administration (Alphandéry E., 2014).

Exposure to air is followed by ultrasonic cell lysis or the use of a laboratory extrusion cell disintegrator (French press), stirring in a shaker, magnetic separation of magnetosomes, or separation by centrifugation with cellular debris removed and multiple rinsing with buffered solutions and deionized water. To customise magnetite for the desired use, an automated MTB culture mode with varying oxygen content can be employed to regulate particle size and, as a result, magnetic properties (Basit et al, 2020).

1.9 NATURE OF MAGNETOSOMES

Magnetosomes are membrane structures seen in bacteria that are magnetotactic (MTB). They are made up of iron-rich magnetic particles wrapped in a lipid bilayer membrane. Each magnetosome can contain 15 to 20 magnetite crystals that create a chain that works as a compass needle to help magnetotactic bacteria navigate across geomagnetic fields and find their preferred microaerophilic habitats. Magnetosomes are invaginations of the inner membrane, not freestanding vesicles, according to new research. Magnetosomes containing magnetite have been discovered in eukaryotic magnetotactic algae, with each cell carrying thousands of crystals.

Magnetosome crystals are chemically pure, have constrained size ranges, have species-specific crystal morphologies, and are arranged in certain ways within the cell. These characteristics suggest that magnetosome production is under strict biological control and is mediated by biomineralization.

Magnetotactic bacteria commonly mineralize either iron oxide magnetosomes containing magnetite (Fe_3O_4) crystals or iron sulphide magnetosomes containing greigite crystals (Fe_3S_4) (Komeili et al, 2006).

1.10 MECHANISM OF MAGNETOSOMES

At least three steps are required for the magnetosome to form:

- The magnetosome membrane is invaginated (MM)
- Magnetite precursors are introduced into the freshly formed vesicle.
- The formation of the magnetite crystal and its development

A GTPase initiates the creation of an invagination in the cytoplasmic membrane. This process is thought to occur in both prokaryotes and eukaryotes.

The external environment must be used to introduce ferric ions into the freshly created vesicles in the second phase. MTB can accumulate large intracellular amounts of Fe^{3+} even when cultivated in a Fe^{3+} deficient media. It's been claimed that they do this by secreting a siderophore, a low-molecular-weight ligand with a high affinity for Fe^{3+} ions, as needed. The "Fe^{3+}-siderophore" complex is then transported to the cytoplasm and cleaved. The ferric ions must subsequently be transformed to the ferrous form (Fe^{2+}), which is stored within the BMP, using a transmembrane transporter that shares sequence homology with the Na^+/H^+ antiporter.

Magnetite crystal nucleation occurs in the final step of the process, thanks to the action of transmembrane proteins with acidic and basic domains. Mms6 is one of these proteins that has been used in the artificial synthesis of magnetite, allowing for the formation of crystals that are uniform in shape and size (Matsunaga et al, 2003).

1.11 MOVEMENT OF MAGNETOTACTIC BACTERIA

Besides using magnetosomes for their movement along the earth's magnetic field. (Nature, Advanced Online Publication, 2005). Bacteria can have a single flagellum (monotrichous) at one end of the cell (polar flagellum), a single flagellum at both ends (amphitrichous), several flagella in a tuft (lophotrichous), or flagella dispersed throughout the cell (amphitrichous) (peritrichous). Amphitrichous, bilophotrichous, and peritrichous flagella underpin complicated magnetotactic motion in the magnetotactic bacteria (Zhang and Wu, 2020).

1.12 PROMINENT LOCATION OF MTBs

The Magnetotactic bacteria are mostly found in the OATZ region of the aquatic source where in due to the earth's magnetic field and depending on the hemisphere they are present in; they will get attracted accordingly. If the bacteria are present in the northern hemisphere, then they will get attracted to the

north pole and the ones present in the southern hemisphere will get attracted to the South Pole. As compared to those found at the equator show no specific attract to one type of pole in other words, they are neutral in magnetic behaviour (Chen et al, 2010).

CHAPTER 2: LITERATURE REVIEW.

Fresh water is essential to life, but it is a limited resource. Fresh water makes up only 3% of the total amount of water on the planet. Glaciers, lakes, reservoirs, ponds, rivers, streams, wetlands, and even groundwater all contain fresh water. Although freshwater ecosystems account for less than 1% of the worlds total surface area, they are home to 10% of all recorded animals and up to 40% of all existing fish species. They are vital to life for a variety of reasons, including providing drinking water, maintaining crops through irrigation, and giving sustenance in the form of fish. Freshwater has also been shown to support the growth of Magnetotactic bacteria, which have proven to be quite useful for a variety of applications (Araujo et al, 2015).

MTB generate magnetosomes, which are intracellular single-domain crystals of magnetite (Fe_3O_4) or greigite (Fe_3S_4) enclosed by a lipid bilayer membrane. Magnetosomes are arranged as single or multiple chains that allow MTB to swim along geomagnetic field lines toward their ideal habitats, which are typically at or near the oxic-anoxic interface (OAI) in aquatic environments (Frankel et al, 1979). Magneto-aerotaxis is the name given to this process. Magnetosomes in MTB have been utilised to recover paleomagnetic and paleoenvironmental information from ancient sediments as magnetofossils. MTBs also serve as natural model bioreactors that can be replicated to produce well-tailored magnetic nanoparticles with improved magnetic characteristics (Lefèvre et al, 2013).

When compared to artificially manufactured magnetite, bacterial magnetosomes have a more regular structure and a restricted particle size distribution. MTB and magnetosome discovery has sparked interest in their possible uses in a variety of sectors, including medical, chemistry, physics, geology, crystallography, mechanics, planetary geology, geology, planetary science, food science, and biotechnology (Yan et al, 2012).

Swimming behaviour, type and number of flagella, cell morphology, form of magnetic crystals, number of magnetosomes, number of magnetosome chains in each cell, composition of nanomineral crystals, and presence of intracellular vacuoles were all determined using this method (Spormann et al, 1984).

Magnetotactic bacteria's iron uptake methods differ from species to species. At neutral pH, Fe^{2+} is extremely soluble, and in aerobic (oxygenated) conditions, Fe^{2+} is readily oxidised to Fe^{3+}. As a result, the majority of iron on Earth's surface is in the form of Fe(III)-containing minerals, that are insoluble at neutral pH (Chen et al, 2010). Certain magnetotactic bacteria are thought to generate iron-binding proteins termed siderophores in order to use solid-phase Fe^{3+}. Bacteria and fungi that live in low iron environments create siderophores, that are iron ion (Fe^{3+}) chelators. The siderophores attach or chelate iron because they have a strong affinity for Fe^{3+}. Proteins inside the bacteria decrease Fe (III), turning the iron to Fe^{2+}, which the magnetosomes subsequently take up (Schüler et al, 1996).

Table 2.1: Phyla of Magnetotactic Bacteria

BACTERIA ↙ ↘				
Gram positive bacteria	Gram positive bacteria ⇩ **MTB** {Prokaryotes-Magnetotactic property (Magnetotaxis)}	PHYLA	Alphaproteo-bacteria	*Manetospirillum magnetotacticum strain MS-1, Magnetovibrio blakemorei strain MV-1, Magnetospirillum magneticus strain MG-T1*
			Deltaproteo-bacteria	*Desulfovibrio magneticus* strain RS-1, *Candidatus magnetoglobus multicellularis, Deltaproteo* bacterium AV-1
			Gammaproteo-bacteria	Vibrio cholerae strain, Gamma proteobacterium BW-2
			Nitrospira	*Candidatus Magnetobacterium bavaricum, Magnetobacterium* ZZL4G12
			Candidate division OP3	Part of the Planctomycetes-Verricomicrobia- Chlamidiae bacterial superphylum

The appearance differs from other types of inclusion bodies reported to occur in bacterial cells, and they are assumed to be responsible for magnetic orientation of this organism, ultrastructural characteristics of magnetotactic bacteria of strain MS-1 with electron-dense, iron-rich particles in its cells were of interest (Abreu F. and Acosta-Avalos D, 2018).

M. magnetotacticum strain MS-1, *M. griphyswaldense* strain MSR-1, and *M. magneticum* strain AMB-1 are the three most prevalent *Magnetospirillum* species discovered in shallow freshwater and sediments. Others include *Magnetospirillum magneticum* MGT-1, *Magnetovibrio* MV-1, *Magnetococcus* sp. MC-1, *Marine magnetic spirillum* QH-2, *Magnetospirillum* sp. WM-1 all of these bacteria belong to the α-Proteobacteria; *Desulfovibrio magneticus* RS-1 belongs to the δ-Proteobacteria as seen in Table 2.1. (Frankel et. al, 1979).

1) *Magnetospirillum magnetotacticum* is a microaerophilic, gram-negative, helical, magnetotactic spirillum. It is isolated from oxic to anoxic transition zones, which are commonly found at the sediment-water interface in many freshwater settings. This bacterium was previously defined under the genus Aquaspirillum and named *Aquaspirillum magnetotacticum* by Robert Blakemore in the 1980s. It belongs to the magnetotactic bacterium (MTB) family, and its motility is determined by the magnetic properties of the environment. *Magnetospirillum magnetotacticum* is one of the most important *Magnetospirillum* species because it was the first to be found and is one of the few that can be cultured (Lefèvre et al, 2012). This is a physiologically, environmentally, and commercially

important microorganism that produces superior single-domain magnetite crystals than those produced industrially. Exploring the evolutionary role of magnetism in higher organisms; creating models for biomineralization; applications as geobiological tracers; models for sediment mineralization, environmental remediation; contrast enhancers in MRI's; wastewater treatment options for elimination of toxic heavy metals and radioactive elements; cell isolation imaging; manufacturing of colourants and magnetic tapes; and magnetic targeting of pharmaceuticals are just a few of its many potential applications (Chen et al, 2018).

2) *Magnetospirillum gryphiswaldense* is a spirilla-shaped microaerophilic gram-negative bacteria that survives in freshwater aquatic habitats with vertical chemical gradients. *M. gryphiswaldense* is categorized as a magnetoactive bacterium (MTB), indicating that it can biomineralize magnetic nanoparticles inside its body. Magnetosomes are organelles that generate and store these nanomagnets. Within the cell, the magnetosomes are positioned to form a microbial 'compass needle,' which reacts with the Earth's magnetic field (Uebe et al, 2019). *M. gryphiswaldense* motility is controlled by connections among its magnetosomes and the earth's magnetic field, which are powered by two revolving flagella at both the end of the cell. This magnetotactic bacterium was discovered in freshwater silt in Greifswald, Germany, in the eutrophic Ryck River. In membrane-bound organelles termed magnetosomes, it assembles up to 60 cubo-octahedral magnetosome particles. Magnetosomes are structured in well-ordered chains along the cellular motility axis, causing the cell to behave like a motile, microscopic compass needle, aligning and swimming parallel to magnetic field lines in search of its optimum oxygen concentration environment (Fdez-Gubieda et al, 2013).

3) *Magnetospirillum magneticum* AMB-1 was in 1963 discovered while examining bog sediment samples, Salvatore Bellini of the University of Pavia described this magnetotactic bacteria for the first time. In the watery environment, he discovered the bacteria with unusual movement and orientation that seemed to follow earth's magnetic field. It has magnetite-filled magnetosomes, utilises flagella for locomotion, and prefers to develop between the aerobic upper and anaerobic lower parts of most waterbodies. *M. magneticum* migrates in the water following earth's magnetic field lines. Within magnetosomes, it actively converts ferrous to magnetite or gregite, contributing to iron cycle. *Magnetospirillum magneticum* is indeed a Gram-negative, aquatic, mesophillic bacteria with a spiral structure. It loves temperatures between 25 and 40 degrees Celsius and is microaerophilic, requiring less oxygen than is available at typical sea level on Earth. Magnetosomes, magnetite-containing vesicle invaginations of the interior cell membrane kept in place by cytoskeletal filaments, are the major structures of importance in *M. magneticum*. Magnetosomes are encased in a lipid bilayer and typically contain 15-20 magnetite crystals, each measuring around 50 nm in length, though this number might vary depending on environmental conditions (Matsunaga et al, 2005). The protein MamK anchors the magnetosomes to the membrane and allows them to align themselves linearly. Under anaerobic conditions, respiratory nitrates reduction allows for the oxygenation of a substrate. Fe(III) could be the terminal electron acceptor through membrane-bound ferric reductase. *Magnetospirillum magneticum* AMB-1, like some other magnetotactic bacteria, produces internal magnetite (Fe_3O_4) crystals enclosed by a membrane (magnetosome). The crystals' consistent size and dispersion show that the mineralization process is under strict biological control.

The bacteria utilizes the ferrous metal as a magnet to arrange itself, and it demonstrates magnetotaxis, which allows it to trace magnetic field lines downhill to areas where it may grow. Usually found around the oxygen - deficient transition zone, that can be found at the silt interface in freshwater or moved upwards through the water column in oceanic semi-anaerobic conditions (Suzuki et al, 2006). When all of the tiny magnetic dipoles present in the cell operate together, the magnetosome chain utilize magnetic responses to rotate the bacteria in the most efficient way. The stationary magnetosome chain is torqued by the magnetic field, allowing it to turn and position the cell with magnetic field lines. The magnetosome directs the bacteria toward the best growing conditions, but flagella are required for motility to get there (Arakaki et. al, 2003).

The development of magnetosomes is tightly regulated by particular gene expression. The majority of the genes are found on the Magnetosome Island, which is a genomically conserved area (MAI) (Lohsse et al, 2011). In practically all MTB species, the MAI has a highly conserved and crucial operon (mamAB), as well as three less conserved operons (mamGFCD, mms6, and mamXY). The lack of magnetosome formation is caused by the deletion of the MAI or mamAB operon. In MSR-1, the mamAB cluster includes factors that are required for magnetite biomineralization. A recombinant Rhodospirillum that generated magnetosomes was created when four gene clusters and feoAB1 were transferred (Uzun et al, 2020).

In M. gryphiswaldense MSR-1, the MamAB operon contains 17 open reading frames corresponding to 16.4 kb of DNA. This operon contains genes required for magnetosome formation as well as genes involved in membrane invagination, iron transport, and magnetite biomineralization. Membrane invagination and magnetosome biogenesis (mamB, E, I, L, and Q), magnetosomal iron transport (mamB and M), magnetite biomineralization (mamE, O, T, P, and S), and magnetosome chain assembly (mamE, O, T, P, and S) are all regulated by the mamAB cluster (mamK and mamJ) as seen in figure 2.1. (Lin et al, 2018).

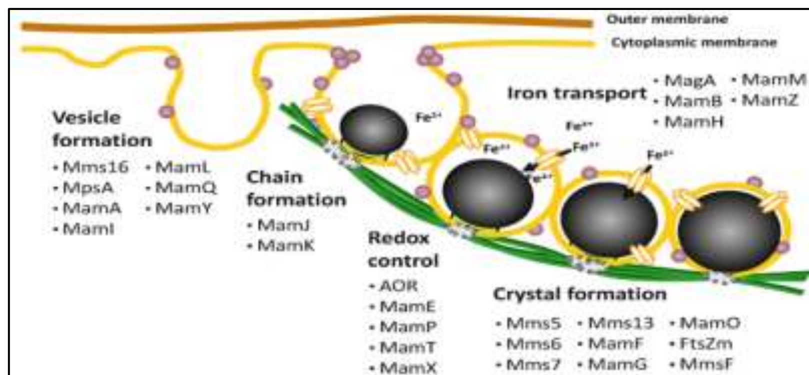


Figure 2.1: Magnetosome formation with the help of various genes

Several processes are involved in the production of magnetosomes, and they can all happen at the same time. They are protein sorting and inner membrane invagination, magnetosome chain alignment, iron uptake and crystal nucleation, and crystal maturation. The α -proteobacterium magnetotactic has all four magnetosome operon that is present in *Magnetospirillum gryphiswaldense* MSR-1. Over 28 distinct proteins are involved in magnetosome formation in *M. gryphiswaldense* MSR-1, and the majority of the relevant genes are found on the MAI. Some of these proteins are homologous to well-known protein

families, including as TPR proteins, CDF transporter, PDZ proteins, proteases, and others (Wang et. al, 2017).

Magnetic fluid hyperthermia was demonstrated using magnetosomes obtained by magnetotactic bacterium *Stenotrophomonas* spp. strain RP-8. Magnetosomes were recovered by sonication from strain RP-8, which was grown in an optimised magnetospirillum growing medium with microaerophilic conditions under a permanent magnetic field. The existence of magnetosomes was discovered using transmission electron microscopy, while the magnetic data was examined using an alternate gradient magnetometer (AGM). Magnetosomes have a maximum Specific Absorption Rate (SAR) of 337.30 W/g at 502 O^e magnetic fields. The magnetic characteristics and heating effectiveness of magnetosomes isolated from strain RP-8 have been used for magnetic fluid hyperthermia study (Gandia et al, 2019).

Because the bacteria live in OATZ, which has vertical chemical gradients, they must constantly look for the best place in the stratified water column to meet their nutritional needs. In these situations, magnetotaxis is expected to provide a significant benefit by boosting chemotaxis efficiency. Because the earth's magnetic field lines in a layered environment operate as vertical passageways due to their orientation, bacteria oriented in the Earth's field reduce a three-dimensional quest to a single value, swimming up-down the stratified column (Lin et al, 2018).

Magnetosomes have also been proposed as detoxifying agents that scavenge reactive oxygen species or provide resistance to UV-B irradiation. As a result, the creation of magnetosomes may aid in the survival strategy in the face of a stressful scenario.

Freshwater sediments have yielded MTB of many morphological kinds, notably bacillus, vibrios, spirilla, cocci, and multicellular forms. MTB is known to create iron oxides and ferrous sulphides, two forms of minerals. Primarily iron oxide-producing MTB has been discovered in freshwater systems. MTB is found in both marine and lake habitats, and it can create both iron oxide and iron sulphide. Magnetic cocci have been detected in mud sediments with a high organic matter content (Oestreicher et al, 2013).

Magnetotactic cocci swim in a preferential direction that correlates to downward movement through geomagnetic field lines alongside local magnetic field lines. Magnetotactic cocci have recently been found at high cell concentrations in the water columns of chemically stratified marine and brackish ecosystems, rather than in the sediments. Cells from a pure culture of a marine magnetotactic coccus, strain MC-1, developed microaerophilic bands in capillary tubes and employed aerotaxis to migrate to a desired oxygen concentration in an oxygen gradient (Zhang et al, 2017).

Cells could migrate either in a manner along the local magnetic field and employed magnetotaxis in combination with aerotaxis, or magneto-aerotaxis, to more successfully migrate to and hold position at their optimal oxygen concentration. Instead of the temporal sensory mechanism utilised by other bacteria in aerotaxis, such as *Magnetospirillum magnetotacticum,* cells of strain MC-1 have an aerotactic sensory mechanism which plays a dual purpose (Gareev et.al, 2021).

Because Co, Mn, Ni, Zn, and Cu are near to Fe, their integration into the magnetite structure of the magnetosome mineral core may be easier. Various mechanisms, such as the creation of protein-metal complexes to adsorb metal ions, their reduction to less harmful forms, or the continuous pumping of metal out of the cell, prevent magnetotactic bacteria's tolerance to oxidative stress, electron transport chain inhibition, antagonising metal uptake, and enzyme mismetallation. The magnetotactic bacterium *Magnetospirillum gryphiswaldense* has a high endurance to metal stress when compared to other magnetotactic bacteria species (Muñoz et al, 2020).

Metal stress tolerance is higher in bacteria with magnetosomes than in bacteria without magnetosomes. The role of magnetosomes in the bacteria's resistance mechanisms to metal stress was discovered using a X-ray absorption near edge structure (XANES) experiment. Additionally, using XANES, it was discovered that both the MTB and the magnetosomes isolated from the bacteria store excess metal in the magnetosomes or other cell compartments. Metal-specific tolerance mechanisms exist. Unlike Co, which is solely found in magnetosomes, Ni is found in cell regions other than magnetosomes. Both magnetosomes and other cell compartments include Mn, Zn, and Cu (Wang et al, 2020).

Magnetotactic bacteria were isolated using a modified nutritional medium using this an aerobic strain of Magnetotactic bacteria (MTB) was isolated and identified. The main source of iron was ferric quinate. The magnetotactic behavior of the isolated strain was studied using macroscopic and microscopic techniques. Motility in the direction of an applied magnetic field confirmed that the isolated strain was magnetotactic. The morphology of the isolated strain was studied using scanning electron microscopy, which revealed a rod-like form. The existence of pure carbon as a main ingredient was validated by electron dispersive X-ray diffraction investigation. Furthermore, the presence of atomic iron and oxygen in the bacterial cells indicated the creation of magnetic material (Hong et al, 2021).

By altering a single iron or oxygen molecule, we may either lead cells to make magnetosomes with tremendous efficiency or not at all and this was done for the MSR-1 Bacterium strain.

The procedure of producing a pure MTB culture for *Magnetospirillum magnetotacticum* requires large-volume fermenters with capacities of 30 to 50 litres. Moreover, regulation of the dissolved iron content and pH of the nutritional medium, as well as a 5% oxygen concentration in the gas phase need to be maintained precisely. Besides, fermentation also can be used for MTB biomass generation without the need of precise gas environment control. Furthermore, flow cytometry, can be used for maintaining the physiology of the Magnetotactic bacteria thus allowing cultivation of MTB to be obtained easily (Balkwill et. al, 1980).

2.1 APPLICATIONS OF MAGNETOTACTIC BACTERIA

The applications of Magnetotactic bacteria are such that on the surface of the magnetosome envelope, bioactive chemicals can be coupled, which is significant for a variety of medical applications. MTB magnetite can be utilized for enzyme immobilization, the production of magnetic antibodies, and the measurement of IgG antibodies. Genes are also delivered into cells via MTB magnetite wrapped with DNA (Jogler et al, 2010). MTB has also been observed to help with the administration of pharmaceuticals to tumors where regular treatments are difficult to reach. The realm of soft magnetic materials such as Si-Fe sheet and amorphous Co-based ribbons may be studied with the use of living MTB. MTB can be used to locate the magnetic pole of rocks containing magnetic minerals. MTB could be phagocytized and magnetically isolated from blood after being absorbed into monocytes and granulocytes (Vargas et al, 2018).

One of major role of MTB's is the possible environmental removal of pollutants from wastewater. Therefore, MTB has been employed to remove organic contaminants, radionuclides, and heavy metals in a number of studies. Besides, they also are effective in pathogen eradication and endocrine disruptor identification (Vargas et al, 2018).

Magnetotactic bacteria's nanomagnets have a lot of promise for magnetically directed medicine delivery. Concerning the physical nature of interactions, which these nanomagnets are capable of, it is crucial for any targeted medication delivery application to be effective. The structure of two magnetic bacterial strains, *Magnetospirillum magnetotacticum* and *Magnetospirillum gryphiswaldense*, was analysed using

bar magnets to define the features of each bacteria in two dimensions: length and width (in microns). Under various circumstances *M. magnetotacticum* underwent morphological alterations and this was observed by performing analysis of variance (ANOVA) (Nudelman et al, 2018). Contrastingly, *M. gryphiswaldense*, showed no morphological alterations under any conditions. *M. magnetotacticum* breadth was found to be substantially higher under control conditions than under any magnetic condition. When only the south poles of the bar magnets (single or couple) were facing the bacteria, the length of *M. magnetotacticum* was drastically reduced. Due this factor it is important that the strain chosen has magnetosomes packed well in the strain so that it becomes easier to choose the right microbial source of nanomagnets (rather than using any strain), trapped inside biological membranes, for potential targeted drug delivery applications requiring enhanced sensitivity to external magnetic fields (Grouzdev et. al, 2017).

Bioremediation, cell imaging, DNA/antigen recovery or detection, drug carriers, enzyme immobilisation, magnetic hyperthermia, and image enhancement of magnetic resonance imaging are some of the applications that use magnetite-producing MTB, magnetite magnetosomes, and/or magnetosome magnetite crystals (Vargas et al, 2018).

It was seen that by using magnetosomes obtained from Magnetotactic Bacteria proved advantageous that when they are given intravenously to mice, they have a natural predisposition to target tumours, according to MRI findings. MRI was used to see magnetotactic bacteria in tumours. A part of the magnetosomes was found to provide T1 (longitudinal relaxation times)-weighted positive contrast, which helped researchers see the magnetotactic bacteria in the tumours better. The T1-weighted MRI signal increases with the increase bacterial concentrations in small magnetosomes, as long as the bacterial concentration stays below a limit of 0.5 1010 cells/mL. The T1-weighted MRI signal falls above 0.51010 cells/mL due to the competing T2 (transverse relaxation times) effect. It was discovered that good contrast agents have very high relaxivities (the inverse of T2 relaxation time, generally abbreviated as r2) and very short T2 values. Magnetosomes are capable of achieving such high r^2 values. Magnetosomes encased in a gel and ferrimagnetic nanoparticles that are not ferridex but have similar qualities to magnetosomes had r2 values of 1175 and 324 mM s1, respectively. These two values are higher than the r2 133 mM s1 reported for chemically generated ferridex nanoparticles, which are currently utilised as MRI contrast agents (Nan et al, 2021).

Magnetosomes obtained from MTB are used in food safety, pathogens in food have been identified using functionalized magnetosomes. The protein A gene was linked to the mamC gene in a recombinant magnetosome produced from cultures of *M. gryphiswaldense* strain MSR-1. The combination was attached to a specific antibody in order to trap *Vibrio parahaemolyticus*, a pathogen that triggers a variety of gastrointestinal and foodborne diseases. 1mg of this compound was capable of capturing 1.74 107 pathogen cells (Jovane et al, 2014).

A magnetosome-polyclonal antibody complex has also been produced to collect Salmonella species from food. This is accomplished by attaching specific antibodies to the surface of magnetosomes using the cross-linking reagent bis(sulfosuccinimidyl) suberate (BS3). 178 g of antibody was bound on 1 mg of magnetosomes in this test. To identify and isolate Salmonella dublin from a sample suspension, fluorescence quantitative-PCR was done (Sannigrahi et al, 2020).

The complex was also used to identify pathogens in food samples. Whenever the pathogen was present in amounts more than 60 CFU/mL, Salmonella was discovered in experimentally contaminated food

samples like milk, eggs, and pork. The complex had a high level of selectivity, since it was unable to collect Vibrio colonies from mixed Salmonella and Vibrio suspensions (Jovane et al, 2014).

The use of magnetotactic bacteria (MTB) to remove heavy metals from wastewater is explored and thus brings nto account of MTB-based treatment method. Due to nano-sized magnetosomes (MS), the MTB has the unusual ability of moving along the periphery of applied external magnetic fields (Schüler et al, 1999). MTBs can collect iron through a compartmentalization process and use it as a component in their metabolism. MTB bioremediation leads to formation of internal metal crystals or the reduction of metal toxicity through precipitation. Superoxide dismutase is a *Magnetospirillum* strain secreted enzyme that converts superoxide to oxygen and peroxide. As an alternative electron accepter, *Magnetospirillum* sp. can utilise perchlorate, oxygen, nitrate, chlorate, nitrite, and nitrous oxide. Magnetotactic bacteria are made up of granules that collect metal ions, indicating that they play a detoxifying role. As a result of heavy metal exposure in magnetotactic bacteria, metabolic intermediates may form, which are then used as primary substrates for cell growth and metabolism. Thus, making it beneficial for the bacteria and the environment as well by utilizing it for its own growth and reducing environmental pollution respectively (Thrash et al, 2010).

2.2 Antigen Recovery/Detection tests utilising Magnetosomes in DNA-

Magnetosomes have been utilised successfully in protein detection techniques. The protein streptavidin was coupled to magnetosomes using biotin groups attached to the magnetosome membrane. These biotin-binding semi-synthetic composite particles could be utilised to link a variety of functional biomolecules, such as biotin - conjugated DNA oligonucleotides or biotin - conjugated antibodies. This method could be useful in serological testing and proteome research (Vargas et al, 2018). Utilizing antibody-functionalized magnetosomes in a surface-independent immunoassay, a variation in an automatable, highly sensitive immuno-PCR (M-IPCR) was developed. Antibody-functionalized magnetosomes were utilised to immobilise hepatitis B antigen in human serum and amplify the signal provided by the detecting complex through magnetic concentration in this technique. The M-IPCR detected HBsAg around 100 times more sensitively than magneto-ELISA, which utilizes synthetic nanoparticles to increase antigen detection in ELISA and was run in tandem with M-IPCR for evaluation (Alphandéry, 2014)

2.3 Use of MTBs in Cancer therapy-

MTB have recently been employed as biological agents, taking use of their flagella's self-propulsion capabilities and the existence of the magnetosome chain. MTBs are thought to be nanobiots that can be led and regulated by exterior magnetic fields and thus generally drawn to hypoxic places like tumors (Banerjee et al., 2017). MTB are not pathogenic; however, they can be genetically modified to transmit and/or express cytotoxic chemicals. The presence of magnetosomes gives the MTB biomedical capabilities for therapy and imaging, such as the capacity to heat under interchanging magnetic fields for magnetic hyperthermia therapy, without having to go through the time-consuming and inefficient process of alienating the magnetosomes from the MTB (Kotakadi et al, 2022).

Magnetic hyperthermia has emerged as one of the most promising developing approaches for cancer therapy since it was first established in 1957 that magnetic particles might be inductively heated to destroy lymphatic metastasis (Kutova et al., 2019). Cancer cells can be led to apoptosis by raising the temperature of the tumour area to 42–45 °C, through the therapeutic window. Thermal ablation at higher temperatures (>50 °C) might lead in a more disruptive death of cancer cells. Furthermore, it has been

discovered that increasing the tumor's temperature boosts the efficacy of adjuvant therapies including chemotherapy and radiotherapy (Johannsen et al., 2007).

Alsaiari and colleagues (Alsaiari et al., 2016) devised an inventive way for vector transfer via MTBs. M. gryphiswaldense was used as a carrier for gold nanoparticle-loaded ssDNA, which can also be used to control the loading and release of the DNA as a bioimaging agent.

Mice with xenografted MDA-MB-231 breast cancer were treated with chains of magnetosomes from *M. magneticum* AMB-1 in a specific magnetic field, which resulted in the tumour disappearing completely after 30 days. In addition, mice with glioblastoma were given magnetosomes coated with poly-l-lysine (Alphandéry et al., 2017). The mice were subjected to 27 30-minute magnetic hyperthermia sessions with a magnetic field of H= 270 O^e and f = 202 kHz. 350 days after the first injection, all of the mice were alive and appeared to be cured. Furthermore, it has been demonstrated that these magnetosomes can sustain anti-tumor action even after extensive disintegration inside tumour cells, making them suitable candidates for long-term hyperthermia therapy (Yukumi et al., 2009).

MTB has been employed to open the blood–brain barrier for brain tumour treatment because of their increased heating potential when used with commercial SPIONs as heat delivering agents. Since the MTB end up being internalized or attached to the cancer cells, local heating effects can give rise to deactivation of the cancer cells (Kuzajewska et al, 2020).

MTBs are considered as nanobiots that can be led and controlled by applied magnetic field and are naturally drawn to hypoxic areas, such as tumours, while preserving the medicinal and imaging capabilities of isolated magnetosomes. Furthermore, unlike the majority of bacteria now being studied in clinical studies for cancer treatment, MTB are not pathogenic but might be modified to deliver and/or express certain toxic compounds (Martel,et al, 2009).

On their membrane surface, magnetosomes have amino groups and glycerol, allowing them to be connected to some other ligand. As a result, magnetosomes can be more effective drug carriers than synthesized magnetic particles, with a greater drug loading ratio. Magnetoliposomes with cis-diammine-dichloro-platinum(II) and magnetosomes have been tested for targeted therapy and controlled release at tumour areas (Kuzajewska et al, 2020).

The use of radiations such as gamma rays, X-rays, and electron beams to weaken or stop cancer cell growth and multiplication is known as radiotherapy. Magnetosomes could also be connected to radioactive isotopes, e.g., utilising chelates, and radioactive-labeled molecules, such as proteins and nucleic acids, to provide better internal radiation of tumours due to their precise targeted delivery (Alphandéry E., 2014).

The biggest group of membrane proteins in the human genome is G protein-coupled receptors (GPCRs). Drug discovery, cancer treatment, endocrine, neurological, and other illnesses all benefit from them. GPCRs have a lot of hydrophobic domains, which makes it difficult to purify them from cells and lose their original shape (Bazylinski et al., 1995). The GPCR gene was expressed in MTB to produce a GPCR with natural shape. The GPCR gene was produced as a chimaera gene that coded for both the GPCR and the anchor protein, which was chosen from magnetite particle membrane proteins of fragments. D1R is a GPCR model that has been fused to Mms16, a magnetosome-membrane-specific protein. GPCRs were also assembled into the lipid membrane of *M. magneticum* AMB-1 magnetosomes (Mathuriya et al, 2016).

Magnetosomes, which are peculiar structures that contain nanometre-sized crystals of magnetite minerals, provide magnetic orientation to magnetotactic bacteria (Bazylinski Although these organelles

have obvious potential for exciting biotechnological applications, the development of such tools has been hampered by a lack of genetically tractable magnetotactic bacteria; however, genetic analysis using two different *Magnetospirillum* species have demonstrated much about the mechanisms of magnetosome biogenesis in the last decade (Mathuriya, 2015).

Chapter 3: Aim and Objectives

AIM

To isolate Magnetotactic Bacteria from Aquatic ecosystems with main preference to freshwater source and check their magnetotactic properties.

Objectives:

- Isolation of Magnetotactic Bacteria from Goan Fresh waters.
- To observe the magnetotaxis property of the obtained isolates.
- To determine the oxygen requirement of the isolates.
- To check the nitrate reduction ability of the isolates.
- Screening of the isolates for EPS and Siderophore production.
- Morphological and structural studies of the magnetotactic bacteria.
- To determine the elemental structures, present in the magnetosomes.

Chapter 4: Materials and Methods

4.1 Sample Collection

Two freshwater sampling sites were selected in Goa, India namely Carambolim Lake and Cujira Stream. The location where the depth of the water is between 10 and 100 cm was selected.
The Physico-chemical parameters prevalent at the site during sampling, such as the water's temperature and pH, were checked. The samples were collected in previously surface-sterilized large plastic jars. The samples were collected in such a way that the water level was reduced to $1/4^{th}$ and the remaining capacity of the container was filled with $3/4^{th}$ of sediment. The container was left soaked in water until it was full, and then the lid was closed tightly.
The samples were then taken to the laboratory the same day and kept in a dry place at a temperature of 25°C for 1-2 months. The lids of the containers were kept slightly loose in order to develop an Oxic-Anoxic Transition Zone (OATZ). OATZ is the zone where the magnetotactic bacteria thrive to a greater extent, and the zone is formed at the interface at the water and sediment layer (Oestreicher *et al,* 2012).

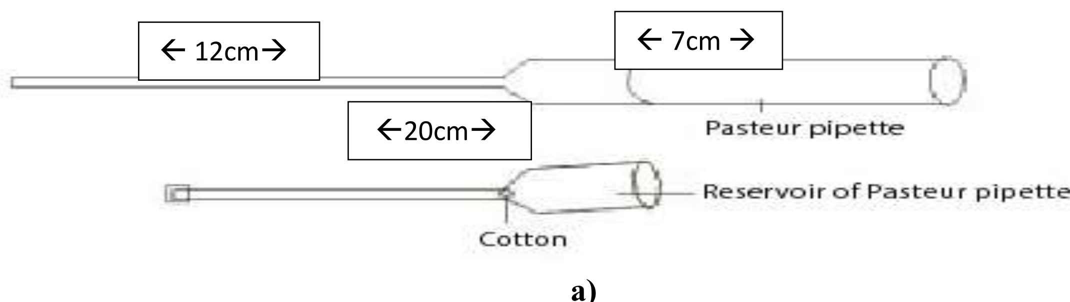
4.2 Sample Enrichment

After incubating the samples in the dark for at least 2 months the samples were removed out from the incubation zone and magnets are placed at opposite ends of the container for a brief period of approximately 30 minutes at the interface of the water and sediment, as this is the zone where the magnetotactic bacteria (MTB) mostly thrive. (Oestreicher *et al,* 2012). Care was taken that the settled sediment is not disturbed and the container is carefully placed on the bench top.
A stage was prepared where the magnets were placed on a support and the container was placed in between the magnets. The South Pole magnet was facing one side of the container, followed by the North Pole on the other side. The magnets were placed depending on the North-South axis of the magnetic compass.
The rectangular magnets with a power of 10G with dimensions of (3.6cm x 2cm x 1.6cm) were used, thus enabling the enrichment of the bacteria to the poles to which it gets attracted.

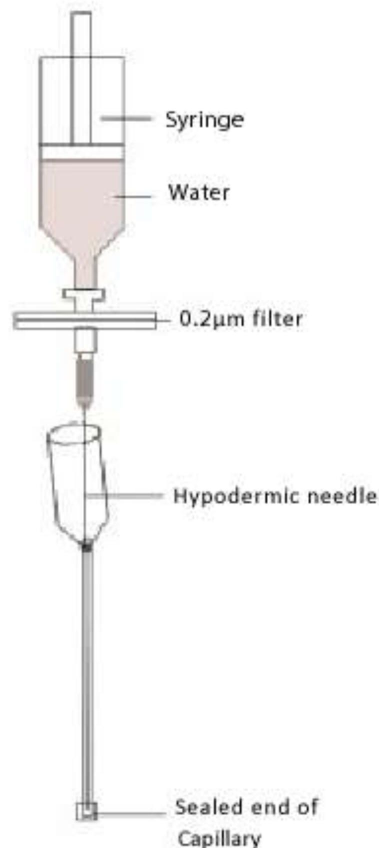
The magnets were placed just 1 cm above the sediment, ensuring that they were in contact with both the water and sediment layer in the container.
For best results, the magnets were placed for 1 hour. After the enrichment period, 5ml of the sample was collected from 1cm above the water-sediment interface using a sterile syringe. The aliquots were transferred to a sterile beaker soon after the collection. This was then used for further purification by Capillary Race Track method.

4.3 <u>Purification by Capillary Race Track Method</u>

In order to purify the magnetotactic bacteria, the aliquot collected from the sample was processed by using a capillary race track method. A Pasteur pipette was autoclaved with a tip sealed at one end and the other side was left open for inserting the sample. 1ml from the 5 ml aliquot was taken and put into the Pasteur pipette, ensuring that the sample reached the edge of the tip; this was done by using a sterile needle attached to a filter syringe of 0.22 micron size to filter out any sediment debris. 1 ml of the enriched sample was taken into the Pasteur pipette through the open end Ensuring that there were no air bubbles trapped in the capillary. A piece of sterile cotton was placed above the sample in the capillary. Then 1ml of the sample next to the poles was collected and put above the sterile cotton. This was done for both poles. This entire procedure was performed aseptically.
This setup was then placed horizontally in the Laminar Air Flow with the south pole of the magnet placed at the tip and the north pole of the magnet placed at the open end of the pipette and vice versa. The assembly was left undisturbed for 30 minutes. In this time the magnetotactic bacteria is thought to be traveling through the cotton and reaches the tip end .
Since we are in the Southern Hemisphere, mainly the pipettes facing the Magnet's South Pole at the tip end will attract the bacteria towards it. Thus, the MTB's will be south seeking. After 30 minutes the magnet was removed and the tip was broken off. Using a sterile syringe, the enriched aliquot expected to have Magnetotactic bacteria were collected. The aliquot was inoculated in Flies Media plates, which were prepared prior to perform capillary race track method. The collection through CRT method is shown in the figure 4.3 below.



a)



b)

Figure 4.3 a) The Pasteur pipette diagram for CRT purification

Figure 4.3 b) Pasteur pipette with the entire setup put together for inoculation.

4.4 Preparation of MTB Specific Media and Isolation

The MTB enriched liquid was inoculated in Flies media, which contains 10mM ferric citrate, Wolfe's Mineral Solution and Sodium succinate. The solid media enabled the growth of the MTB which was obtained after CRT purification. This was then spread plated and kept in the dark at room temperature for 48 hours.

In the media the ferric citrate present was used as reductant, electron mediator, and iron source. Depending on the utilization of the media components the bacteria will grow and show its characteristics by morphological identification. Isolated colonies were obtained from the spread plate which is then streaked on fresh media plates and kept for same time period and temperature.

Sodium succinate, ammonium chloride and ferric citrate are the three significant factors which help in enhancing cell growth and magnetosome production.

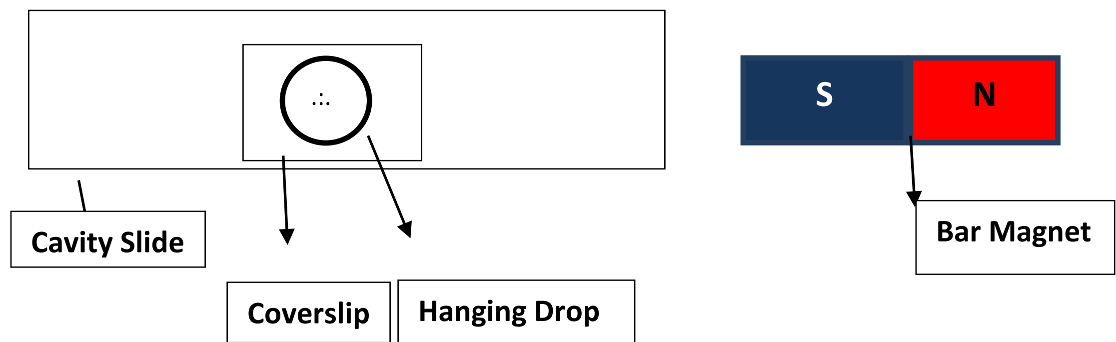
The 100µl inoculum was spread plated from the CRT purified liquid, distinct isolated colonies were selected for further studies.

4.5 MTB Observation by Light Microscopy

To observe the magnetic movement of the MTB, video microscopy was done, wherein a drop of 0.8% saline was placed on a cover slip. A platinum loop was taken and heat flamed before touching the colony, once the loop cooled, it was touched to the colony and a smear was prepared using a drop of saline.

Silicone grease was applied to the coverslip at the 4 corners with the help of a toothpick. The cavity slide was taken and the concave side was placed over the center of the drop without allowing it to spread.
The slide was immediately turned over so that the drop is now in a hanging position as seen in the figure 4.5. The slide was placed immediately under the light microscope and observe it under 100 x magnification.
The magnets were placed on the opposite sides of the slide and cells residing at the edge of the drop were observed for the characteristic movement. The bacteria will move towards the south pole of the magnet. Due to the magnetic moment the bacteria will show rotating movement towards the edge of the drop. Even after the magnet has been removed it will show some moving behaviour towards the edge.

Figure 4.5: Hanging Drop Technique for viewing the bacterial movement.



4.6 Morphological Identification

Identification was carried out based on the morphological characterization and microscopic observation. Morphological characterization included the study of the colony characteristics such as size, shape, colour, opacity, margin, elevation and consistency. Microscopic observation was done by staining the colonies on the slide for Gram staining method, thus determining the Gram property.

4.7 Gram Staining

Once the morphology of the selected isolates was obtained, next it was gram stained to check if the bacteria are gram positive or negative. This was done to distinguish their cell wall composition. Gram positive bacteria have a thick peptidoglycan layer thus gets stained by crystal violet stain whereas for gram negative bacteria since they have a thin peptidoglycan layer the primary stain get washed out with the help of the decolouriser and the it then get stained with the secondary stain that is safranin.

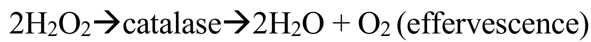
4.8 KOH String Test

The KOH test, like the Gram stain response, was based on variations in bacterial cell wall chemistry. Gram negative cell walls were broken down in the presence of potassium hydroxide. The peptidoglycan layer of gram-negative bacteria's cell walls was easily dissolved by KOH. The gram-negative cell wall was disintegrated, causing the cell to lyse and release its contents, including the DNA. As a result, viscid chromosomal material is liberated, thickening and stringing the bacterial solution. When contacted, the viscous and the solution cling to the loop.
Gram positive bacteria, on the other hand, are unaffected by KOH because their cell walls have a stronger peptidoglycan coating. As a result, the cells were not lysed, the DNA is not released, and there is no viscosity.
This is a simple and rapid (< 60 s) non-staining technique with 3% potassium hydroxide.

A KOH positive result is indicative of a string formation when the loop is touched to the culture suspended in 3% KOH. A negative is no formation of a string (Powers et al, 1995).

4.9 Catalase Test

Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). Catalase breaks down hydrogen peroxide into water and oxygen.



$2H_2O_2 \rightarrow catalase \rightarrow 2H_2O + O_2$ (effervescence)

A rapid formation of oxygen bubbles was observed upon adding a small inoculum of the organism to hydrogen peroxide. Weak bubble production indicated lack of catalase enzyme.

Catalase production was tested using 30% H_2O_2. Catalase positive samples showed immediate formation of effervescence as compared to catalase negative samples.

Catalases catalyse the breakdown of H2O2 in bacteria, which helps them defend against oxidative stress. Catalases are also engaged in a variety of biological activities, including cell growth and differentiation, as well as the generation of metabolites. In some cells, a lack of this enzyme can allow hydrogen peroxide to build up to toxic amounts (Chester B., 1979).

4.10 Capsule Staining

Most of the colony isolates obtained was having a mucoid nature and it was seen that most of the mucoid, gram-negative bacteria tend to produce capsules. Capsules are protective structures with various functions, including but not limited to adherence to surfaces and other bacteria, protection from desiccation, and protection from phagocytosis.

Simple staining methods do not work with capsules because of their primarily non-ionic composition and ability to reject stains; instead, capsule staining uses a negative staining approach that stains the cells and the background, leaving the capsule as a transparent halo around the cells (Breakwell et al, 2009).

4.11 Oxidase test

An internal oxidase enzyme is produced by organisms that have cytochromes. The oxidation of cytochrome c is catalysed by this oxidase enzyme. Organisms with cytochrome c in their respiratory chains were oxidase positive, turning the reagent blue/purple. Organisms that lack cytochrome c in their respiratory chain do not oxidise the reagent, leaving it colourless within the test's limits, and were therefore oxidase-negative.

Cytochrome oxidase is present in oxidase-positive bacteria. N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride, the test reagent, functions as an artificial electron acceptor for the enzyme oxidase. The coloured chemical indophenol blue is formed when the reagent is oxidised.

When the culture was streaked on the filterpaper soaked in 1% reagent solution the Oxidase producer is identified by the change in colour of the paper at the region of streak. There was a colour change within 10 secs of streaking the colony. Sometimes delayed results can also be obtained (P Jurtshuk Jr et al, 1976).

4.12 Hugh Leifson Test

Saccharolytic bacteria breakdown glucose in one of two ways: fermentatively or oxidatively. Fermentation produces relatively powerful mixed acids, which can be identified in a standard fermentation test medium. However, the acids generated during oxidative breakdown of glucose are exceedingly weak and few, necessitating the use of Hugh and Leifson's OF medium, which is a more sensitive oxidation fermentation medium. The medium was created by raising the glucose concentration which leads to elevated levels of acid production during the fermentation process.

The tubes were made in duplicates where in one set the medium was covered with mineral oil and the other set was left open. The colony stabbed in the medium if they utilize glucose anaerobically will result

in change in colour to yellow in the tubes with mineral oil. If the bacteria utilize glucose aerobically then the tubes without mineral oil will change colour. If not, then there would be no change in colour of the media (Lemos et al, 1985).

4.13 Thioglycollate Broth Test

Thioglycollate broth is a media used to test bacteria's aerotolerance. The growth ingredients required for bacterial proliferation are dextrose, pancreatic digest of casein, yeast extract, and L-cystine. The oxygen is reduced to water by sodium thioglycollate and L-cystine. In an anaerobic environment, Resazurin is a colourless indicator. The agar helps maintain stratification of organisms growing in separate layers of the broth by slowing oxygen diffusion.

Strict anaerobes can grow in this medium even under aerobic circumstances thanks to L-cystine and sodium thioglycollate. The reducing agent sodium thioglycollate neutralises the harmful effects of mercurial preservatives and peroxides generated in the medium, encouraging anaerobiosis and making the medium appropriate for heavy metal testing. The broth was put in screw cap tubes, covering 3/4th of the tube. This was incubated at 37°C for 48 hours and checked for growth turbidity in respective regions in the tube (Doyle et al, 1968).

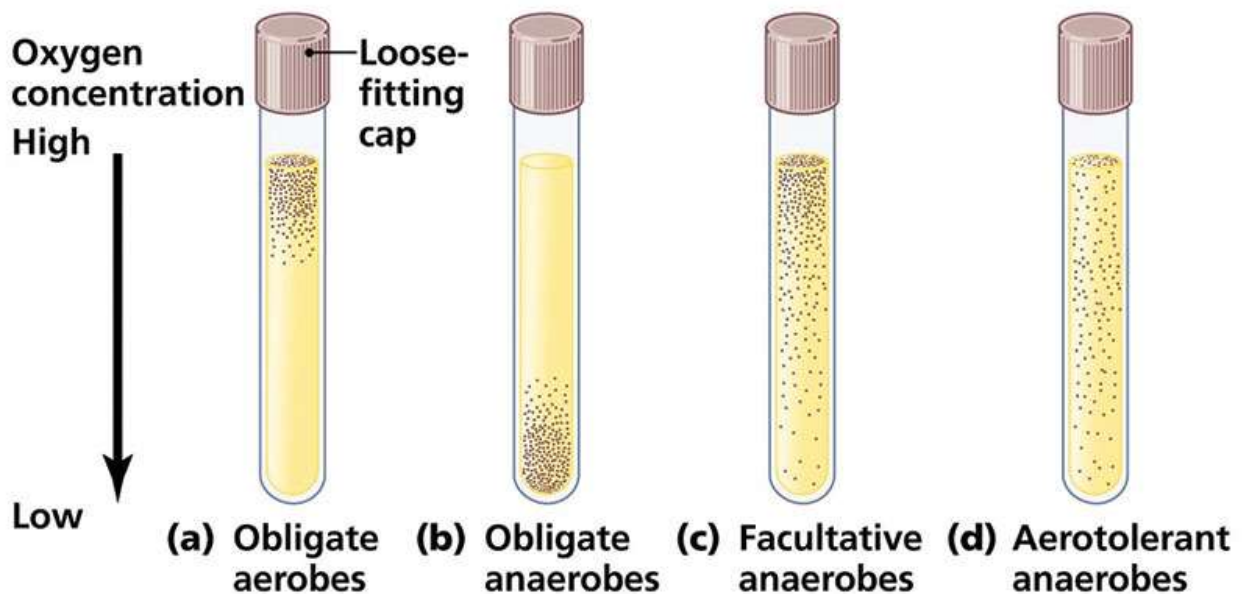


FIGURE 4.13: The location of different type of bacteria depending on their oxygen requirement.

4.14 Nitrate Reduction Test

This test is done to check if the organism is able to reduce nitrate to nitrite. The nitrites in the medium will generate nitrous acid if the organism has reduced nitrate to nitrite. Sulfanilic acid reacts with nitrous acid to generate diazotized sulfanilic acid when added. This forms a crimson product when it combines with the alpha-naphthylamine (water soluble Azo-dye). As a result, if the medium turns red after the nitrate reagents are added, it is termed a positive nitrate reduction result.

If the medium does not become red when the reagents are added, the organism was either unable to decrease the nitrate or was able to denitrify the nitrate or nitrite to form ammonia or molecular nitrogen. As a result, the test will require another step. Add a pinch of zinc powder to the mix. If the tube becomes red

after adding the zinc, this indicates that there was unreduced nitrate present. As a result, the second step's red colour is a bad result.

The result was called a positive complete if the medium does not become red after adding the zinc powder. There was no nitrate to decrease if no red colour appears. Because no nitrite was present in the media, denitrification occurred, resulting in the formation of ammonia or molecular nitrogen. The change in coloration occurs in seconds which results in easy detection. The broth had to be incubated with the culture for 24 hours (Conn et al, 1919).

4.15 <u>Siderophore Production</u>

The isolates were tested for siderophore production by growing them on King's B medium supplemented with Chrome Azurol S dye. The King's B media was autoclaved separately and the CAS dye is added after cooling the media. The agar plate is spot inoculated and incubated at room temperature for 48 hours. After the incubation period there is yellow halo ring formed around the colony. The colonies that produce the zones are siderophore producing isolates. The diameter was measured to check how much of iron from the media is sequestered (G. Ames et al, 1989).

4.16 <u>Detecting EPS Production</u>

Congo Red Agar was used for detecting exopolysaccharide (EPS) production by inoculating the isolates on the agar. After an incubation period of 24 hours at room temperature black colonies were formed where the isolate grew. The media was prepared by suspending 0.8g of Congo red powder in the remaining media components. If the isolates changed its coloration to black this indicates that the bacteria produce EPS (Roper et al, 2007).

4.17 <u>Indole Test</u>

Indole test demonstrates the ability of certain organisms to decompose tryptophan to indole which accumulates in the medium. Tryptophan is hydrolysed to tryptophanses to produce three possible end products such as pyruvic acid, ammonium and indole.

Kovac's reagent was used as an indicator which contains hydrochloric acid and 4-p-dimethylamino benzaldehyde. On reacting with indole, it produces a ring thus indicating a positive result. The colour is formed because amyl alcohol is not water soluble thus forming an oily layer over the broth. Th e isolates were grown in F.M. broth for 48 hours and then a stab needle is taken and dipped in the broth and stabbed directly in the tube. This is checked for indole utilization by putting few drops of Kovac's reagent, which gives coloured ring formation within seconds (MacWilliams MP, 2009).

4.18 <u>SIM Test</u>

The medium containing ferrous ammonium sulphate and sodium thiosulfate, both of which act as markers for hydrogen sulphide generation (H2S). When ferrous sulphide, a black precipitate, is formed when ferrous ammonium sulphate reacts with hydrogen sulphide gas, it is detected by hydrogen sulphide production. This medium's casein peptone is high in tryptophan. Tryptophan is converted to indole by organisms that have the enzyme tryptophanase. After incubation of the inoculation medium, Indole detection is achieved by adding Kovac's reagent. Indole reacts with p-dimethylaminobenzaldehyde to form a red band at the medium's top. After adding Kovac's reagent to a negative indole test, there is no colour change, that is Kovac's reagent is yellow.

A decreased quantity of agar in the medium creates a semi-solid structure that allows bacterial movement to be detected. Motile organisms spread from the stab line into the media, causing turbidity or cloudiness. Non-motile bacteria's growth was limited along the stab line, leaving the surrounding medium clear (Darkoh C, 2015).

4.19 Voges Proskauer Test

Voges-Proskauer Test is used to detect the presence of acetoin. It determines whether the organism produces acetyl methyl carbinol from the glucose fermentation. Acetyl methyl carbinol is the product of butylene glycol which mainly involves glucose utilization. The formation of a brown ring upon addition of Barritt's reagent indicating positive results which the absence of a ring indicated negative result (Barry AL, 1967).

4.20 Citrate Utilization Test

Citrate utilization test is mainly used to determine the ability of organisms to utilize sodium citrate as its carbon source and inorganic ammonium dihydrogen phosphate as its fixed nitrogen source. Alkaline carbonates, bicarbonates and ammonium hydroxide are the end products formed when an organic acid such as citrate is used as a carbon and energy source, and ammonium salts are used as nitrogen source. Originally the tubes are green in colour and after incubation for 24 hours at room temperature the tubes changed the colour to Prussian blue colour, this was because of the raise in pH of the medium above 7.6, probably due to production of alkaline carbonates and bicarbonates (Vaughn RH et al, 1950).

4.21 Scanning Electron Microscopy (SEM)

The isolates showing positive results in majority of the tests were then further analyzed by Scanning Electron Microscopy using Carl-Zeiss Scanning Electron Microscope at Goa University.

The isolates were grown in respective medium and centrifuged at 10,000rpm for 8 minutes. The supernatant was discarded and the pellet was washed twice with sodium phosphate buffer. The obtained cell pellet was resuspended in 2ml of sodium PO_4 buffer. A loopful of culture was taken on a piece of glass slide and air dried. The smears were fixed with 2.5% glutaraldehyde and kept overnight. Next day, treatments of 10mins each was given using 20%, 30%,40%,50%, 60%, 70%, 80%, 90% and 100% of ethanol. The sample was air dried. The slide pieces were then sputter coated with gold and then processed for SEM analysis.

4.22 X-Ray Diffraction Analysis

XRD can be used to determine the phase of metal/ element present inside the magnetosomes. Fresh isolates were grown in F.M. + N.B. The cells were harvested by centrifuging at 7000rpm for 10 minutes followed by distilled water wash. The pellet was then resuspended with 5 ml alkaline lysis buffer and stored in the refrigerator. Next day the samples are subjected to 15 minutes of sonication to burst open the cells. After sonication the sample was centrifuged. The procedure resulted in a black colour compound that was deposited at the bottom. The pellet was then washed twice with 70% ethanol and allowed to air dry to remove any form of moisture and stored at -20°C till further processing. The final product obtained was then lyophilized, crushed into powder form, and then sent for XRD analysis.

4.23 Scanning Transmission Electron Microscopy

The Scanning Transmission Electron Microscope (STEM) works in a similar way to a light microscope. The main distinction is that light microscopes use light rays to focus and make images, whereas the STEM employs an electron beam to concentrate on the object and produce images.

In comparison to light, which has a long wavelength, electrons have a shorter wavelength. The wavelength of light reduces as resolution power grows in a light microscope, yet when the electron lights the specimen, the resolution power increases, increasing the wavelength of the electron transmission in the STEM.

Because electrons have a wavelength of around 0.005nm, which is 100,000 times shorter than light, STEM has a 1000-fold greater resolution than a light microscope.

The STEM, for example, can be used to examine the interior structures of the tiniest particles, such as virion particles.
The electron gun produces a stream of electrons that the magnetic condensing lens causes to fall across the object.
The beam is partially transmitted and partially diffracted depending on the angle of incidence. The image is formed by recombining both of these beams at the E-wald sphere. The phase contrast image is the result of combining the two images. An amplitude contrast must be obtained in order to boost the image's intensity and contrast. This can only be accomplished by employing the transmitting beam, which eliminates the diffracted beam. The observation wavelength for the isolates were done at 100-500nm.

Chapter 5 RESULTS AND DISCUSSION

A) RESULTS OBTAINED

5.1: Sample Collection and Enrichment and Isolation of MTB

The samples were collected from two different freshwater sources as mentioned in table 5.1 and the pure isolates obtained after repeated streaking are shown in figures 5.2.

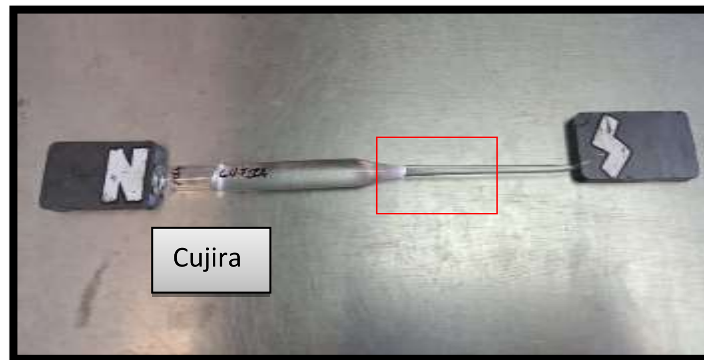
Figure 5.1a: Carambolim Lake **Figure 5.1b: Cujira Stream**

The samples were enriched using bar magnets as shown in the figure No. 5.1c

Figure 5.1c) Enrichment of the MTB using bar magnets.

A- Shows the magnet placed at the north pole of the container

B- Shows the magnet placed at the south pole of the container.
C- Layer of black precipitate formed by the Magnetotactic bacteria.
Figure 5.2: The Figures 5.2a and 5.2b show the isolates obtained after CRT purification. Figures 5.2 c-j show the streaked plates for isolating pure cultures.



Key: N- North pole, S- South pole

FIGURE 5.1: Purification of Magnetotactic bacteria through Capillary Race Track method. The highlighted region was where the Magnetotactic bacteria will get attracted and collect towards the South Pole of the magnet.

Isolation of MTB:

After enrichment of the sample by Capillary race track method, the enriched samples were spread plated and incubated for 48 hours. Majority were translucent colonies with some having unique color such as brown, blue and yellow. In this manner a total of 6 isolates were obtained from both the sampling sites. The obtained isolated colonies were purified by repeated sub culturing and used for further studies. Fig No.5.2a and b depicts the isolated colonies. Fig No. 5.2c-j are the purified isolates.

Figure 5.2a

Figure 5.2b

Figure 5.2c

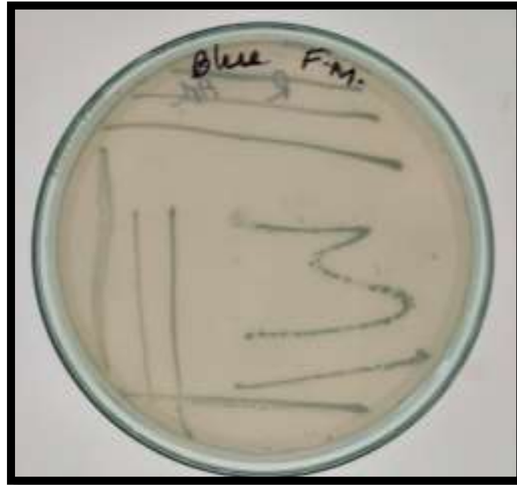


Figure 5.2d

Figure 5.2e

Figure 5.2 f

Figure 5.2g

Figure 5.2h

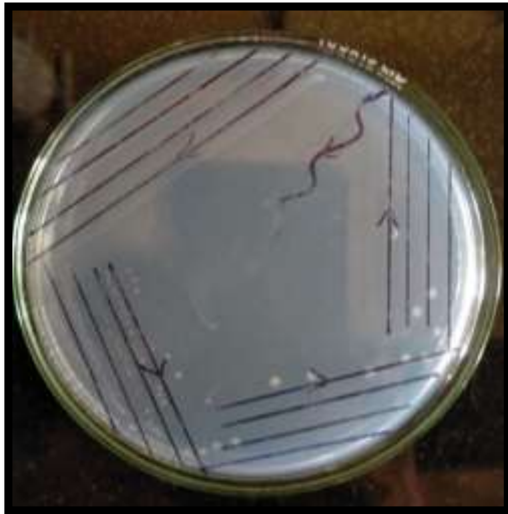


Figure 5.2i

Figure 5.2j

5.2: Morphological Characteristics

Morphological characteristics were studied by using light microscopy. The colony characteristics of the selected isolates are summarized in table 5.2

Table 5.2: Colony Characteristics of the CRT purified colonies obtained from two different freshwater sources and grown on Flies Media (F.M.).

Characteristics/Colony → ↓	ARDM1	ARDM2	ARDM3	ARDM4	ARDM5	ARDM6
Temperature	29°C	29°C	29°C	29°C	29°C	29°C
Time	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours
Colour	Brown	Blue	White	White	Orange	Yellow
Opacity	Translucent	Translucent	Translucent	Translucent	Translucent	Translucent
Size(cm)	0.1cm	0.2cm	pinpoint	0.3cm	0.2cm	0.4cm
Form	Irregular	Circular	Irregular	Circular	Circular	Circular
Elevation	Raised	Umbonate	Raised	Flat	Convex	Flat
Margin	Undulate	Wavy	Undulate	Wavy	Entire	Entire
Consistency	Butyrous	Butyrous	Slimy	Slimy	Slimy	Butyrous
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Media	F.M.	F.M.	F.M.	F.M.	F.M.	F.M.
Sample Form	Water	Water	Water	Water	Water	Water
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Gram Character	Gram -ve	Gram -ve	Gram -ve	Gram +ve	Gram +ve	Gram -ve
Source of Isolate	Cujira	Caramboli m	Caramboli m	Cujira	Cujira	Caramboli m

Note: Gram -ve = Gram Negative, Gram +ve= Gram Positive

5.3 Gram Staining and KOH String Test

Table 5.3: Determination of the Gram Character with the help of Gram staining and KOH string test.

Isolate/Test→ ↓	Gram Staining	KOH String Test	Interpretation
ARDM1	Gram -ve short rods	+	Gram -ve short rods
ARDM2	Gram -ve short rods	+	Gram -ve short rods
ARDM3	Gram -ve short rods	+	Gram -ve short rods
ARDM4	Gram +ve cocci	-	Gram +ve cocci
ARDM5	Gram +ve cocci	-	Gram +ve cocci
ARDM6	Gram -ve short rods	+	Gram -ve short rods

Key= +: Positive, -: Negative

Note: Gram -ve = Gram Negative, Gram +ve = Gram Positive

Important Observation: It was observed that majority of the isolates had Gram -ve character and were having a short rod shape, whereas there were two isolates that were having Gram +ve character and were cocci in shape.

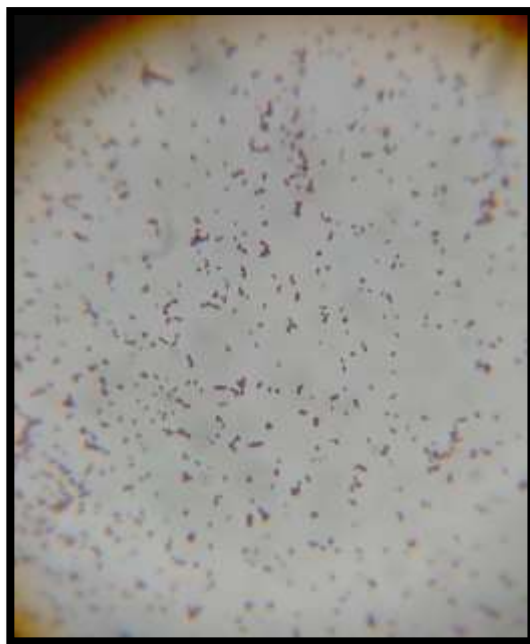


Figure 5.3 a

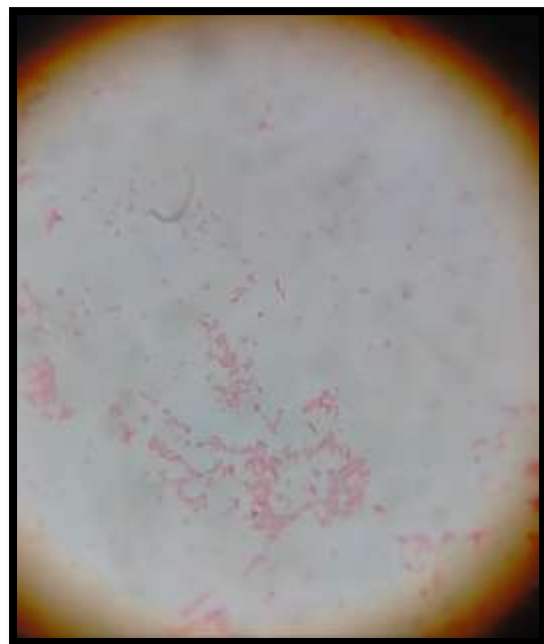


Figure 5.3b

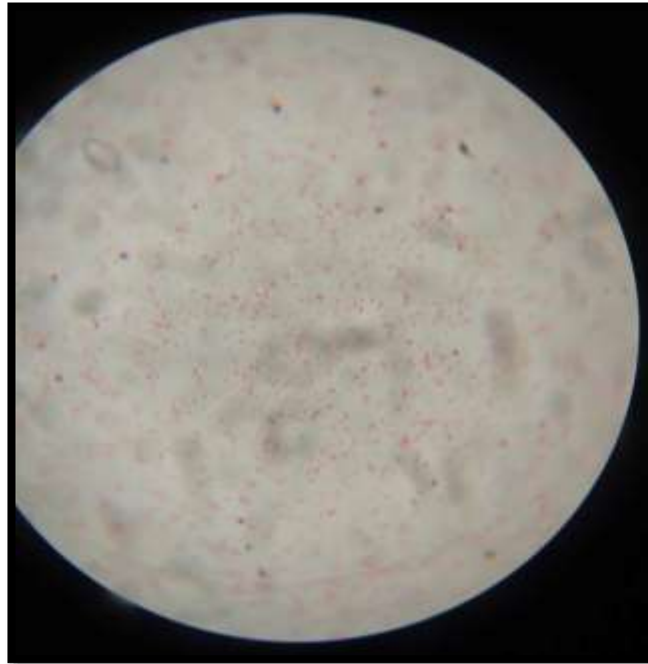


Figure 5.3 c

Figure 5.3: a and c show Gram -ve short rods and b shows Gram+ve short rods

Figure5.3d

Figure 5.3d shows the string formed, thus indicates that isolates were Gram Negative as compared to no string string formation and so are Gram Positive.

5.4 Capsule Staining

Table 5.4: To determine if the isolates produce capsule by capsule staining.

Isolates	Result Obtained
ARDM1	+
ARDM2	+
ARDM3	+
ARDM4	-
ARDM5	-
ARDM6	+

Key= +: Present, -: Absent

Note: Positive result was indicative of capsule formation whereas negative result was indicative of no capsule formation. Out of all the isolates only two isolates did not produce capsules as seen in figure 5.4.

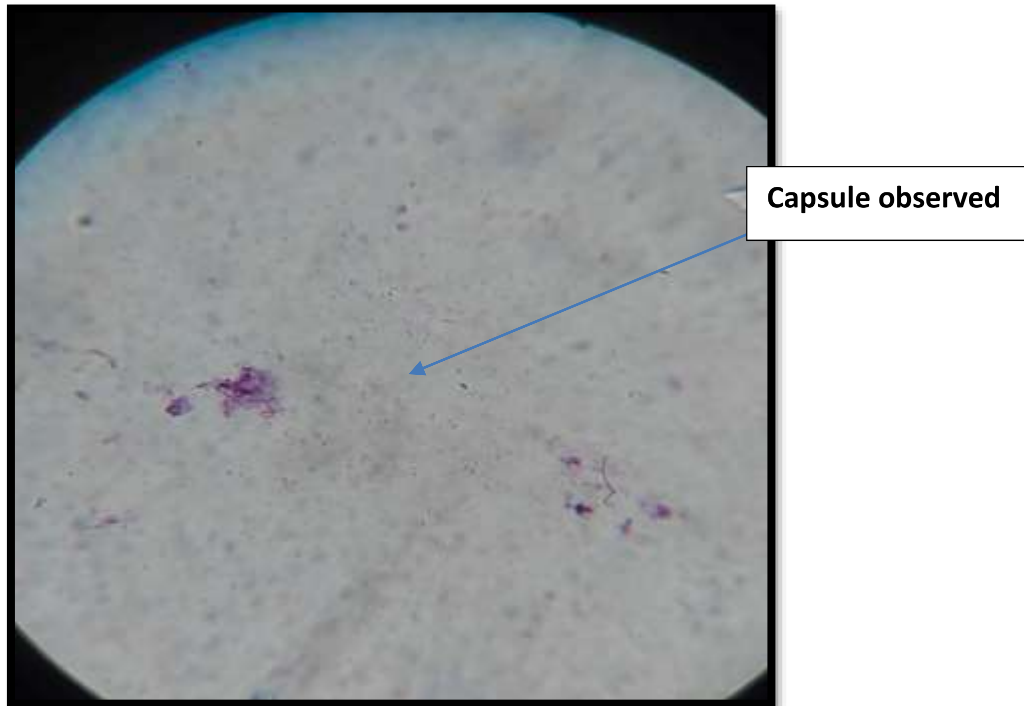


Figure 5.4

5.5 Studying Magnetotaxis of the isolates using Microscopy

The motility of the bacteria was checked with the help of the light microscope by placing the bar magnet at the edge of the drop from both sides and the characteristic movements was observed using video microscopy. Since the magneto tactic bacteria are motile they will move to the pole to which they are attracted to, in this case it is the South Pole. In order to rule out the possibility of any other form of magnetic components in the inoculum the magnetotaxis property of the isolates was checked. The magneto taxis behavior shown by the isolates is summarized in table 5.5.

Table 5.5: Magnetotaxis behavior shown by the isolates.

Isolates	Motility Result
ARDM1	**Motile**
ARDM2	**Motile**
ARDM3	**Motile**
ARDM4	**Motile**
ARDM5	**Motile**
ARDM6	**Motile**

Note: The motility of the bacteria was checked with the help of the light microscope by placing the bar magnet at the edge of the drop from both sides as performed in section 4.5.

5.6 Hugh-Leifson's Test

Table 5.6: To determine the oxidative- fermentative nature of the isolates by Hugh-Leifson's Test.

Isolate	Result obtained		Interpretation
	Tube without	**Tube with Mineral Oil**	

	Mineral Oil		
ARDM1	Yellow	Yellow	Microaerophile
ARDM2	Yellow	Yellow	Microaerophile
ARDM3	Pale-Yellow	Yellow	Microaerophile
ARDM4	Yellow	Green	Strict aerobe
ARDM5	Pale-Yellow	Yellow	Microaerophile
ARDM6	Yellow	Yellow	Microaerophile

Note: Two sets of tubes were made with one tube having a layer mineral oil and the other is without the oil. There is a change in colour to yellow of the media with the tubes with a layer of oil, which are indicative of anaerobes and the ones which do not show are a result of being aerobic in nature as seen in figures 5.6. it was observed that there was colour change in both the tubes for most of the isolates as they used up the glucose as their carbon source thus the isolates were fermentative in nature.

Figure 5.6a

Figure 5.6b

Figure 5.6: Tubes showing growth depending on their oxygen requirement.

5.7 Nitrate Reduction Test

Table 5.7: Determination of Nitrate reduction.

Isolate	Results obtained
ARDM1	+
ARDM2	+
ARDM3	+
ARDM4	-
ARDM5	+
ARDM6	+

Key: +: Positive, -: Negative

Note: The tubes showed red colouration on addition of sulphanilic acid and alpha-naphthylamine thus indicating the reduction of nitrate to nitrite by the isolates (shown in table 5.7) as seen in figure 5.7.

Figure 5.7

Figure 5.7 showing detection of nitrate reduction by change in colour to red.

5.8 Catalase Test

Table 5.8: Determination of the presence of Catalase enzyme in the isolates

Isolate	Results obtained
ARDM1	+
ARDM2	+
ARDM3	+
ARDM4	+
ARDM5	-
ARDM6	+

Key: +: Positive, -: Negative

Note: The presence of catalase enzyme in 5 of the isolates except one was detected by the presence of effervescence when the sample was inoculated on H_2O_2 seen in figure 5.8 a and b.

Figure 5.8a: Effervescence formed by the isolates

Figure 5.8b: Effervescence formed by the isolates

5.9 Oxidase Test

Table 5.9: Determination of Oxidase in the isolates

Isolate	Results Obtained
ARDM1	+
ARDM2	+
ARDM3	-
ARDM4	-
ARDM5	-
ARDM6	+

Key: +: Present, -: Absent

Note: The isolates that showed positive result for oxidase enzyme possess cytochrome oxidase (an iron hemoprotein) that was mainly used in the bacterial electron transport chain as seen in figure 5.9 a, b and c.

Figure 5.9a

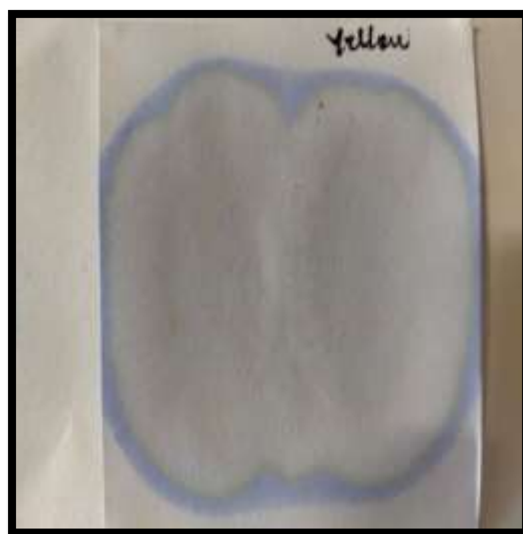


Figure 5.9b

Figure 5.9c

5.10 Indole Test

Table 5.10: Determination of Indole production

Isolate	Result Obtained
ARDM1	-
ARDM2	-
ARDM3	-
ARDM4	-
ARDM5	-
ARDM6	-

Key: -: Negative

Note: Since there was no accumulation of indole in the media by the breakdown of tryptophan, thus the result obtained was negative with no red ring formation as seen in figure 5.10 a, b.

Figure 5.10a

Figure 5.10b

5.11 H_2S Production

Table 5.11: Determination of H_2S production by the isolates.

Isolate	Results Obtained
ARDM1	+
ARDM2	+
ARDM3	-
ARDM4	-
ARDM5	-
ARDM6	+

Key: +: Present, -: Absent

Note: The presence of blackish brown coloration in the media was indicative of H_2S production by the isolates. This coloration was noticed at the stabbed region and surrounding as observed in figure 5.11.

Figure 5.11

5.12 Thioglycollate Broth Test

Table 5.12: Determination of the nature of bacteria based on their Oxygen Utilization.

Isolate	Results Obtained
ARDM1	Microaerophilic
ARDM2	Microaerophilic
ARDM3	Microaerophilic
ARDM4	Aerobic
ARDM5	Microaerophilic
ARDM6	Microaerophilic

Note: The turbidity in the broth was observed towards the middle and end of the tube and not towards the top end thus indicating that they were microaerophiles as compared to the one that was showing aerobic growth as seen in figure 5.12 a, b and c.

Figure 5.12a **Figure 5.12b** **Figure 5.12c**

5.13 Citrate Test

Table 5.13: Citrate Utilization determination

Isolate	Results Obtained
ARDM1	+
ARDM2	+
ARDM3	+
ARDM4	+
ARDM5	+
ARDM6	+

Key: +: Positive

Note: Upon utilization of Citrate by the bacteria the media which was originally green in colour changed to Prussian blue thus indicating there was change in pH to alkaline nature and that bicarbonates, ammonium hydroxide was produced as the end product as seen in figure 5.13a, b and c.

Figure 5.13a **Figure 5.13b** **Figure 5.13c**

5.14 Methyl Red Test

Table 5.14: To determine if the isolates can ferment glucose by Methyl Red Test.

Isolate	Results Obtained
ARDM1	+
ARDM2	+
ARDM3	+
ARDM4	+
ARDM5	+
ARDM6	+

Key: +: Positive

Note: The isolates utilizing glucose will change the colour of methyl red from yellow to red thus indicating the production of mixed acids. As seen in Figure 5.17.

5.15 Voges- Proskauer Test

Table 5.15: Determination of the presence of acetoin for Glucose utilization by Voges-Proskauer Test.

Isolate	Result Obtained
ARDM1	-
ARDM2	-
ARDM3	-
ARDM4	-
ARDM5	-
ARDM6	-

Key: -: Negative

Note: The isolates as they did not produce acetyl methyl carbinol did not produce a brown ring which was indicative of glucose fermentation. As seen in the figure 5.17.

5.17 Biochemical Strip Detection

Isolates	Indole	MR	VP	Citrate	Glucose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose
ARDM 1	-	+	-	+	+	+	-	-	-	+
ARDM 2	-	+	-	+	+	+	-	-	-	+
ARDM 3	-	+	-	+	+	+	-	-	-	+
ARDM 4	-	+	-	+	+	+	-	-	-	+
ARDM 5	-	+	-	+	+	+	-	-	-	+
ARDM 6	-	+	-	+	+	+	-	-	-	+

Table 5.17: Test results obtained by inoculating isolates on a biochemical strip.

Isolate	Adonitol	Arabinose
ARDM1	-	V
ARDM2	-	V
ARDM3	-	-
ARDM4	-	-
ARDM5	-	V
ARDM6	-	V

Key: +: positive, -: negative, V: 11-80% positive

Note: The results obtained from isolating the isolates in the biochemical strip was recorded in Table 5.17 as shown in figure 5.17.

Figure 5.17: Biochemical strip showing positive results for various tests

5.18 EPS Production

Table 5.18: Determination of Exopolysaccharide Production.

Isolate	Results Obtained	Interpretation
ARDM1	**Blackened Colony**	+
ARDM2	**Blackened Colony**	+
ARDM3	**No Blackening of the colony**	-
ARDM4	**Blackened Colony**	+
ARDM5	**Blackened Colony**	+
ARDM6	**No Blackening of the colony**	-

Key: +: Positive, -: Negative

Note: The change in colour of the isolate was indicative of the production of Exopolysaccharide as compared to those isolates that did not show blackening of the colony as seen in figure 5.18.

Figure 5.18: Black colouration of the colonies thus indicating the production of EPS

5.19 Siderophore Detection

Table 5.19: Siderophore Production detection by Halo formation of the isolates.

Isolate	Colony Diameter (cm)	Zone Diameter (cm)	Result Obtained
ARDM1	**0.6**	**2.5**	**2.5**
ARDM2	**0.5**	**2**	**2**
ARDM3	**-**	**-**	**-**
ARDM4	**0.4**	**1.2**	**1.2**
ARDM5	**0.3**	**0.6**	**0.6**
ARDM6	**0.4**	**1.5**	**1.5**

Note: The diameter of the zones indicates the amount of Iron the isolate had sequestered thus giving Halo Yellow zones around the isolate as seen in figure 5.19a as compared to the control plate in figure 5.19b.

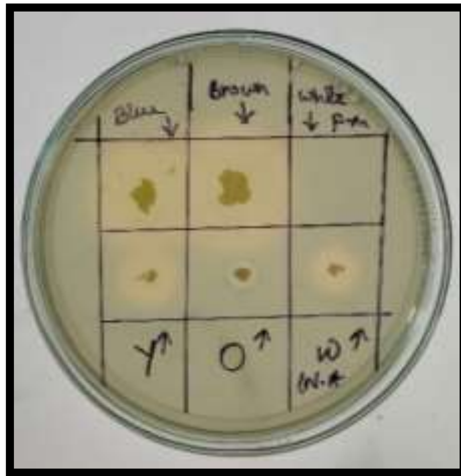


Figure 5.19a

Figure 5.19b

5.20: Scanning Electron Microscopy (SEM)

The surface morphology of the colonies was observed by SEM analysis. The instrument used was by Carl Zeiss (Figure 5.20c) and the bacteria was observed at a magnification of 2.00 KX as seen in figure 5.20a and b of isolate ARDM1.

Figure 5.20a: Rod shaped structure observed for the bacteria

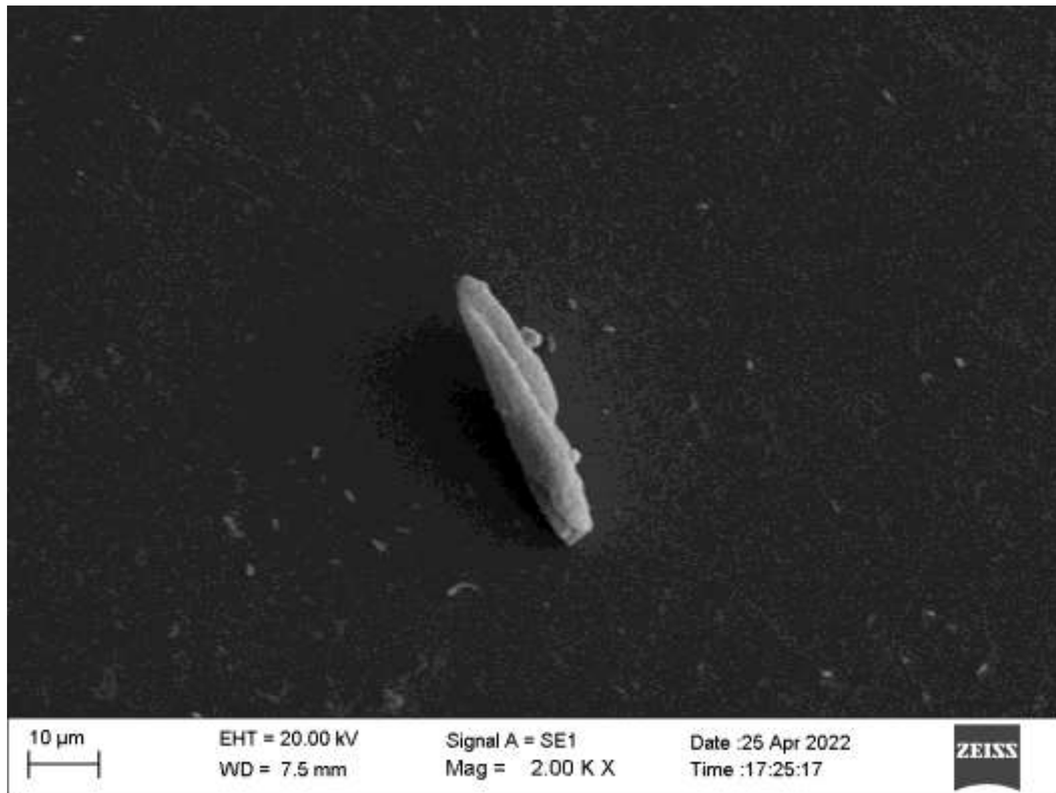


Figure 5.20b: Rod shaped structure observed for the bacteria

Figure 5.20c: instrument used for SEM analysis

5.21: X-Ray Diffraction Analysis (XRD)

The XRD profile of the magnetosomes isolated from ARDM1 was recorded.

The element present in magnetosomes was found to be Perovskite . Perovskite is an element mainly used in the degradation of organic pollutants. The peak was observed at 32 with an intensity of more than 1000 at 2Θ degree as seen in figure 5.21a and compared to the standard XRD graph for Perovskite shown in figure 5.21b. The profile was observed on Rigaku instrument for XRD analysis (figure5.21c).

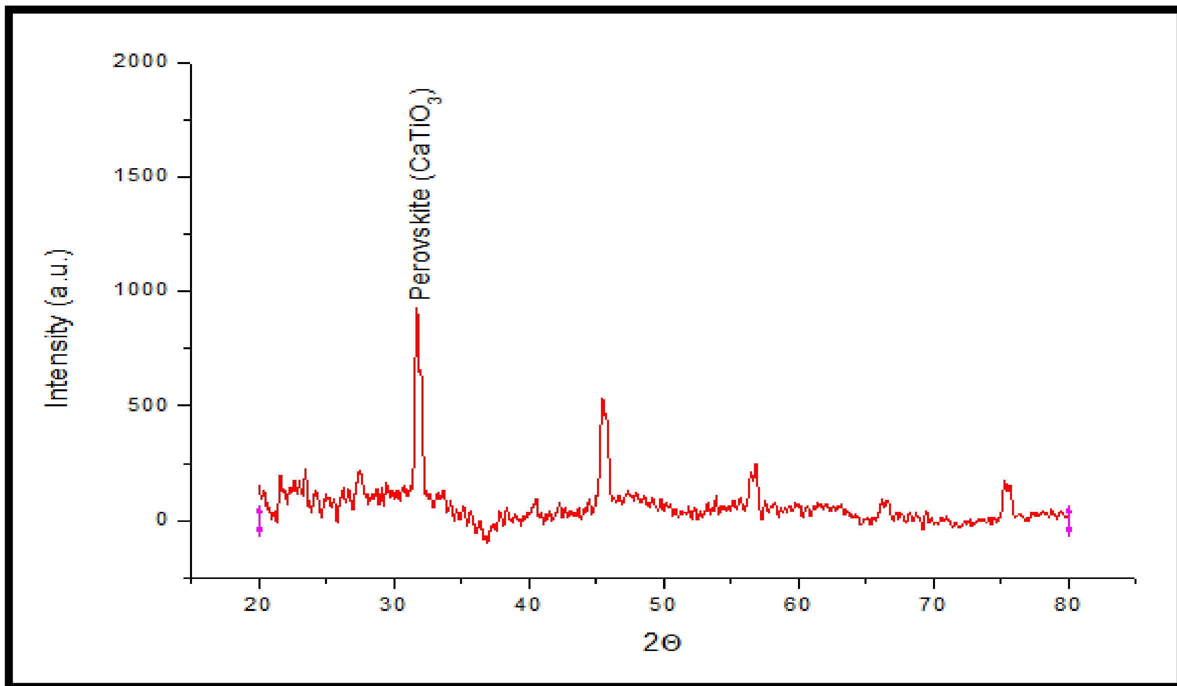


Figure 5.21a: Perovskite profile obtained from XRD analysis for sample ARDM 1

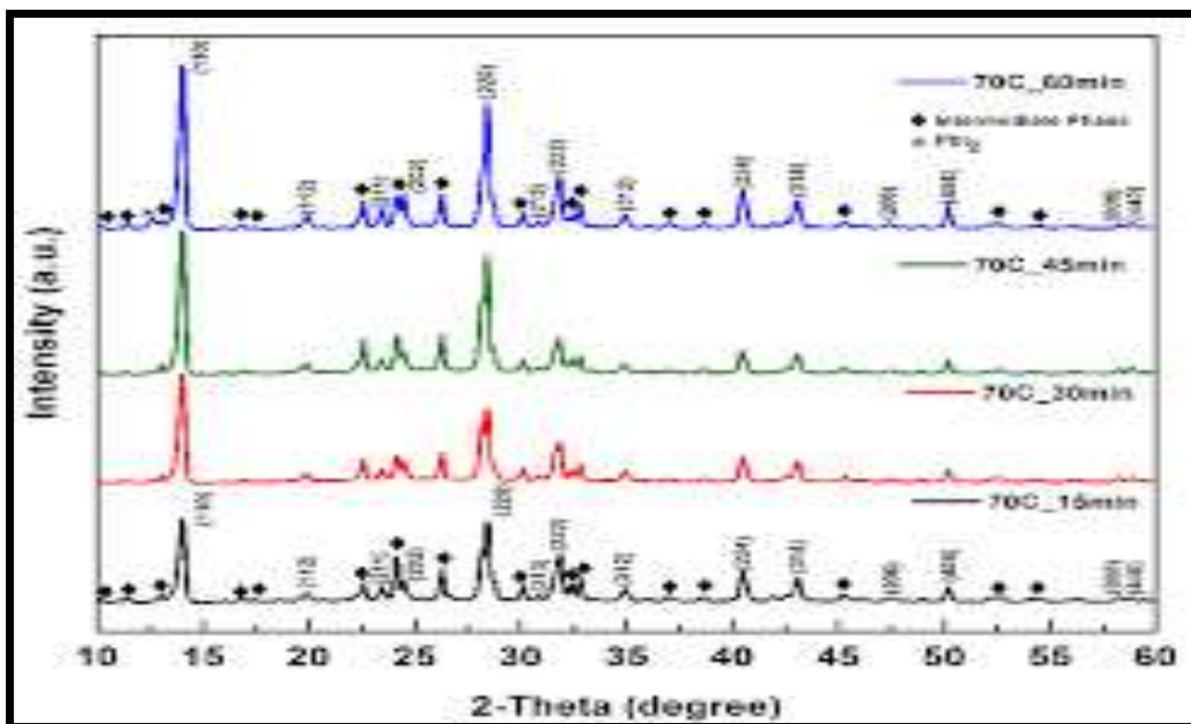


Figure 5.21b: Standard graph for Perovskite Peaks

Figure 5.21c: Rigaku Instrument used for XRD

5.22: STEM (Scanning Transmission Electron Microscopy)

STEM analysis was used to observe the inner components of the magnetotactic bacteria and the magnetosomes were aligned in a linear fashion along the length of the MTB. The isolate was observed at a magnification of 30,000-60,000x at 1-3µm (figure 5.22b and c) with the 5.22c showing presence of flagellum. These images were observed on Quanta FEG 250 as shown in figure 5.22a. The presence of magnetosomes was observed for isolate ARDM1.

Figure 5.22b: Quanta FEG 250 for STEM Analysis

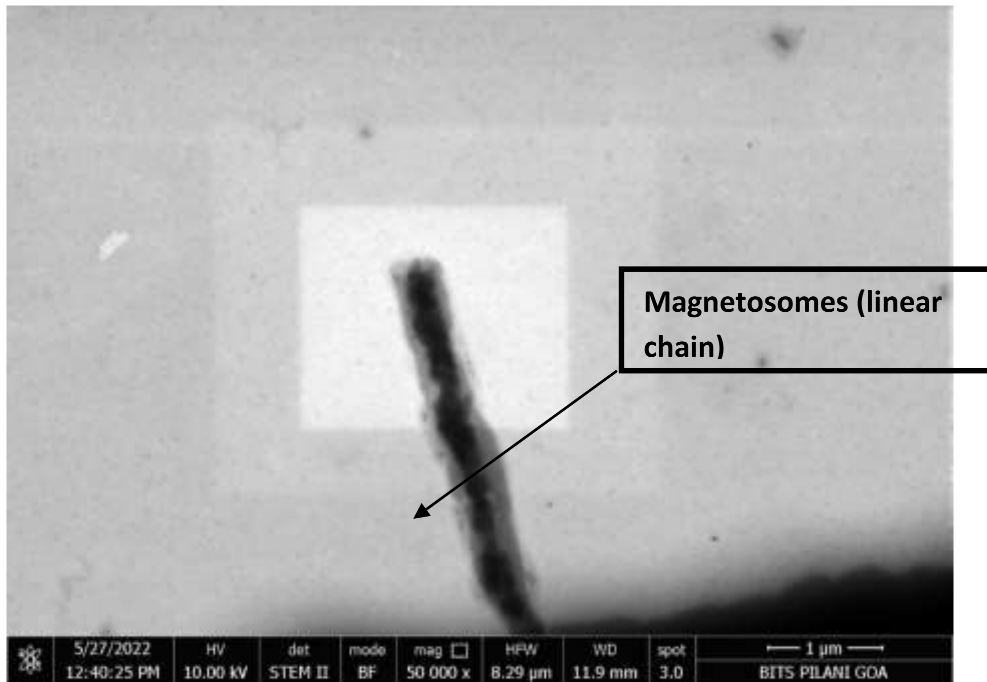


Figure 5.22b: Magnetosomes visualized in a linear fashion upon observation through STEM analysis

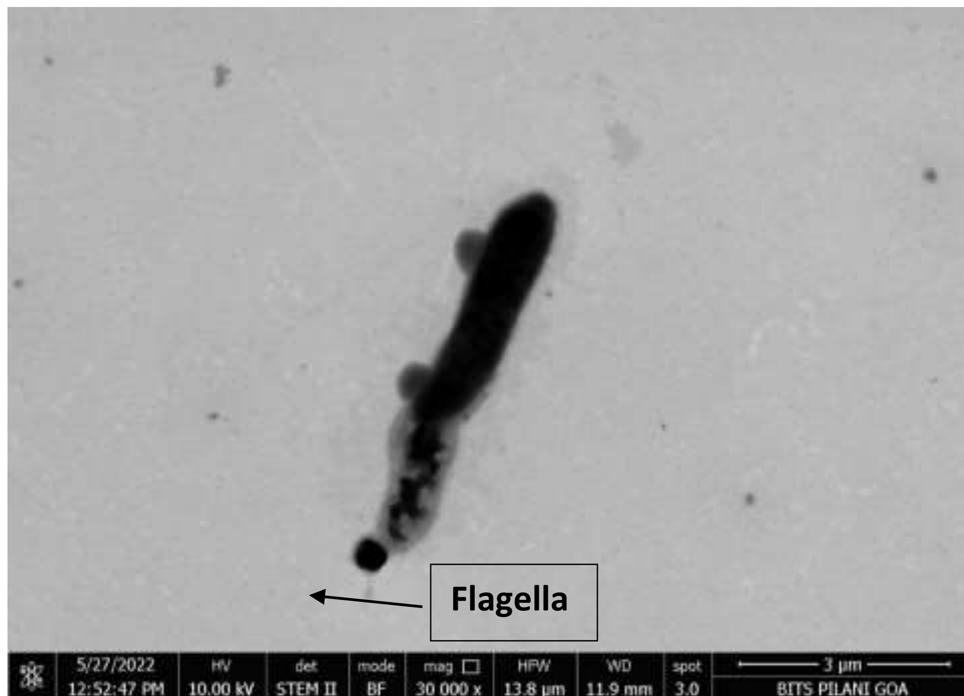


Figure 5.22c: Flagella seen that helps in locomotion of MTB

B) DISCUSSION

With the help of the Physico-chemical parameters obtained from the freshwater sources it was easy to maintain the conditions required for the growth of the bacteria in the laboratory conditions. Determining the Geographical locations, it was convenient to locate which pole the bacteria would greatly get attracted to.

Taken note of all the on-site parameters of the location, it was organized in table 1. It was found that the pH was at neutral and the temperature was between 25°-29°C and thus it was favourable for the growth of Magnetotactic Bacteria. The salinity of all freshwater sources is be zero% anything above is indicative of pollution. The conditions are very important for the growth of the bacteria as observed by Jajan *et al*, 2019.
By performing certain tests and upon observation of the surroundings of the freshwater sources, it was seen that there was some sort of elemental accumulation. The reasons for this could be due to surface-runoffs, leaching of mine tailings into the ground water, Municipal, industrial, and agricultural waste, wastewater and fertiliser run-off, power generation, heavy industries, automobiles, and other sources all contribute to freshwater pollution. The mining leachate was the main source of contamination as to which certain elements got accumulated in the sediment. Inspite, of all this the pH remained neutral that is 7 thus indicating that the contamination did not alter the conditions of the freshwater. A reason for this can be maybe due to the fact that magnetosomes can sequester certain elements and can even degrade organic pollutants (Pola et al, 2020). Not much research is done as to how this degradation is actually occurs.
The brief period of 2 months of incubation of the samples in dark lead to successful formation of the Oxic-Anoxic Transition Zone at the interface of the water and Sediment and this is the region where the Magnetotactic Bacteria are evident to be found. As the containers remained closed for a period of 2 months, the production of H_2S was noticed as the oxygen got depleted which is seen in anaerobic and microaerophilic organisms. The OATZ was formed after all the sediment particles have settled and the sample was kept undisturbed, which formed just below the surface of the sediment layer. From outside of the walls of the container a layer of Black precipitate was seen forming which is indicative of the Magnetotactic Bacteria (Lefèvre and Bazylinski, 2013).
After the incubation period the samples were taken and magnets were placed at the sides of the container which was a major source of MTB attraction. Based on the type of magnetic field whether it is south pole or north pole the bacteria will get attracted to it.
Once the inoculum from the container was obtained the sample was purified by Capillary Race Track Method. The method allowed the sample to be purified rom any other type of bacteria that might come along with the sample by passing through a filter and the magnetotactic bacteria will get attracted to the tip end of the pipette where the magnet was placed. The isolates obtained by CRT had high affinity to the external magnetic field indicating the presence of magnetosomes (Oestreicher *et al,* 2012).
This purified sample was then inoculated onto Flies Media Plates, thus giving the bacteria space for growing as isolates. The colonies obtained were then further streaked to obtain isolated colonies. After a brief incubation of 48 hours the isolates were checked for their morphological analysis. With some isolates have very unique features such as colour. The size of colonies ranged from pinpoint to 0.4cm, having butyrous texture, translucent in nature. The main highlighting feature was the colour as such type of colonies have not yet been reported in any of the studies done so far regarding this particular bacterium (Figure 5.2c, d and h).
With the help of Video Microscopy, it was seen that the bacterial isolates move under the influence of magnet. Not all showed this kind of movement, when it was placed at the edge of the drop when performing hanging drop method. Out of 6, 4 showed prominent behaviour when the magnet was placed. Other bacteria move with the help of some sort of locomotory organ but magnetotactic bacteria move

with the help of magnetosomes. The movement was called magnetotaxis and this was studied by *Sharma et al., (2007).*
The isolates were then checked for their Gram Character and 4 were Gram negative and 2 were Gram Positive. This was further confirmed by performing KOH String Test. Since the KOH results corelated to the Gram staining technique, thus the Staining was performed rightly. Majority of the Magnetotactic Bacteria are found to be Gram Negative as also studied by Wang *et al*, (2020).
Majority of the isolates were having short rod shape and since it was grown on Magnetotactic Bacteria specific media there was no doubt of the growth of other forms of bacteria in the media.
Thioglycollate and Hugh Leifson Test were the two tests done to check the oxidative nature of the bacteria. It was found out that 5 out of 6 isolates were microaerophiles and one was a strict aerobe. This coincides with the OATZ condition for the growth of Magnetotactic bacteria as seen in Flies *et al*, (2004).
Nitrate Reduction was seen in all the isolates where in nitrate was reduced to nitrite and then finally to molecular nitrogen which is another property of OATZ conditions. Since the level of oxygen is very low nitrate is used as an electron acceptor rather than oxygen. Due to this property present in magnetotactic bacteria it is able to bioremediate nitrate contaminants. Since the level of oxygen is low this favors the formation of magnetosomes. SIM media was used to check the presence of H_2S production which was seen in 3 isolate tubes.
Catalase is an enzyme that reacts in the presence of free oxygen radicals. These radicals are a by-product of metabolism as studied by Schüler, Frankel, (1999).
Citrate utilization also was checked as the pH of the media changed, this was recorded in all 6 of the isolates, similarly indole test was done which did not show any result in any of the isolates. Methyl red showed positive results in all of the wells and Voges-Proskauer showed no result in any of the wells (Table 5.17). Similar study was also reported by Waghmare et al, (2018).
By using King's B media, Siderophore Production was checked (Table 5.19), which is another property of the bacteria to sequester iron. Due to this the pH of the freshwater source doesn't change as normally the salinity of the freshwater is zero. This comes under Bioremediation as these bacteria can degrade organic pollutants from the water source studied by Tan *et al*, 2018.
Extracellular macromolecules secreted as a tightly bound capsule or loosely attached slime layer by microbes are known as exopolysaccharides (EPS). This was observed in 5 out of 6 isolates tested positive for EPS production (Table5.18). As the concentration of nutrients at the OATZ is very little, with the accumulation of EPS the nutrient balance is maintained thus enables the bacteria to survive and grow. Just the detection of EPS was done but was never tested for their functional role, therefore making it another point to ponder on and study.
The Biochemical strip helped to confirm the present of glucose, lactose and sucrose with some detection of arabinose being utilized by the bacteria. Methyl red and Citrate was also confirmed using this technique whereas Indole and Voges-Proskauer turned out to be negative.
The structural morphology of the isolates was observed by Scanning Electron Microscopy analysis and the internal components such as magnetosomes was studied by performing STEM analysis. The elemental structure of the isolates showed the presence of Perovskite an organic pollutant degrading element, which are found specifically in magnetosomes thus concluding that the isolates are Magnetotactic Bacteria. This was observed in a study done by Tan *et al*, 2018.

To confirm majority of the tests done a concluding biochemical strip was inoculated with the culture for 24 hours and the reagents were added accordingly as shown in Figure 5.17. with all these tests and findings with the SEM (figure 5.20), STEM (figure 5.22), XRD (figure 5.21) and sequencing helps in the identification of the bacteria and confirming that the isolates were in fact magnetotactic bacteria.
In conclusion, it can be said that the Freshwater Sources in Goa are a good source of Magnetotactic Bacteria and that these microorganisms are a potent source of various applications one main being that of Bioremediation.

Chapter 6: SUMMARY

2 Sampling sites were chosen from Goa as Fresh Water Sources and the sample was collected in plastic containers of 1L capacity. These were first wiped with 70% alcohol to remove any sort of contaminants and then washed with sterile distilled water.
The containers were taken in biohazard bags to the freshwater collecting site and by holding the container with the use of gloves the water and sediment was collected in a $1/4^{th}$:$3/4^{th}$ ratio and stored in a dark space at 25°C for a period of 2 months undisturbed and the lid was left slightly loose to facilitate the growth of microaerophiles and create an OATZ condition. During the course of time a blackish layer forms which indicated low levels of oxygen in the container.
Enrichment was done by placing the container under the influence of magnets with both poles at opposite ends of the container. Aliquots from both the poles were collected. This was then subjected to CRT purification to remove any sort of debris or other bacteria. A Pasteur pipette and a bar magnet was used for this purpose. The magnet attracts the Magnetotactic Bacteria towards the tip of the pipette depending on the pole it gets attracted to. This was done for 30 minutes undisturbed and placed horizontally at the work space. The tip was broken off and then with a sterile needle and syringe the aliquot was collected and inoculated on Flies Media.
The isolates that grew on the media the media had varied morphology and characteristics, which were then checked for their motility by observing under the microscope and placing the magnet at the edge of the drop by hanging drop method, thus determining the magnetotaxis property of the bacteria under the influence of applied magnetic field. This was seen in all the isolates and was then subjected to further analysis.
Gram Staining showed the Gram Negative and positive character of the bacteria and this was confirmed by performing the KOH string test.
Hugh-Leifson test and Thioglycollate Broth Test helped to determine the respiratory nature of the bacteria and it being microaerophilic.
Nitrate reduction was seen in all isolates.
4 out of 6 isolates tested positive for the presence of capsules formation.
5 isolates showed the production of siderophores by yellow halo formation.
4 isolates showed the production of Exopolysaccharides (EPS).
Scanning Electron Microscopy confirmed the rod-shaped morphology of the isolates, STEM showed the presence of magnetosomes by observing the internal structure, XRD analysis was done to determine to elemental structure of the magnetosome thus revealing the presence of perovskite in the bacteria.
Magnetotactic bacteria is one such microorganism whose capability has not yet been explored fully. So far, the main aspect to which they have been looked into are their bioremediation property to sequester organic pollutants. There are various applications which are yet to be studied and exploited.

Chapter 7: CONCLUSION

The study done to check the presence of magnetotactic bacteria was the first report done from sampling sites that are from Carambolim Lake and Cujira Stream.

This report was done by isolating the magnetotactic bacteria in suitable OATZ conditions. Hanging drop technique and Agar plate method were used to confirm MTB isolation. When the MTB was placed in a magnetic field on a semisolid material, it migrated strongly towards both magnetic poles. Different biochemical tests such as catalase, oxidase, sugar fermentation, Gram's nature, nitrate reduction, and others were used to identify isolated MTB.

Various isolation and culturing methods were used to obtain pure cultures of the MTB. Since they require specific conditions to grow, they were kept and maintained at stable conditions in the work place. Due to this, various studies can be done and their fastidious metabolism can be looked upon.

This study also shows how the MTB survive and thrive due to their magnetotaxis property which guide them in OATZ conditions. Even though their oxygen requirements are still unknown as the amount required by each type shows variation.

Since the isolation of MTB itself is cumbersome let alone the purification methods, many molecular approached have been studied but due to their incomplete knowledge they have not been in use. As the reports have not been further looked upon, it has hindered the study of this potential group of bacteria.

Chapter 8: FUTURE PROSPECTS

Magnetotactic Bacteria are a group of Gram- negative and Gram-positive bacteria and are mostly microaerophilic in nature. This group of bacteria have been of massive interest to scientists around the world due to their discovery of novel genera and that they are able to sequester various toxic pollutants. The current report gives the elemental structure and morphological analysis by performing XRD and SEM along with presence of magnetosomes visualized through STEM.

The future prospects of this current study are as follows:

- Magnetotactic Bacteria can be used as Anti-Cancer Agents
- Magnetotactic Bacteria can be applied in Drug Delivery
- To show the presence of plasmid and determine if the genes that cause magnetosome development are DNA coded or plasmid borne.
- To collect magnetosomes and use them in the immobilisation of enzymes
- Bioremediation of various metals from metal contaminated sites and even from e-waste.
- Creating the Biocomputer of the Future with Magnetotactic Bacteria.
- Use of magnetotactic bacteria and their magnetosomes in magnetic hyperthermia and contrast enhancement of magnetic resonance imaging.

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