CO_2 fixing lithotrophs from a highly haloalkaline environment of the Lonar lake

HEM LATA
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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

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Certificate of Examination

This is to certify that the dissertation entitled "CO_2 fixing lithotrophs from a highly haloalkaline environment of the Lonar lake" submitted by **HEM LATA (Reg. No. MS15138)** for the partial fulfillment of BS-MS dual degree program of the Indian Institute of Science Education and Research Mohali 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.

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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 acknowledgment 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.

HEM LATA
(MS15138)

Date: June 22, 2019

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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MES	Microbial electrosynthesis
BES	2-bromoethanesulfonate
SEM	Scanning electron microscope
WLP	Wood–Ljungdahl pathway
3HP-4-HB	3-Hydroxyproppionate/4-hydroxybutyrate cycle
CBB	Calvin-Benson-Bassham cycle or reductive pentose phosphate
3-HP	3-Hydroxyproppionate bicycle
rTCA	Reductive tricarboxylic acid cycle
D-4-HB	Dicarboxylate/4-hydroxybutyrate cycle

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Abstract

Chemolithoautotrophic microorganisms play an important role in carbon and energy metabolism in different environments through the fixation of CO_2 using inorganic energy sources such as hydrogen (H_2) and hydrogen sulfide (H_2S). These microbes are also of interest for the development of CO_2-based bioproduction technologies. Acetogenic bacteria belong to this group. These are obligate anaerobes that reduce CO_2 into acetate and conserve energy *via* the Wood–Ljungdahl pathway (WLP). These microorganisms have been reported from various natural but mostly normal environments. Not much is known about their ecology and diversity in extreme environments, such as saline soda lakes. Such environments host unique microbial communities that possess exceptional metabolic capabilities due to their adaptation to harsh growth conditions.

In the present study, intending to improve our understanding of CO_2 fixing microorganisms in extreme environments, I attempted the enrichment of chemolithoautotrophic bacteria from a highly saline and alkaline environment of the Lonar lake with CO_2 and H_2 as the only carbon and energy sources, respectively. The experiments were conducted under 2% salinity and 7 or 9.6 pH conditions. The enrichment of chemolithotrophs was tracked by monitoring the growth via OD measurements and the production of organic acids *via* HPLC. At pH 7, the production of acetic acid (467.4 ± 7.4 mg/L) and formic acid (307.8 ± 3.4 mg/L) suggested successful enrichment of the CO_2 fixing chemolithotrophs. In the case of pH 9.6 condition, only formic acid (88.9 ± 1.3 mg/L) production was observed. It suggests the enrichment of novel microbes, which are capable of growing and conserving energy by fixing CO_2 and H_2 into only formic acid. It is an interesting observation, which warrants further investigations. A unique growth pattern comprising of black colored aggregates and thread-like structures was observed in the culture flasks. Microscopic characterization revealed the presence of oval-shaped bacteria aligned in long chains in these structures.

In conclusion, successful enrichment of the chemolithotrophic microorganisms was achieved from the sediment samples of an extreme haloalkaline environment in this work. Further characterization through metagenomics is expected to reveal the most abundant CO_2 fixing lithotrophs in the enriched cultures.

Chapter 1: Introduction

The study of carbon dioxide (CO_2) fixing microorganisms from different environments is desired not only to understand their role in global carbon cycling but also to find out efficient strains for bioproduction from CO_2. Due to industrialization and the burning of fossil fuels, the global CO_2 concentration has reached an unprecedented level. Over the past few decades, rising CO_2 levels in the atmosphere have contributed to several environmental issues such as global warming, rise in sea-level, and ocean acidification (Douglas & Frank, 2007). Hence, there is an urgent need to develop sustainable approaches to reduce the effect of CO_2 on the environment. One of the strategies to achieve it is the production of commodity chemicals through microbial CO_2 fixation (Salehizadeh, Yan, & Farnood, 2020).

1.1 Microbial CO_2 fixation

Carbon fixation or carbon assimilation is the process of converting inorganic carbon into organic compounds by microorganisms. It can be done efficiently by photosynthetic and chemosynthetic microorganisms, which possess different metabolic pathways (Claassens, 2016). To date, six metabolic pathways have been identified for CO_2 fixation. These include Calvin-Benson Bassham (CBB or reductive pentose phosphate) pathway, 3-Hydroxypropionate bicycle (3-HP/malyl-CoA cycle), 3-Hydroxypropionate/4-hydroxybutyrate cycle (3HP-4-HB) in aerobic, and Wood-Ljungdahl (or reductive acetyl-CoA) pathway, Dicarboxylate/4-hydroxybutyrate cycle, reductive tricarboxylic acid (rTCA) cycle in anaerobic microorganisms (Salehizadeh et al., 2020). Among these, the CBB pathway has been recognized as the main pathway for CO_2 fixation in photosynthetic microbes. These CO_2 fixation pathways contribute, directly or indirectly, to the growth and survival of different microbial groups in diverse habitats (Osmond, Winter, & Ziegler, 1982).

1.2 Chemolithoautotrophy

In chemolithoautotrophy, organic compounds are synthesized through CO_2 fixation using chemical energy obtained from the oxidation of inorganic compounds such as sulfur, NH_4^+, Fe^{2+}, H_2S, H_2, etc. (Dimitry Yu Sorokin & Kuenen, 2005). Different types of chemolithotrophic microorganisms include sulfur-oxidizers, nitrifiers, hydrogenotrophs, iron-oxidizers, acetogens, and methanogens (Dimitry Yu Sorokin & Kuenen, 2005). These microorganisms are present abundantly in those environments where darkness prevails, competition with the photosynthetic organism is eliminated, and inorganic substrates are present. Such environments include marine or freshwater sediments, subsurface soils, hydrothermal vents, methane seeps, terrestrial caves, and hot springs (Nakagawa & Takai, 2008). Acetogens, one of the prominent groups of chemolithoautotrophic microorganisms, utilize CO_2 and H_2 to conserve energy and produce acetate as the main organics *via* the Wood–Ljungdahl pathway. These are among the most diverse bacteria. Nearly 100 species of acetogens are known that are phylogenetically classified into 23 distinct genera (Rake et al., 2008; Schuchmann & Müller, 2016). These microbes play a vital role in the global carbon cycle, with the formation of nearly 10^{13} kg of acetate in anaerobic environments (Yang, 2018). Besides chemolithoautotrophy, their metabolic features include heterotrophic growth with carbohydrates, lactate, and different alcohols (Schuchmann & Müller, 2016). The unique metabolic capability of acteogens to convert different substrates into acetate links fermentation with acetoclastic methanogenesis. They develop syntrophic associations with hydrogenotrophic bacteria such as methanogens and sulfur-reducing bacteria, which use acetate as a substrate (Schuchmann & Müller, 2014; Sorokin, 2018). Acetogenic bacteria thus play an essential role in the anaerobic food web.

1.1 Chemolithoautotrophic microorganisms in extreme environments:

The study of chemolithoautotrophy in extreme ecosystems can provide clues to the earliest biological communities on earth or potential extra-terrestrial life (Nakagawa & Takai, 2008). Hence, exploring extreme environments for chemolithoautotrophs is of interest. The extremophile group of microbes includes thermophiles,

hyperthermophiles, alkaliphiles, halophiles, and halo-alkaliphiles. Among various extreme environments, the high-temperature environments have been mostly investigated for chemolithoautotrophs thus far (Nakagawa & Takai, 2008). Thermophilic chemolithoautotrophs dominate environments such as hot springs (>70°C) and include *Thermodesulfobacterium hydrogeniphilum, Thermodesulfatator indicus,* and *Geothermobacterium ferrireducens* (Nunoura, Oida, Miyazaki, & Suzuki, 2008) species. Evidence for acetogenic bacteria in soda lakes, which possess two extreme conditions (due to high salinity and alkalinity), is limited. Among the acetogenic bacteria, the extremely haloalkaliphilic genera of *Halanaerobiales* belong to closely related genera *Natroniella* (able to convert alcohols to acetate) and *Fuchsiell*a (H_2 to acetate) (Dimitry Y. Sorokin, Banciu, & Muyzer, 2015). These microbes are capable of surviving in hypersaline conditions. The members of *Clostridiale*s from genera *Tindallia* (T.*magadiensis)* and *Natronincola (N. histidinovorans)*, can use alcohols and amino acids and are active at moderate salinity. The low salt-tolerant acetogens are closely related to the first characterized lithotrophic acetogen *Clostridium aceticum* (Dimitry Y Sorokin, 2018). Few acetogenic bacteria show syntrophic association with hydrogenotrophic bacteria that utilize acetate, VFAs (volatile fatty acids), and alcohols to produce CO_2 or other intermediates and H_2 (Dimitry Y. Sorokin, 2018). Among *Clostridia,* most of the syntrophs collected from soda lakes are the novel members of the order *Syntrophomonadales* (Dimitry Y Sorokin, 2018).

The literature review suggests that we barely know about the microbial ecology and diversity of chemolithoautotrophs in the hypersaline and alkaline or haloalkaline environments. The pure culture isolates of haloalkaliphilic chemolithotrophs are scarcely available in the culture repositories. Such microorganisms are promising catalysts for developing CO_2-based bioproduction processes such as microbial electrosynthesis (MES). MES is an electricity-driven bioproduction process from CO_2 using microorganisms as biocatalysts (Rabaey & Rozendal, 2010). The ohmic losses that occur due to poor charge transfer through the standard electrolyte solution reduce the efficiency of the MES process (Oren, 2011; Alqahtani et al., 2019). The microbes enriched from the haloalkaline environments

are adapted to saline conditions. Therefore, their use as biocatalysts in MES systems saline electrolytes can help to reduce the ohmic losses, leading to the efficient movement of ions, thereby improving the efficiency of the overall process. Furthermore, studying such microbes in extreme conditions would help to elucidate and understand their role in the biogeochemical cycling of carbon.

In this context, this study aimed to investigate CO_2 fixing lithotrophs from the haloalkaline habitat. For this purpose, we selected a hypersaline and alkaline lake named Lonar lake as the sampling site. It was originated around 52000 years ago and is the only meteorite impact crater lake which is situated in the basaltic rock. This lake is a remarkably well-preserved depression in the Deccan Plateau located in Buldhana district (Maharashtra, India) (Antony et al., 2013). In soda lake, alkalinity and salinity are characterized by the presence of sodium carbonate and other salts formed by evaporative concentration (Jones, Grant, Duckworth, & Owenson, 1998). This lake system has been mostly studied to understand the broad microbial diversity, and the isolation of novel microorganisms is limited mainly to aerobic microorganisms (Paul et al., 2016; Antony et al., 2013; Wani et al., 2006). To the best of our knowledge, the CO_2 fixing lithotrophs have not been reported from this lake system thus far. To this end, the objective of this study was to enrich and characterize haloalkaliphilic chemolithoautotrophic microorganisms from the Lonar lake sediments.

Chapter 2: Materials and Methods

2.1. General experimental conditions

All chemicals were purchased from Sigma Aldrich or Merck. The gases were purchased from Sigma Gases Private Limited (Delhi). The enrichment experiments were conducted using 100 ml serum bottles with 40 ml working volume under strict anaerobic and aseptic conditions.

2.2 Sediment sampling and characterization

Sediment samples were collected in the amber-colored plastic sampling bottles from a depth of up to ~1 ft. from three random sampling sites located at the periphery of the Lonar Lake. The sampling was conducted in the monsoon season in August 2019. They were stored in airtight bottles at 4 ºC.

2.3 Enrichment medium and microbial inoculum:

A modified M9 medium with 2 % salinity and two different pH conditions, viz.,7, and 9.6 were used as the enrichment culture medium. It contained sodium hydrogen phosphate Na_2HPO_4 (4.33g/l); sodium phosphate monobasic NaH_2PO4 (2.69g/l), ammonium chloride NH_4Cl (0.3g/l), potassium chloride KCl (0.13g/l) and sodium 2-bromoethane sulfonate (2g/l) as described in Appendix 1. The growth medium was supplemented with trace elements (20 ml/l), vitamins (10 ml/l), L-cysteine, and sodium bicarbonate (5g/L). Sodium bicarbonate served as a carbon source. L-cysteine was used as a reducing agent that allows maintenance of the negative redox potential in both anoxic and normoxic conditions (Rymovicz et al., 2011). Sodium 2-bromoethanesulfonate (BES) was added to the medium to inhibit the growth of methanogens. Resazurin (0.5 ml/l) was used as a redox indicator. It turns pink in the presence of oxygen and turns into translucid at anoxic conditions when E_h is <-110 mV (Wagner et al., 2019). $Na_2S.9H_2O$ (0.5 g/l) was added to the medium as a reducing agent. The medium preparation procedure as follows. All the ingredients except bicarbonate, vitamins, cysteine, and sulfide were dissolved in distilled water.

The mixture was boiled for 15-20 minutes and then cooled to the room temperature under 100% N_2 gas sparging condition. The medium was then transferred to the serum bottles and subjected to gassing-degassing cycles with N_2: CO_2 (80:20) to create anaerobic conditions. Serum bottles were sealed with butyl rubber stoppers and crimp seals and were then autoclaved. Vitamins, trace elements, a reducing agent (Na_2S. $9H_2O$), and $NaHCO_3$ were added to the medium just before inoculation.

The microbial inoculum was prepared by mixing the sediment samples in M9 buffer solution. It was subjected to sonication and centrifugation for 10 minutes. Then the suspension was used to inoculate the serum bottles containing the growth medium (10% v/v). After inoculation, the headspace of the bottles was filled with H_2:CO_2 (80:20) or pure H_2 (100 %) at a maximum pressure of 1.5 bar. H_2 was the only energy source provided to the microorganisms.

2.4 Enrichment experiments:

The serum bottles hosting the enrichment medium and inoculum were incubated at 30°C under shaking conditions of 30 rpm. The batch mode experiments were performed at least in triplicates. Liquid samples from the bulk phase were analyzed daily for pH, OD, and metabolites, i.e., CO_2 fixation organic products. Biotic (with inoculum but no carbon and energy sources) and abiotic (no inoculum but with carbon and energy sources) control experiments were also performed under the same conditions.

2.5. Measurement and Analysis:

The following parameters were monitored during the enrichment experiments.

a) pH: The changes in the pH of the growth medium were monitored using a pH meter (Jenway).

b) Growth: Microbial growth was monitored by measuring optical density at 600 nm wavelength (OD_{600}) using a UV-VIS spectrophotometer (PhotoLab 7600 UV-VIS).

c) Organics: Filtered liquid samples (0.22 μm) were used for this purpose. Volatile fatty acids and alcohols from C-1 to C-4 carbon chain length were analyzed using

HPLC (Agilent Infinity 1260) equipped with a RI detector and Agilent Hi- Plex H C-18 column, at 50 °C. 5 mM H_2SO_4 at a flow rate 0.5ml/min was used as a mobile phase.

2.6. Microscopic observation of the enriched culture

2.6.1. Bright-field microscopy:

The enriched culture was stained using a Gram staining kit (Sigma Aldrich) (Appendix 3 B). It was then observed using a Light Microscope (Nikon Eclipse E200).

2.6.2. Fluorescence Microscopy:

The LIVE/DEAD BacLight Bacterial Viability staining Kit (Thermo Fischer Scientific) was used for this purpose. The stains used were SYTO 9 dye (3.34 mM, 300 µL solution in DMSO) and propidium iodide (20 mM, 300 µL solution in DMSO). Equal volumes of these dyes were mixed thoroughly. Bacterial smear was prepared on a clean glass slide inside the anaerobic work station (Don Whitley Scientific Ltd. England). To it, 3 µl of the dye mixture was added and incubated in the dark condition for 15 minutes. The excess dye was washed with distilled water, and it was then covered with a coverslip. Images were taken using a Fluorescence Microscope (Eclipse Ts2 Nikon), and image processing was done using FV10I 1.2C (01.02.03.01) software.

2.6.3 Scanning electron microscopy (SEM):

For SEM, the cell suspension was filtered through a 0.22 µm polycarbonate membrane filter (Isopore TM, Merck Millipore Ltd.) using a 13 mm Swinny Syringe Filter holder (Merck Millipore). Cells were fixed using 2% glutaraldehyde and paraformaldehyde solution and incubated at 4 °C for 12 hours. Further post-fixation was done using 1 % osmium tetraoxide for 90 minutes. The samples were dehydrated in a graded ethanol series (20, 40, 60, 80, 100%), and air-dried overnight in the desiccator. The fixed samples were gold coated using JEOL JEC-1600 Auto-Fine

Coater (JEOL Ltd., Japan) at 20 mA for 35 seconds and observed under the SEM microscope (JEOL JSM-6010PLUS/LS, JEOL Ltd., Japan).

2.7 DNA extraction and metagenomics of the enriched culture:

Genomic DNA was extracted from the sediment sample and the enriched cultures using a FastDNA SPIN Kit (MP Biomedicals, France) following the manufacturer's protocol. Eluted DNA was stored at -20°C for extended periods or 4°C until use. The purity and concentration of the DNA samples were determined using nanodrop (Thermo Scientific™ NanoDrop 2000). The isolated DNA samples have been sent for the next-generation sequencing of V3-V4 regions of 16s rRNA at Illumina HiSeq template (Outsourcing Bioserve, A Reprocell company, India)

Chapter 3: Results and discussion

3.1 Enrichment of the halophilic CO_2 fixing lithotrophs at pH 7

The enrichment of CO_2 fixing microorganisms was monitored by analyzing OD and organics production. The OD data of the first enrichment culture is shown in Figure 1. Initially, the optical density was 0.205 ± 0.04 (mostly due to turbidity arising from the fine sediment particles) and the pH was 7.1 ± 0.034. On consecutive days, OD increased and reached to 0.400 ± 0.025. The pH decreased slightly to 6.9 ± 0.085 (Figure 1).

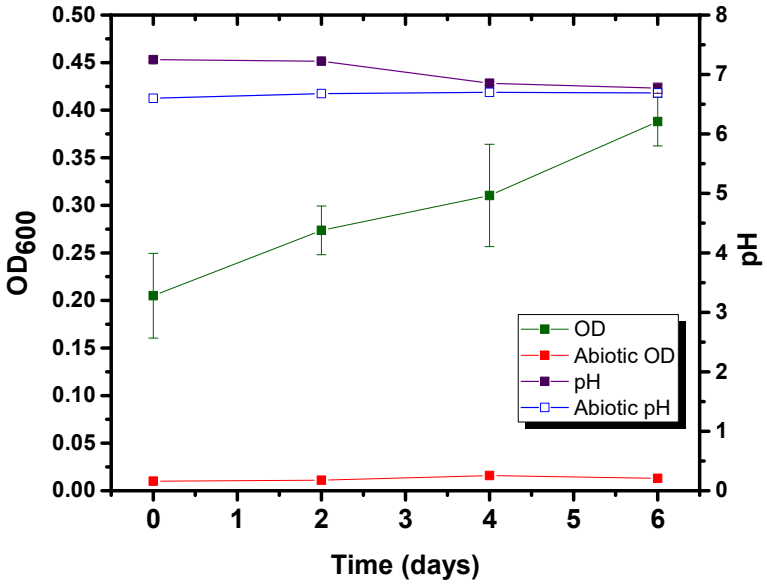

Figure 2:Growth and pH profiles observed during the initial enrichment cycle.

After 48 hours of incubation, black coloured small thread-like structures started emerging in the bulk phase. With time, these filaments formed a filamentous network and large black aggregates at the bottom of the serum bottle (Figure 2). With a slight disruption or movement of the bottles, these filaments got mixed in the medium and reappeared again under the static conditions. In the case of abiotic control and biotic control, no growth or such filamentous structures was observed. The increase in

optical density and aggregate formation in the test serum bottles suggested the growth of microbes.

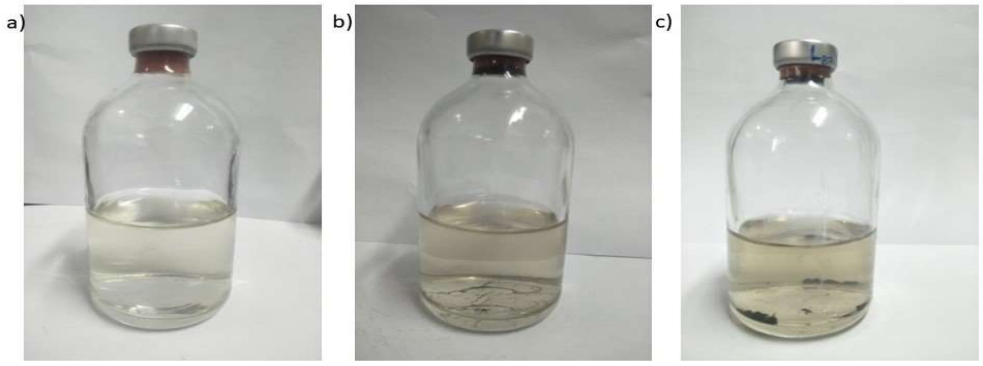

Figure 2: Growth pattern observed at a) 0 h, b) 48 h, and c) 144 h during the enrichment experiments.

The culture that was enriched in the initial enrichment cycle (Figures 1 and 2) was used as an inoculum for the subsequent enrichment and confirmation experiments. The liquid samples from the bulk phase were analyzed for growth, pH, and organic acid production. The growth curve of the enriched culture for three batch cycles is shown in Figure 3. For instance, the initial OD was 0.0423 ± 0.002 and it reached up to the final OD of 0.173 ± 0.023 on the 8^{th} day in the first batch cycle. It followed similar trends but with lower OD values in the subsequent batch cycles. The enriched culture showed a similar growth pattern as observed in the initial enrichment cycle (Figure 4). No growth or increase in OD was observed in the case of both biotic and abiotic controls.

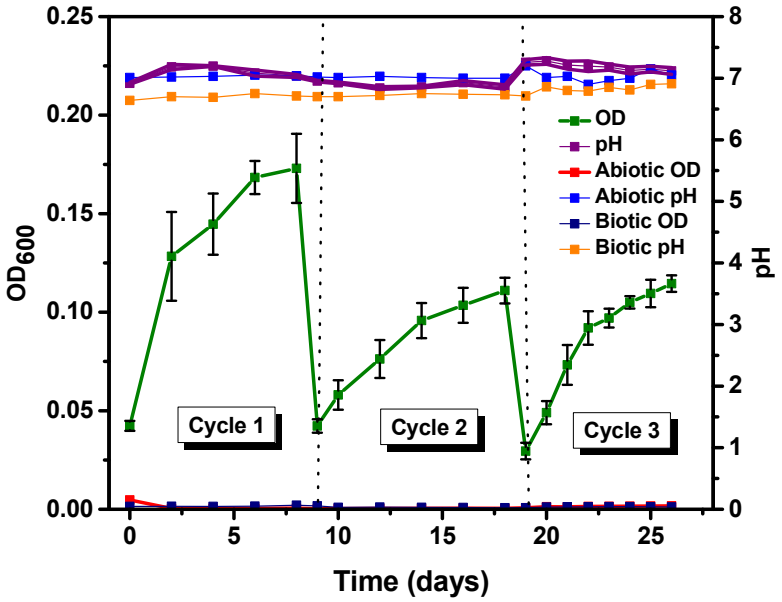

Figure 3: Growth and pH profiles of enriched CO_2 fixing culture at pH 7.

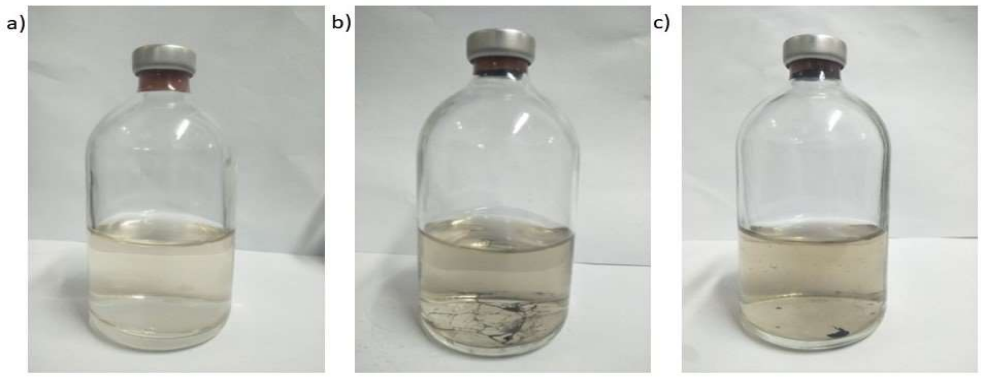

Figure 4: Growth pattern of the enriched culture observed at a) 0 h, b) 48 h, and c) 168 h.

The analysis of the liquid samples for organics revealed the production of acetic acid and formic acid by the enriched culture (Figure 5). The concentration of these organic acids increased rapidly on the 2nd day and remained at a similar level from day 3. The production of organic acids confirmed that the enriched microbes were able to grow by fixing CO_2 and H_2 into organics. Therefore, the enriched culture is chemolithoautotrophic. The maximum concentrations of acetic acid and formic acid produced were 467.41± 7.40 and 307.81± 3.39 mg/L, respectively (Figure 5 and

Table 1). The OD data correlated well with the organics production data in all batch cycles. In abiotic as well as biotic controls no organic acids were observed. In each cycle, the production of acetic acid was higher than formic acid. Formic acid is an intermediate product in the Wood-Ljungdahl pathway. It is normally produced transiently by acteogens. However, in the case of halophilic enriched culture in this study, it was produced in a considerable amount, which suggests the enrichment of novel chemolithotrophs.

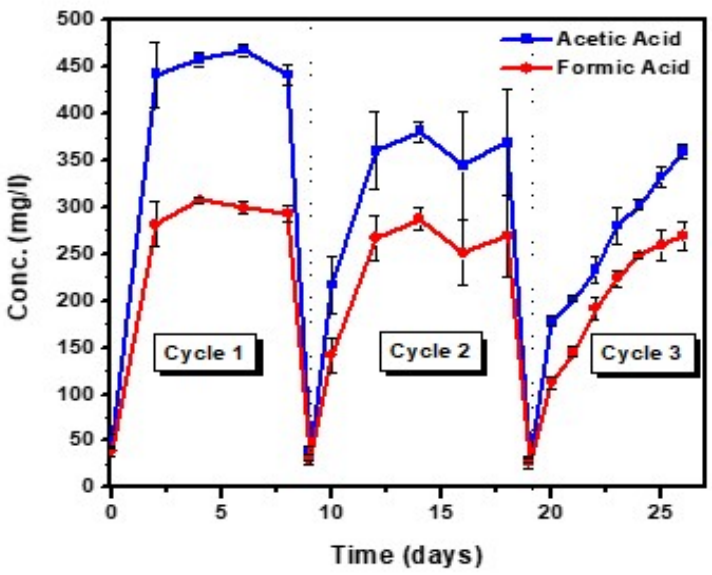

Figure 5: Organic acid production by the enriched CO_2 fixing culture at pH 7.

Table 1: Maximum organic acid production by the enriched CO_2 fixing lithotrophic culture in three batch cycles.

Organic acid (mg/L)	Batch cycle 1	Batch cycle 2	Batch cycle 3
Acetic acid	467.41± 7.4	380.81± 11.28	302.75± 5.0
Formic acid	307.81± 3.4	287.92± 12.54	269.36± 5.4

3.2 Microscopic observation of the enriched culture at pH 7

The aggregates formed in the enrichment cultures were analyzed using different microscopic techniques. The bright-field microscopic examination was performed under an oil immersion objective at 100X. It revealed the oval-shaped morphology of microbial cells in the enriched culture (Figure 6). Gram staining revealed the Gram-negative nature of the enriched culture.

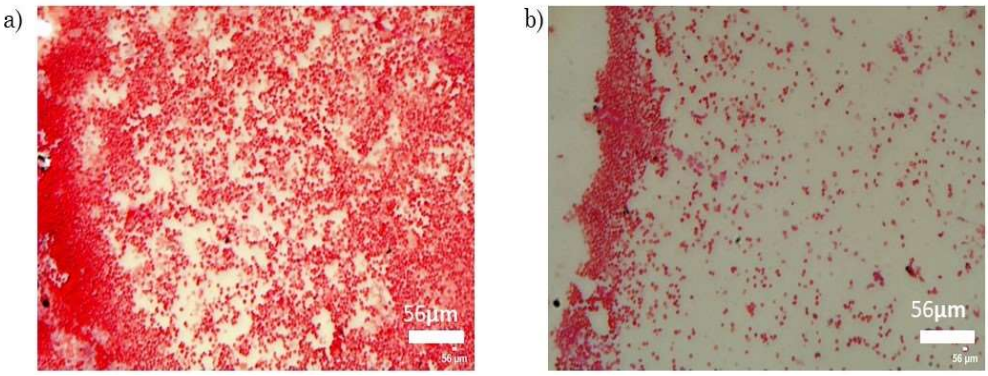

Figure 6: Bright field microscopic images of the enriched culture at pH 7.

The fluorescence microscopy with nucleic acid staining confirmed the growth of microorganisms (Figure7a) in the form of thread-like filaments (Figure 7b). The presence of green fluorescence (SYTO 9) indicates the existence of bacteria cells with intact cell membranes while, in bacteria with damaged membranes, red fluorescence is observed (Figure 7c). The SEM of the enriched culture further confirmed the presence of long thread-like filaments (>1 mm long and about 10 µm thick) (Figures 7e& f). Magnified image of these long threads revealed the presence of chains of oval-shaped bacteria inside these filaments (Figure 7g). This type of unique growth pattern can be a survival strategy of these microbes under nutrient-limited conditions. These observations warrant further investigations.

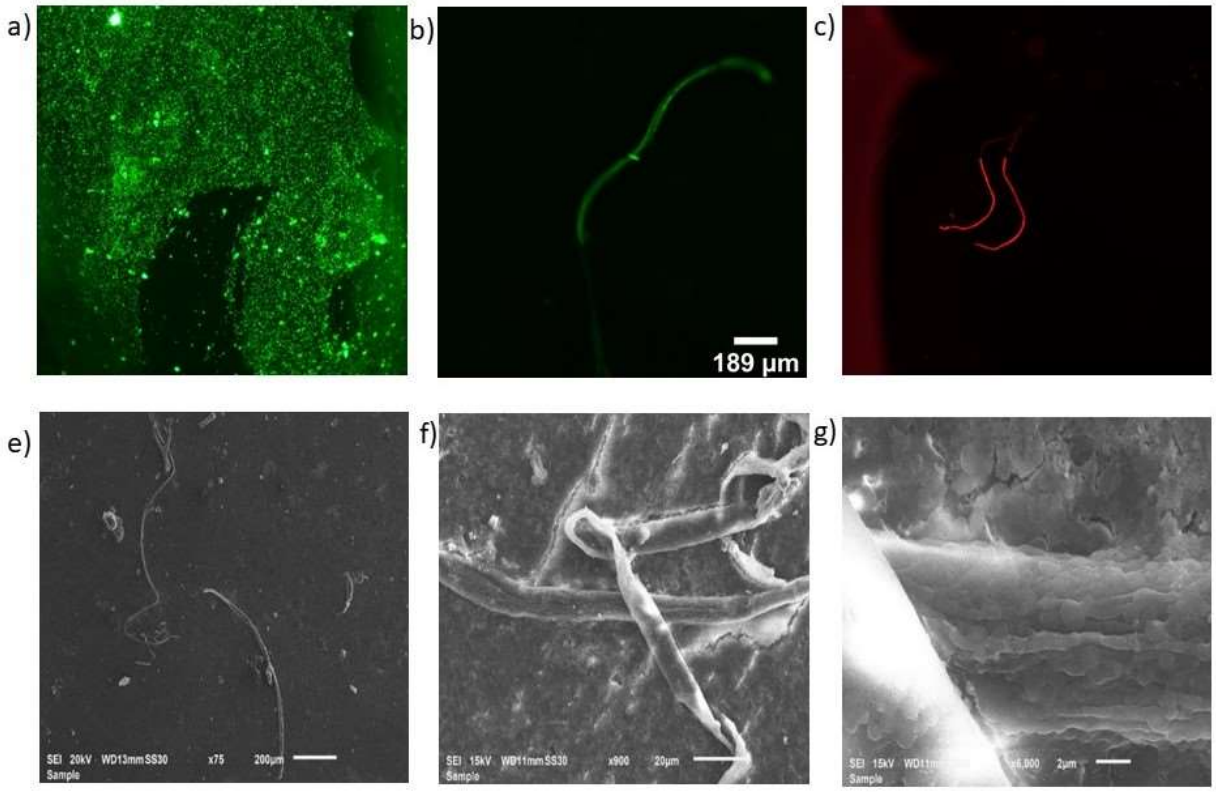

Figure 7: Fluorescence (a to c) and SEM (e to g) images of enriched culture at pH 7.

3.3 Microbial community composition in the enriched culture

The DNA reading recorded in nanodrop for the inoculum source and enriched culture was 100 ng/µL and 350 ng/µL, respectively (as described in Appendix 4). These samples have been sent for metagenome sequencing and analysis. At the time of thesis submission, the data was not received.

3.4 Enrichment of CO_2 fixing bacteria at pH 9.6

The growth curve shows that the initial OD of the culture was 0.335 ± 0.13 (Figure 8). On consecutive days, an increase in growth accompanied by a decrease in pH was observed. The OD reached up to 0.415 ± 0.14 on the 7th day. In abiotic control, no growth was observed and pH remained stagnant throughout the enrichment. No growth was observed in the case of abiotic and biotic controls.

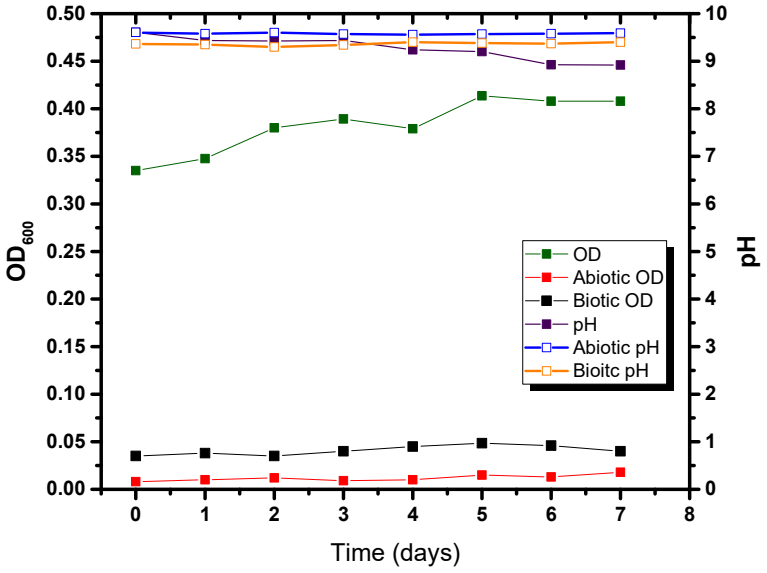

Figure 8: Growth and pH profiles of the enriched culture at pH 9.6.

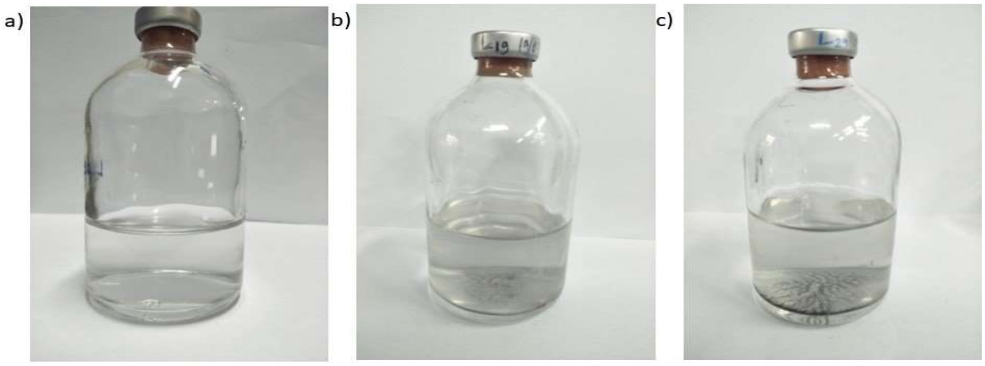

Figure 9: Growth pattern of the enriched culture observed at a) 0 d, b) 5 d, and c) 10 d.

After 5 days of incubation, some black coloured particles started emerging in the enrichment cultures (Figure 9b). With time these black aggregate became denser and settled down at the base of the serum bottle (Figure 9c). In abiotic control as well as biotic control experiments, black aggregates and growth were not observed. These observations indicate the growth of haloalkaliphilic CO_2 fixing chemolithotrophic microorganisms at pH 9.6 condition. The OD data also suggest that it is a slow-growing culture.

The organics production by the enrichment culture at pH 9.6 was analyzed only for 4 days. Interestingly, only formic acid was produced by this culture. On the first day, its concentration increased rapidly (55.57 ± 0.55 mg/L). It reached up to 88.93 ± 1.35 on the 4th day (Figure 10). The formic acid production at pH 9.6 is lower than at pH 7. This might be due to the low solubility of the gases (Oswald et al., 2018) and carbonate fixation as sodium carbonate at a highly alkaline pH condition in the medium. Further tests are needed to confirm these observations. Organic acid production was not observed in abiotic control and biotic controls. The production of only formic acid while growing suggests the possibility of novel chemolithoautotrophs and carbon fixation pathways under the haloalkaline conditions.

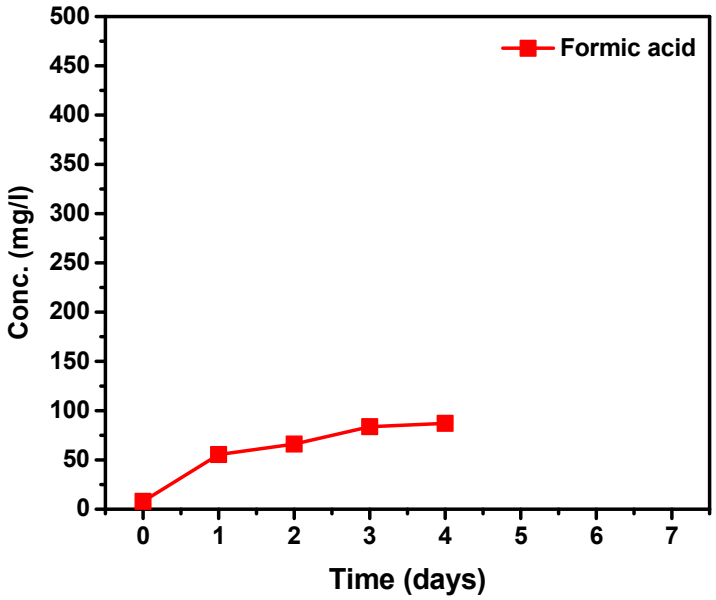

Figure 10: Organic acid production by the enriched culture at pH 9.6.

Conclusions and future perspectives

Successful enrichment of CO_2 fixing chemolithotrophic microbes was achieved at 2% salinity and pH 7 condition. The enriched culture at pH 7 culture was fast-growing and showed an aggregate-type growth pattern. Microscopic analysis revealed that the microbial cells align themselves in chains to form long thread-like filaments. It could be their survival strategy to grow under nutrient-limited conditions. The production of organic acids confirmed that the enriched culture fixed CO_2 and H_2, and thereby its chemolithoautotrophic nature. The enriched culture at 2% salinity and pH 9.6 culture was slow-growing and formed fewer aggregates. Interestingly, this culture produced only formic acid as the CO_2 fixation product. Further tests to confirm these observations and detailed characterization of the enriched culture through metagenomics are warranted. Follow-up work is expected to enhance our knowledge of this novel microbial group, which has implications for strengthening extreme microbiology and developing electrochemistry-driven CO_2 bioconversion technologies.

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Appendix 1

Table 2: Composition of modified M9 medium

Components	Concentration (g/L)
Na_2HPO_4	4.33
NaH_2PO4	2.69
NH_4Cl	0.30
KCl	2.69
$C_2H_4BrNaO_3S$ (BESA)	2
Resazurin	0.3 ml

Note: In the case of enrichment at pH 9.6, sodium carbonate (Na_2CO_3, 1.18M) was added to increase alkalinity.

Table 3: Vitamin solution

Vitamin Solution	Concentration
Biotin	2 mg
Folic acid	2 mg
Pyridoxine-HCl	10 mg
Thiamine-HCl $\times 2\ H_2O$	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
D-Ca-pantothenate	5 mg
Vitamin B_{12}	0.10 mg
p-Aminobenzoic acid	5 mg
Lipoic acid	5 mg
Distilled water	1000ml

Table 4: Trace metal solution

Trace elements	Concentration
Nitriloacetic acid	1.5 g
$MgSO_4 \times 7 H_2O$	3 g
$MnSO_4 \times H_2O$	0.5 g
NaCl	1 g
$FeSO_4 \times 7 H_2O$	0.1 g
$CoSO_4 \times 7 H_2O$	0.18 g
$CaCl_2 \times 2 H_2O$	0.1 g
$ZnSO_4 \times 7 H_2O$	0.18 g
$CuSO_4 \times 5 H_2O$	0.01 g
$KAl(SO_4)_2 \times 12 H_2O$	0.02 g
H_3BO_3	0.01 g
$Na_2MoO_2 \times 2 H_2O$	0.01 g
$NiCl_2 \times 6 H_2O$	0.03 g
$Na_2SeO_3 \times 5 H_2O$	0.03 g
$Na_2WO_4 \times 2 H_2O$	0.04 g
Distilled water	1000 ml

Appendix 2

Glycerol stock preparation:

- Prepare a 50% glycerol solution by diluting 100% glycerol in distilled water. Glycerol act as cryoprotectant that forms hydrogen bonds with water molecule which enhance super-cooling and dissipate the ice crystal formation(Bhattacharya & Prajapati, 2016).
- Flush 50% glycerol with N_2 or N_2:CO_2 and autoclave the stock to maintain anaerobic conditions.
- Then, prepare 15% glycerol stock from 50% glycerol by diluting with buffer (i.e. Basal medium containing M9 modified media).
- Store the culture in cryo vials at -80°C.

Appendix 3

A. Prepare a Slide Smear:

- Transfer filamentous aggregate of bacterial culture on a slide using an inoculation loop. If suspended culture is to be taken from serum bottle, then the first centrifuge, and then transfer the culture onto a slide.
- Spread the culture with an inoculation loop to an even thin film over a circle.
- Place the slide and allow air to dry and fix it over a gentle flame while moving the slide circularly to avoid localized overheating. The heat applied allows the cells to adhere to the glass slide and allow the subsequent rinsing of the smear with water possible without significant culture loss.

B. Gram Staining:

- Gently flood the smear with Gram's crystal violet Solution (act as primary stain) and allow it to remain it for 60seconds.
- Tilt the slide and gently wash with tap water from a faucet or a plastic water bottle.
- Gently flood with Gram's iodine solution and let stand for 60 seconds. The addition of iodine (act as mordant) will fix the violet stain in gram-positive bacteria.
- Tilt the slide slightly and gently rinse the iodine solution with tap water or distilled water using a wash bottle. The smear will appear as a purple on the slide.
- Decolorize with Gram's Decolorizer Solution until the blue dye no longer flows from the smear. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated.
- Immediately rinse the smear with water.
- Gently flood with safranin to counter-stain and let stand for 60 seconds
- Gently rinse the smear with tap water or distilled water using a wash bottle and remove the excess water by using blot with bibulous paper, or by shaken slide and air-dried.
- Examine the smear using a microscope under oil-immersion.