Isolation Of Magnetotactic Bacteria From Goan Waterbodies

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PREFACE

The primary objective of this study was to isolate Magnetotactic bacteria from different Goan waterbodies and extract magnetosomes for bioremediation studies. Magnetotactic bacteria, although less explored, can be immensely useful due to the production of fairly pure magnetite or greigite magnetosomes inside them having a lipid membrane surrounding them. They can be used in various environmental, biomedical, and biotechnological fields. Hence, isolating them from different environmental conditions and identifying them can be of great use for new, developing modern science.

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ABBREVIATIONS USED

OATZ	Oxic Anoxic Transition Zone
MTB	Magnetotactic bacteria
Fe_3O_4	Magnetite
Fe_3S_4	Greigite
CRT	Capillary Race Track
STEM	Scanning Transmission Electron Microscopy
XRD	X-ray Diffraction
µ	Micron
cm	Centimetre
µl	Microlitre
mM	Millimolar
rpm	Revolutions Per Minute
pH	Hydrogen ion concentration
RT	Room Temperature
%	Percentage
°C	Degree Centigrade
Mam	Magnetosome membrane
Mms	Magnetosome membrane specific
CDC	Centre for Disease Control
psi	Pounds per square inch
MSR-1	*Magnetospirillum gryphiswaldense*
AMB-1	*Magnetospirillum magneticum*
MS-1	*Magnatospirillum magnatotacticum*
MV-1	*Magnetovibrio*
MC-1	*Magnetococcus sp.*

ABSTRACT

Magnetotactic bacteria (MTB) are a special group of gram-negative, microaerophilic species of bacteria that sequester iron from the environment. The iron is either in the form of Magnetite (Fe_3O_4) or Greigite (Fe_3S_4) depending upon which species of MTB sequesters it. The magnetite and/or greigite is stored in a unique organelle called Magnetosome, which has a lipid layer around it. The magnetosome confers Magnetotaxis property to the MTB, which allows them to orient themselves in the presence of Earth's geomagnetic field and move to a nutrient-rich zone with the help of flagella.

The TSDM and ARDM2 cultures were isolated from water-sediment samples by capillary race track method using magnets and grown in Flies's media enriched with ferric citrate. Both TSDM and ARDM2 showed positive results for the siderophore test, indicating that both strains sequestered iron from the media. The strains also showed magnetotaxis properties when placed in an external magnetic field, indicating that the cultures were indeed magnetotactic bacteria, which was later confirmed by STEM analysis, which showed the presence of Magnetosomes inside both TSDM and ARDM2 cells.

CHAPTER 1

INTRODUCTION

CHAPTER 1

1.1 **INTRODUCTION**

Magnetotactic bacteria (MTB) are unique prokaryotes that have the ability to positively respond to the Earth's geomagnetic field due to the presence of a distinct organelle called a Magnetosome. Magnetosomes confer Magnetotaxis property to MTB which helps them to get a sense of direction and travel to the Oxic-Anoxic Transition Zone (OATZ) with the help of flagella in both the Northern and Southern hemisphere (Wang et al., 2020). The arrangement of the flagella can be monotrichous (polar), bipolar or lophotrichous with either one or two bundles of flagella (Lefèvre et al., 2011).

MTB were first reported by Salvatore Bellini in 1963, when he observed them moving towards the north pole. Later in 1975, Richard Blakemore independently rediscovered them and also observed magnetosomes under the microscope (Blakemore, 1975).

According to preliminary research, MTB preferentially swim northward in parallel with the geomagnetic field lines (north-seeking: NS) from the Northern Hemisphere, whereas those from the Southern Hemisphere preferentially swim southward in antiparallel with the geomagnetic field lines to the magnetic pole (south-seeking: SS). Magnetosomes can be made up of magnetic mineral crystals like magnetite (Fe_3O_4) or greigite (Fe_3S_4), enveloped by a bilayer membrane composed mostly of phospholipids, called the magnetosome membrane, that contains various proteins not present in the cytoplasmic and outer membranes (OMs) and are unique to MTB (Prabhu & Kowshik, 2016) . The most predominant phospholipids found in the magnetosome membrane of *Magnetospirillum* species

are phosphatidylserine, phosphatidylglycerol, and phosphatidylethanolamine (Lefèvre et al., 2011). Around 10 to 50 magnetosomes are found per cell, but about 600-1000 magnetite magnetosomes were found in Candidatus M. bavaricum isolated from Lake Chiemsee (Germany)(Jogler et al., 2010). MTB being very diverse can be found in vibrions, rods, cocci, spirilla or multicellular forms. They can be classified in the following phyla: *Proteobacteria, Nitrospirota, Omnitrophota, Latescibacterota, Planctomycetota, Nitrospinota, Hydrogenedentota, Elusimicrobiota, Fibrobacterota, Riflebacteria, Bdellovibrionota, UBA10* (Gareev et al., 2021).

All the phyla of the magnetotactic bacteria have a region in their genome called Magnetosome island. This region contains genes that are responsible for magnetosome formation. The genes present in this region include *mam (magnetosome membrane), mms (magnetosome membrane specific), mtx (magnetotaxis).* The proteins encoded by these genes are localized on the magnetosome membrane or help facilitate the magnetotxis motility behaviour (Abreu & Acosta-Avalos, 2020).

Abiotically generated magnetic nanocrystals may be chemically synthesized, although this process is challenging when it comes to the distinctive characteristics of biogenically created magnetosomes. These features include a significant degree of crystallographic perfection, a permanent magnetism, a limited, single magnetic domain, a nanosized range, and the existence of a biocompatible lipid bilayer around each mineral particle. All these unique characteristics have great potential in biotechnology and medicine such as contrast for nuclear magnetic resonance (NMR), in cell separation assays, as drug carriers and in the destruction of tumour cells by hyperthermia (Araujo et al., 2015).

1.2 **AIM AND OBJECTIVES**

AIM

To isolate Magnetotactic bacteria from Goan water bodies

OBJECTIVES

1. Isolation and identification of Magnetotectic bacteria from Goan waterbodies.
2. Isolation and characterisation of magnetosomes.
3. Application of Magnetotectic bacteria.

1.3. **HYPOTHESIS**

In this present study, the following things are hypothesised :

1. Using appropriate isolation and culture techniques, Magnetotactic bacteria can be successfully isolated from different Goan waterbodies.
2. Magnetosomes can be successfully isolated from the magnetotactic bacteria and characterised.
3. Magnetotactic bacteria may have Lead metal bio-absorption properties which can be determined by characterizing the magnetosomes extracted from MTB cultured in lead-enriched media by X-ray diffraction.

1.4. **SCOPE**

Magnetotactic bacteria are a diverse group of bacteria having the magnetotaxis property which allows them to orient themselves in the direction of Earth's geomagnetic field. This property is due to the presence of magnetosomes in the cell. Magnetosomes are either made up of magnetite or greigite, which have a lipid layer around them. These magnetosomes have many applications in various fields like environmental science, biomedical science and biotechnology.

The extracted magnetosomes can be used as biosensors for detecting toxins and in drug delivery, as the magnetosomes have a lipid layer having different proteins and ligands attached to it. The magnetosomes can be used as anti-tumour agents and can be used to destroy cancerous cells by magnetic hyperthermia.

Due to their great metal absorption capacity, the magnetotactic bacteria can be used to detect and recollect heavy metals from wastewater. They can also be used in cell separation techniques due to their unique magnetotaxis property.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

REVIEW OF LITERATURE

Magnetotactic bacteria (MTB) although quite ubiquitous in nature are found to be very strenuous to cultivate and maintain in pure culture (Basit et al., 2020). This is due to unknown growth requirements, presumptive metabolic diversity, specific culture conditions and media components required to grow the isolated culture as well as for the magnetosome formation (Araujo et al., 2015). Till now only few have been maintained in a pure culture. These strains include *Magnetospirillum gryphiswaldense* (MSR-1), *Magnetospirillum magneticum*(AMB-1), *Magnetospirillum magneticum* (MGT-1), *Magnatospirillum magnatotacticum* (MS-1), *Magnetovibrio* (MV-1), *Magnetococcus sp*. (MC-1) belonging to *α-Proteobacteria* (Yan et al., 2012).

The magnetotactic bacteria are found to biomineralize two minerals - Iron oxide i.e. Magnetite (Fe_3O_4) or Iron sulphide i.e. Greigite (Fe_3S_4) (Bazylinski & Frankel, 2004). Almost all of the magnetite-producing known MTB are found in freshwater and are mesophilic. However, they can also be found in various extreme environmental conditions.

(Lefèvre et al., 2010) discovered MTB in Great Boiling Springs, Nevada. Populations of a moderately thermophilic magnetotactic bacterium HSMV-1 surviving in a temperature range of 32 to 63°C were found. A chain of bullet-shaped magnetite magnetosomes was biomineralized by these gram-negative, vibrioid - helicoid shaped cells. The bacteria possessed a single polar flagellum. Phylogenetically, through 16S rRNA sequencing the organism was found to be a member of the phylum *Nitrospirae.*

(Lefèvre et al., 2011) also reported obligate alkaliphilic and hypersaline, helical MTB strain ML-1, isolated from Mono Lake in Sierra Nevada Mountains in California. The optimal pH and salinity for its growth was found to be 9-9.5 and 68-70 ppt respectively and did not grow when the pH was 8 or lower . It biomineralized bullet shaped Magnetite magnetosomes and was found to be related to non-magnetotactic *Desulfonatronum thiodismutans* upon 16S rRNA gene sequencing and phylogenetic analysis.

(Stolz et al., 1986) isolated MTB from Santa Barbara Basin in the eastern Pacific from 598m depth and temperature of 8˚C. The bacteria isolated were of different shapes, having comma, rod-shaped, and coccoid morphology. Each type of bacteria produced 10-20 Magnetite crystals having a cuboidal, rectangular, or irregular shape. (Petermann & Bleil, 1993) also isolated psychrophilic and piezophilic MTB from 1000m deep South Atlantic Ocean. The strains isolated from the deep ocean were incubated at 25˚C for 12hrs and it was seen that the number of MTB and its motility declined significantly but when incubated at 2˚C the culture survived indicating that the isolated MTB strains were psychrophilic (Bazylinski & Lefèvre, 2013).

Magnetotectic bacteria have also been isolated from various Indian aquatic bodies. KEEM4 isolate was isolated from freshwater Keetham Lake, Agra, Uttar Pradesh. The cells were found to be gram-negative and expressed motility due to the presence of a single polar flagellum. They showed a resemblance with *Magnetospirillum gryphiswaldense*. The results of this investigation showed that the isolated MTB can tolerate and reduce selenate oxyanion under microaerobic conditions. This is a useful feature for this strain, as selenate reduction has not been documented in other MTB (Singh et al., 2018).

VITRJS1 strain was isolated from Pulicat Lagoon, a saltwater lake in Andhra Pradesh. Based on the 16S rRNA gene sequencing analysis the strain was identified as *Magnetospirillum.* The magnetosomes extracted from the isolate were measured as 42 nm in size and the shape was found to be cubo- octahedran. The heavy metal removal capacity of the bacteria was assessed which indicated the maximum removal of Cobalt from the sample (Revathy, T, 2015).

MTB were also isolated from Tamiraparani River, Tamil Nadu, having rod and oval shapes, gram-negative in nature.

Some of the well-studied magnetotactic bacteria are *Magnetospirillum gryphiswaldense* (MSR-1), *Magnetospirillum magneticum*(AMB-1), and *Magnetovibrio blakemorei.*

- *Magnetospirillum gryphiswaldense* (MSR-1) was obtained from Ryck River, Greifswald, Germany. The cells were helical and 0.7 by 1 to 20 μm in size and had a single monotrichous flagellum on both the poles. The cells contained 0-40 magnetosomes of 42nm in diameter having cubic-octahedral shape. The MSR-1 strain was more aerotolerant than the previously known *A. magnetotacticum.* 16S rRNA sequencing and phylogenetic analysis proved that the bacteria belonged to α-*Proteobacteria.* It was also proved that repeated subculturing of the culture resulted in the loss of magnetotactic property of the bacteria (Schleifer et al., 1991).
- *Magnetovibrio blakemorei* (MV-1^T) is a marine euryhaline, mesophilic magnetotactic bacteria and was collected from sulfide-rich sediments in a salt marsh near Boston, Massachusetts. It grew well on salinity of more than 10%. The cells were gram-negative and had vibrioid to helicoid morphology with a

single monotrichous flagellum. The cells had a single chain of hexa-octahedral shaped magnetite magnetosomes.

The MV-1^T cells were found to be microaerophilic but could also survive in anaerobic conditions. It exhibited chemolithoautotrophic growth on thiosulfate and sulfide and oxygen acted as final electron acceptor in microaerophilic conditions and nitrous oxide in anaerobic conditions.

The genome of MV-1^T was determined to be of 3.7 Mb in size and no extra chromosomal elements like plasmids were found in the bacteria. Upon phylogenetic analysis based on 16S rRNA sequencing, it was determined that the bacteria belonged to class *Alphaproteobacteria* and is a member of *Rhodospirillaceae* family (Bazylinski et al., 2013).

- *Magnetospirillum magneticum* (AMB-1) was isolated from Koganei in Tokyo, Japan. AMB-1 strain was s 0.4-0.6 μm in diameter and cell length was over 3 μm. The colonies formed on solid agar medium was blackish- brown in colour but eventually turned white to colourless after eight days. The cells were helical in shape and had single flagella at both the ends of the cell. The cells were oxidase positive and were found to be microaerophilic. The stain could grow in 5 – 8.2 pH range. The cells were also able to grow in medium containing a-ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, lactate, acetate, propionate, n-butyrate and β-hydroxybutyrate (Matsunaga & Kamiya, 1987).

2.1 Nutrient Medium for Magnetotactic bacteria

Culturing magnetotactic bacteria in a medium requires a variety of organic and inorganic compounds. The carbon source can be different carboxylic acids like

acetic acid, succinic acid, malic acid, lactic acid etc. (Wang et al., 2020) (Liu et al., 2010). Iron source is the important component of the media as it is essential for magnetosome formation. Ferric gallate, ferrous sulphate or ferric citrate can be used as iron source and has shown to significantly increase the magnetosome production. Mineral elements like magnesium, copper, cobalt, manganese, zinc, aluminium are also required for cell growth, magnetosome size, number and alignment of the chain. Zinc acts as cofactor for many enzymes and helps stabilize proteins (Wang et al., 2020). Nitrogen source is also one of the important components for magnetosome formation. Ammonium and nitrate can be used as nitrogen sources, although it was noted that presence of nitrate (4 mM) significantly increased magnetosome formation in *Magnetospirillum gryphiswaldense* (MSR-1) however, higher concentration of 10-20 mM decreased the magnetosome formation but did not hinder the growth of the bacteria (Yan et al., 2012).

2.2 Characteristics of Magnetosome

An internal, membrane-bounded magnetic mineral crystal found inside a prokaryotic cell is known as a bacterial magnetosome. Significant attention is paid to the structure, size, and morphology of the magnetic mineral crystal, all of which suggest that the biomineralization of the bacterial magnetosome is strictly regulated by chemical, biological, and genetic factors. Therefore, as opposed to a physiologically triggered mineralization process, the biomineralization of magnetosomes is thought to be a biologically regulated process. The majority of magnetosome forming genes being present in a genomic region called Magnetosome Island (MAI). This special region has been observed in all of the MTB isolated and studied so far although the size and gene content were seen

varying across different species isolated so far (Murat et al., 2010). The loss of MAI region leads to formation of non-magnetic bacteria which primarily lacks crystal and magnetosome vesicle formation as demonstrated in *Magnetospirillum magneticum* (AMB-1) strain (Komeili et al., 2006).

The mechanism of magnetosome formation can be divided into following stages.

1) Magnetosome vesicle formation

   The cytoplasmic membrane of prokaryotic bacteria has a lipid membrane surrounding it. Magnetosome membrane as seen in many *Magnetospirillum* species is also made up of 3-4 nm thick lipid membrane. All the fatty acids, glycolipids, phospholipids, sulfolipids and proteins found in cytoplasmic membrane were also detected in magnetosome membrane indicating that the magnetosome vesicle formation could be due to the invagination in cytoplasmic membrane. MamB, MamI, MamL, and MamQ proteins are responsible for magnetosome membrane formation in AMB-1 strain (Murat et al., 2010). MamB may function as a landmark protein and be in charge of attracting MamL, Q, and Y at the membrane's invagination site in MSR-1 (Dieudonné et al., 2019) and MamI in AMB-1.

2) Iron internalisation

   Siderophore released by MTB and transport proteins help sequester iron in the cell (Bazylinski et al., 2014). Fe(II) being highly soluble in water is taken up by MTB non specific mechanism but Fe(III) take needs Iron chelaters ligands like siderophores for its internalization. MamB, MamH, MamM, MamZ genes helps in take up of Iron (Dieudonné et al., 2019). MamV is seen in only in few MTB species that can transport iron to the formed vesicles.

The maturation of magnetite crystal and its size determination is done by MamC, MamD, MamG, MamF, MamR, MamS, Mms6, and MmsF genes (Islam et al., 2018). MamK and MamJ proteins identified in *Magnetospirillum gryphiswaldense* (MSR-1) were seen to be managing in magnetosome assembly in the cell (Wang et al., 2020).

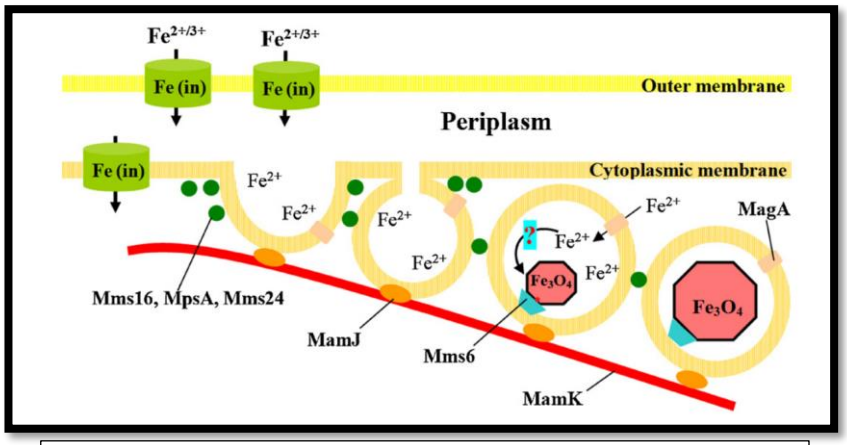

Fig.: 2.1 Magnetosome formation

Courtesy: https://doi.org/10.1016/j.micres.2012.04.002.

2.3 Applications of Magnetotactic bacteria

Magnetotactic bacteria have an immense potential in the field of environmental sciences, medicine and biotechnology due to the production of highly stable and pure magnetite or greigite magnetosomes. Magnetosomes being enveloped by a lipid membrane containing a variety of proteins and ligands, it gives them an edge over chemically produced crystals as they have to be specially coated and stabilized with dextran or PEG for them to be used in biotechnological field (AlphandÃ©ry, 2014).

2.3.1 Application of Magnetotactic bacteria in environmental sciences

Water pollution due to heavy metal contamination, presence of radionuclei and organic pollutants have been a major problem since many years faced by the world.

Release of all these things in the water not only destroys the vegetation but also pose a serious threat to human and animal life.

The use of magnetotactic bacteria for removing these contaminants and recovering the heavy metal has been studied using MTB strains. Elemental Au was successfully recovered from contaminated water with the help of the biosorption property of *Magnetospirillum gryphiswaldense* MSR-1 by (Cai et al., 2011). *M. magneticum* AMB-1 as well showed an affinity for Au^{3+} ions and recovered 100% of the Au present in the medium (Tanaka et al., 2009). MTB has also shown adsorption capacity towards Cr^{6+} ions (Qu et al., 2014). Metal ions like Cd2+, Pb2+, Ni2+, Fe3+, Fe2+, Mn2+, and Hg can also be recovered from wastewater with the help of MTB (Wang et al., 2020). A recovery rate of 40% of Plutonium was seen in MTB as reported by (Bahaj et al., 1998).

2.3.2. **Application of Magnetotactic bacteria in drug delivery**

Magnetotactic along with extracted magnetosomes have been tested for drug delivery. Because of the presence of numerous chemical groups and proteins on the magnetosome membrane, drug like doxorubicin was attached to it and tested for its anti-cancer property in hepatic cancer. Attaching the drug to the magnetosome not only increased the anti-tumour capacity by 8% but also reduced the toxity decreasing the mortality from 80% to 20% (Sun et al., 2008).

Magnetococcus marinus strain MC-1 strain was used to transport nanoliposomes, loaded with drug into the hypoxic region in colorectal tumour in mice (Felfoul et al., 2016). It was seen that seen that the bacteria was still alive after injecting it into the tumour was regarded as safe as no immune response was observed when the cells were present inside the tumour cells (Vargas et al., 2018).

2.3.3 **Magnetic hyperthermia**

Magnetosomes extracted from *Magnetospirillum magneticum* (AMB-1) were used to kill tumour cells by magnetic hyperthermia. MDA-MB-231 breast cancer cell line induced in the mice was injected with AMB-1 magnetosome chain and was placed under 20 mT magnetic field at a frequency of 198 kHz for 20 min. This lead the temperature of the tumour cells to reach 43˚C and completely eradicated the tumour in 30 days while individual magnetosomes also showed anti-tumour properties (AlphandÃ©ry, 2014) .

2.3.4 **Cell separation with the help of MTB**

(Matsunaga.,1987) proved that granulocytes and monocytes are able to ingest MTB by phagocytoses. After phagocytoses, the researchers were able to separate out and concentrate about 95% of the leucocytes from the sample when placed in magnetic field (Matsunaga & Kamiya, 1987).

2.3.5. **Use of MTB in food safety**

SM developed a polyclonal antibody-magnetosome combination to extract *Salmonella* species cells from food. The cross-linking reagent bis(sulfosuccinimidyl) suberate (BS3) was used in place of genetic engineering procedures to bind certain antibodies to the magnetosome surface. In this investigation, 1 mg of magnetosomes were immobilized with 178 μg of antibody. According to fluorescence quantitative PCR, the capture effectiveness was as high as 87% when utilized to identify and separate *Salmonella dublin* from a test solution. Using this complex, pathogen detection in food samples was also established. When the pathogen was present in experimentally contaminated food

items (such as milk, eggs, and pork), it was possible to identify Salmonella at quantities more than 60 CFU/mL (Li et al., 2010).

CHAPTER 3

METHODOLOGY

CHAPTER 3

METHODOLOGY

3.1 Sample Collection

Zuari riverside, Loutolim, Carambolim Lake and Ribandar salt pan, Goa, were selected as the sampling sites. The water sample was collected from 10-15m deep water along with the top 3-4cm sediment layer from the water, as suggested by (Ambiganandham et al., 2015), in a pre-sterilized, transparent container. The container was kept soaked in the water until it was completely filled and the cap was immediately closed. Care was taken not to get any air bubbles in the water sample. The physiochemical parameters, such as pH and temperature, were checked.

The water-sediment sample was then taken to the laboratory and was kept in a dry, dark place for 2-3 months of incubation at room temperature. The container lid was loosely closed to develop an Oxic-Anoxic Transition Zone (OATZ) (Lefèvre & Bazylinski, 2013) .

3.2 Sample Enrichment

After incubating the sample for 2 months, the sides of the container were attached with magnets just above the sediment-water interface and kept undisturbed for 1 hour, as mentioned by (Oestreicher et al., 2012). Care was taken not to disturb the container while attaching the magnets. This will allow the motile magnetotactic bacteria to swim towards the magnetic pole and accumulate at the sides of the container where the magnet is attached.

After 1 hour of incubation, the water near the side of the container where the magnet was attached was collected in a sterile syringe and kept for further purification by the capillary race track.

3.3 Isolation Of Magnetotactic Bacteria By Capillary Race Track Method

For the purification and isolation of the MTB, the capillary race track method was used as suggested by (Wolfe et al., 1987). A Pasteur pipette was sterilized by autoclaving at standard temperature and pressure. The narrow end of the pipette was then sealed by placing it on a high flame. A syringe was filled with water from the water-sediment sample and was passed through a 0.22µm filter directly into the Pasteur pipette up till the narrow portion of the pipette was filled. A sterile cotton plug was inserted at the end of a narrow portion of the pipette just above the water. The MTB-enriched water sample was then added to the broader end of the pipette just above the cotton. The pipette was then kept on a horizontal surface, and a magnet was attached to both ends of the pipette so that the opposite poles of the magnets faced each other. The setup was kept undisturbed for one hour to attract the MTB.

After one hour, the narrow tip of the Pasteur pipette was broken and the MTB accumulated at the tip was spread plated on Flies medium (Flies et al., 2005).

The isolated colonies were then individually streaked onto new Flies medium agar plates.

The entire capillary race track procedure was carried out in clean, sterilized laminar airflow (LAF).

3.4 Screening For Magnetotaxis Property

Video microscopy was done to check the isolated bacteria's magnetotaxis property. A drop of sterile 0.85% saline was put on a clean grease-free coverslip. The isolated bacterial colony was then touched with a sterile nichrome loop and then mixed in the drop of saline. Silicone grease was applied to the corners of the coverslip. A clean cavity slide was placed on top of the coverslip such that when the slide was turned, the saline drop hanged inside the cavity. The prepared slide was then observed microscope at 100x magnification.

The magnets were placed at the edge of the drop, opposite poles facing each other. The response of the MTB towards the magnetic field was checked at the edge of the saline drop. The characteristic movement of the MTB towards the south pole was checked.

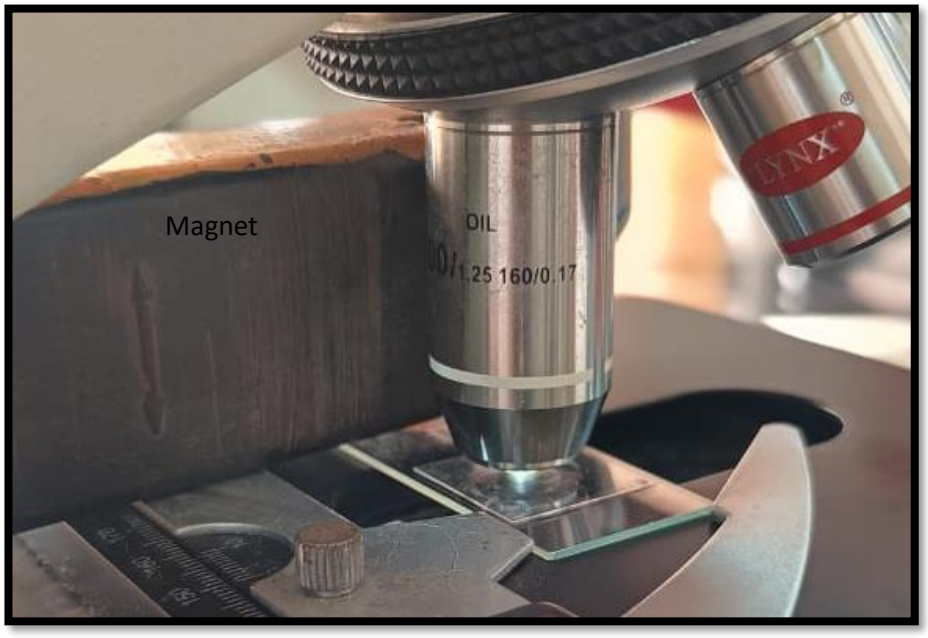

Fig.: 3.1 Visualisation of MTB by hanging drop method by placing magnet near the slide

3.5 Morphological Identification

The isolated bacterial colonies were characterised based on their colony characteristics such as size, shape, colour, opacity, margin, elevation etc. as directed by Centre for Disease Control (CDC).

The gram character was also identified of the isolated TSDM and ARDM2 cultures by following the standard Gram staining procedure.

3.6 Hugh Leifson Test

The Hugh Leifson test was carried out by following the standard procedure given by (Hanson, 2008).

Hugh Leifson basal media was prepared and sterilized by autoclaving at 121˚C at 15psi pressure. Glucose was autoclaved separately and then added to the media before pouring. The media was poured into the tubes and kept in an upright position. The TSDM and ARDM2 cultures were stabbed into the solidified agar tubes (two tubes of each culture). One of the tubes of each culture was overlayed with mineral oil to block the airflow to the culture and to produce anaerobic conditions, while the other was kept without adding the oil. The tubes were incubated for 4 days at room temperature to check the results.

Yellow colour change in the oil overlayed tubes indicates that the cultures are anaerobic. If the non-oil overlayed tubes show yellow colour change, it indicates that the culture is aerobic and able to oxidatively ferment the substrate.

3.7 Siderophore Test

King's B media supplemented with Chrom Azurol S (CAS) dye was prepared. After cooling, the CAS dye was autoclaved separately and added to the media. The agar plate was spot inoculated with the TSDM and ARDM2 culture and kept for incubation at room temperature for 48 hours. The formation of yellow hallow formation around the colony indicated a positive result (Louden et al., 2011)

3.8 Citrate Utilization Test

Simmons citrate agar was autoclaved and slants were prepared. TSDM and ARDM2 cultures were streaked onto the slants and kept for incubation at room temperature for 48 hrs. Colour change from green to blue indicates positive result (MacWilliams, 2009) .

3.9 Biochemical Tests

3.9.1 Catalase Test

A catalase test procedure was carried out to check if the isolated ARDM2 and TSDM cultures produce catalase enzyme as suggested by (Reiner, 2016). 30% hydrogen peroxide was directly added onto the grown bacterial colonies on agar plate. Immediate release of oxygen bubbles indicates positive results whereas no effervescence indicates negative result.

3.9.2 Oxidase Test

Oxidase discs were streaked with TSDM and ARDM2 cultures. The blue colour formation within 10 sec of streaking indicates positive result.

3.9.3 **Hilmvic Biochemical Test Kit**

50µl of ARDM2 and TSDM cultures were inoculated in each of the tests provided in the kit. The kit included Indole test, Methyl red test, Voges Proskauer's test, Citrate utilization test, Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, Sucrose tests.

3.9.3.a **Indol Test**

1-2 drops of Kovac's reagent were added to well no. 1 to check the results after 24 hrs. of inoculation. The development of pink colour within 10 seconds indicated positive reaction whereas no colour change indicates negative result.

3.9.3.b **Methyl Red Test**

1-2 drops of Methyl Red reagent were added to well no. 2 to check the results after 24 hrs. of inoculation. The red colouration indicated a positive result whereas the yellow colouration indicated a negative result.

3.9.3.c **Voges Proskaeur's Test**

1-2 drops of Baritt reagent A and Baritt reagent B was added to well no. 3 after 24hrs of incubation. Pinkish colour development within 5-10 minutes indicated positive results whereas no colour change indicated negative results.

3.10 SIM Media Test (Sulphide Indole Motility)

SIM media was taken, autoclaved and dispensed in test tubes. TSDM and ARDM2 cultures were directly stabbed into the stabs prepared with the help of sterile nichrome needle. The tubes were kept for incubation for 48 hrs at room temperature. To check the indole production, Kovac's reagent was added to the tubes. Pink ring formation indicates positive indole result. Black colour formation in the tube indicates sulphide production and diffused line of stab spreading in the tube indicates positive motility results.

3.11 Stem Analysis

ARDM2 and TSDM cultures were inoculated in fresh Flies' liquid media and allowed to grow for 20 days. The 20-day-old cultures were then added directly to the grid and analysed using a scanning transmission electron microscope (STEM) to detect magnetosomes in the cells. The samples were magnified at 60000X and 100000X magnification.

3.12 Magnetosome Extraction

40 ml of ARDM2 and TSDM were taken in 50ml centrifuge tube and centrifuged at 8000 rpm for 15 minutes. The pellet collected was then washed twice with sterile distilled water by centrifuging it at 8000 rpm for 15 minutes. The pellet was then suspended in 10ml of 1X PBS buffer and sonicated for 20 minutes at 3 s/time to lyse the cells. The sonicated cells were then centrifuged at 8000 rpm for 15 minutes. The pellet formed was then washed twice by centrifugation with sterile distilled

water and then with 70 % ethanol at 8000 rpm for 15 minutes. The pellet containing magnetosomes were dried then stored at -80˚C until further use (Basit et al., 2020).

3.13 Checking the Lead bio-absorption property of MTB by culturing them in Lead contaminated media.

To check the presence of Lead in the magnetosome, the TSDM and ARDM2 cultures were grown in Lead supplemented media which was prepared by adding 10mM of $PbCl_2$ in Flies media. $PbCl_2$ was autoclaved separately and then added to the broth. 1% bacterial culture was then added to the media.

3.14 X-Ray Diffraction Analysis Of Magnetosomes

The isolated magnetosomes were washed with deionised water, dried and then sent for XRD. All the magnetosomes extracted from Flies medium as well as Flies medium supplemented with Lead were analysed by XRD.

CHAPTER 4

ANALYSIS AND CONCLUSION

CHAPTER 4

4.1 ANALYSIS AND CONCLUSIONS

4.1.1 Sample Collection And Enrichment

Water-sediment sample was collected from the Zuari River. The sediment sample along with water was collected from 10-15 cm deep water in a starile transparent plastic container. The containers were then kept undisturbed in a dry, dark place for 2 months. The sampling site is shown in Fig. 4.1.

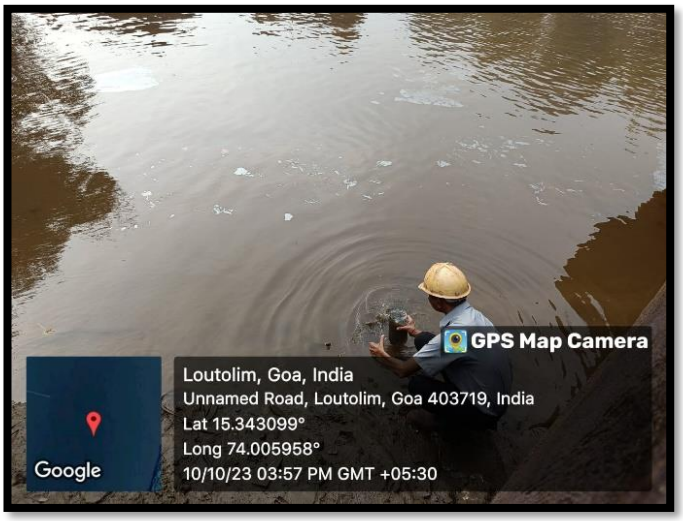

Fig.: 4.1 Sample collection from Zuari river

For the enrichment of the bacteria, magnets were attached to the outer walls of the container just besides the water-sediment interphase and kept undisturbed for 1 hours. The magnets were attached such that opposite poles of the magnet faced each other on either side of the container as shown in the fig. 4.2

Fig.:4.2 Magnets attached to the sides of the container for enrichment

The motile magnetotactic bacteria, which might have accumulated at the south pole of the magnet, was isolated by using the capillary rate track method(Wolfe et al., 1987). The narrow end of the sterile Pasteur pipette was filled with filter-sterilized water taken from the sample container. A sterile cotton plug was plugged at the base of the wider end of the pipette and then filled with the bacteria-enriched sample water. The pipette was kept on the horizontal surface. Magnets were placed at both ends of the Pasteur pipette, as shown in Fig: 4.3, and kept undisturbed for one hour. The narrow tip of the pipette was broken, and the water sample was spread and plated on a Flies agar medium.

The entire capillary race track procedure was carried out in Laminar Air Flow.

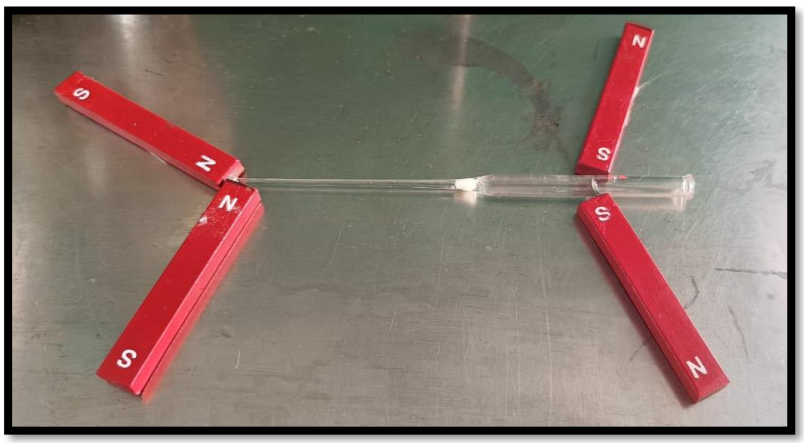

Fig.: 4.3 Capillary Race Track set up for MTB isolation

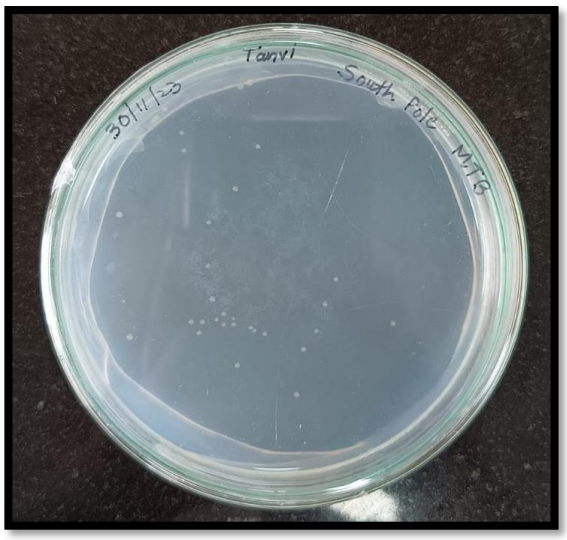

Fig: 4.4 Bacterial colonies spread plated on Flies Media.

All the colonies were screened for magnetotaxis property by placing the cells in magnetic field and visualising them under light microscope. Out of 28 colonies seen in fig 4.4, only one TSDM was seen reacting to the magnetic field. The bacteria showed rotating movement in the presence of external magnetic field.

ARDM2 culture was isolated by Ashlyn Rodrigues using similar procedure explainer above. Fig 4.5 and 4.6 shows TSDM and ARDM2 colonies on Flies agar.

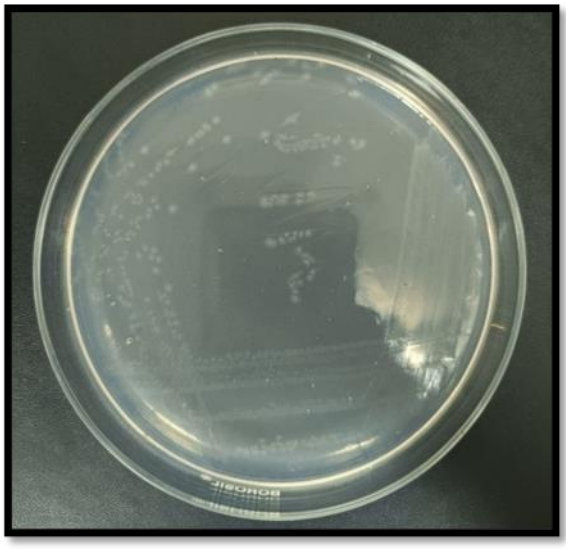

Fig.: 4.5 Isolated TSDM culture on Flies agar plate.

Fig.: 4.6 Isolated ARDM2 culture on Flies agar plate.

4.1.2 Morphological Characteristics

Colony Characteristics	MTB Species	
	TSDM	ARDM2
Temperature	27˚C	29˚C
Time	96 hrs	48 hrs
Colour	white	White eventually turning brown
Opacity	Translucent	Translucent
Form	irregular	Circular
Elevation	Flat	Raised
Margin	Udulate	Entire
Consistency	Dry	Butyrous
Media	Flies media	Flies media
Sample form	Water	Water
Motility	Motile	Motile
Gram character	Gram -ve	Gram -ve

TSDM and ARDM2 colony characteristics were studied and the observations are mentioned in table:4.1

Gram Staining

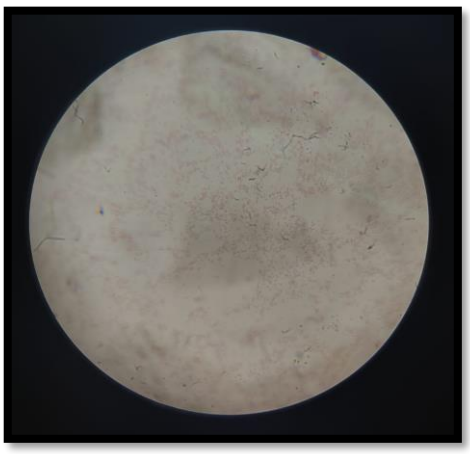

Fig.: 4.7 TSDM under 100x magnification

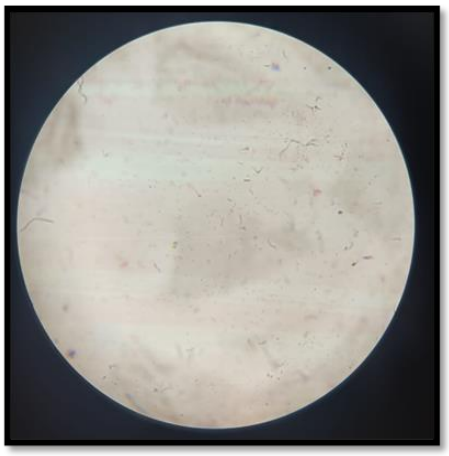

Fig.: 4.8 ARDM2 under 100x magnification

Both TSDM and ARDM2 were found to be Gram-negative as seen in fig. 4.7 and fig. 4.8. TSDM were oval in shape whereas ARDM2 were short rods.

4.1.3 Hugh Leifson's Test

Hugh Leifson's test was done to determine whether the gram-negative TSDM and ARDM2 bacteria utilised the carbohydrate by oxidative fermentation or by anaerobic fermentation or are nonsaccharolytic.

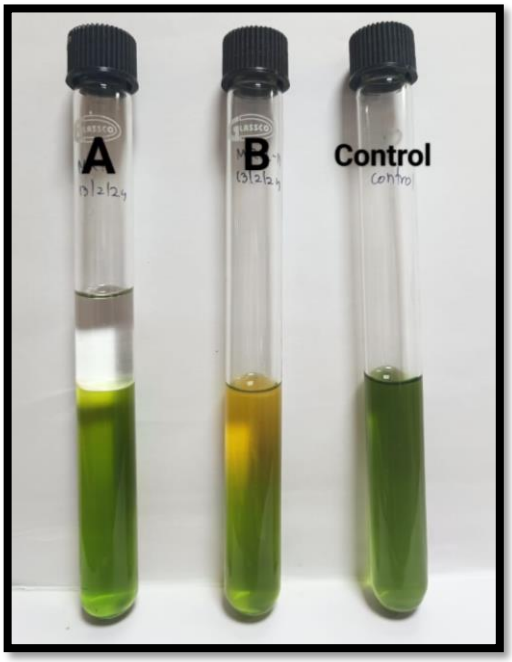

Fig.: 4.9 TSDM tubes A and B alongside control.

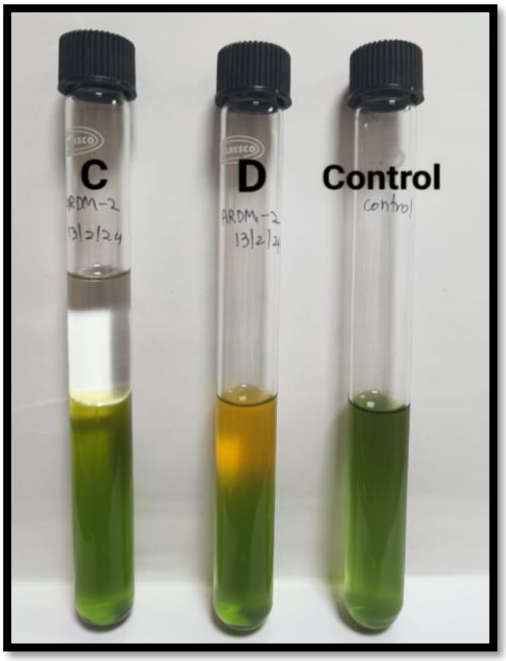

Fig.:4.10 ARDM-2 tubes C and D alongside control

Yellow colour change was seen in the TSDM B in fig.4.9 and ARDM-2 D in fig.4.10 tubes indicating that both the cultures utilise the carbohydrate by oxidative fermentation. The acids produced during the fermentation decreased the pH and turned the Bromothymol blue indicator change its colour from green to yellow. This result was in alignment with what was reported in literature as other strains of MTB like MTB - *Magnetospirillum magneticum* (AMB-1) (Matsunaga & Kamiya, 1987), *Magnetovibrio blakemorei* (MV-1T) (Bazylinski et al., 2013) were also found to be microaerophilic in nature.

4.1.4 Siderophore Test

Siderophore test was done to detect the uptake of ferric iron by TSDM and ARDM2 bacteria from the environment. Both TSDM and ARDM2 produced a yellow hallow round it indicating a positive siderophore test result as seen in fig.4.11 and fig.4.12.

A siderophore is a ligand deployed by the bacteria to scavenge iron from the environment. CAS-CTAB complex binds to ferric iron to give blue-green colour to the media. Siderophore released by the bacteria chelates the iron and removes it from the CAS-CTAB complex giving yellow coloured hallow around the bacterial colony.

MTB are suggested to be sequestering iron by deploying siderophore by (Wang et al., 2020)

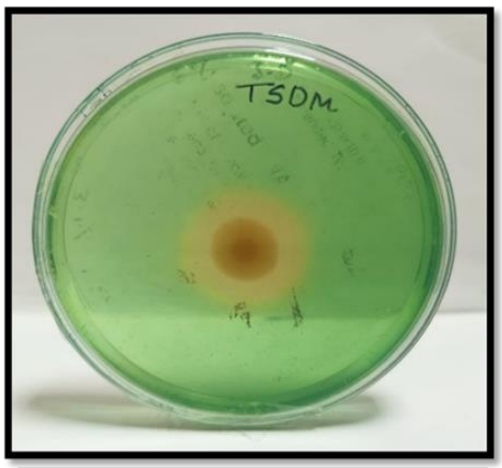

Fig.:4.11 TSDM on CAS agar media

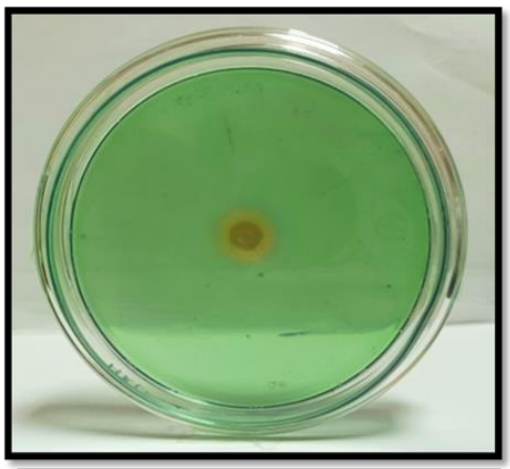

Fig.:4.12 ARDM2 on CAS agar media

4.1.5 Citrate Utilization Test

Colour change was seen in the tube containing ARDM2 indicating that it is positive for citrate utilization test as seen on fig.4.13. The Bromothymol blue indicator

changed its colour from green to blue due to alkaline pH due to the formation of Sodium carbonate which is formed from the combination of Sodium, CO_2 and water. This proves that the bacteria possess citrate permease enzyme which allows it to take in citrate and utilize it as its carbon source.

TSDM did not utilize citrate as its carbon source indicating that it does not possess citrate permease enzyme which facilitates citrate inside the cell.

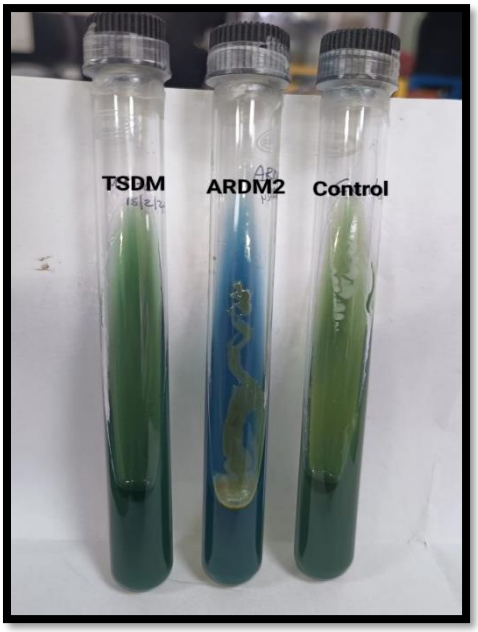

Fig.:4.13 TSDM and ARDM2 bacteria streaked on Simmon citrate agar

4.1.6.a. Catalase Test

TSDM was found to be catalase positive for catalase test whereas ARDM2 showed negative results as seen in fig.4.15 and fig.4.14. The positive result was indicated by immediate release of oxygen bubbles due to break down of hydrogen peroxide into oxygen and water by Catalase enzyme present in TSDM. *M. gryphiswaldense* and *M. magneticum* AMB-1 are Catalase negative (Miner, 2016) which is in accordance with what is seen in ARDM2.

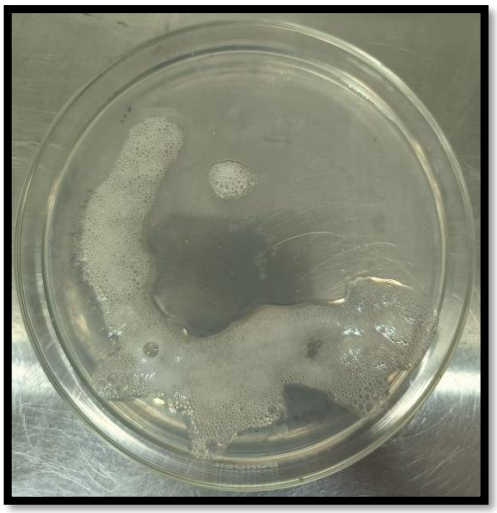

Fig.:4.14 ARDM2 upon addition of H_2O_2

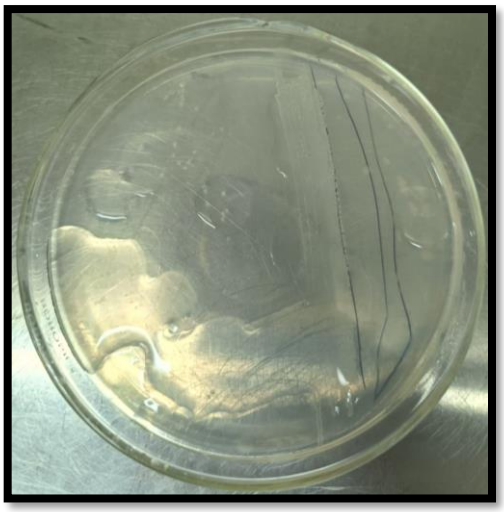

Fig.: 4.15 TSDM upon addition of H_2O_2

4.1.6.b <u>Oxidase test</u>

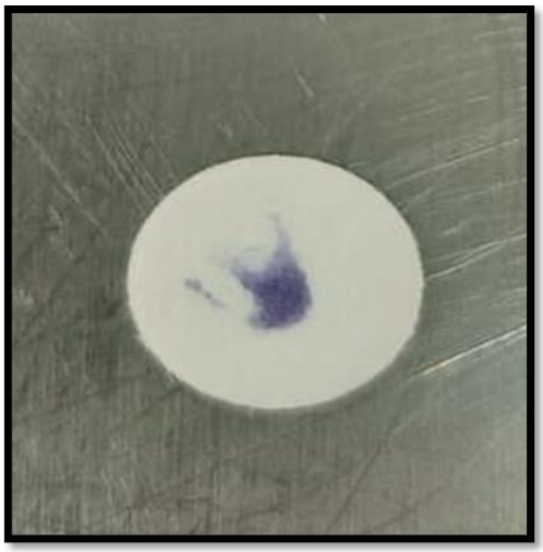

Fig.:4.16 ARDM2 streaked on Oxidase test disk

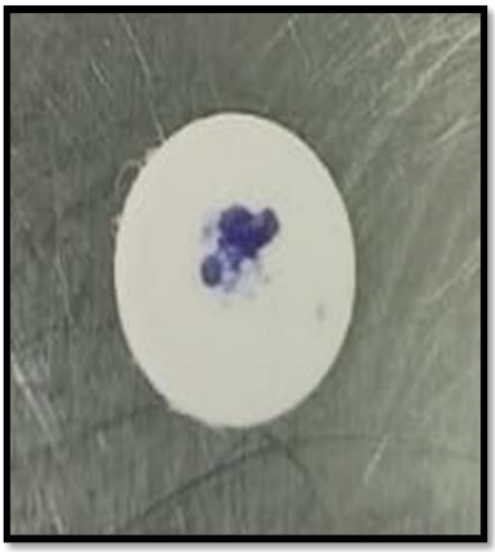

Fig.: 4.17 TSDM streaked on Oxidase test disk

Both ARDM2 and TSDM streaked disks turned blue within 10 seconds of streaking indicating positive results as seen in fig.4.16 and fig.4.17. Cytochrome oxidase oxidised N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (test

reagent) which acts as an electron acceptor turning it to indophenol blue which is blue in colour. The positive results suggests that both ARDM2 and TSDM cultures utilize oxygen as their final electron acceptor. This result aligns with what is reported in the literature. *Magnetospirillum magneticum* (AMB-1) was also reported oxidase positive (Matsunaga & Kamiya, 1987).

4.1.6.c Hilmvic Biochemical Test Kit

Biochemical tests were carried out for TSDM and ARDM2 bacteria whose results are depicted in table: 4.2 .

No.	Test	TSDM	ARDM2
1	Indole	-ve	-ve
2	Methyl Red	-ve	+ve
3	Voges Proskauer's	-ve	-ve
4	Citrate Utilization	-ve	+ve
5	Glucose	-ve	+ve
6	Adonitol	-ve	-ve
7	Arabinose	-ve	-ve
8	Lactose	-ve	-ve
9	Sorbitol	-ve	-ve
10	Mannitol	-ve	-ve
11	Rhamnose	-ve	-ve
12	Sucrose	-ve	-ve

Table 4.2 HilMViC biochemical test kit test results of ARDM2 and TSDM culture

TSDM showed negative results for all the tests indicating that it does not utilise any sugars present in the kit, does not utilise citrate and does not utilise glucose to produce stable acids. ARDM2 showed positive results for Methyl red, citrate utilisation and glucose test indicating that it can utilise glucose as well as citrate.

4.1.7 Sim Test

Both TSDM and ARDM2 showed positive results for motility (fig.4.18 and fig.19).

Only TSDM was found to be positive for Indole test which was indicated by the formation of reddish-pink ring formation after the addition of Kovac's reagent as seen in fig.4.18. Tryptophanase enzyme present in the bacteria converts tryptophan into indole. Indole then reacts with Kovac's reagent to form rosindole which is reddish in colour. No black colour formation was seen in both the tubes indicating that both the cultures did not produce hydrogen sulphide.

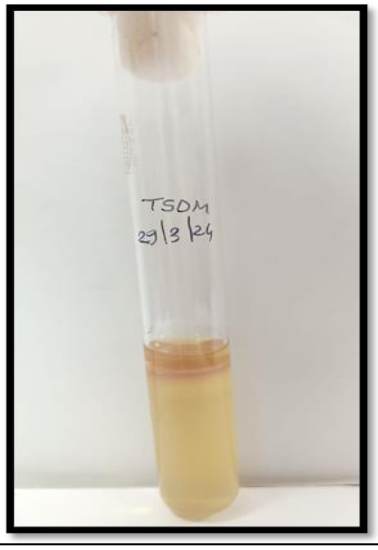

Fig.:4.18 TSDM inoculated in SIM media tube

Fig.:4.19 ARDM2 inoculated in SIM media tube

4.1.8 Stem Analysis

Both TSDM and ARDM2 showed the presence of Magnetosome in the cell. Both the bacteria had a single polar flagellum as seen in fig.4.20 and fig.4.21.

Fig.: 4.20 Quanta FEG 250 for STEM analysis

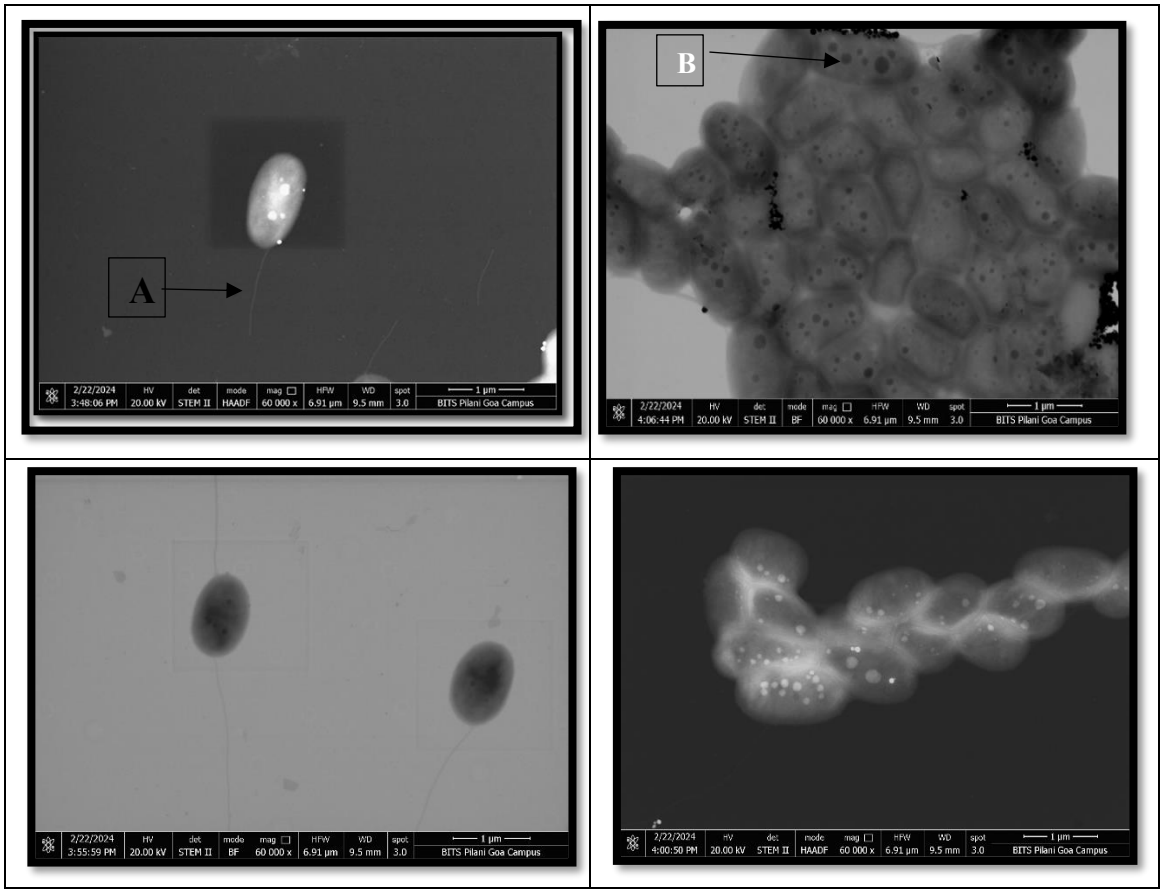

Fig.:4.21 TSDM bacteria under 60000X magnification
A = Flagellum B = Magnetosome

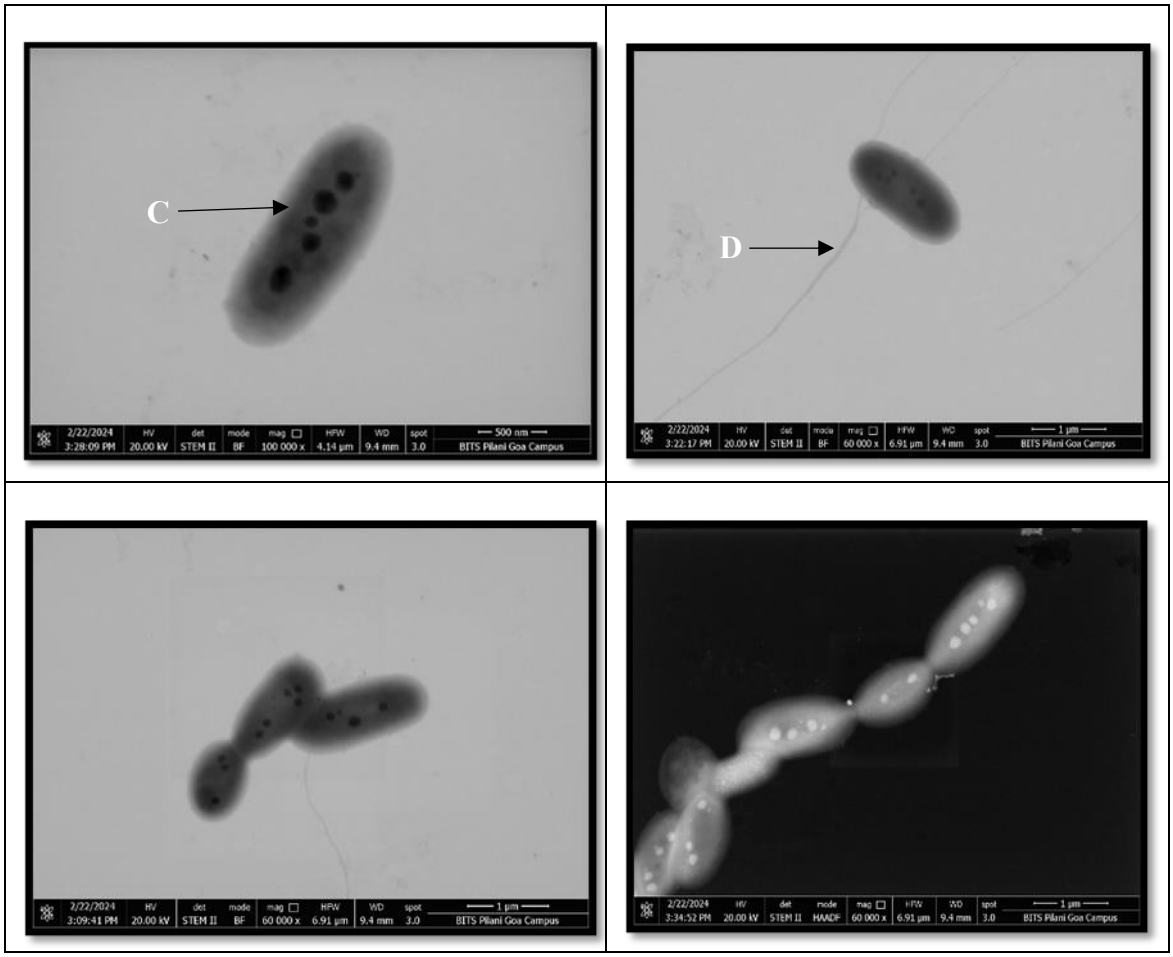

Fig.:4.22 ARDM2 bacteria under 60000X magnification
C= Magnetosome D = Flagella

Scanning Transmission Electron Microscope (STEM) is a microscopic imaging technique used to detect the structure and composition of a thin specimen. An electron beam is focused on the specimen where the transmitted electrons are detected by bright field (BF), annular dark field (ADF), and high-angle annular dark field (HAADF) detectors giving high-resolution and highly magnified images.

4.1.9 Characterisation Of Magnetosomes

The ARDM2 and TSDM bacteria grown in Lead enriched media were lysed by sonication and magnetosomes were extracted from them by washing the cell lysate multiple times. The magnetosome samples were then analysed by X-ray diffraction (XRD). The intensity peaks were seen at 30˚ for both ARDM2 and TSDM extracted magnetosomes. The peaks at 30˚ correspond to 220 crystal plane of Fe_3O_4 as seen in the literature by (Alfredo Reyes Villegas et al., 2020). No lead peaks were seen in the graphs indicating that ARDM2 and TSDM might not sequester Lead in magnetosomes.

Fig.: 4.23 Extracted Magnetosomes

Fig.:4.23 indicates the black-coloured magnetosomes accumulated at the sides of the centrifuge tube after washing with distilled water.

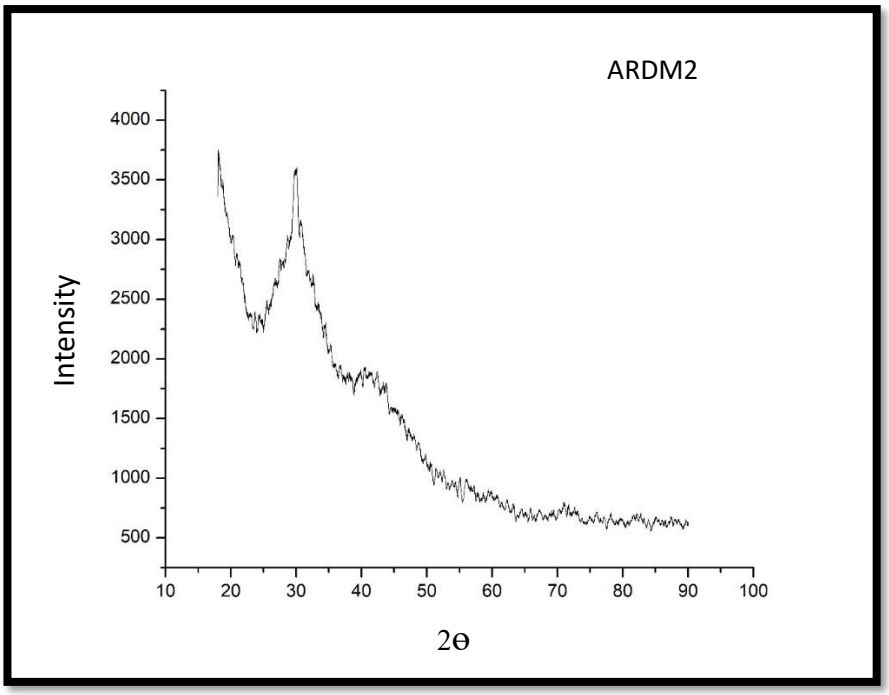

Fig.:4.24 XRD graph of ARDM2 magnetosomes showing diffraction peak at 30˚

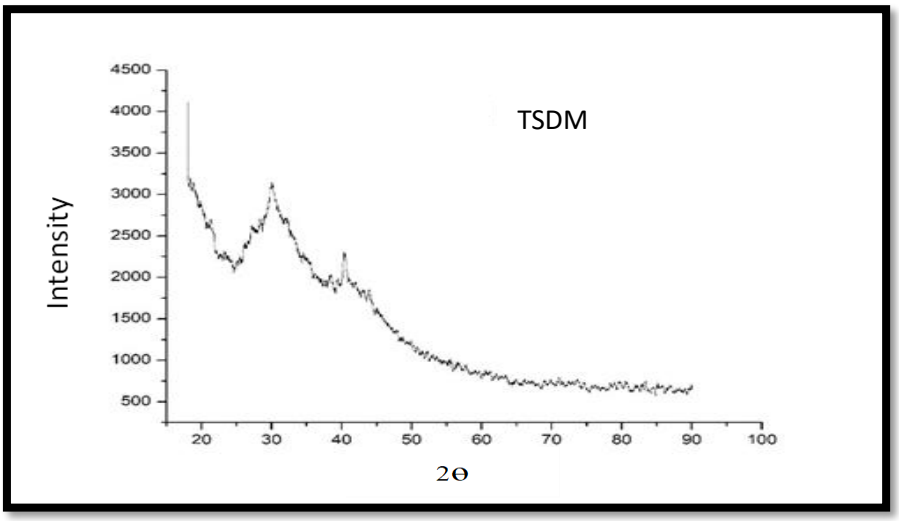

Fig.:4.25 XRD graph of TSDM magnetosomes showing diffraction peak at 30˚

4.2 CONCLUSION

TSDM magnetotactic bacteria was successfully isolated from the Zuari River. The water-sediment sample from Zuari River was collected and kept for 2 months of incubation, after which the TSDM bacteria was isolated by the capillary race track method as suggested by (Wolfe et al., 1987). ARDM2 was isolated by Miss Ashlyn Rodrigues from Carambolim Lake using a similar procedure. The isolated TSDM bacteria and ARDM2 bacteria were then grown on Flies agar medium supplemented with ferric citrate, the media composition given by (Flies et al., 2005). Both the bacteria were then screened for their magnetotaxis property by video microscopy by attaching the magnets near the slide. Both TSDM and ARDM2 were seen responding to magnetic fields. They showed rotational movement in the magnetic field compared to their random movement without a magnetic field, as suggested by (Vincenti et al., 2019). The cultures were then screened for different biochemical tests like Oxidase, Catalase, and HilMViC test kit, and it was found that both TSDM and ARDM2 were Oxidase positive, requiring oxygen as their final electron acceptor as seen in other well-studied MTB like *Magnetospirillum magneticum* (AMB-1). Only TSDM was positive for the Catalase test, whereas ARDM2 was negative for the catalase test, as seen in *M. gryphiswaldense* and *M. magneticum.* Catalase enzyme protects the bacteria from reactive oxygen species formed during the metabolic activity of the bacteria in aerobic respiration. TSDM culture did not utilise any of the sugars provided in the biochemical test kit, indicating that the carbon source for the cultures was sodium succinate incorporated in the Flies media. ARDM2 was able to utilise glucose. Upon STEM analysis, both TSDM and ARDM2 cultures showed the presence of magnetosomes and polar flagella in them like what is reported in the literature by

(Wang et al., 2020). The magnetosomes were extracted by sonication following the procedure given by (Basit et al., 2020) and characterised by XRD. The extracted magnetosome samples from both cultures showed a peak at 30˚ which was similar to the 220 crystal plane of Fe_3O_4 as seen in the literature by (Alfredo et al. et al., 2020), which may indicate that the extracted crude sample contained Magnetite in it.

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Appendix I

1. Flies Media

COMPONENTS	g/L
Potassium Dihydrogen Phosphate	0.068g
Magnesium Sulphatc, Heptahydrate	0.108g
Ammonium Chloride	0.1g
Sodium Succinate	0.166g
Ferric Citrate (10mM)	2ml
Trace Element Solution	5ml
Agar	2%
Distilled Water	IOOOml
pH	7±0.2

NOTE: Dissolve all the ingredients except ferric citrate and trace element solution in the order given and make sure the pH is at 7. Autoclave the trace element solution and ferric citrate solution separately from the remaining ingredients and add this to the basal medium after autoclaving at 121°C for 20minutes.

2. Trace Element Solution

COMPONENTS	g/L
Nitrilotriacetic acid	1.50g
$MgS0_4$ x$7H_20$	3.oog
$MnS0_4$	0.50g
NaCl	1.oog
$FeS0_4$ x $7H_2O$	0.10g

$CoS0_4$ x $7H_2O$	0.18g
$CaCl_2$ x $2H_2O$	0.10g
$ZnSO_4$x $7H_2O$	0.18g
$CuSO_4$ x 5 H_2O	0.01g
$KAI(SO_4)_2$ x 12 H_2O	0.02g
H_3BO_3	0.01g
Na_2MoO_4 x 2 H_2O	0.01g
$NiC1_2$ x 6 H_2O	0.03g
Na_2SeO_3 x 5 H_2O	0.30g
Na_2WO_4 x 2 H_2O	0.40g
Distilled Water	IOOOml
pH	7±0.2

NOTE: Dissolve Nitriloacetic acid first and adjust the pH to 7 with KOH and then add the remaining components. Autoclave at 121˚C for 20 mintutes.

3. Ferric Citrate Solution (10nM)

COMPONENTS	**g/L**
Ferric Citrate	3.0 g
Distilled Water	1000ml

4. King's B Media

COMPONENTS	**g/L**
Proteose Peptone	20,0g
Dipotassium hydrogen phosphate	1.50g
Magnesium sulphate, Heptahydrate	1.50g
Agar	20g
Distilled Water	1000 ml

5. CAS Dye Composition

Reagent A- Chrome Azurol S dye	18.15mg in 4ml distilled water
Reagent B-HexaDecylTriMethyl	21.87mg in 3m1 distilled water
Reagent C- $FeCl_3$	0.01622g in 3ml distilled water

NOTE: Reagent A is mixed with Reagent B, one this is mixed then to this Reagent C is added and a dark blue colour dye is obtained. This is then autoclaved at 121°C for 20 minutes. cooled and then added to the media.

6. Hugh Leifson Media

COMPONENTS	**g/L**
Peptone	2.0g
Sodium Chloride	5.0g
Dipotassium Phosphate	0.3g
Glucose (Dextrose)	10.0g
Bromothymol Blue	0.03g
Agar	3.0g
Distilled Water	IOOOml
pH	7.1

NOTE: Suspend 20.33g in 1000ml distilled water. Heat till boiling, to dissolve the medium completely. Dispense into testtubes in duplicate for aerobic and anaerobic fermentation. Sterilize by autoclaving at 115°C for 20 minutes. Cool the tubes containing medium in an upright position (stabs).