Haloalkaliphilic cable bacteria: Investigations of sulphide-oxidizing filamentous microorganisms from a highly saline and alkaline environment

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Certificate of Examination

This is to certify that the dissertation titled “Haloalkaliphilic cable bacteria: Investigations of sulphide oxidizing filamentous microorganisms from a highly saline and alkaline environment” submitted by Mr. Ramandeep Singh (Reg. No. MS15061) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rachna Chaba

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Dr. Sunil A. Patil
(Supervisor)

Dated: May 04, 2020

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sunil A. Patil at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Ramandeep Singh

(Candidate)

Dated: May 04, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sunil A. Patil (Supervisor)

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Ramandeep Singh

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Notations

BES	Bioelectrochemical System
CA	Chronoamperometry
CV	Cyclic Voltammetry
EAB	Electroactive Bacteria
EAM	Electroactive Microbes
EET	Extracellular Electron Transfer
EPS	Extracellular polymeric substances
MET	Mediated electron transport
LDET	Long Distance Electron Transfer
SEM	Scanning Electron Microscopy
SHE	Standard Hydrogen Electrode
SRB	Sulphate Reducing Bacteria
SRM	Sulphate Reducing Microorganisms
TEA	Terminal Electron Acceptor
PI	Propidium iodide
COD	Chemical oxygen demand

Contents

Abstract

Electron transfer is an integral part of the aerobic and anaerobic respiratory processes in different life forms. Microorganisms gain energy by transferring the metabolically produced electrons to the terminal electron acceptors through a series of electron transfer chain components located in the cell membrane. In aerobic conditions, oxygen acts as the ultimate electron acceptor, while in anaerobic conditions, microorganisms use different soluble or insoluble electron acceptors, other than the oxygen. These include, for instance, fumarate, Fe (III) or Mn (IV), sulphate and nitrate. In the case of insoluble or solid-state electron acceptors, some microorganisms use a unique mode of electron transfer referred to as extracellular electron transfer (EET) to achieve their respiratory processes in anaerobic conditions. Such microorganisms are termed as Electroactive Microorganisms (EAMs). A select filamentous microbial group named as cable bacteria has been reported to achieve its respiratory processes by using distantly placed electron donor and acceptor molecules, for instance, at anoxic-oxic interfaces. They perform a unique mode of EET, referred to as Long Distance Electron Transfer (LDET), to link sulphide oxidation to oxygen or nitrate reduction reactions separated over centimetre distances.

The study of microorganisms capable of EET and LDET provides important insights into the dynamics of electron transfer processes and their interactions with the environment, e.g., biogeochemical cycling of various elements. They can also be explored for some applications. For instance, the nanowires produced by such microorganisms bear high conductivity, comparable to copper wire, thus making them a potential candidate for the development of bio-electronics. These microbes can reduce the sulphide toxicity in soils and aid in agriculture. They can also outcompete methanogens in anaerobic environments, and thereby affect the release of methane - a potent greenhouse gas. The cable bacteria have been studied mainly from the marine and freshwater habitats so far. To broaden the understanding of the cable bacteria in extreme environments, this study focused on investigating them in a haloalkaliphilic environment. To this end, two different cultivation approaches, namely, serum flask and electrochemical, were used for the enrichment of sulphide-oxidizing cable bacteria in a highly saline (20 g/L) and alkaline (pH 9.5) growth medium. It helped to understand the microbial growth pattern with soluble and insoluble terminal electron

acceptors. The electrochemical cultivation technique resulted in the enrichment of filamentous bacteria but at different length distribution in the reactors. For instance, larger size filaments (up to 200 µm) were dominant in suspension, whereas smaller size filaments (in the range of 5-10 µm) were observed at the electrode surface. In the case of serum flask cultivation, mostly single cells and a few smaller size filaments were observed. The enriched bacteria were capable of linking sulphide oxidation with the electrode and nitrate reduction in electrochemical and serum flasks approaches, respectively. The light, fluorescence, and scanning electron microscopy observations revealed the presence of long filaments, thereby confirming the growth of haloalkaliphilic filamentous bacteria.

This study, for the first time, reports on the electrochemical cultivation of filamentous microbes in the extreme saline-alkaline environment and thereby validates their presumably ubiquitous presence in diverse interfacial environments. It also opens up the possibilities of understanding their LDET processes and a role in the biogeochemical cycling of sulphur, nitrate, oxygen, and Fe-bearing minerals in extreme environments. Further characterization of the enriched filamentous bacteria via metagenomics approach would help to understand their phylogenetic lineage and functional genes.

Chapter 1

Introduction

1.1 Microbial respiratory processes

Respiration is an essential physiological process by which all living organisms produce energy to grow and achieve essential metabolic functions. It is classified into two types, namely, aerobic and anaerobic respiration (Buckel *et al.,* 2018). In aerobic respiration, oxygen acts as the ultimate electron acceptor. In contrast, in anaerobic respiration, different soluble or insoluble compounds/minerals other than the oxygen such as fumarate (carbon-based soluble acceptor), Fe (III) or Mn (IV) (both soluble and insoluble forms), sulphate and nitrate (non-carbon based soluble acceptors) act as the terminal electron acceptors (TEA) (Pandey *et al., 2016)*. Different microorganisms can inhabit both aerobic and anaerobic environments by respiring on both soluble and insoluble TEA. In the habitats where microorganisms get the easily available electron acceptors, they can achieve respiration easily. However, in the habitats where soluble electron acceptors are depleted, some microbes tend to use insoluble molecules, if present, to achieve respiration. The microorganisms possessing a unique capability of using insoluble electron acceptors via extracellular electron transfer (EET) processes are termed as electroactive microorganisms (EAMs) (Krake *et al.,* 2015; Patil *et al.,* 2012). EAMs harbour a special chain of outer membrane components or proteins, to transfer the electrons in or out of the cell. Different microbial groups utilize different strategies to transfer electrons extracellularly. For instance, *Geobacter* spp. use specific outer membrane c-type cytochromes and conductive pilli to achieve EET. Microbes like *Shewanella* spp. either use direct electron transfer via membrane-bound cytochromes or use electron shuttles such as flavins, to complete EET (Logan *et al.,* 2019; Lovely, 2012; Kiran and Patil, 2019). The electron transfer to and from the easily available or closely placed electron acceptors and donors is termed as short-distance electron transfer, which is common in aerobic and anaerobic environments. Whereas the electron transfer to and from the distantly placed electron acceptors and donors is termed as long-distance electron transfer (LDET). A special microbial group, which grows in the form of long filaments, and referred to as cable bacteria, has been reported to achieve its

respiration by using LDET under such conditions. For instance, they link sulphide oxidation (anoxic phase) to oxygen reduction (oxic phase) reactions that are separated over centimetre long distances at oxic-anoxic interfaces (Reguera *et al.*, 2018*).*

1.2 Cable Bacteria

Cable bacteria, first reported in 2012 (Pfeffer *et al*., 2012), are filamentous bacteria that grow vertically by linking substrate oxidation at one end of the filament to oxygen/nitrate reduction at the other end of the filament via electron transport over centimetre distance (Fig. 1) (Bjerg *et al.*,2015). This type of electron transport is termed as the Long-Distance Electron Transport (LDET) and was first proposed in 2010 (Nielsen *et al.*, 2010; Nealson, 2010).

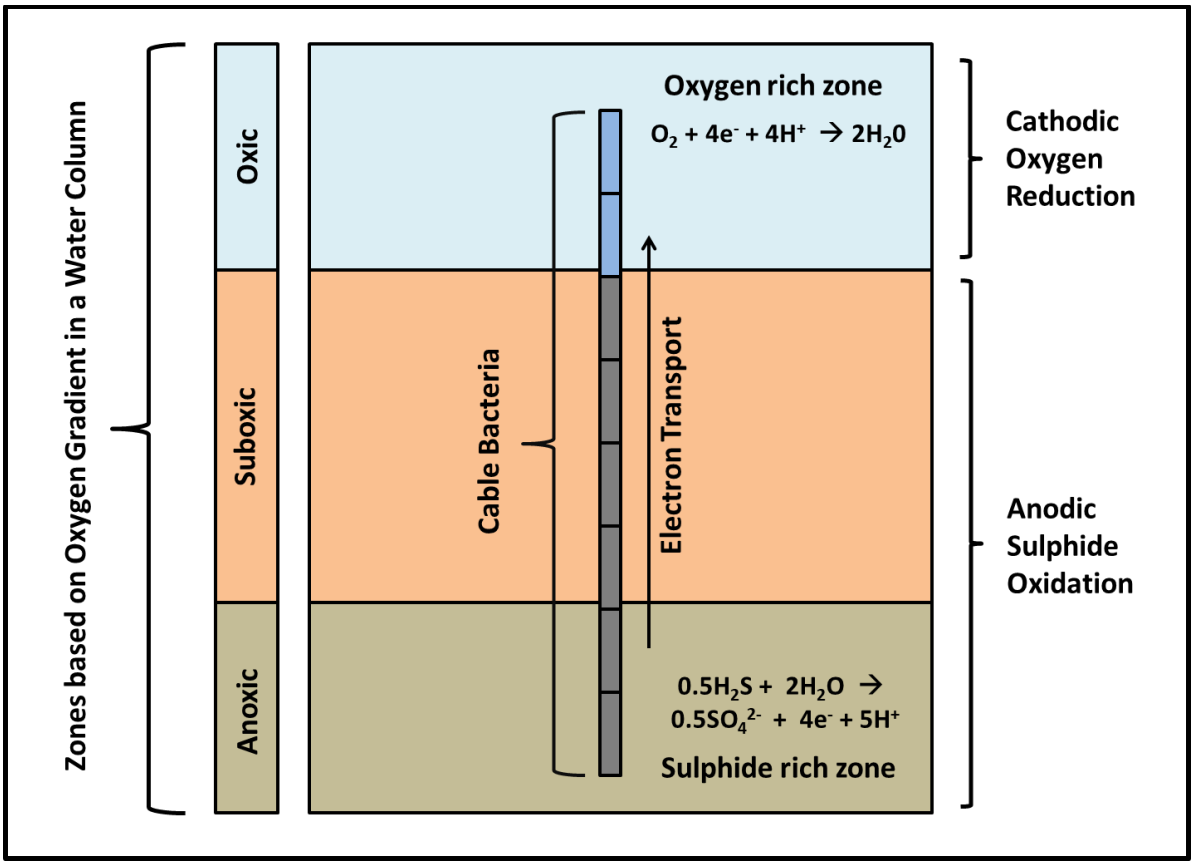

Figure 1: Schematic illustration of the metabolism of the cable bacteria. Cable bacteria complete sulphide oxidation by dividing it into two half-reactions performed by different ends of the cable. The end in the sulphide-rich zone oxidizes sulphide, and the harvested electrons are transported to another end, where it reduces the terminal electron acceptor such as oxygen (Burdorf *et al*., 2017).

LDET provides cable bacteria with a unique ability to harvest and transfer electrons between the donor and acceptor molecules present at far-off locations and provide a survival advantage over other microbes under such conditions.

For the cable formation, single cells join together which are wrapped in a common sheath that contains periplasmic fibres to form the electric conduit in the filament (Fig. 2) (Meysman *et al.,* 2019; Reimers *et al.,* 2017). In unicellular as well as multicellular organisms, individual cells maintain their energy needs by utilizing electron donor or acceptor present in their vicinity. However, in cable bacteria, several single cells work together to transfer electrons across the length of the filament, to meet their energy needs, which is unique to this organism. In a single cable, around 10^4 cells can be present (Meysman, 2018*).* The most interesting characteristic of this organism is that the cells at one end take up electrons, i.e. act as the anodic half of the reaction, and cells at other end deliver electrons to the TEA, i.e. act as the cathodic half of the reaction (Fig. 1) (Meysman *et al.*, 2015*).*

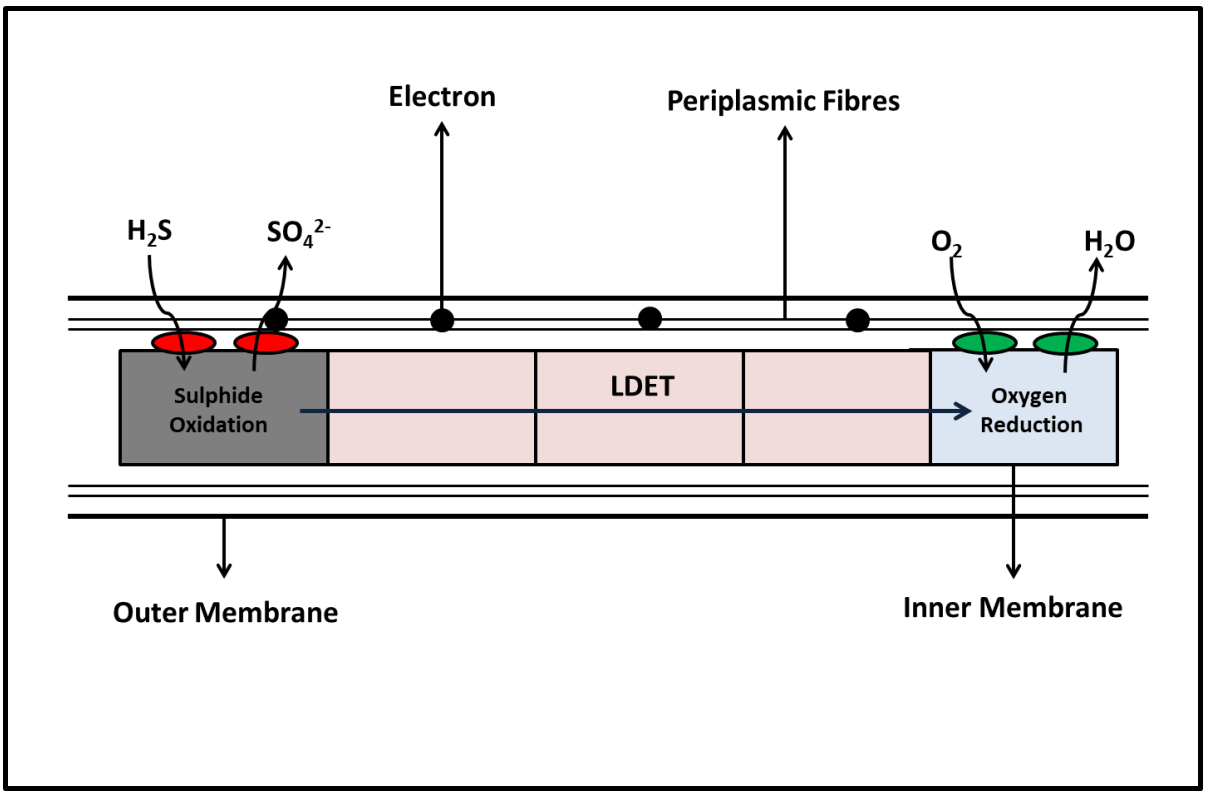

Figure 2: Schematic of Long-distance electron transport (LDET) in Cable Bacteria. Sulphide oxidation leads to the gain of electrons in the anodic half of the cable. These electrons are then docked onto the periplasmic fibre, which transports electrons to the cathodic half of the cable. Finally, electrons leave the periplasmic fibre and move into the cell and reduce oxygen (Meysman *et al.*, 2019).

These bacteria have been reported to be found in diverse habitats (Larsen *et al.*, 2015; Malkin *et al.*, 2015 ; Risgaard-Petersen *et al.*, 2015), from marine to freshwater aquatic to shallow waters so far. Cable bacteria belong to the Desulfobulbaceae family in the delta-proteobacteria class of bacteria (Pfeffer *et al*., 2012), which are known to contain sulphate-reducing or sulphur-disproportionating species (Kuever, 2014). Probably as they inhabit different environments and can be found globally, they bear a higher probability of being involved in the global biogeochemical cycling of carbon, sulphur, nitrogen, iron and other elements (Meysman *et al.*, 2015; Sulu-Gambari F *et al.*, 2016). They can also be linked to agricultural applications, to overcome the sulphide contamination in previously contaminated fields, aiding in plant growth (Martin *et al.*, 2019). Recently, they have been reported to reduce methane emissions from the rice fields, which account for approximately 11% of global methane emissions (Scholz *et al*., 2020). They do so by increasing the sulphate

content in the soil by sulphide oxidation, which aids in the growth of sulphate reducing bacteria. The sulphate reducing bacteria then outcompetes methanogens, thereby reducing the methane emissions up to 93% compared to the rice fields without cable bacteria (Scholz *et al*., 2020). Since after a decade of research, they have been termed as natural wires, and can probably be used in bioelectronics (Teske, 2019).

Single isolated filaments of cable bacteria have been studied via different microbial cultivation approaches and used by different research groups to characterize conductive properties (Meysman *et al.,* 2019*).* For enriching and understanding the EET capabilities of such microorganisms, an electrochemical cultivation approach can also be used (Chiranjeevi *et al.,* 2020). In this approach, an electrode is used as the non-exhaustible source of electron acceptor or donor for the microorganisms. Though cable bacteria have been studied using various aerobic and anaerobic cultivation approaches, they have not been enriched using electrochemical cultivation approach to the best of our knowledge. Moreover, whether they are also present in extreme environments is not known. Exploring extreme habitats for cable bacteria may provide an insight into diverse habitats or ubiquitous nature of this novel microbial group and probably unravel novel LDET components. This study thus aimed at investigating the cable bacteria from a halo-alkaline environment, i.e. a highly saline and alkaline habitat of the Lonar Lake (Maharashtra, India). For this purpose, we attempted three different enrichment and cultivation approaches, viz. electrochemical using insoluble electron acceptor, anaerobic serum flask using soluble electron acceptor, and microcosms mimicking oxic-anoxic interface environment. In the electrochemical approach, the anode was used as the non-exhaustive solid-state insoluble TEA. In serum flask approach, we tried enrichment with $FeCl_3$ and nitrate soluble electron acceptors, and in the case of the microcosm, oxygen was used as the soluble electron acceptor. The growth of enriched microbes was monitored by various microscopic techniques and the oxidized or reduced products.

Chapter 2

Materials and methods

2.1 Sediment Sampling and characterization

Sediment samples, from the shallow part of the Lonar Lake, were collected in August 2019 using 50 ml falcon tubes without disturbing the sediment integrity. Five sampling sites were chosen along the periphery of the Lake to have varying water levels, from muddy to shallow waters. From each site, 7-8 samples were taken by plunging inverted falcons perpendicularly into the soil and then capping to avoid air diffusion. Each falcon tube contained sediments along with 3-5 ml of overlaying water. After reaching the laboratory, all samples were stored at 4°C. Sediment samples were then characterized for several chemical parameters, *viz.*, sulphide, sulphate, ammonia, nitrate, and phosphate using standard protocols as per APHA 2012 (Rice *et al.,* 2012) besides pH and salinity.

2.2 Enrichment of sulphide-oxidizing cable bacteria

Before starting the enrichment experiments, microbial growth medium and sediment inoculum were prepared**.** The growth medium containing buffer and micronutrients along with sulphide and/or acetate as an energy source and with 9.5 pH and 20 g/L salinity was used (Table 1). For inoculum preparation, the sediment samples were mixed well and kept anaerobic by sparging with 99.9% nitrogen for at least 10 minutes. For enriching haloalkaliphilic sulphide-oxidizing cable bacteria, the following three cultivation approaches were used.

Table 1: Microbial Growth Medium with 9.5 pH and 20 g/L salinity.

Components	Concentration (g/L)
NaH_2PO_4	2.69
Na_2PO4	4.33
NaCl	20
Na_2CO_3	4.2
KCl	0.13
NH_4Cl	0.30
Vitamins*	12.5 mL/L
Trace Metals**	12.5 mL/L

(Note: for * & **: refer appendix)

2.2.1. Electrochemical enrichment approach

To enrich and grow the cable bacteria capable of sulphide oxidation using a solid-state insoluble terminal electron acceptor via EET, a single-chambered, three-electrode configuration reactor system connected to a potentiostat (VMP3, Biologic Science Instruments, France) was used (Fig. 3) (Patil *el al.*, 2011). It hosted graphite anode and cathode electrodes with almost equal surface area, i.e., 16.485 cm^2 and an Ag/AgCl reference electrode (3.5M KCl, 0.205 V vs. SHE (standard hydrogen electrode). Titanium wire was used to connect graphite electrodes to the potentiostat channels. Before use, both anode and cathode were subjected to acid-alkali treatment followed by drying at 60 °C overnight for removing any impurities from the electrode surfaces. As an electrolyte, 200 ml of growth medium supplemented with 10 mM acetate (for initial 15 days of the experiment) and 5 mM

sulphide was used. Acetate was provided mainly as the carbon source to promote initial microbial growth. The medium lacked any soluble electron acceptor. The whole setup was made anaerobic by sparging with 99.999% inert nitrogen gas for 20 minutes.

Before starting the main experiments, control cyclic voltammetry (before inoculation and after inoculation) was conducted to check for any redox-active components at the electrode surface and in the electrolyte. A standard potential of 0.170 V *vs.* Ag/AgCl was polarized at the anode (working electrode) using chronoamperometry technique. The anode acted as the only terminal electron acceptor to support the microbial respiratory process in this approach. After the initial 15 days of growth experiments, the reactors were operated with only sulphide (without acetate) as a sole energy source. On completion of each batch cycle, the reactors were replenished with a fresh growth medium. The decrease in sulphide and an increase in sulphate concentrations were measured at regular intervals to correlate the bioelectrocatalytic current production to sulphide oxidation directly. The microbial growth at the electrode surface and in the bulk phase was checked by various microscopic techniques, as discussed in section 2.3.

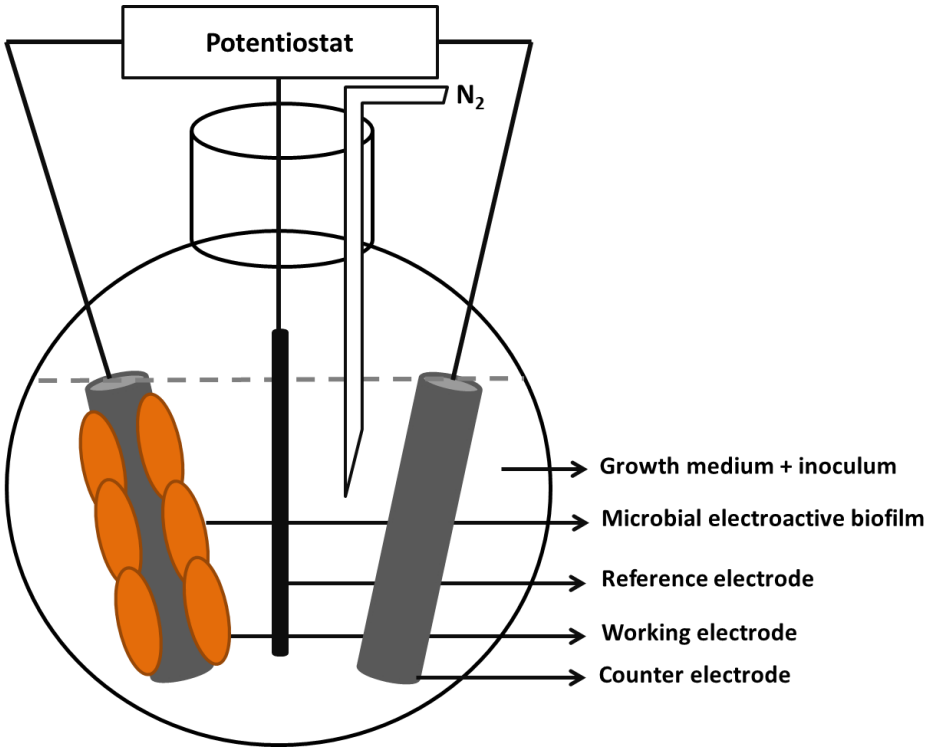

Figure 3: Schematic of the single-chambered three-electrode configuration electrochemical setup.

For control experiments, reactors with only medium and without inoculum but connected to the potentiostat (abiotic control), and with medium and inoculum but not connected to the potentiostat (biotic control) were operated for 10 days and the current vs. time profile was recorded.

Cyclic Voltammetry

Cyclic voltammograms (CVs) in the potential range of -0.4V to 0.6 V vs. Ag/AgCl were recorded before starting the enrichment experiments, to check the occurrence of any undesired electrochemical reaction happening in the same potential range (Labelle *et al.*, 2005; Harnisch *et al.*, 2012).

2.2.2. Serum flask enrichment approach with different soluble electron acceptors

In this case, to enrich and grow the cable bacteria capable of sulphide oxidation using soluble terminal electron acceptors, 100 ml serum flasks filled with 40 ml of the growth medium were used as reactors. Here, 10 mM acetate was provided as the carbon/energy source along

with 5mM sulphide as another energy source. A duplicate set of serum flasks were set-up with different terminal electron acceptors, *viz.* nitrate (KNO_3) and ferric chloride ($FeCl_3$). Before inoculation, serum flasks, filled with 40 ml were sparged with N_2, and sealed with butyl rubber, and aluminium crimp. Then the flasks were processed through 25 cycles of gassing and degassing and were followed by autoclaving. Then they were inoculated using 4 ml of the sediment inoculum solution and incubated undisturbed at 30℃ for a month. After the incubation period, the microbial growth was analysed using different microscopic techniques as discussed in section 2.3 (Nealson *et al.*, 2016; Rowe *et al.*, 2017; Muller *et al.*, 2020). A biotic control experiment was conducted to check the microbial growth without providing any electron acceptor. While to check abiotic sulphide oxidation, another control experiment without providing any inoculum was also conducted (abiotic control).

2.2.3. Microcosms- mimicking natural conditions for growing the enriched microbial consortia

To check whether the enriched filamentous bacteria are able to grow or not in the natural conditions from where they are enriched several microcosm reactors (Fig. 4) with oxic-anoxic interface conditions were used. Approx. 200 g of sediment sample was autoclaved and filled in sterile 50 ml falcon tubes up to a depth of 3-4 cm. The sediment surface was covered by a filter paper to hold the inoculum close to the upper sediment surface. Then the pre-enriched microbial culture from the bulk phase of the electrochemical system was added over that filter. A thin layer of autoclaved sediment was then placed over the filter and was followed by filling the reactor with 10 ml of autoclaved growth medium. A triplicate set of falcon reactors was incubated aerobically for each growth condition as described below.

1. Autoclaved sediment along with medium only, no inoculum (abiotic control).

2. Autoclaved sediment along with medium but without sulphide and inoculated (biotic control).

3. Autoclaved sediment along with medium, inoculum and supplemented with 5mM sulphide.

4. Autoclaved sediment along with medium, inoculum and supplemented with 10mM acetate.

5. Autoclaved Sediment along with medium, inoculum and supplemented with 5mM sulphide sediment and 10mM acetate.

After inoculation, they were incubated undisturbed at 25 °C for a month. After the incubation period, the microbial growth was analysed using Light microscopy as discussed in section 2.3.

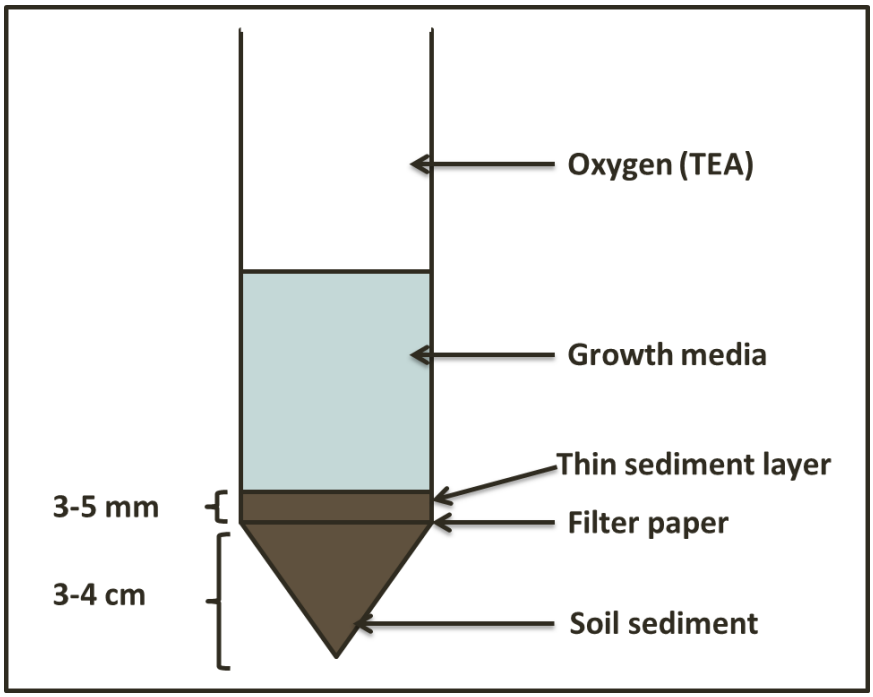

Figure 4: Schematic of the microcosm setup.

2.3. Microbial growth assessment and visualization

Different microscopic techniques were used to visualize the microbial growth in electrochemical, serum flask and microcosm reactors.

2.3.1. Light Microscopy:

It helped to reveal the cell morphology and growth pattern of the enriched microbial communities. For this, replicate samples were harvested and stained using gram staining kit (Sigma-Aldrich) at the end of each experiment. It was done according to the manufacturer's

instructions with a little bit of modification. For instance, the microbial samples were dried in a hot air oven over 60°C for 2 minutes, instead of heat dried, to prepare a smear over the slide.

2.3.2. Fluorescence Microscopy:

It was also used to confirm the microbial growth (via DNA staining), and growth pattern at the end of enrichment experiments by using Live-Dead Backlight staining Kit (Thermo Scientific). It uses two different nucleic acid staining dyes, SYTO9 and Propidium iodide (PI). SYTO9 stains cells with both damaged as well as the intact plasma membrane and shows green fluorescence. At the same time, PI is only able to penetrate damaged membranes giving red fluorescence and reducing SYTO9 fluorescence when both are present. So, SYTO9 fluorescence gives the proportion of live cell in the PI fluorescence background.

2.3.3. Scanning Electron Microscopy:

The microbial samples (suspension from all enrichment experiments and the anode electrode from electrochemical enrichment) were fixed overnight in a fixative solution of 2% glutaraldehyde and 2.5% paraformaldehyde by incubating at 4 ºC. The samples were then dehydrated using different ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) sequentially for 20 min in each solution. It was followed by drying of the samples in a silica desiccator overnight. Then the samples were coated with gold nanoparticles by JEOL JEC-1600 Auto-Fine Coater (JEOL Ltd., Japan) at 20 mA for 45 seconds and finally were observed using a JEOL JSM-6010PLUS/LS Scanning Electron Microscope (JEOL Ltd., Japan) (Khan *et al.,* 2014).

Chapter 3

Results and Discussion

3.1. Sediment characteristics

The chemical characterization of sediments revealed the presence of organic and inorganic components (Table 2). The presence of high sulphide concentration can support the growth of sulphide oxidisers. Sulphate and nitrate, which can act as the terminal electron acceptors in anaerobic conditions, are also present in high concentrations. These, along with a high concentration of organic matter, suggest the favourable conditions for the growth and survival of diverse microorganisms in the lake sediments.

Table 2: Sediment characteristics

Parameter	August 2019
pH	9.6 ± 0.2
Salinity (g/L)	14.33 ± 1.0
Conductivity at 24^0C	22.03 ± 1.5
Ammonia (mg/L)	23 ± 0.1
Phosphate (mg/L)	47.23 ± 2.6
COD (mg/L)	526.43 ± 13.4
Sulphate (mg/L)	62.83 ± 1.2
Nitrate (mg/L)	222.43 ± 7.0
Sulphide (mg/L)	102.97± 13.81

3.2. Enrichment of sulphide oxidising microorganisms

3.2.1.1. Electrochemical enrichment

The control CVs before the start of enrichment experiments helped us to find out the potential safe window without any redox activities, for the targeted sulphide oxidation reaction (Fig. 5). No redox-active peaks were seen in the CV, thereby suggesting the lack of any soluble redox-active components in the medium or adsorbed species at the electrode surface that can be involved in the electron transfer process.

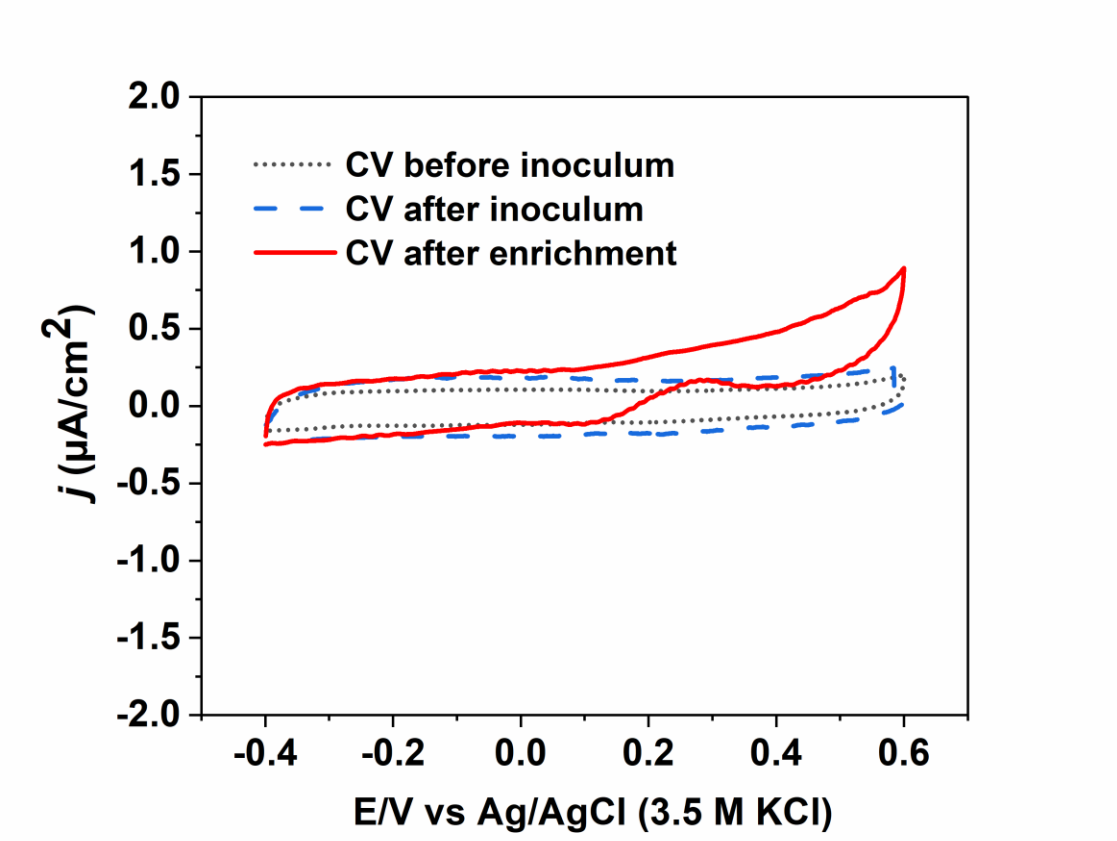

Figure 5: Representative cyclic voltammograms (CVs) recorded before and after inoculum show no electrochemical redox activities within the selected potential range. The CV recorded at the end of enrichment experiments shows a redox activity at a mid-point potential of 0.191 V vs. Ag/AgCl.

On the third day after starting the electrochemical enrichment experiments with acetate and sulphide, a gradual increase in the bioelectrocatalytic current generation was observed (Fig. 6). After 15 days of operation, no further increase in current density was observed. After

medium replacement with the only sulphide, a slight increase in the current generation, followed by a gradual decrease was observed. The purpose of providing sulphide was to promote the activity of sulphide oxidizing bacteria in the reactors. Although the current level is low, it confirms the enrichment of sulphide oxidizing microorganisms in the reactor. Some filamentous growth was seen visually in the reactors. These filaments got detached from the anode even on slight disturbances during the sampling events. No current generation was recorded in the control experiments which confirmed the bioelectrocatalytic current generation was due to the microbial sulphide oxidation and outward EET from cells to the anode electrode.

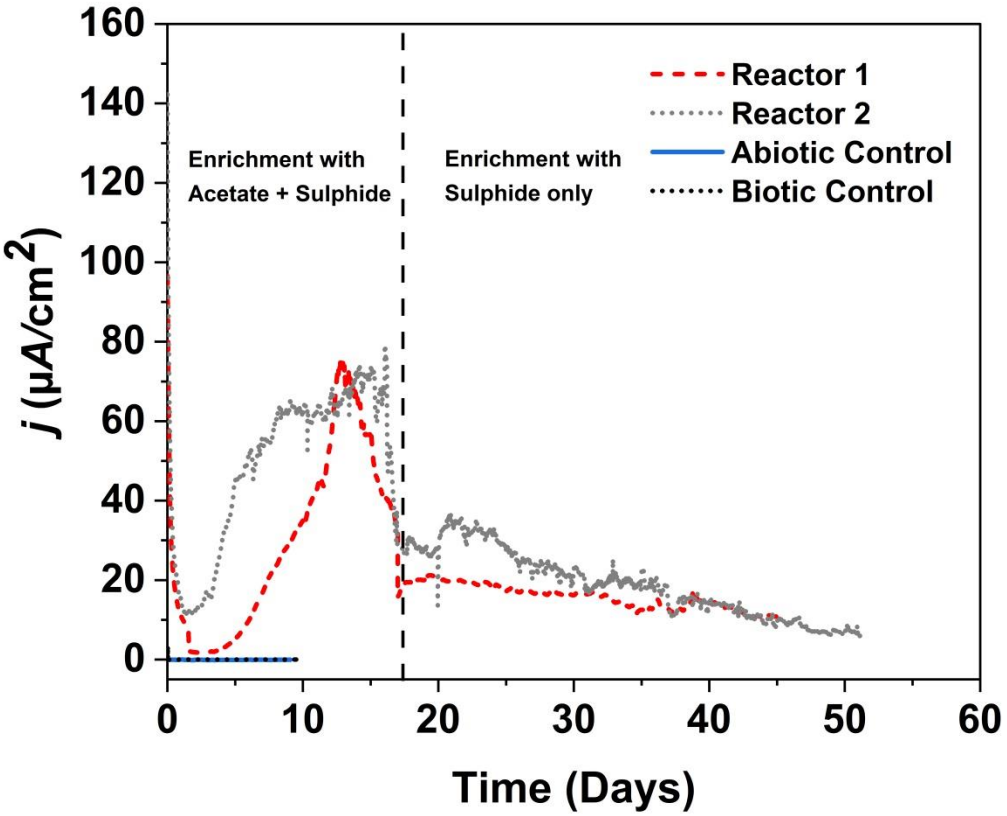

Figure 6: The current generation profiles during the enrichment experiments under two different conditions, viz. with and without acetate in a batch-mode operated electrochemical setup. The data for the biotic and abiotic control experiments are also shown.

A gradual increase in sulphate concentration was observed in these reactors (Fig. 7), which further confirms the sulphide oxidation reaction. A decreasing trend in the sulphide

concentration was also observed along with the increase in sulphate concentration. However, the data was not consistent. Sulphide can be precipitated at higher pH (Nielsen *et al.*, 2008) resulting in difficulties for the detection of actual concentration without disturbing the system, thus leading to the fluctuating trend. Hence, sulphate production data as a proxy for sulphide oxidation/consumption is reported. Sulphide was spiked once within the operation period in a third separate reactor to see the effect on current density. It showed an increase in current density (Fig. 8), thereby confirming the growth of sulphide oxidizing bacteria in at the electrode surface and in the bulk phase.

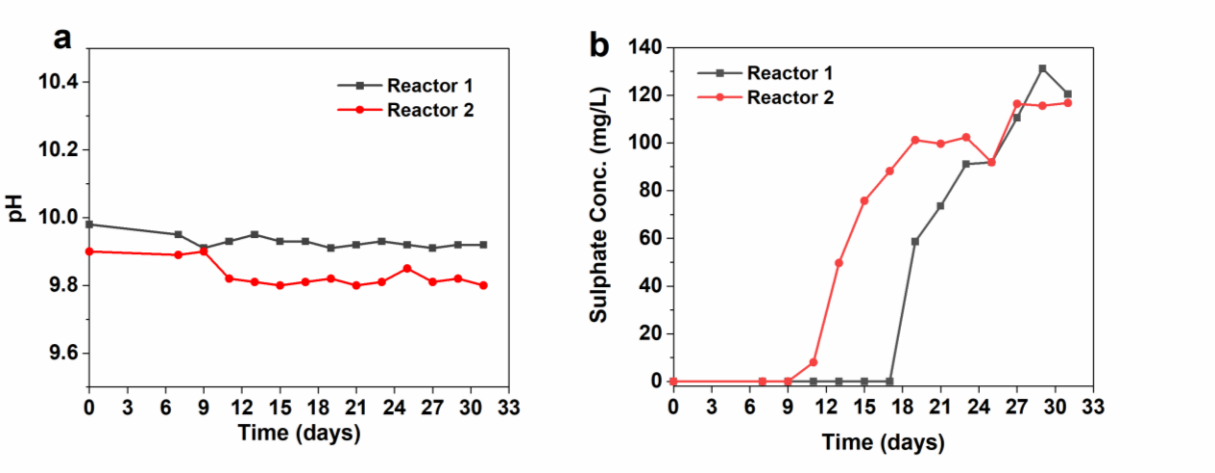

Figure 7: a) pH profiles, and b) incremental trend in the sulphate concentration in two replicate electrochemical setups.

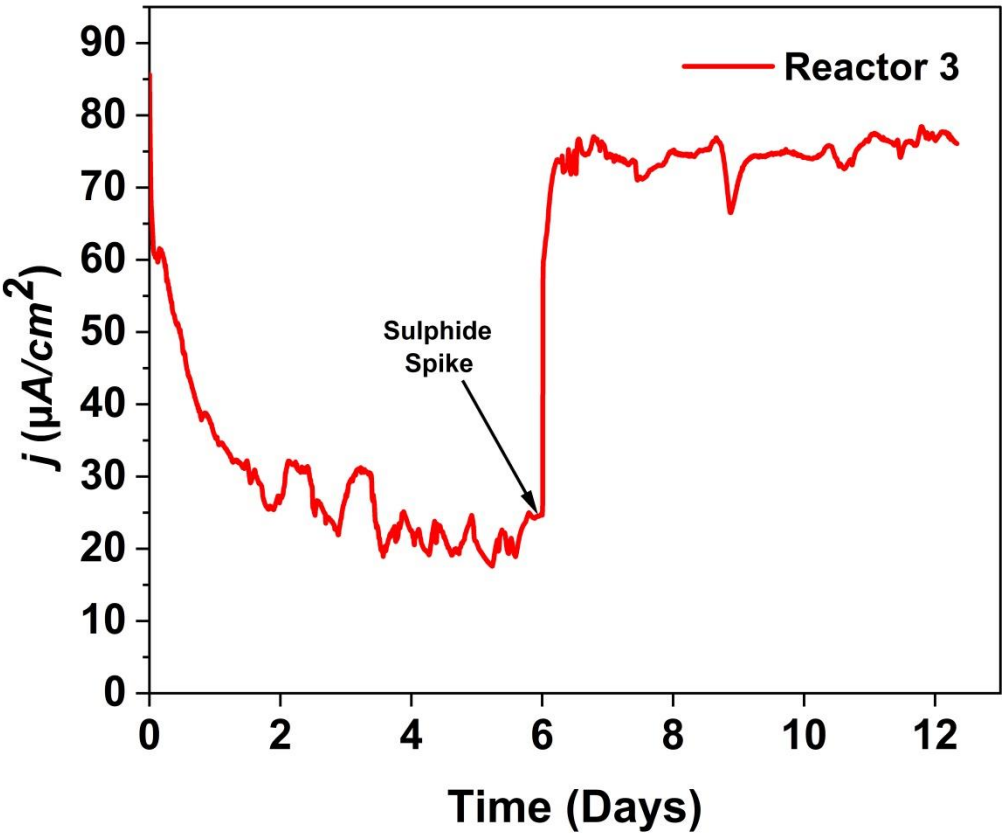

Figure 8: Effect of sulphide spike on the bioelectrocatalytic current density.

As expected, a decrease in the pH over time was also observed, but it was not very pronounced and can be attributed to the fact that only a small amount of sulphide was being oxidised. It correlated well with the increase in sulphate concentration with a small pH change at the same time period (Fig. 7). The low sulphide consumption can be linked to the low biomass concentration since no carbon source was added (thus no growth) during these tests in the reactors.

3.2.1.2. Microbial growth assessment and confirmation through microscopic observations

Light Microscopy: Entangles filaments were observed in the bulk phase of the electrochemical enrichment reactors (Fig. 9). These were longer as compared to the filaments attached to the electrode, observed *via* SEM of the anode. This discrepancy in filament length

can be linked to the availability of terminal electron acceptor. Cable bacteria form long length filaments in natural conditions to link distantly placed electron donor and acceptor. In electrochemical reactors, for the filaments attached to the anode, the terminal electron acceptor is readily available, and hence the shorter filaments were enriched at the anode. Whereas for the filaments growing in suspension, the anode is distantly placed and hence longer size filaments got enriched in suspension.

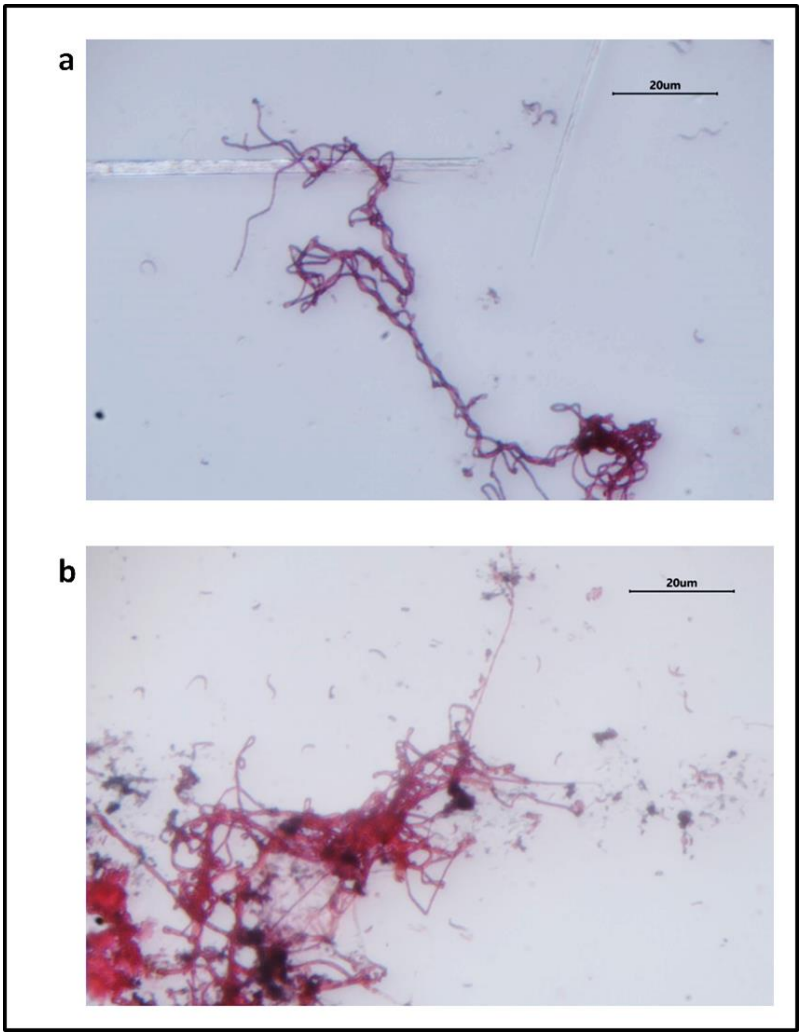

Figure 9: Light microscopic images of filamentous bacteria from the suspension of electrochemical reactors (100X).

Scanning Electron Microscopy: Long filaments, as well as single rod-shaped cells, were observed in the SEM imaging of both the suspension and anode surface samples (Figs. 10 and 11). The contrast in the sizes of bacteria, longer in suspension and shorter on the electrode, can be attributed to the electron acceptor unavailability in suspension and availability as the electrode surface, respectively.

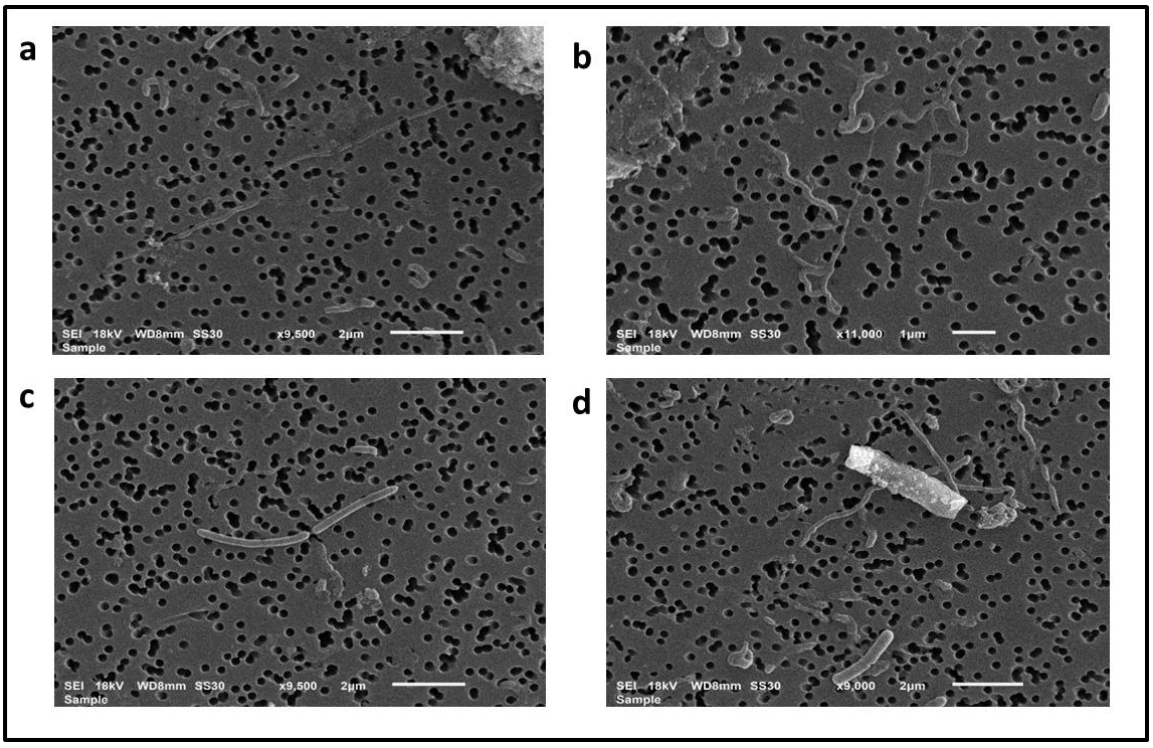

Figure 10: SEM images of microbial samples from electrochemical reactor suspension showing filamentous as well as rod shaped microbial morphologies.

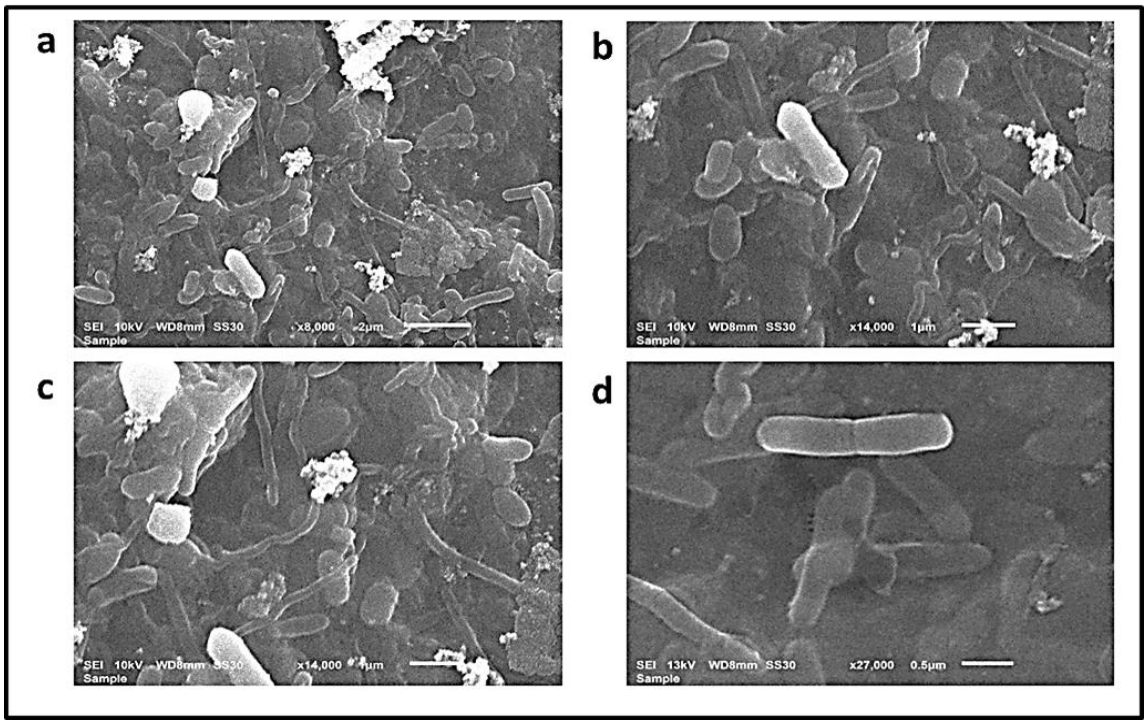

Figure 11: SEM images of microbial samples from the anode surface showing mixed microbial growth in the form of single rod-shaped cells and short filaments.

Fluorescence Microscopy: It confirmed the growth of long filaments in the suspension from the electrochemical enrichment experiment (Fig. 12).

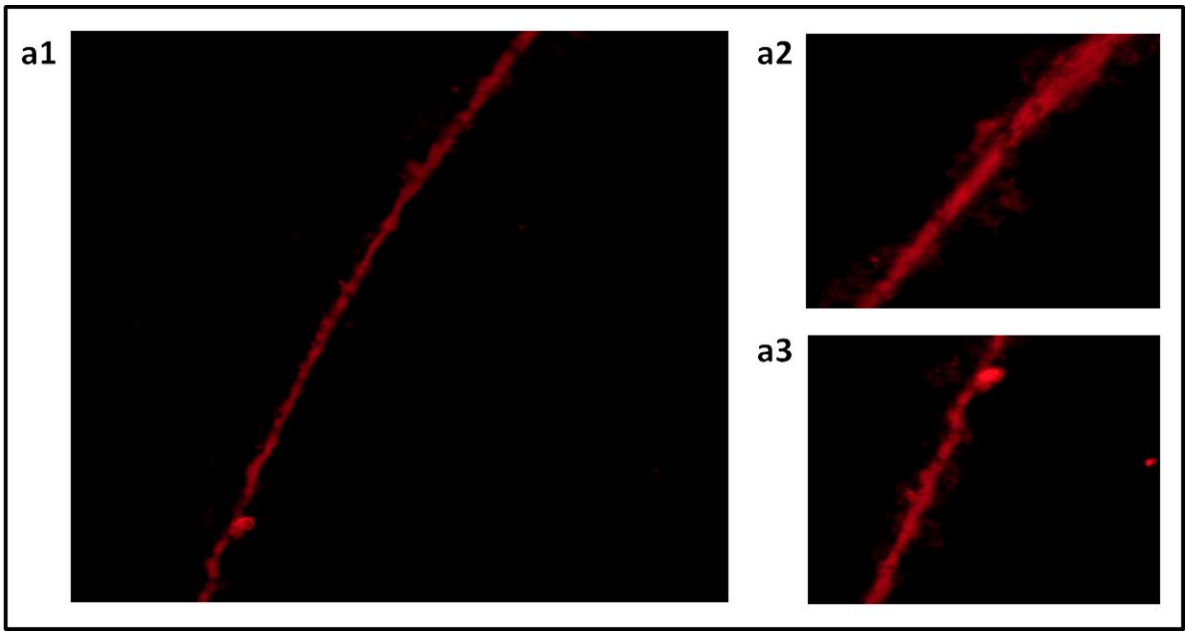

Figure 12: Fluorescence microscopic images of single filament using SYTO9 at 40 X from the electrochemical reactor suspension.

3.2.2.1. Serum flask enrichment

In case of the enrichment with $FeCl_3$ as the terminal electron acceptor, no microbial growth was observed. With nitrate as the terminal electron acceptor, microbial growth was observed, which directly correlated with the decrease in nitrate concentration. The nitrate concentration dropped to zero within 32 days of the enrichment experiment. The same behaviour was again confirmed by cultivating the enriched culture in triplicate serum flasks (N1, N2, and N3) (Fig. 13). Neither nitrate reduction nor microbial growth was observed in the case of abiotic and biotic control experiments. These results suggest the growth of sulphide oxidizing bacteria using nitrate as the terminal electron acceptor.

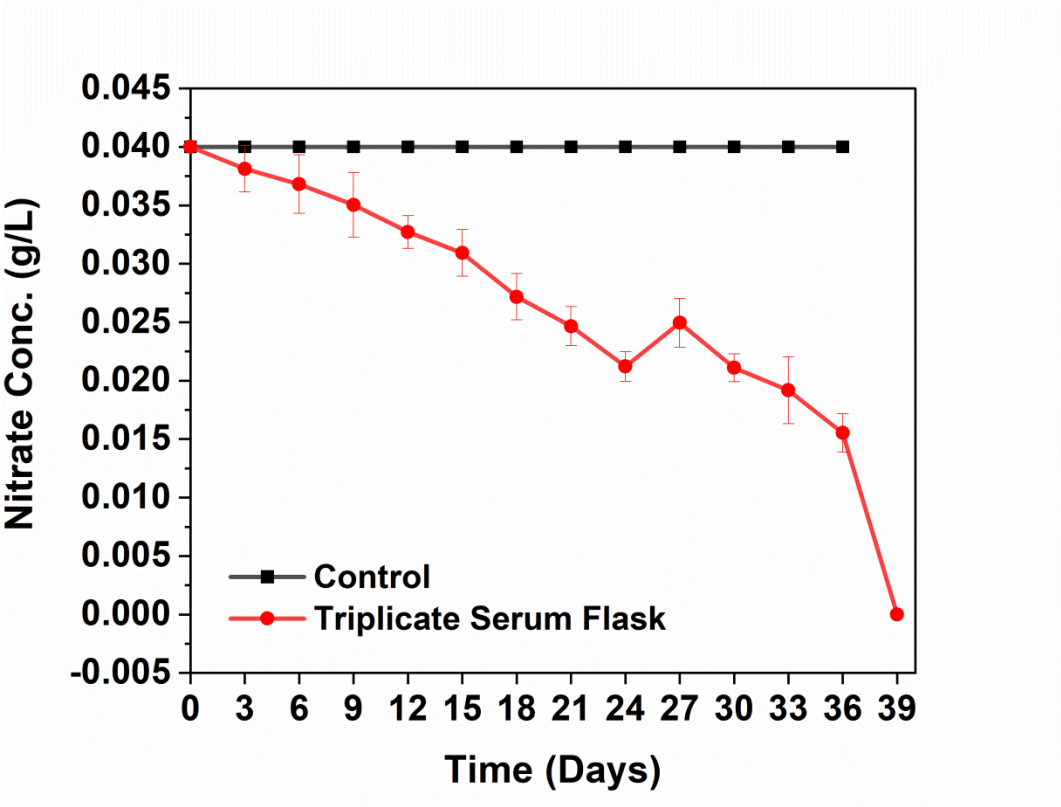

Figure 13: Nitrate concentration profile in the triplicate serum flasks inoculated with the enriched culture. (Electron donor source: sulphide).

3.2.2.2. Microbial growth assessment and confirmation through light microscopy

Mostly small rod-shaped cells and very few shorter filaments were observed in the case of serum flask enrichment (Fig. 14). The presence of small rod-shaped cells indicates that the filament formation may be an adaptive feature and not a characteristic feature of these bacteia. It occurs when electron donor and acceptor are placed far apart from each other. In serum flasks, nitrate is readily available, and hence, the formation of longer cables/filaments is not necessary to achieve respiration.

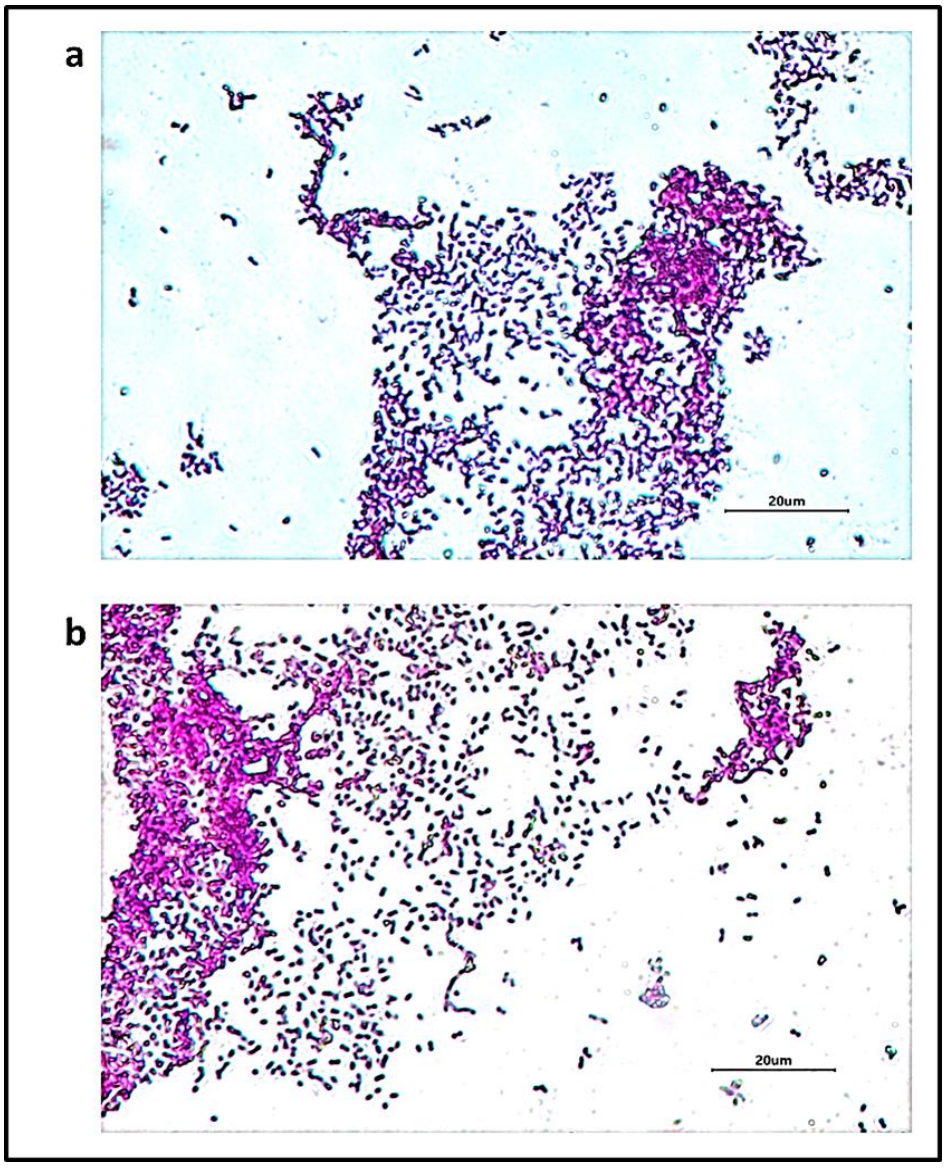

Figure 14: Light microscopic images of the suspension from the serum flask enrichment with nitrate as the terminal electron acceptor.

3.2.3. Cultivation of the enriched culture in microcosms

Entangled and single filaments were observed only in the microcosms in which both acetate and sulphide were provided (Fig. 15). No growth was observed in abiotic and biotic controls. In case when the only sulphide was provided, growth was not observed since no carbon source was provided while when the only acetate was provided, growth was expected but not observed. These observations suggest that both acetate and sulphide are needed for the cultivation of sulphide oxidizing filamentous bacteria.

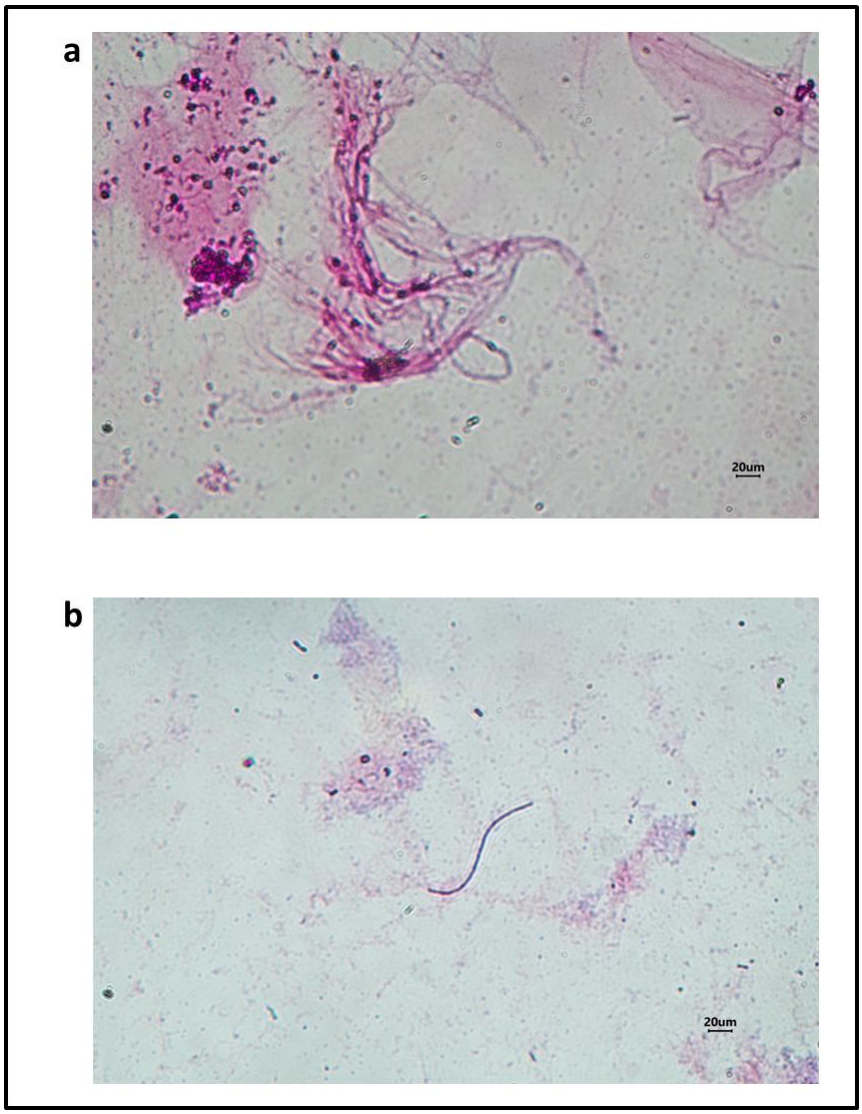

Figure 15: Light microscopic images of microbial samples at 100X from the microcosm inoculated with the enriched culture, a) Entangled filaments, and b) Single filament.

Chapter 4

Conclusions and Future Perspectives

Both the electrochemical and serum flask cultivation approaches resulted in successful enrichment of longer as well as shorter filamentous bacteria. The incremental sulphate production and bioelectrocatalytic current generation data in the electrochemical enrichment experiments support the microbial sulphide utilization as an energy source through its oxidation and electron transfer to the anode electrode. The microscopic observations revealed the growth of longer filaments in suspension, and shorter filaments and single cells growth at the anode surface. The serum flask enrichment with a soluble electron acceptor condition resulted in the abundance of small rod-shaped bacteria and very few filamentous bacteria.

By reporting on the haloalkaliphilic filamentous bacteria for the first time, this study suggests the presence of such sulphide-oxidising bacteria, most likely to be cable bacteria, in extreme environments. It also demonstrates, for the first time, that the electrochemical enrichment of sulphide-oxidizing filamentous bacteria is possible. Microscopic observations revealed the adaptive growth feature of these bacteria, i.e. they can be present in cable or single-cell forms. It suggests the dependency of the filament length on the distance between electron donor and acceptor. The larger the distance between donor and acceptor, the longer will be the filament size. Similarly, the lesser the distance, shorter will be the filament size.

Future work should focus on testing the enriched culture from the serum flask and microcosm experiments for its ability to use the solid-state electron acceptor. In order to confirm that the enriched sulphide oxidiser is cable bacteria, 16sRNA metagenome sequencing and fluorescence microscopy needs to be done. Further work on these filaments using transmission electron microscopy (TEM) would probably reveal the internal structures of these filaments.

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Appendix

*Vitamin Solution composition (Patil *et al.*, 2015)

S. No.	Component	Concentration (mg/L)
1.	Sodium ascorbate	10
2.	Biotin	4
3.	Folic acid	4
4.	Pyridoxine hydrochoride	20
5.	Thiamine hydrocloride	10
6.	Riboflavin	10
7.	Nicotinic acid	10
8.	DL-calcium pantothenate	10
9.	Vitamin B12	0.2
10.	p-aminobenzoic acid	10
11.	Lipoic(thioctic) acid	10
12.	Myo-inositol	10
13.	Choline chloride	10
14.	Niacinamide	10
15.	Pyridoxal hydrochloride	10

16. Tungstate- Selenium solution Composition: 0.1mM Na_2WO_4 + 0.1mM Na_2SeO_3 in 20mM NaOH

**Trace Metal Solution composition (Patil *et al.*, 2015)

S. No.	Component	Concentration (g/L)
1.	Nitrilotriacetic acid (dissolve with KOH; pH 6.5)	1.5
2.	$Mg_2Cl_2.6H_2O$	3.0
3.	$MnCl_2.2H_2O$	0.5
4.	NaCl	1
5.	$FeCl_2$	0.1
6.	$CoCl_2$	0.1
7.	$CaCl_2.2H_2O$	0.1
8.	$ZnCl_2$	0.1
9.	$CuCl_2$	0.01
10.	$AlCl_3.6H_2O$	0.01
11.	H_3BO_3	0.01
12.	$Na_2MoO_4.2H_2O$	0.01

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