Anaerobic haloalkaliphilic electrotrophs: Nitrate and Sulphate reducing microorganisms possessing extracellular electron transfer capabilities from a highly saline-alkaline environment

Srishti

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Certificate of Examination

This is to certify that the dissertation titled “Anaerobic haloalkaliphilic electrotrophs: Nitrate and sulphate reducing microorganisms possessing extracellular electron transfer capabilities from a highly saline-alkaline environment” submitted by Ms. Srishti (Reg. No. MS15032) 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

Dr. Anoop Ambili

Dr. Sunil A. Patil
(Supervisor)

Dated: May 4, 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.

Srishti

(Candidate)

Dated: May 4, 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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Notations

BES	Bioelectrochemical System
CA	Chronoamperometry
CV	Cyclic Voltammetry
DIET	Direct Interspecies Electron Transfer
EAM	Electroactive Microorganisms
EET	Extracellular Electron Transfer
MFC	Microbial Fuel Cells
NRB	Nitrate Reducing Bacteria
OD	Optical Density
PEM	Proton Exchange Membrane
SEM	Scanning Electron Microscopy
SHE	Standard Hydrogen Electrode
SRB	Sulphate Reducing Bacteria
TEA	Terminal Electron Acceptor

Contents

Abstract

Electromicrobiology deals with the study of extracellular electron transfer (EET) processes between microorganisms and insoluble, solid-state electron donors or acceptors, and their roles in different environments. Different microorganisms are known to possess EET capabilities to fulfil their respiratory and metabolic requirements in various environments. EET seems to be a widespread metabolic trait by which microorganisms use multivalent metal ions associated with minerals and other insoluble compounds as either a sink or source for the electrons. It is classified into two types, *viz*. outward EET (from cells to electron acceptor) and inward EET (from an electron donor to cells). The microorganisms bearing such unique capabilities are termed as Electroactive Microorganisms (EAMs). Based on the type of EET, EAMs are further categorized into two groups, namely, exoelectrogens and electrotrophs. The microorganisms possessing outward EET are termed as exoelectrogens, whereas the microorganisms which take up electrons from an extracellular electron donor to uphold their metabolic processes are termed as electrotrophs. Exoelectrogens and outward EET mechanisms have been well documented and understood, whereas very little is known about the electrotrophs and the inward EET mechanisms they possess. Furthermore, not much research has been conducted on the electrotrophic microorganisms. Only a handful of pure cultures of electrotrophs are known to date, including, nitrate or sulphate reducing microorganisms. More importantly, the extreme environments have been barely explored for such microorganisms, which can be of interest for biotechnological applications.

This study aimed at investigating the EET capable anaerobic nitrate-reducing bacteria (NRB) and sulphate-reducing bacteria (SRB) from a hypersaline-alkaline soda lake (Lonar Lake, Maharashtra, India), which is known to host a wide diversity of haloalkaliphilic microorganisms. Two different approaches were used for this purpose. The first one was based on the use of electrochemical cultivation, wherein the electrode, i.e., cathode poised at a specific electric potential, was used as the source of electrons. The second approach involved the enrichment in serum flasks with soluble electron donor source and further testing of the enriched culture for its ability to draw electrons from the cathode via EET for growth. A highly saline (20 g NaCl/L) and alkaline (9.5 pH) growth medium supplemented with either acetate (10 mM) or bicarbonate (10 mM) was used for enrichment experiments. The successful enrichment of NRB was achieved via both the enrichment approaches. However, SRB was enriched via the electrochemical approach only. Particularly the increase

in the cathodic reduction current confirmed the enrichment of both electrotrophic NRB and SRB. The cyclic voltammetry recorded with the enriched NRB revealed two redox-moieties with the formal potentials of -0.622 V and -0.433 V *vs.* Ag/AgCl. Further, the decrease in nitrate and sulphate concentrations in the electrochemical reactors confirmed the growth of NRB and SRB, respectively. In the case of serum flasks, the increase in optical density, and the decrease in nitrate concentration confirmed the enrichment of NRB. Its EET capability of this culture remains to be checked via the electrochemical cultivation approach. Microscopic analysis of the enriched cultures revealed the abundance of oval-shaped cells in all cases.

These results indicate the successful enrichment of the electrotrophic NRB and SRB from the sediment samples of an extreme halo-alkaline environment. Their EET capabilities will be confirmed via additional electrochemical tests. Further characterization of the enriched cultures through 16S rRNA metagenome sequencing is envisioned to understand the dominant haloalkaliphilic electrotrophs. It will be followed up by isolation of the novel electrotrophs and identification and characterization of the observed redox-moieties involved in the inward EET processes. Detailed understanding of the haloalkaliphilic electrotrophic microorganisms is expected to increase our existing knowledge of this novel microbial group and electron uptake mechanisms, which have implications for strengthening electromicrobiology discipline and developing microbial electrochemistry-driven biotechnologies.

Chapter 1

Introduction

1.1. Electromicrobiology

Electromicrobiology is an emerging (Environmental) Microbiology sub-discipline that explores the electrochemical interactions or electron transfer processes between microorganisms and solid-state electron donors or acceptors as well as its implications in the environment (Lovley, 2012). The involved electrochemical interactions or electron transfer, either inward or outward of the microbial cell and from or to the solid-state electron donor or acceptor, is known as Extracellular Electron Transfer (EET). A wide diversity of microorganisms is known to possess the EET trait. Examples include several species belonging to *Shewanella* and *Geobacter* genera, which use EET to sustain their respiratory or metabolic processes in various environments (Coursolle *et al.*, 2010; Nealson *et al.*, 2017). Electromicrobiology can be pursued and applied using bioelectrochemical systems (BES). The electrochemical systems utilizing microbial catalysts for different applications based on anodic and cathodic reactions or processes are termed as microbial BES (Lovley, 2006; Rabaey *et al.*, 2007).

1.2. Bioelectrochemical systems (BES)

Microbial BES can be of different types, viz. Microbial Fuel Cell (MFC), Microbial Electrosynthesis Cell (MES), Microbial electrolysis cell (MEC), Microbial Solar Cell (MSC), and Microbial desalination cell (MDC) based on the target process and application (Katz *et al.*, 2003). The basic working principle of BES is substrate oxidation either electrochemically or microbially linked to transfer of liberated energy (electrons) to the electrode (anode) and later to the cathode via an external circuit where they are used to reduce several oxidized substrates *viz*. sulphate, nitrate, CO_2, heavy metals, etc. (Bajracharya *et al.*, 2016). BES can be used for various biotechnological applications like H_2 production, wastewater treatment, electricity production, value-added chemical production, CO_2 sequestration, and bioremediation of heavy metals, polyaromatic hydrocarbons, dyes, etc. The microbial catalysts that possess EET capabilities and used

widely in BES are generally termed as electroactive microorganisms (EAM) (Lovley, 2012; Nealson *et al.*, 2016). These are cost-efficient (can be generated from inexpensive feedstock), eco-friendly (less waste generation and no hazardous compounds are used), and are sustainable (self-repair and replicate) than the conventional or chemical catalysts (Ali *et al.*, 2018; Chen *et al.*, 2008).

1.3. Electroactive microorganisms (EAM)

EAM possess either inward or outward EET or both capabilities. Some of them also possess the capabilities to transfer electrons to another microorganism, either belonging to the same or other species directly without the involvement of any soluble mediator. This process of transferring electrons between two microbial species is known as Direct Interspecies Electron Transfer (DIET) (Logan *et al.*, 2019; Rotaru *et al.*, 2014; Kato *et al.*, 2012). Among EAM, the microorganisms capable of transferring electrons extracellularly to the solid terminal electron acceptors such as mineral oxides or electrodes are known as Exoelectrogens (Lovley, 2012). There are several known examples of exoelectrogens (Kiran *et al.*, 2019). The most well-studied EAM include *Shewanella* and *Geobacter* spp. Exoelectrogens can achieve EET via direct and indirect/mediated mechanisms. The direct EET involves electron transfer through either cell membrane-bound proteins or cytochromes or conductive nanowires and pili. The indirect/mediated EET involves the electron transfer with the help of some reduced mediators or compounds such as flavins, H_2, etc. (Lovley, 2008). Due to the presence of these electronic properties, these biofilms and associated components are known to act as supercapacitors, transistors and possess metal like conductivities (Malvankar *et al.*, 2011; Malvankar *et al.*, 2012).

The microorganisms that can achieve inward EET, i.e., capable of drawing electrons from the solid electron donors such as reduced minerals or electrodes to achieve their respiration and other metabolic processes, are termed as Electrotrophs. These microbes can use the gained energy (electrons) to reduce oxidized substrates like Carbon Dioxide, Nitrate, Sulphate, etc. Hence, they can be used to treat nitrate, sulphate or heavy metals containing wastewaters and to produce some value-added chemical products such as short-chain fatty acids from CO_2 (Lovley, 2011; Lovley, 2012). Although the modes of electron transfer (either direct or mediated) are postulated for inward EET, the

components that are involved in EET remain poorly understood (Rosenbaum *et al.*, 2011; Song *et al.*, 2018).

1.3.1. Electrotrophic microorganisms

Some pure and several mixed cultures, CO_2, O_2, and heavy metal ions reducing microorganisms have been reported to uptake electrons directly from the cathode (Kiran et al. 2019). However, most remain poorly studied for EET mechanisms and components. Some of the prominent electrotrophic microorganisms include sulphate-reducing bacteria (SRB) and nitrate-reducing bacteria (NRB) (Su *et al.*, 2012; Cordas *et al.*, 2008; Tingzhen *et al.*, 2019).

Sulphate-Reducing Bacteria (SRB)

SRB group belongs to prokaryotes that use SO_4^{2-} as a terminal electron acceptor (TEA) to achieve their respiration in diverse anoxic environments and plays a significant role in the global cycling of carbon and sulphur (Jørgensen, 1982). Most of the known sulphate-reducing microbial communities belong to *Deltaproteobacteria*, *Nitrospirae*, *Firmicutes*, *Thermodesulfobacteria* phyla among bacteria and *Euryarchaeota*, *Crenarchaeota* phyla among archaea (Rabus *et al.*, 2006; Muyzer *et al.*, 2008). Some haloalkaliphilic SRB isolated from soda lakes across the world belong to *Desulfonatronovibrio*, *Desulfonatronum* and *Desulfonatronospira* genera (Pikuta *et al.*, 1998; Sorokin *et al.*, 2008; Sorokin *et al.*, 2008; Sorokin *et al.*, 2011). A study on enriching haloalkaliphilic SRB using only high-throughput 16S rDNA sequencing revealed the dominance of different genera such as *Halanaerobium*, *Halothiobacillus*, *Desulfonatronum*, *Syntrophobacter*, *Fusibacter*, etc. (Zhou *et al.*, 2015). SRB are known to convert sulphate to sulphide by oxidising organic substance or hydrogen (dissimilatory sulphate reduction). As shown in reaction R1, the whole process involves eight electrons to convert sulphate to sulphide through sulphite as an intermediate (Agostino *et al.*, 2018). After entering the cell membrane, sulphate is converted to APS (Adenosine 5′-phosphosulfate) by ATP (Adenosine Triphosphate), which is then converted to sulphite and finally to sulphide using APS reductase and sulphite reductase complex, respectively (Peck *et al.*, 1959; Lampreia *et al.*, 1994; Fike *et al.*, 2016).

$$SO_4^{2-} + 8e^- + 10H^+ \leftrightarrow H_2S + 4H_2O \quad \text{------}\gg \text{R1}$$

Nitrate-Reducing Bacteria (NRB)

NRB group uses NO_3^- as the terminal electron acceptor in an anoxic environment to complete their respiration and contribute significantly in the global nitrogen cycle by recycling nitrate to nitrite (R2) (Winnerberger, 1982). A few studies on mixed culture and pure biofilms of electrotrophic NRB have been reported (Tingzhen *et al.*, 2019). The pure culture isolates of electrotrophic NRB includes *Thiobacillus denitrificans* (Yu *et al.*, 2015), *Pseudomonas alcaliphila* (Su *et al.*, 2012), and *Pseudomonas aeruginosa (*Niepa *et al.*, 2017). While the mixed culture biofilms of NRB were found rich with the species of *Thiobacillus* (Pous *et al.*, 2014), *Rhodocyclales* and *Burkholderiales* (Gregoire *et al.*, 2014). Some studies have reported on switchable electroactive biofilms dominated with *Geobacter* spp. that is capable of complete denitrification. Such biofilms can perform both oxidation and reduction by polarity reversion process (Lianga *et al.*, 2019; Pous *et al.*, 2015).

$$NO_3^- + 2e^- + 2H^+ \leftrightarrow NO_2^- + H_2O \qquad \text{------>} \; R2$$

As it has been studied that nitrate crusts and certain microorganisms are responsible for the decrementing material surfaces in cultural heritages (Webster *et al.*, 2006; Gioventu *et al.*, 2011; Troina *et al.*, 2013), various NRB such as *Pseudomonas denitrificans*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Pseudomonas pseudoalcaligenes*, and *Paracoccus denitrificans* are used to remove nitrate crusts from the surface of stony materials (Ranalli *et al.*, 1996; Ranalli *et al.*, 2003). Haloalkaliphilic NRB can also be used to treat and to ensure no further microbial growth on material surfaces, even in neutral conditions (Romano *et al.*, 2018).

Both nitrate and sulphate are toxic to humans. For instance, nitrate can cause ‘blue baby syndrome’ in infants. Nitrate can also be converted to other toxic compounds that can be carcinogenic to humans (Winnerberger *et al.*, 1982; Claudio *et al.*, 2005). While the inappropriate sulphate levels in drinking water can cause diarrhoea and dehydration in humans and possess a severe risk to infants (Pineau, 2008; Silva *et al.*, 2012). Different conventional approaches including precipitation, adsorption, nano-filtration, ion exchange, etc. and electrochemical treatment, ion exchange, reverse osmosis, electrodialysis and heterogeneous have been used for sulphate and nitrate removal, respectively (Ashane *et al.*, 2018; Park *et al.*, 2009; Gupta, 2016; Reinsel 2015).

It has been reported that high concentrations of sulphide are even toxic to SRB (Kalyuzhnyi *et al.*, 1997; Reis *et al.*, 1992). In haloalkaliphilic conditions, hydrogen sulphide gas is converted into sulphide ions that are not capable of entering microbial cells (Mora-Naranjo *et al.*, 2003). Removal of sulphate at these conditions using extremophiles has an advantage over normal conditions (Jing, *et al.*, 2013; Wu *et al.*, 2009).

Although several studies have reported on nitrate and sulphate removal in BES, only a few pure cultures of electrotrophic SRB and NRB are known to date. Furthermore, very little is known about the inward EET mechanisms they possess. Importantly, the extreme environments have barely been explored for such microorganisms. Studying such microorganisms will not only add to the existing knowledge about electrotrophs and their EET mechanisms but would also probably have implications to astrobiology (Shrestha *et al.*, 2018; Babu, *et al*, 2015; Dopson, *et al*, 2016). Therefore, in this study, we aimed to explore a highly saline and alkaline environment of Lonar Lake, located at Buldhana District, Maharashtra, India for enriching and isolating electrotrophic SRB and NRB. The sediments of this lake have been reported to be rich in sulphate and nitrate content (Borul *et al.*, 2012; Sengupta *et al.*, 1997; Misra *et al.*, 2009; Koshy *et al.*, 2012). It is a hypersaline soda lake that supports a wide diversity of haloalkaliphilic microorganisms (Borul *et al.*, 2012; Sengupta *et al.*, 1997). For enriching haloalkaliphilic electrotrophs, we used two different anaerobic enrichment approaches. The first one was based on the use of electrochemical cultivation, wherein the electrode, i.e., cathode poised at a certain electric potential, is used as the source of electrons. The second approach involved the enrichment in serum flasks with mainly a soluble electron donor source and further testing of the enriched culture for its ability to draw electrons from the cathode electrode via EET for growth. It was then followed by the understanding of their EET capabilities and morphological features *via* electrochemical and microscopic techniques, respectively.

Chapter 2

Materials and methods

2.1. General experimental conditions

All chemicals and gases were obtained from either Sigma-Aldrich or SRL chemicals. For electrochemical and serum flask enrichments, all the reactors were operated at 23±2 ºC and 30±2 ºC, respectively (Kumar *et al.*, 2013). All the experiments were conducted in at least triplicates under strictly anaerobic conditions. All the electric potentials, either applied or reported, are with respect to 3.5 M KCl Ag/AgCl reference electrode (0.205 V *vs.* SHE (standard hydrogen electrode)). The pH and salinity of the microbial growth medium were maintained at 9.5 and 20 g/L throughout the experiments.

2.2. Sediment sampling and characterization

Sediment samples from a depth of up to ~1 ft. from three sampling sites located at the periphery of the Lonar Lake were collected in the amber-coloured plastic sampling bottles. The sampling was conducted in the monsoon season in August 2019. This reduced the possible physiochemical variation that could be present in the samples due to the location variations (Table 2). When not in use, all bottles were stored airtight at 4 ºC. The sediment samples were analyzed for different physical and chemical parameters by following the standard analytical protocols as per APHA 2012. These include pH, salinity, conductivity, ammonia, COD, phosphate, sulphate, and nitrate.

2.3. The experimental set-up, growth medium and inoculum source

For the enrichment of electrotrophic microorganisms, serum flask and electrochemical cultivation approaches were used. Before starting the enrichment experiments, the inoculum was prepared by mixing the sediment samples in the modified M9 medium (Patil *et al.*, 2015) (Table 1) followed by sonication and centrifugation for 10 minutes. Then the suspension was used to inoculate the serum flasks and electrochemical reactors.

Table 1: Microbial growth medium composition (9.5 pH and 20 g/L salinity).

S. No.	Components	Concentration (g/L)
1.	NaH_2PO_4	2.69
2.	Na_2PO4	4.33
3.	NaCl	20
4.	Na_2CO_3	4.2
5.	KCl	0.13
6.	NH_4Cl	0.30
7.	Vitamins*	12.5 ml/L
8.	Trace Metals**	12.5 ml/L

(Note: for * and ** refer to appendix)

Serum Flask experiments: 100 mL capacity serum flasks were used for the experiments (Figure 1). The total working volume for cultivating the microorganisms was fixed at 40 mL. It contained a modified M9 medium with pH 9.5 and salinity 20 g/L as a growth medium (Table 1). It was amended with either solid iron particles (Fe^0) or acetate (10mM) as the source of electrons. In the case of Fe^0 condition, bicarbonate served as the carbon source whereas, in the case of acetate, it served as both the carbon and electron source. For the enrichment of nitrate or sulphate reducing microorganisms, the medium

was supplemented with 100 mg/L of either nitrate or sulphate, which acted as the terminal electron acceptor. No other electron acceptors were provided in the medium. Flasks filled with the growth medium were sparged with 99.999% pure inert N_2 gas for at least 10 minutes to make it completely anaerobic. Then the flasks were sealed with butyl rubber stoppers and crimp seals followed by autoclaving.

Along with the main enrichment experiments, two controls were also set up. The biotic control lacked the electron acceptor but contained all other components, including inoculum, whereas the abiotic control contained all components but the microbial inoculum. The main experiments were conducted in three fed-batch cycles. The sampling and analysis were done every 24 hours for the carbon source, pH, optical density (OD_{600}), nitrate, sulphate, nitrite, and sulphide concentrations. In the case of enrichment using Fe^0, the serum flasks were incubated undisturbed at 30˚C. After successful enrichment of 3.5 months of incubation, turbidity, $Fe^{2+/3+}$ concentration, and sulphide concentration were analysed using standard protocols as per APHA 2012 (Greenberg, 1992).

Figure 1: Serum Flask

Electrochemical reactor setup: A two-chambered electrochemical reactor setup with a three-electrode configuration was used to enrich electrotrophic microorganisms that are capable of drawing electrons from the electrode (cathode) to reduce nitrate or sulphate (Figure 2) (Patil *et al.*, 2012). The cathodic chamber hosted a graphite-working electrode with a projected surface area of (7.0125 cm^2) and an Ag/AgCl reference electrode. The anodic chamber contained a Mixed Metal Oxide coated titanium electrode, which is an

efficient catalyst for water oxidation. A Proton Exchange Membrane (PEM) was used to separate both the anodic and cathodic chambers, and thereby the respective reactions (Figure 2). Both the electrodes and PEM were pre-treated before the installation in the system by following standard protocols. For instance, the acid-alkali treatment method was used to remove any kind of impurities present over the electrodes (Feng *et al.*, 2010). The PEM treatment was done by heating it at 60-70°C for one hour each in distilled water, 2% H_2O_2, distilled water, H_2SO_4 solution (4.9 mL in 100 mL distilled water) followed by distilled water sequentially. Titanium wire was used as the current collector and to establish connectivity with the potentiostat channels.

The same microbial growth medium, as used in the serum flask experiments, was used in the electrochemical reactors. The working electrode (cathode) acted as an analogue to the electron donor, and only bicarbonate was used as the carbon source.

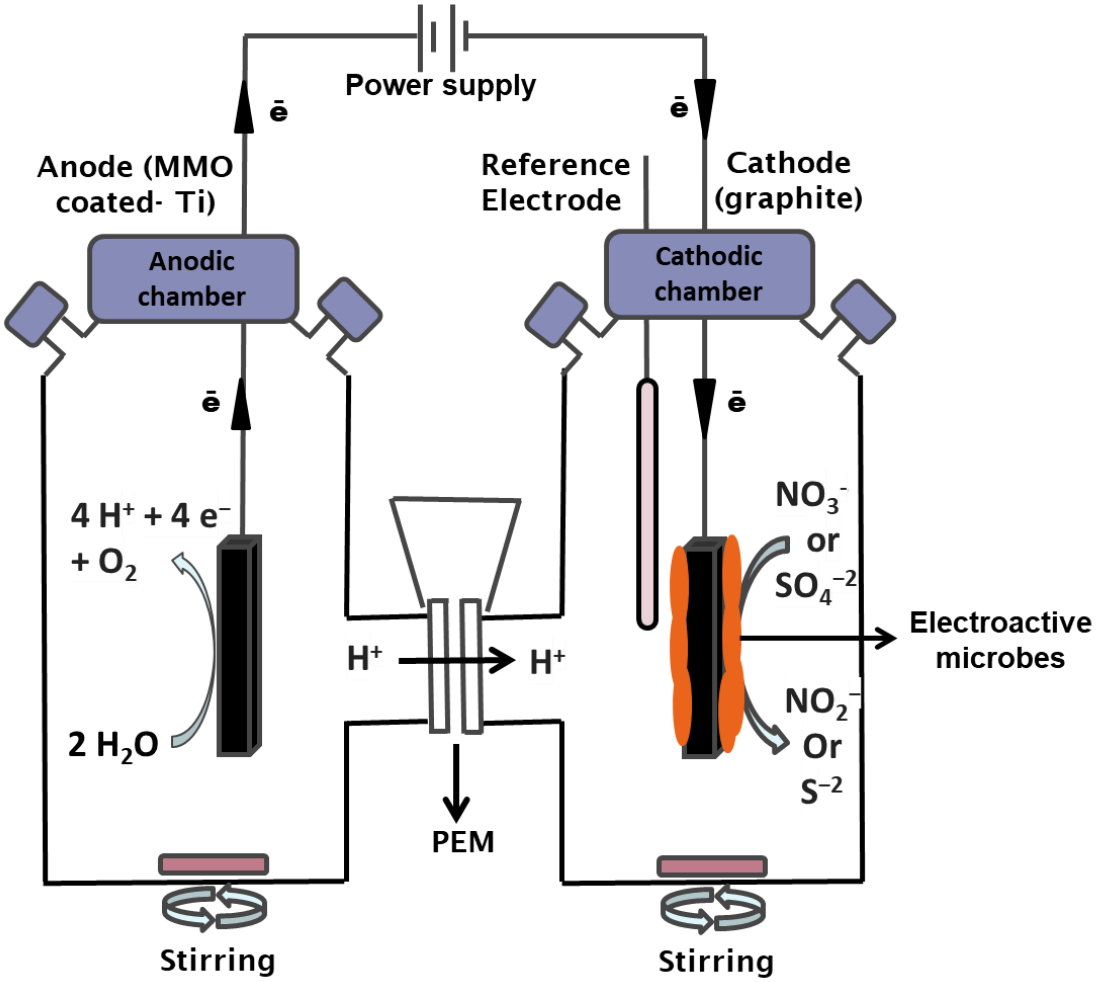

Figure 2: Schematic of the two-chambered electrochemical reactor setup. The proposed anodic and cathodic reactions are depicted.

2.4. Electrochemical enrichment of haloalkaliphilic nitrate and sulphate reducing electrotrophs

The electrochemical enrichment experiments were conducted with the aid of a potentiostat (VMP3 multichannel electrochemical workstation, BioLogic Science Instruments, France). The cathodes were poised at the potential of -0.300 V and -0.755 V *vs.* Ag/AgCl for the enrichment of NRB and SRB, respectively, and the cathodic current drawn by the microorganisms as a function of time was recorded using Chronoamperometry (CA) technique. Before starting CA, cyclic voltammograms (CV) were recorded at two different conditions, namely before and after inoculation to check for any redox-active components in the medium and at the electrode surface and also to understand the development of any redox activity at the electrode after the experiments (Harnisch *et al.*, 2012; Labelle *et al.*, 2005; Carmona *et al.*, 2011). For this purpose, a potential window from -1.0 V to 0.0 V and a scan rate of 1 mV/s were chosen. After the completion of one batch cycle, the reactors were replenished by a fresh complete growth medium.

For testing the EET capabilities of the enriched NRB in the serum flask experiments conducted with acetate and Fe^0 as the electron donors, two more electrochemical reactors were started. Two control experiments, abiotic-connected (without inoculation but applied with the desired potential) and biotic-unconnected (inoculated but without any applied potential), were also conducted to confirm any electrochemical and microbial activity at the same applied potential and conditions. The current density data is presented by normalizing the absolute current with the projected surface area of the working electrode. Analysis of pH, decrease in nitrate or sulphate concentration, and increase in sulphide concentration was done at a regular interval of every 24 hours.

2.5. Characterization of enriched electrotrophic microorganisms

2.5.1. Microscopy

Two microscopic techniques, *viz.*, light and scanning electron microscopy (SEM) were used to confirm the growth and morphology of electrotrophic microorganisms.

Light Microscopy

Microbial samples at different growth stages were stained using gram staining kit (Sigma-Aldrich) according to manufacturer instructions. For this, a drop of the sample was heat-fixed to prepare a smear over the slide. Then the smear was flooded with crystal violet solution for 1 minute, followed by rinsing with water. Then the Iodine solution was allowed to retain for 1 minute, followed by rinsing with water. Later, the slide was flooded with decolourizer for 1-5 seconds, followed by rinsing with water, incubation for 1 minute with safranin and again rinsed off with water. Excess water was removed with the help of tissue paper, and the slides were observed under a Light Microscope (Nikon).

Scanning Electron Microscopy

The samples were passed through 0.2 µm PC isopore membrane filters (Sigma-Aldrich), and the retained cells on filters were fixed overnight in a mixed fixative solution of 2% glutaraldehyde and 2.5% paraformaldehyde at 4 ºC. The samples were dehydrated using different ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) sequentially for 20 minutes in each. This was followed by sample drying 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 analyzed using a JEOL JSM-6010PLUS/LS Scanning Electron Microscope (JEOL Ltd., Japan).

2.5.2. Electrochemical characterization

Cyclic voltammetry as performed before and after inoculating the reactors, and at the end of the enrichment experiments to find out the role of enriched electrotrophic biofilm in current drawn from the cathode (Harnisch *et al.*, 2012; Labelle *et al.*, 2005; Carmona *et al.*, 2011).

Chapter 3

Results and Discussion

3.1. Physicochemical properties of sediment samples

A high pH condition, as reported in the literature, was observed in the collected sediment samples (Table 2). The variation in salinity of the Lonar Lake has also been reported by several other research groups. It ranges from 5 to 24 g/L (Borul, 2012). This might be because of the variations in the seasons and sampling locations. The low salinity level of the sediments collected in the monsoon season in this study might be due to the more inflow of freshwater in the lake.

Table 2: Sediment Characteristics

Parameters	August 2019
pH	9.6 ± 0.2
Salinity (ppt or g/L)	14.3 ± 1.0
Conductivity (mS/cm) at 24ºC	22.0 ± 1.5
Ammonia (mg/L)	2.2 ± 0.1
COD (mg/L)	526.4 ± 13.7
Phosphate (mg/L)	47.2 ± 2.6
Sulphate (mg/L)	62.8 ± 1.2
Nitrate (mg/L)	222.4 ± 7.0

The high Chemical Oxygen Demand (COD) values suggest the eutrophic nature of the lake. Various soluble ions, including sulphate (SO_4^{2-}), ortho-phosphate (PO_4^{3-}), ammonium ion (NH_4^+), and nitrate (NO_3^-) were also present in considerable amounts. The presence of, in particular, SO_4^{2-} and NO_3^- suggests their availability as electron acceptors under anoxic conditions in sediments. Acetate was found to be the dominant short-chain organic acid in the sediment samples.

3.2. Enrichment of the haloalkaliphilic nitrate-reducing microorganisms

3.2.1. Enrichment in serum flask reactors

In the first batch enrichment cycle, visible turbidity in the bulk phase was observed in the inoculated flasks with acetate as an electron donor. A continuous increase in OD was observed during the incubation period of 6 days (Figure 3). It correlated with the decrease in the concentration of nitrate (Figure 4). After the depletion of nitrate in medium, 10 % of the enriched culture was transferred to the fresh medium in subsequent batch cycles. In the second and third batch cycles, the serum flask reactors followed similar trends for OD and nitrate concentration (Figures 3 and 4). The pH remained almost constant at around 9.6-9.7 throughout the batch enrichment experiments. Neither increase in OD nor decrease in the nitrate concentration was observed in the control experiments. These observations suggest the enrichment of nitrate-reducing microorganisms in the main experiments.

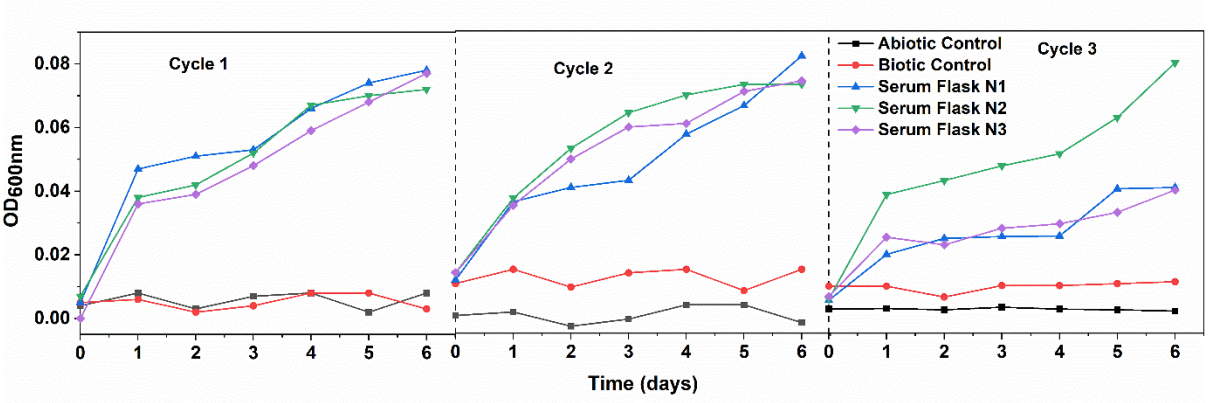

Figure 3: Turbidity or OD_{600nm} variation throughout the three batch cycles of the serum flask enrichment experiments. (Electron donor: acetate, TEA: nitrate)

In the case of SRB enrichment using serum flask approach with acetate as the electron donor, neither increase in optical density nor decrease in sulphate concentration was observed.

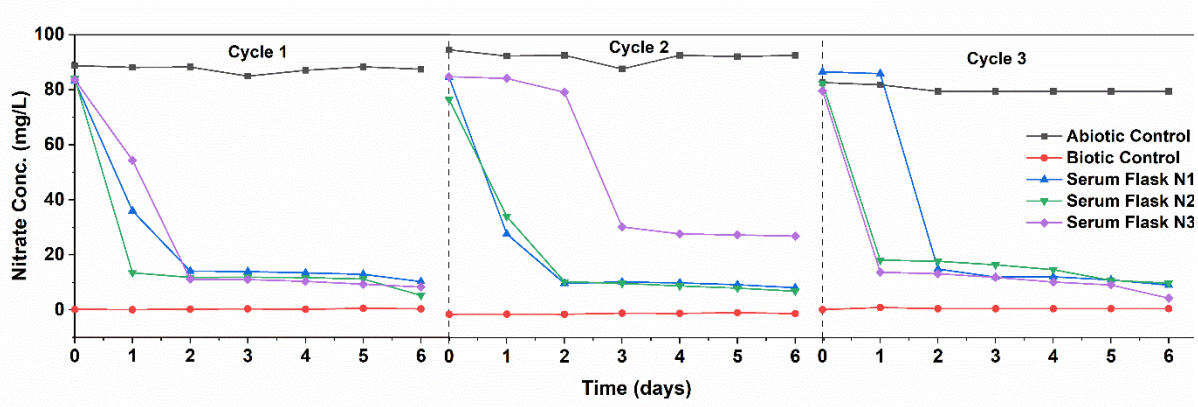

Figure 4: Nitrate concentration profiles in three batch cycles of the serum flask enrichment experiments.

As electrotrophs are very slow-growing microorganisms, so in the enrichment experiments using Fe^0, two serum bottles with nitrate and sulphate were left incubated and undisturbed for 3.5 months. At the end of 3.5 months, turbidity was observed in both cases. The growth medium started to turn in greenish or greenish-black colour in the case of NRB and SRB within the second week of the incubation period, respectively. The appearance of green color and increased Fe^{2+} concentration suggested the growth of NRB using solid iron particles as an energy source. While black color and increased sulphide concentration also suggested the growth of SRB using solid iron particles as an energy source.

3.2.2. Electrochemical enrichment of nitrate and sulphate reducing electrotrophs

It has been shown that electrotrophs can be grown by forcing the microorganisms with EET capabilities to take up electrons from the solid electrode, in the absence of any soluble and easily available electron donors, to reduce components like nitrate and sulphate (Su *et al.*, 2012; Cordas *et al.*, 2008; Lovley, 2011). The microbial growth at the electrode can be monitored by observing the electric current (which is the result of the electron transfer process) as a function of time under a fixed potential using the CA technique. It took almost 1.5 months for the microorganisms to produce any observable

reduction current from the cathode electrode in the case of sulphate electron acceptor condition (Fig 5). Even after 2.5 months of incubation, the reduction current kept on increasing. The reactor has been kept undisturbed to allow and acclimatize the growth of electrotrophic microorganisms over the cathode surface. After the completion of the first cycle, the reactor will be replenished with fresh microbial growth medium for further enrichment cycles. So far, a maximum current density of around – 25 µA/cm^2 has been achieved (Fig. 5). The earlier studies on enriching SRB using the electrochemical approach have reported that the pure biofilm of sulphate reducing bacteria *Desulfovibrio desulfuricans* 27774 strain can produce a maximum current density of -27µA cm^{-2} (Cordas *et al.*, 2008) while other reported biofilms are known to produce reduction current densities which are less than 1 µA cm^{-2} (Logan, *et al.*, 2019). Moreover, different studies showed that DET performing electrotrophs were able to generate maximum current densities of -23.64 µA cm^{-2} (Su *et al.*, 2012) and -80 µA cm^{-2} (Toshiyuki *et al.*, 2018). These observations indicate the enrichment of the haloalkaliphilic sulphate reducing electrotrophs via electrochemical cultivation approach.

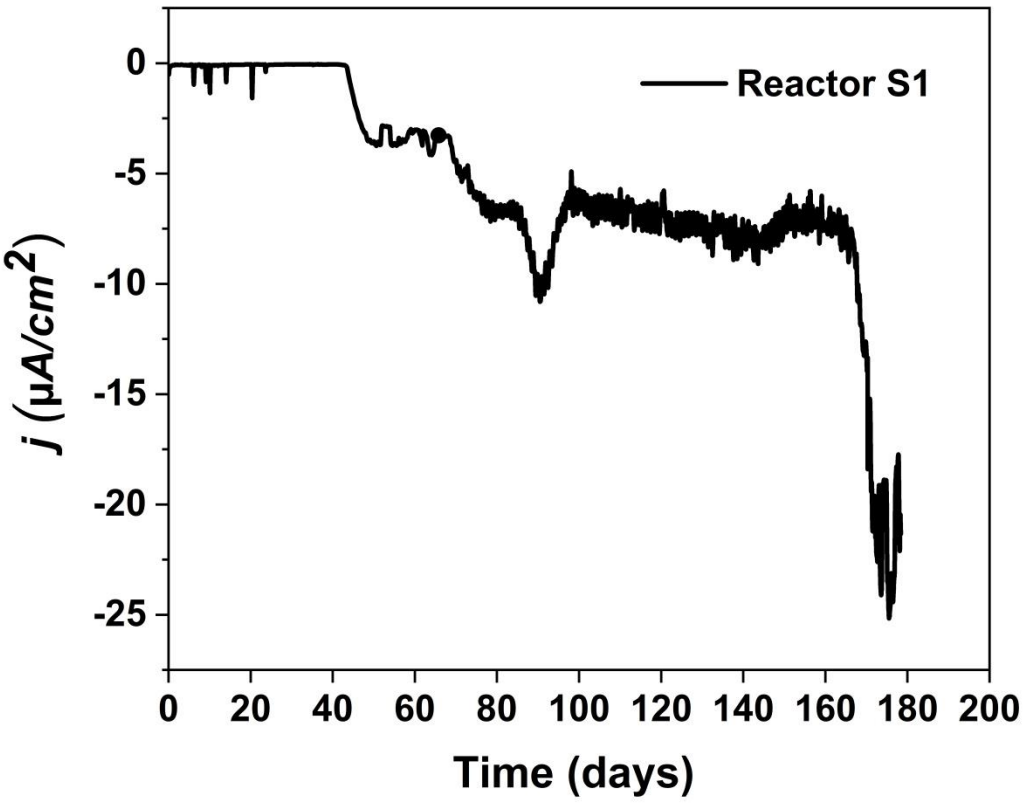

Figure 5: Chronoamperometric profile showing the increase in bioelectrocatalytic reduction current in a sulphate fed reactor.

In the case of nitrate, the start-up in the cathodic reduction current generation was comparatively faster. A maximum current density of – 25 µA/cm^2 was produced in this case (Figure 6). The increase in the current generation was linked to a decrease in nitrate concentration. These observations indicate the electrochemical enrichment of NRB at the cathode surface. Two replicate reactors have been set up to check the reproducibility of electrotrophic NRB using the enriched inoculum from the electrochemical reactor 1.

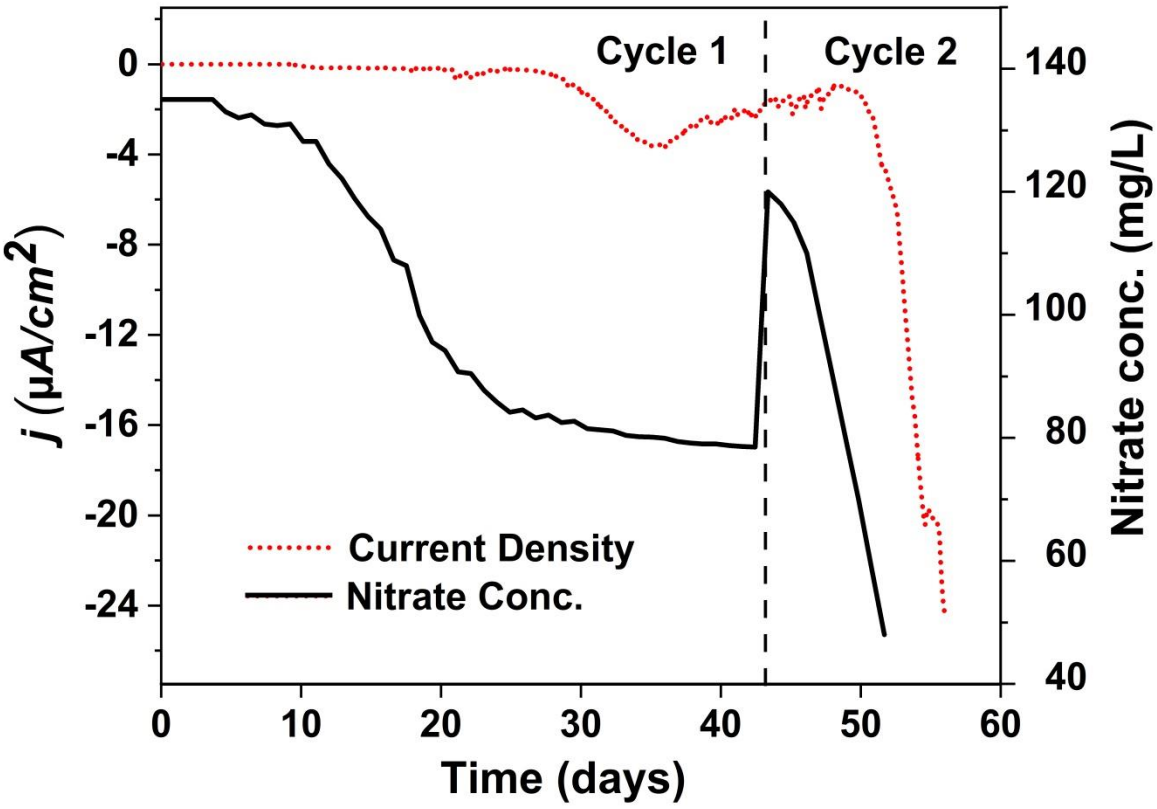

Figure 6: Chronoamperometric and nitrate concentration profiles showing the increase in the bioelectrocatalytic reduction current in the nitrate fed electrochemical enrichment reactor.

In the abiotic (uninoculated-connected) and biotic (inoculated-unconnected) control experiments, no increase in the reduction current was observed (Fig. 7). It thus confirms that the reduction current generation in the main experiments was due to the enrichment of electrotrophic nitrate-reducing microorganisms at the cathode.

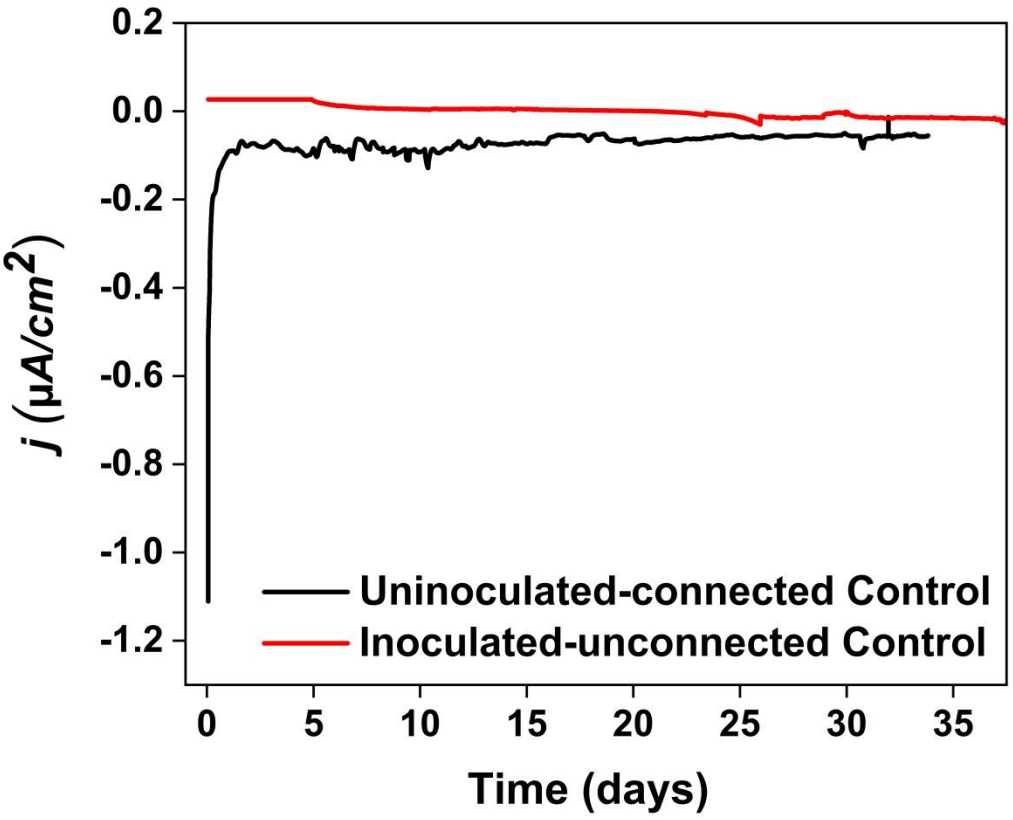

Figure 7: Chronoamperometric graph showing almost negligible or zero change in the reduction current at an applied cathode potential of -0.3 V for the control experiments with nitrate.

3.3. Characterization of enriched haloalkaliphilic electrotrophic microbes

3.3.1. Microscopy

The bulk-phase samples from the serum flask and electrochemical reactors were analysed using two different microscopic techniques.

Light Microscopy

It revealed the presence of small oval-shaped microbial cells in the enrichment cultures. Some representative microscopic figures at different resolutions and observation scales from the serum flask and electrochemical reactors are shown in Figures 8 and 9, respectively.

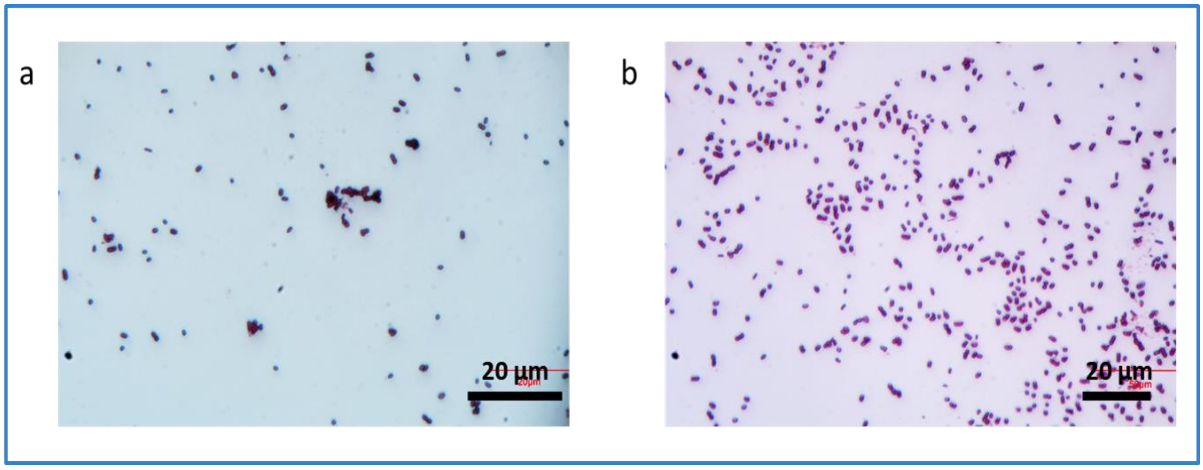

Figure 8: Representative light microscopic images of the enriched nitrate-reducing microorganisms in the serum flask reactors.

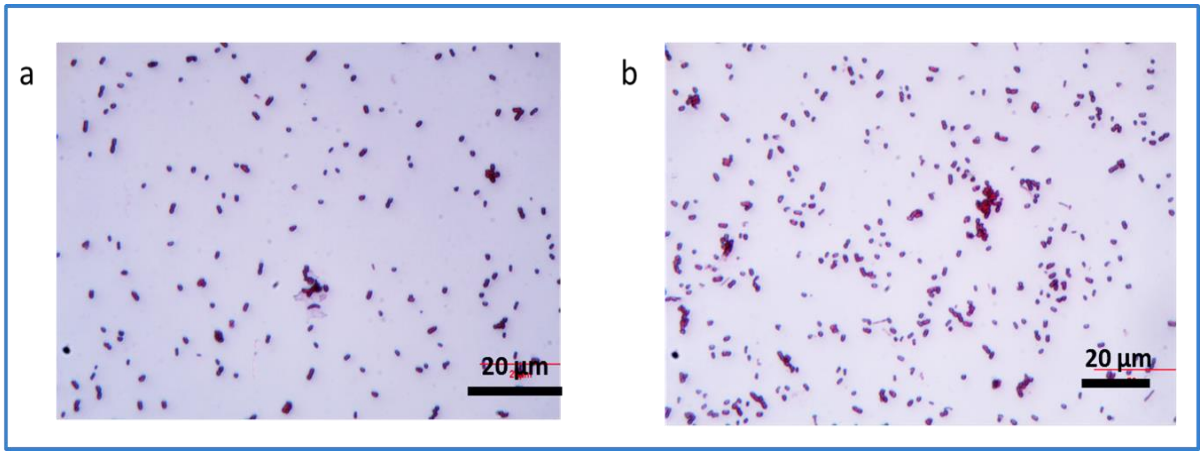

Figure 9: Representative light microscopic images of the enriched nitrate-reducing microorganisms in the electrochemical reactors.

Scanning Electron Microscopy

The SEM analysis of the bulk phase samples confirmed the presence of small rod or oval-shaped microbial cells in the enrichment cultures (Figures 10 and 11). The SEM of the cathode surfaces will be conducted at the end of the experiments to confirm the formation of electrotrophic biofilms.

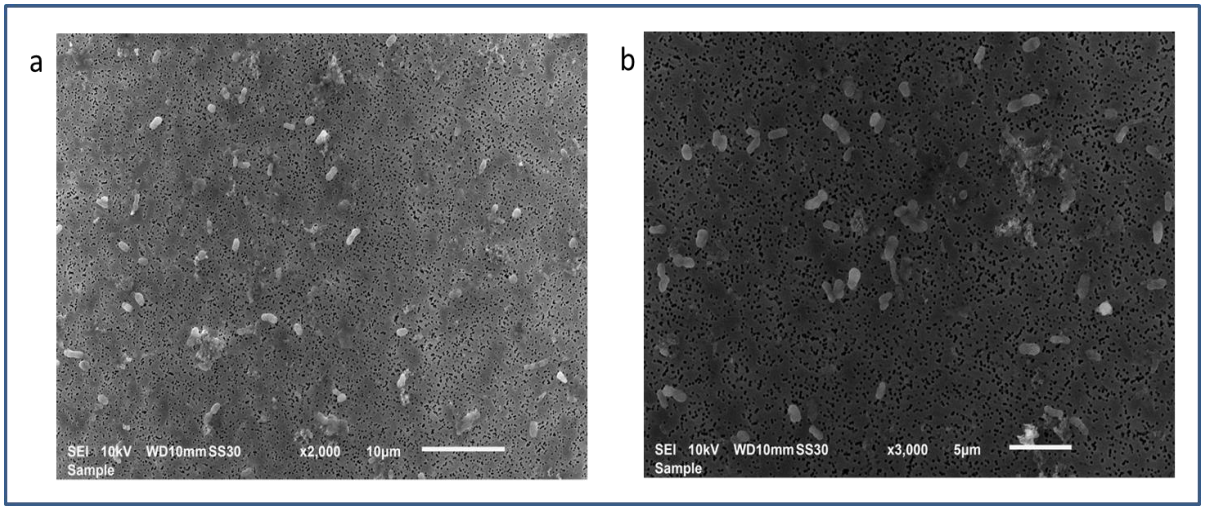

Figure 10: Representative SEM images of the enriched nitrate-reducing microorganisms in the serum flask reactors.

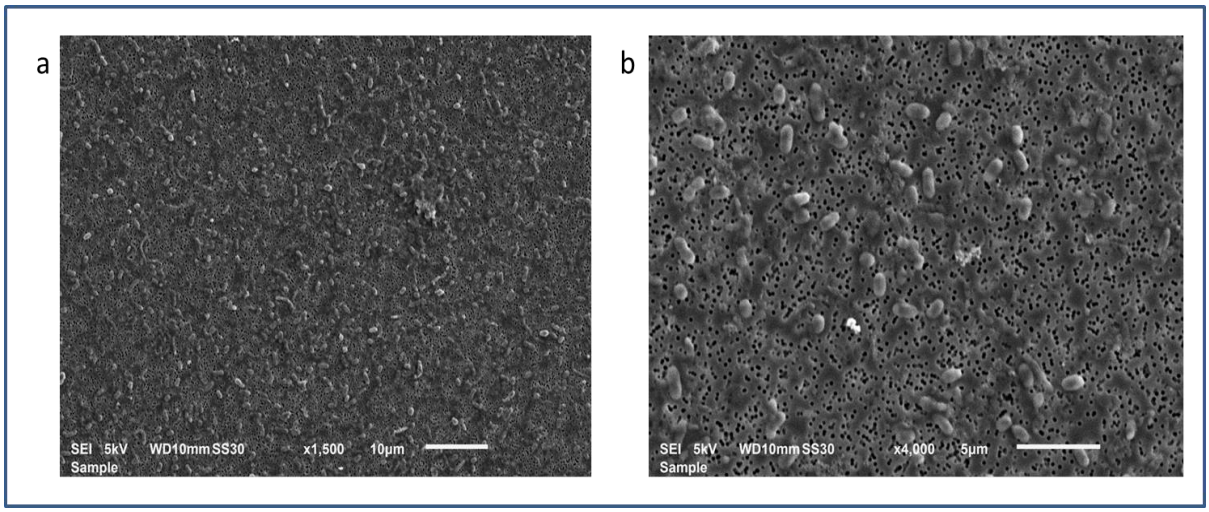

Figure 11: Representative SEM images of the enriched nitrate-reducing microorganisms in the electrochemical reactors.

3.2.2. Electrochemical characterization

Both the cyclic voltammograms, before and after inoculum, confirmed the absence of any redox-active components in the used growth medium as well as at the cathode surface in the case of sulphate and nitrate fed reactors (Figures 12 and 13). It also helped us to find out the safe potential window to conduct our desired enrichment experiments both for the nitrate-reducing and sulphate-reducing electrotrophs. The CVs recorded after/during the enrichment of SRB revealed the presence of one redox-active peak (E_1) with mid-

potential of -0.409 V, *vs.* Ag/AgCl (Figure 12). More importantly, an increase in the reduction current was observed in this CV trace compared to control CVs. These observations suggest the role of microbial electrocatalysis in current drawn from the cathode. Earlier, for some electrotrophic SRB redox-peaks at 0.1 V and -0.2 V *vs.* Ag/AgCl (Su *et al.*, 2012) have been reported. However, the redox moieties or components have not been identified or characterized to date. The involvement of the observed redox peak and associated redox-active components in the direct electron uptake process of enriched SRB from the cathode needs to be investigated further.

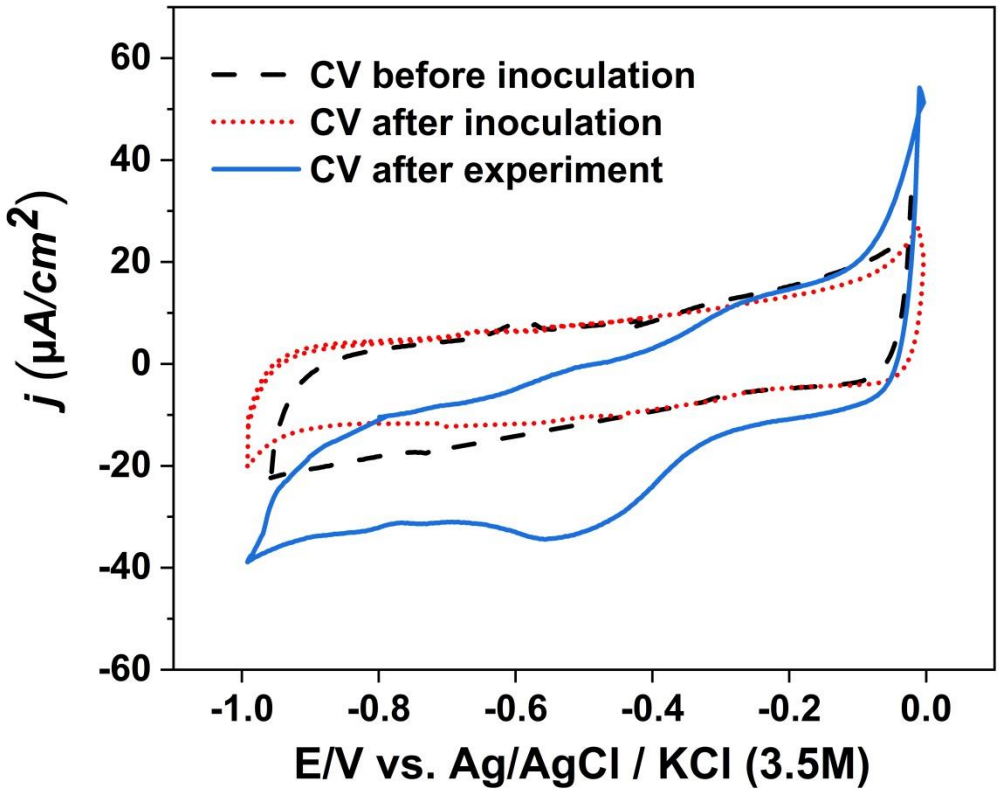

Figure 12: Representative cyclic voltammograms before and after inoculation, and during the enrichment experiment in the sulphate fed electrochemical reactor.

While in the case of nitrate condition, the CV recorded after two months of enrichment experiment revealed the presence of two redox-active peaks (E_1 and E_2) with mid-point potentials of -0.622 V and -0.433 V *vs.* Ag/AgCl (Figure 13). The presence of these two redox-moieties can be attributed to the cell membrane-bounded proteins or components that are most likely involved in inward EET or the cathodic electron uptake mechanism.

However, it needs to be investigated further through additional experiments. The CV results suggest the probability of direct electron uptake by both sulphate and nitrate-reducing microorganisms. No reports are available in the literature concerning the involvement of such proteins/components in the direct electron uptake by SRB and NRB to the best of my knowledge. So, further research, including confirmation, isolation, identification, and characterization of such proteins or components is envisioned. The 16S rRNA metagenomic sequencing of the enriched cultures at the end of experiments would reveal the dominant electrotrophic NRB and SRB.

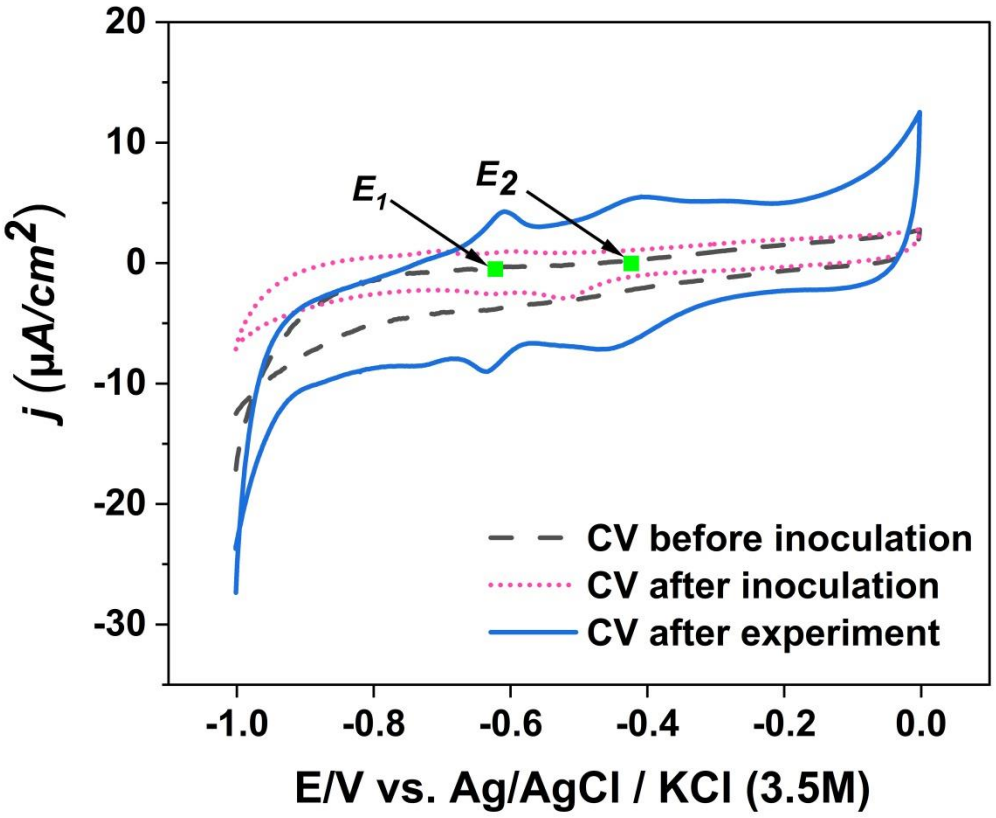

Figure 13: Representative cyclic voltammograms recorded before and after inoculation, and during the enrichment experiment for the nitrate fed electrochemical reactor.

Chapter 4

Conclusions and future prospects

In this study, the enrichment of haloalkaliphilic electrotrophs that can uptake the electrons directly from solid-state electron donors and reduce substrates like nitrate and sulphate was successfully demonstrated. The NRB was enriched through both serum flask and electrochemical enrichment approaches, whereas SRB was enriched only through the electrochemical approach. The enriched NRB was able to reduce nitrate completely within 7 days of the growth cycle in the serum flask experiments. Both NRB and SRB enriched electrochemically, were able to draw current from the cathode, and achieved up to -25 $\mu A/cm^2$ and -25 $\mu A/cm^2$ current densities, respectively. The increase in the reduction current correlated well with the decrease in the electron acceptor concentration. The cyclic voltammograms recorded during the enrichment experiments revealed the redox-active peak with a mid-point potential of -0.409 V for SRB and two redox-active peaks with mid-point potentials of -0.622 V, -0.433 V for NRB. The electrochemical data suggests the enrichment of electrotrophic NRB and SRB at the cathodes. Microscopy of microorganisms enriched in the suspension of electrochemical reactors confirmed their oval-shaped morphology. Further microscopic analysis of the enriched NRB and SRB at the cathode surfaces will be conducted at the end of enrichment experiments.

The enrichment of haloalkaliphilic electrotrophic NRB and SRB suggests the presence of the EET capable microorganisms in such extreme habitats. Further work on isolation and characterization of the enriched electrotrophs, their EET mechanisms, and the cell membrane-associated components involved in the electron uptake process using a multidisciplinary toolkit would strengthen the extreme electromicrobiology discipline. These microorganisms can also be used for the bioelectrochemical removal of nitrate or sulphate from the contaminated waters or wastewaters.

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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