Production of High Value Chemicals from CO_2 through Interlinking of Microbial Electrosynthesis and Yeast-based Processes

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A dissertation submitted for the partial fulfilment of
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Certificate of Examination

This is to certify that the dissertation titled **"Production of High Value Chemicals from CO_2 Through Interlinking of Microbial Electrosynthesis and Yeast-based Processes"** submitted by **Mr. Ravineet Yadav** (Reg. No. MS14146) 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. Anoop Ambili	Dr. Rachna Chaba	Dr. Sunil A. Patil (Supervisor)

Dated: 24 April, 2019

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.

Ravineet Yadav
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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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Ravineet Yadav

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Notations

MES	Microbial Electrosynthesis
CCS	Carbon Capture and Storage
CCU	Carbon Capture and Utilization
OD_{600nm}	Optical Density at 600 nm Wavelength
TCD	Thermal Conductivity Detector
FID	Flame Ionization Detector
CV	Cyclic Voltammetry
CA	Chronoamperometry
HIS	Histidine
LEU	Leucine
URA	Uracil
LYS	Lysine
TRP	Tryptophan
MET	Methionine
SD	Synthetically Defined
COD	Chemical Oxygen Demand

Contents

Abstract

The increase in global Carbon Dioxide (CO_2) emissions is causing climate change, which in turn is poised to trigger disastrous consequences. To curtail the rising industrial CO_2 emissions and avert its associated effects, various measures ranging from technological advancements to policies are being considered. Microbial Electrosynthesis (MES) is one of the emerging technologies for the conversion of CO_2 into multi-carbon chemicals. MES utilizes microbes as catalysts for the electricity-driven conversion of CO_2 to useful chemicals and fuels in bioelectrochemical systems. Although it offers several advantages over other CO_2 utilization approaches, difficulty in the production of high carbon chain length and thus high-value chemicals is one of the key challenges. In order to increase the value of chemicals being produced from CO_2, two strategies, Viz., genetic engineering or synthetic biology and interlinking of different processes, are being considered. In this work, we demonstrate that high-value chemicals such as Sclareol (C_{20}) could be produced from CO_2 (C_1) by interlinking MES with the yeast-based processes. In the first process, CO_2 was converted to acetate by using *Clostridium ljungdahlii* via MES. Up to **478.25 mg/L** organics production at the rate of **0.442±0.0512 g/L/day** was achieved in bioelectrochemical systems. The acetate containing spent media was then fed to the genetically modified *Saccharomyces cerevisiae* as the sole carbon and energy source. Although this yeast grew slowly with acetate, it produced up to **6.364 mg/L** sclareol. Further optimization studies of both the MES and yeast-based processes are needed to increase the production of acetate and sclareol, respectively.

Chapter 1: Introduction

1. Present Day Scenario of Carbon Dioxide (CO_2) Emissions

The property to emit and absorb thermal radiation makes Carbon Dioxide (CO_2) a Greenhouse Gas. Such gases cause 'greenhouse effect' which is responsible for making earth's temperature habitable, without which the earth's surface temperature would have been -18^o C.[1] With the advent of the industrial revolution, the CO_2 emissions from fossil fuels burning, industrial emissions and other anthropogenic activities have increased tremendously, which in turn is causing planetary warming effect. The global warming has a range of ecological, physical and health impacts, including extreme weather events (such as floods, droughts, storms, and heatwaves); sea-level rise; altered crop growth; and disrupted water systems.[2] With a continuous increase in the CO_2 emissions, the current global CO_2 atmospheric concentration has already escalated above 400 ppm[3], making it a serious matter of concern for the future. Limiting the CO_2 emissions induced climatic changes and associated risks is therefore highly desirable.

2. Existing/Proposed Technologies for reducing Carbon Footprint

Broadly, two approaches are being considered and extensively researched for reducing the carbon footprint in the atmosphere. These are Carbon capture and storage (CCS) and Carbon capture and utilization (CCU). In these approaches, CO_2 is first captured and then either stored (CCS) or utilized through different methods (CCU). Usually, there are two kinds of sources for CO_2 emissions: (i) Point sources such as power plants and industrial production sites, and (ii) Diffuse sources such as transport and construction activities.[4] For the implementation of these approaches the source with large and concentrated CO_2 emissions could be used.[4]

2.1 Carbon Capture and Storage (CCS) -

In CCS waste CO_2 is trapped from large point sources by using different methods, after which it is liquified, transported and buried or stored in either deep underground saline aquifers or disused oil fields (Fig 1).[5] This approach is very expensive due to the high costs associated with gas scrubbing, transport systems, and complete plant setup. Moreover, the long-term consequences of carbon storage are still unclear.[5]

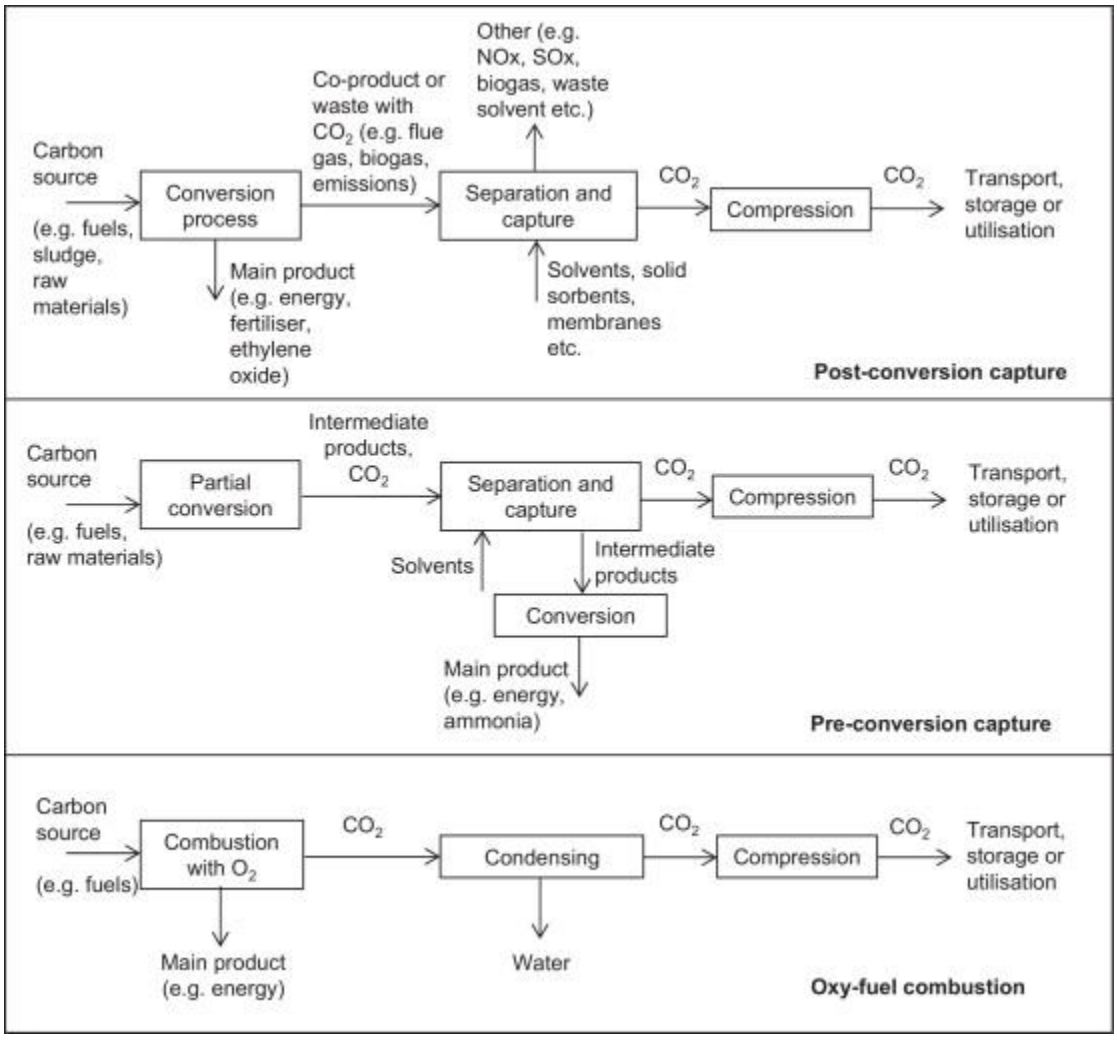


Fig 1 The different methods of carbon capture.[6]

2.2 Carbon Capture and Utilization (CCU) -

In CCU approach, the captured CO_2 emissions can be utilized 'as is' or to produce valuable chemicals and fuels via different processes. This approach not only facilitates CO_2 utilization from the point sources, but it also helps to close the carbon cycle. As the products from CO_2 utilization can be sold, it is an advantage of CCU over CCS.[6] CO_2 being a renewable resource, its utilization through CCU technologies offers an additional advantage over petrochemical feedstocks as well. Several methods are proposed and being tested for the utilization of CO_2. The major ones are discussed below.

- **Chemical catalysis**

Some major fine and commodity chemicals that can be synthesized from CO_2 include salicylic acid, urea, methane, methanol, carbon monoxide, diphenyl carbonate, oxalic acid etc.[7] Limitations of this approach include the challenges associated with the use of chemical Catalysts, the requirement of non-renewable energy, and costs of the synthesis process.[7]

- **Electrocatalysis**

Using electricity and electrocatalysts, several compounds can be produced from CO_2 such as syngas, methanol and formic acid.[4] This could be achieved in an electrochemical setup consisting of two electrodes where water is oxidized to O_2 at the anode while CO_2 and protons are converted at the cathode to the desired products. With the help of applied electric potential and reaction temperature, this process can be controlled. However, CO_2 being highly oxidized substrate, its reduction reaction requires high overpotential, which means requirement of high energy input.[4, 8] In addition to the difficulty in producing specific chemicals, the production of long carbon chain products and high cost associated with the electrocatalysts are some of the major limitations of this approach.[4, 8] Nevertheless, it is being implemented at the commercial scale in Iceland for the production of methanol.[4]

- **Biological utilization**

Three biological routes offer the opportunity to utilize CO_2. These include microalgae/cyanobacteria cultivation, syngas fermentation and microbial electrosynthesis (Table 1). Microorganisms like algae and cyanobacteria can produce oils from consuming CO_2. These oils can be extracted and later on converted to fuels and chemicals.[4] Syngas fermentation is based on the use of acetogens that use the Wood-Ljungdahl pathway to ferment CO or fix CO_2 (as discussed in section 3.3).

Table 1: Biological Methods of Carbon Utilization.[9]

Existing/ Proposed Technologies	Major reported products	Advantages	Key challenges
Algae cultivation	Biodiesel, proteins for animal or human feed, some pigments	High productivity per unit surface area	Require large resources (land, water), fouling, scalability issues, depends on sunlight availability, downstream processing costly
Cyanobacteria cultivation	Ethanol, butanol, 2,3 butanediol, lactate	Better genetic manipulation tools available than algae, higher photosynthetic efficiency than plants	Productivity is low, less genetic manipulation tools available; production of higher carbon chain length chemicals is difficult.
Syngas Fermentation	Ethanol, butanol, acetate, methane,	Advantages over chemical process include requirement of lower temperature, higher	Requirement of external hydrogen, inhibition of organisms, Limitation of Gas-liquid mass transfer,

	butyric acid	reaction specificity	genetic manipulation of microbial systems still not fully developed
Microbial Electrosynthesis	Acetate, methane, Butyrate	Conversions at ambient conditions, less land, water and nutrients, direct way to supply energy/reducing equivalents to microbes, positive environmental footprint.	Production at high titer and rates, product profile is limited, genetic manipulation of microbial systems still not fully developed

3. Microbial Electrosynthesis (MES)

3.1 Microbes and Extracellular Electron Transfer

The ability of certain microorganisms to transport electrons in and out of the cell to uphold metabolic processes is well known. M.C. Potter was the first person to investigate that the microbial disintegration of organic matter is accompanied by liberation of electrical energy.[10] The microbial electron transfer mechanisms allow the transfer of electrons from an electron donor which is at the lower potential to an electron acceptor which is at higher potential, to gain energy. Microorganisms maximize their energy gain by selecting the available electron acceptor with the highest potential.[11] In those environments where there is the absence of soluble electron acceptors, microorganisms can either go for fermentation where they reduce the internal electron acceptors or can use solid electron acceptors by transporting electrons outside of their cell.[11] This particular electron transfer mechanism, known as Extracellular Electron Transport (EET) has gained attention since past decades and has also led to a newly emerging field of microbial electrochemistry and technology.[11] Bioelectrochemical Systems (BES) are used

to study different applications of microbial electrochemistry. The coupling of the microbial metabolism to an electrode through EET resulted in a rapidly evolving scientific field, which is described as microbial electrocatalysis, where microorganisms are used in an engineered environment to facilitate a wide range of different applications.[12] These include, e.g., microbial fuel cells, bioremediation, biomining, microbial electrosynthesis, etc. Microbial electrosynthesis has gained the interest of researchers for chemicals and fuels synthesis from CO_2.

3.2 Microbial electrosynthesis – Electricity-driven Platform for Bioproduction

The knowledge of microorganisms being able to accept electrons from the electrodes has been exploited for the production of chemicals from CO_2 and electrons derived from the cathode. This electricity driven bioproduction of chemicals using microorganisms capable of accepting electrons from the cathode is referred to as microbial electrosynthesis (Fig. 2). In this process, CO_2 is reduced by microorganisms either through direct electron uptake from the cathode or through the utilization of H_2 produced at the cathode. At the anode, water oxidation takes place and results in the release of protons which gets diffused to the cathode via proton exchange membrane ultimately getting reduced to H_2.[13]

Knowledge on the extracellular electron transfer from the microbes to the anode is abundant, but there is limited information on the electron transfer mechanisms from the cathode to the microbes. With the available information there could be three ways through which microbes can uptake electrons from the cathode: (i) Direct electron uptake from the cathode, (ii) through the redox mediators, and (iii) through the oxidation of electrochemically produced energy carriers such as hydrogen (shown in Fig 3).[12]

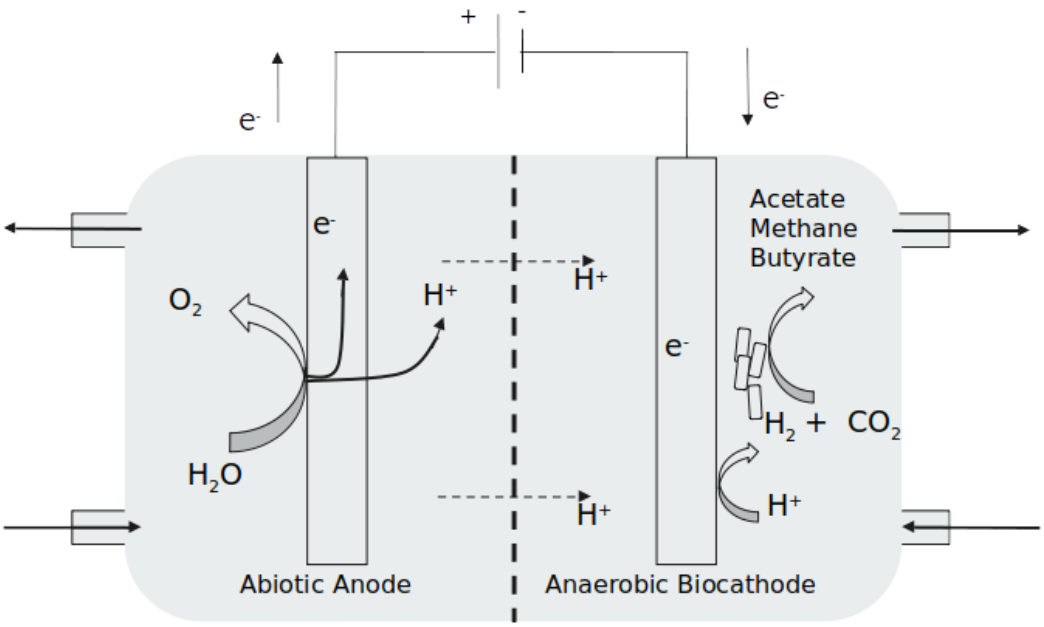


Fig 2: A schematic illustration of the Microbial electrosynthesis process.

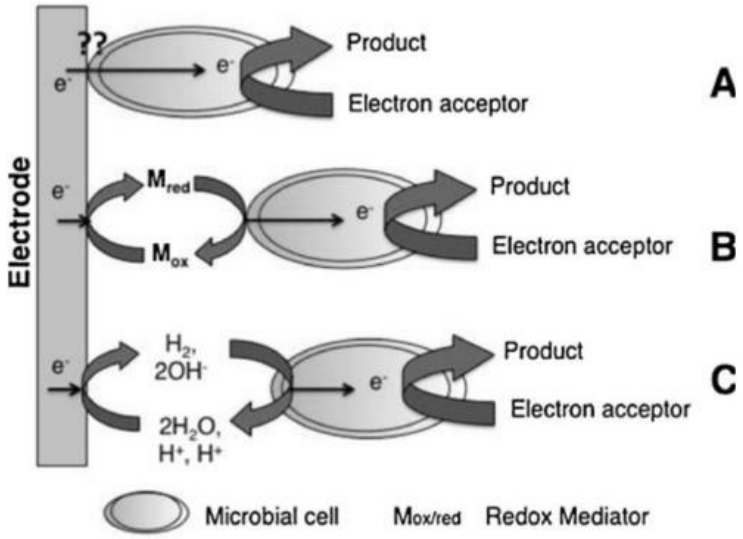


Fig 3: Electron transfer mechanisms from the cathode to microorganisms - A) Direct electron uptake from the cathode, B) Electron shuttles mediated uptake, and C) Hydrogen oxidation by microorganisms.[12]

The electric potential at the cathode determines the direct electron transfer based or hydrogen-based production in MES processes (Fig 4).

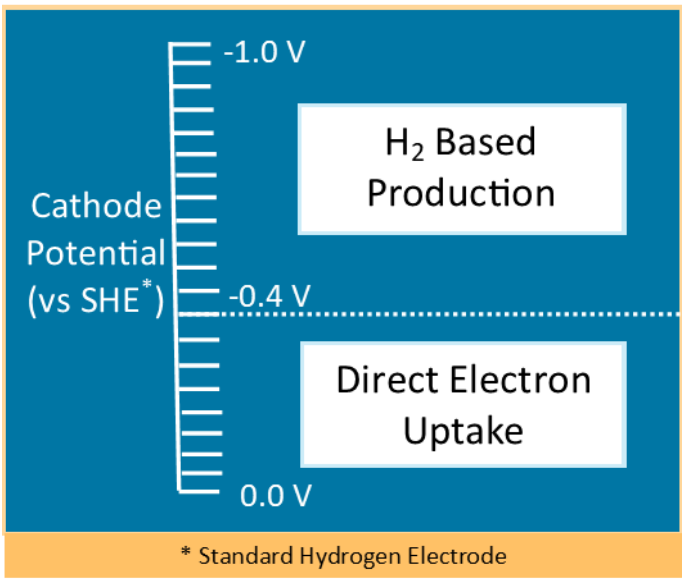


Fig 4: H_2 based or Direct electron based production depends on the applied potential at the cathode.

3.3 Microorganisms used for catalyzing the conversion of CO_2 into chemicals in MES

A special group of microorganisms known as acetogens uses Wood-Ljungdahl pathway (also known as reductive acetyl-CoA pathway) to fix CO_2 under anaerobic conditions (Fig. 5). It is simple and linear as compared to cyclic CO_2 fixation pathways in other organisms.[13] Two molecules of CO_2 are reduced or fixed to 1 molecule of acetate using 4 molecules of H_2 via two branches (eastern and western) in this pathway. Carbon monoxide can also be used through this pathway, which is exploited in the syngas fermentation process. The acetate production is linked to energy conservation in acetogens.

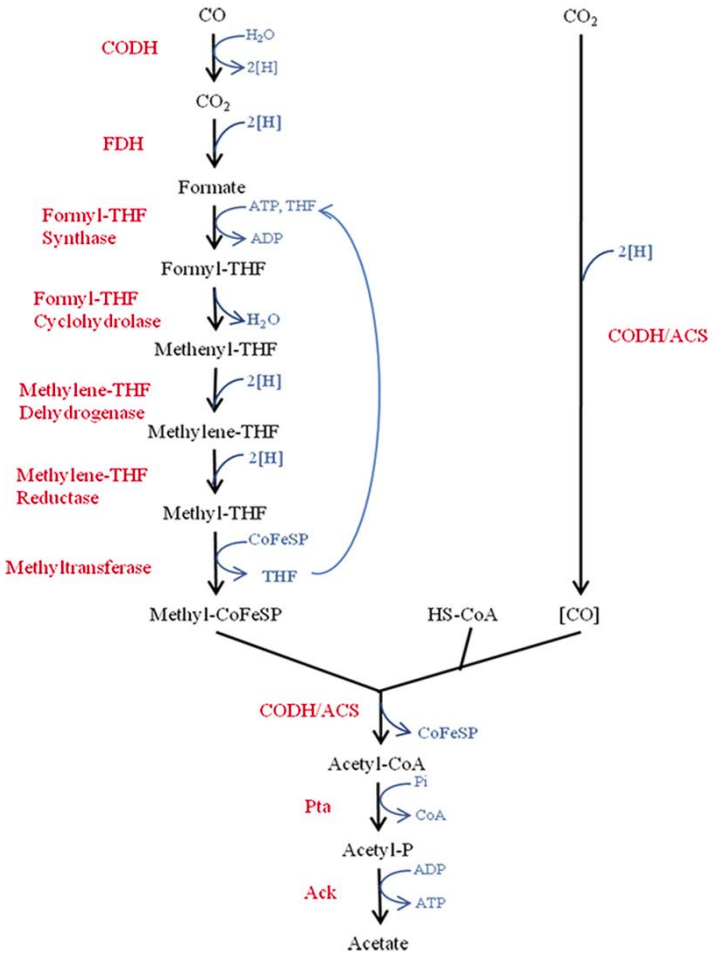


Fig 5: Wood Ljungdahl Pathway in Acetogens.[19]

In some recent studies, it has been shown that certain acetogens can utilize the electrons from the cathode to fix CO_2.[14, 15] Nevin et al. (2010) demonstrated for the first time that *Sporomusa ovata* produces acetate from CO_2 at -0.4 V vs. SHE applied potential at the cathode.[15] Later on, in 2011, the same research group demonstrated that *Clostridium ljungdahlii* is also capable of producing acetate from CO_2 using electrons from the cathode.[16]

Based on the type of microbes used, the major products being produced using MES are mostly methane and acetate. The use of methanogens in the MES results in the production of methane as a major product while acetate is the major product when acetogens are used. Some studies have shown the use of mixed microbial cultures for acetate production and have reported stable and consistent high acetate concentrations

with pre-enriched acetogens.[17] The only limitation with the use of mixed culture is the production of acetate as the major product while there have been some instances where butyrate is also reported but in trace amounts.[20]

There have been some genetic and metabolic engineering approaches applied to *C. ljungdahlii.*[18] The application of metabolic engineering techniques for MES would help develop better bioproduction processes.

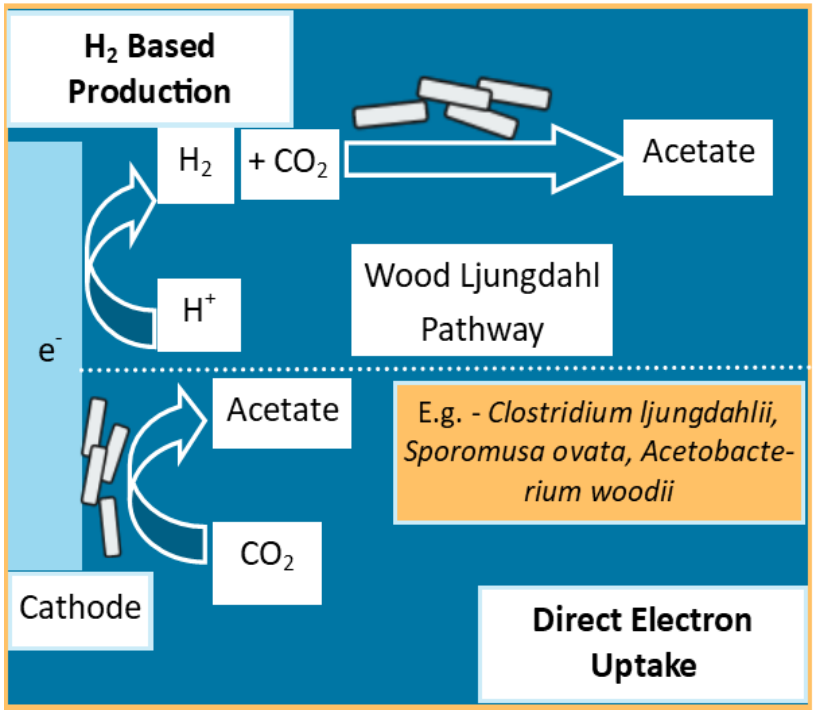


Fig 6: General overview of the MES using Acetogens

3.4 Current status of MES

It offers the most direct way to provide energy in the form of electrons or reducing equivalents for bioconversion processes. Several advantages as listed in Table 1 make the MES approach appealing for CO_2 utilization. The process has some key challenges which need to be addressed. These include e.g., (i) production of multi-carbon chain length chemicals, (ii) production of specific products at high titer and rate, and (iii) expanding the product profile to compete with the catalytic processes. Two approaches are envisioned for the production of multi-carbon chain length chemicals. First is the genetic

engineering or synthetic biology and second is the interlinking of two or more processes. Herein, we considered the second approach of linking MES with the yeast-based process to produce sclareol, a 20-carbon chain length highly valuable chemical.

4. Sclareol and its production using *S. cerevisiae*

4.1 Background information

The Isopentenyl pyrophosphate (IPP) pathway is well studied in *S. cerevisiae,* which is also known as the terpenoid biosynthetic pathway. Terpenoids are natural products from plants with much chemical diversity, market value, and applications.[22] They can be produced in *S. cerevisiae* naturally or by expression of heterologous genes from plants. Synthesizing the non-native terpenes in *S. cerevisiae* is favored over other microbes because of its compatibility for expressing cytochrome P450 enzymes from plant origin.[22] Engineering *S. cerevisiae* for terpenoid synthesis is highly feasible and have several advantages. Sclareol is a naturally occurring bicyclic terpenoid found in some plants like *Nicotiana sp.*, *Salvia sclarea,* etc. with a chemical formula of $C_{20}H_{36}O_2$ (Fig. 7). This chemical has a high value in the cosmetics and perfumes industry. It is also used in the wine industry as a flavoring agent for vermouths, wines, and liquors. Its estimated annual production is around 100 tons, and approximate value is around the US $ 15 - 18 million.[23] Sclareol and similar terpenoids have been extracted from the plants from several years. With the increase in the exploitation of these plants, they are becoming endangered. The chemical synthesis of these products is inefficient and usually results in a large amount of waste generation.[24] Thus, engineering microbes for the production of chemicals like sclareol has become a promising solution to protect endangered plants and prevent pollution from chemical synthesis.[24]

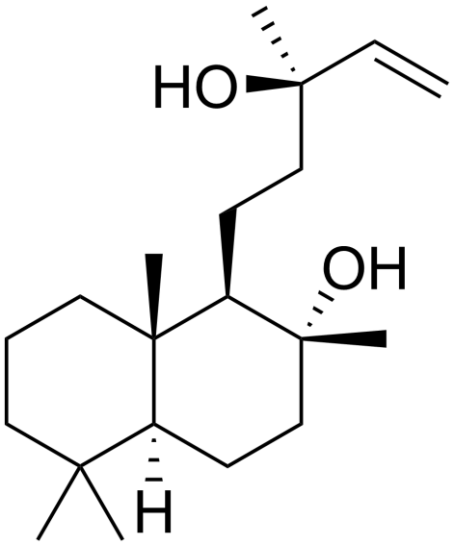


Fig 7: Sclareol structure

4.2 Engineering Sclareol Biosynthesis in *S. cerevisiae*

The Isoprenoid pathway of yeast produces Geranylgeranyl pyrophosphate (GGPP) naturally. In plants, synthesis of sclareol involves two dipernoid synthases for the conversion of GGPP to sclareol, i.e., Labdane diphosphate synthase (LPS) which converts GGPP to labda-13-en-8- ol diphosphate (LPP) and Sclareol synthase (SS) for the conversion of LPP to sclareol (Fig. 8).[25]

Fig 8: GGPP to Sclareol pathway

These have been identified earlier, but there is a need for the higher catalytic activity enzymes for better production of sclareol from *S. cerevisiae.* For which the genes were codon optimized and synthesized for LPS enzyme and SS enzyme which were from plant *Criscus criticus* and *Salvia sclarea,* respectively, the selection was done to identify the novel enzymes with the highest activity. (Patent filing in the process by Prof. A.K. Bachhawat and team, IISER Mohali)

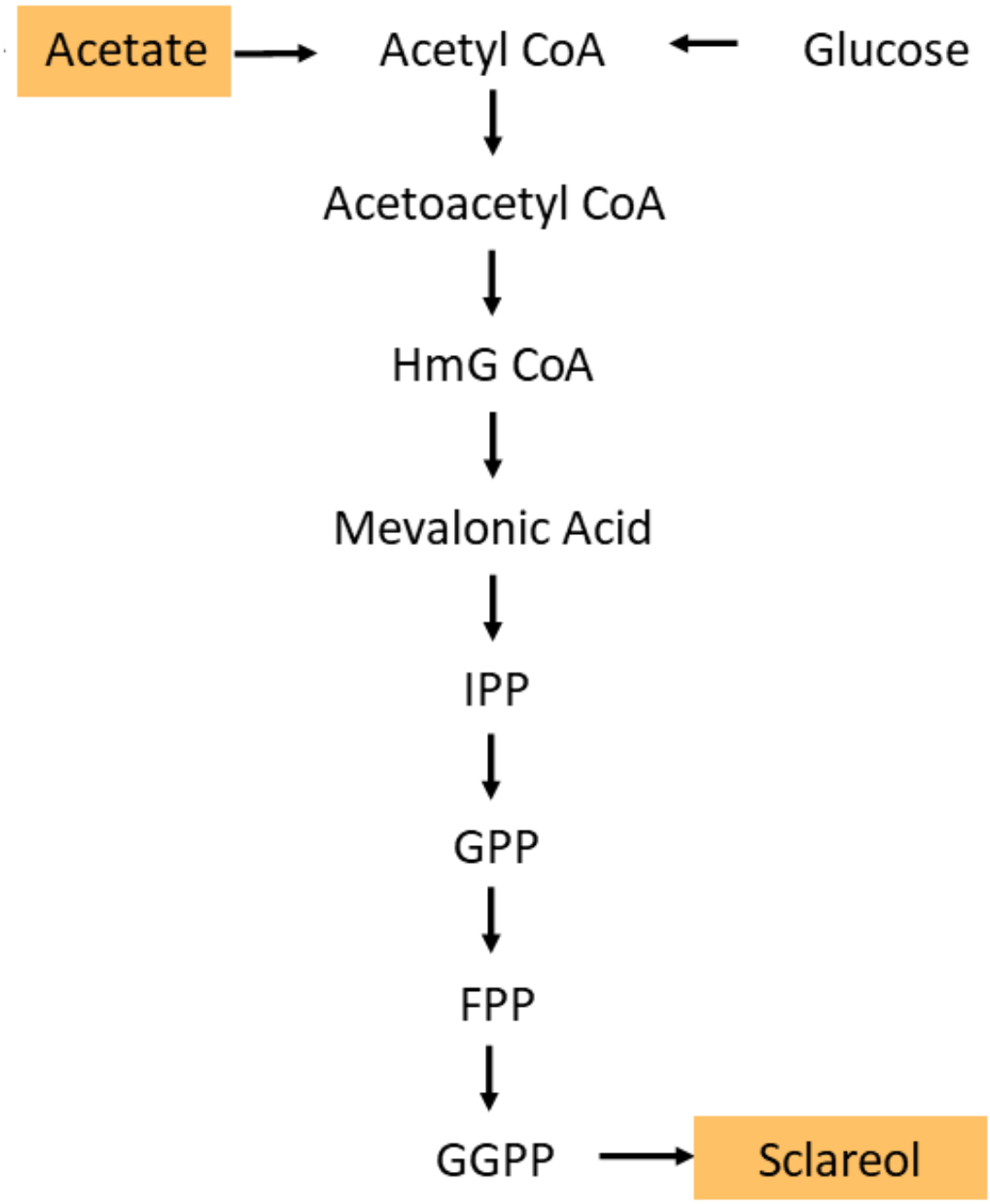


Fig 9: Engineered Sclareol Biosynthetic pathway in *S. cerevisiae*

HmG CoA	3-hydroxy-3-methylglutaryl-CoA
IPP	Isopentenyl pyrophosphate
FPP	Farnesyl pyrophosphate

Acetate is in the upstream of the sclareol biosynthetic pathway. As discussed earlier, acetate can be easily produced from CO_2 using acetogen such as *Clostridium ljungdahlii* via MES process. To add further value to the platform chemical acetate produced from CO_2, its conversion to sclareol via yeast-based process was tested in this thesis work.

5. Objective

To test bioconversion of CO_2 into Sclareol (C_{20}) via acetate (C_2) through interlinking of MES and yeast-based processes.

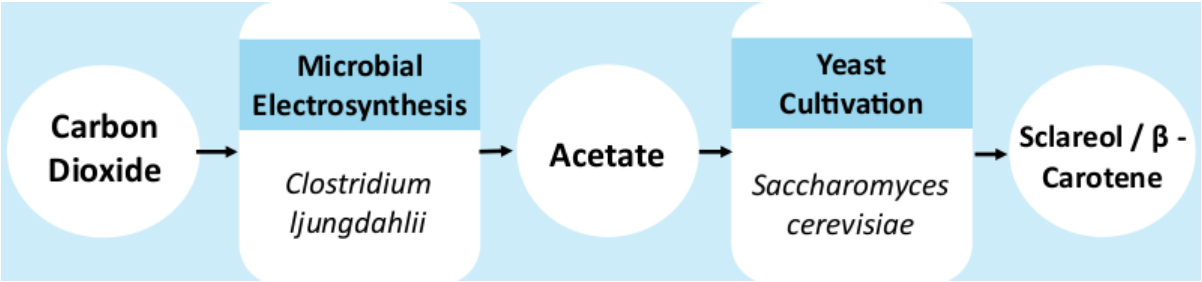


Fig 10: Schematic of Interlinking of microbial electrosynthesis and yeast-based processes

Chapter 2: Materials and Methods

1. Microorganisms and Cultivation Methods -

Clostridium ljungdahlii (DSM 13528) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and was cultivated using standard DSMZ medium 879 (Appendix 1) at 37^0C. It is a gram-positive, rod-shaped anaerobic bacteria. Routine maintenance of the strain was done anaerobically in 50 mL serum bottles with 20 mL medium containing fructose as the Carbon and Energy source and with 80% H_2 and 20% CO_2 headspace (1 bar pressure). Inoculum for the MES tests was prepared in serum bottles by growing the *Clostridium* culture on H_2 and CO_2 (80:20, 1 bar pressure) with 0.1 g/L Yeast Extract in the same medium devoid of fructose. Anaerobic media preparation and culturing techniques were followed for *Clostridium ljungdahlii.*

For the inoculum preparation and growth experiments on acetate with wild type strain, *S. cerevisiae* was grown on yeast Synthetic Defined (SD) medium. Growth assays on different acetate concentrations for carotenoid strain were also done on a slightly modified DSMZ medium 879. For final experiments, modified *S. cerevisiae* was grown on the spent media with an additional nitrogen source and the required amino acids for the auxotrophic strain, i.e., HIS, LEU and URA.

2. MES Experiments with *C. ljungdahlii*-

2.1 Cell Culture and Inoculum Preparation

C. ljungdahlii was grown autotrophically on H_2:CO_2 (80:20) in DSMZ medium 879 in 50 mL serum bottles with 20 mL medium working volume. Yeast Extract concentration was reduced to 0.1 g/L, other carbon sources, i.e., fructose and cysteine were omitted from the medium. Only bicarbonate served as the carbon source in these experiments. The culture was incubated at 37^oC for two days under static conditions. 10 mL culture with OD 0.371

(5% of the final catholyte volume in reactors) was used as the inoculum for the MES experiments.

2.2 Reactor Setup

Two-chambered custom-made Round Bottom Flask Reactors of 250 mL total volume with 5 necks were used for the MES tests with *C. ljungdahlii.* Anode compartment was a bottomless Hungate tube, which was fit in the centermost neck of the reactor. Nafion membrane (Sigma-Aldrich) was fixed at the bottom of the Hungate tube with the help of partly open rubber stopper and was sealed with the help of Aluminum crimp seal. It separated the anode and cathode electrodes and thus the respective reactions. Butyl rubber stoppers were used to seal the necks and maintain anaerobic conditions in the reactors. Three-electrode configuration was used in all MES experiments. Graphite rod with a projected surface area of 16.485 cm^2 was used as the cathode, whereas mixed metal oxide coated Titanium rods and Ag/AgCl (3.5 KCl, Biologic Science Instruments) were used as the anode and the reference electrode, respectively. Electrodes and membrane were treated before setting up the reactor (Appendix 2). Titanium wire was used to establish the connection between electrodes and potentiostat cables. All reactor components were autoclaved separately and assembled later in the Biosafety Cabinet. Gas inlet and outlet were fixed to the cathode chamber. N_2:CO_2 was continuously purged at a rate of 0.05 mL/min in the catholyte using Gas flow meters to maintain anaerobic conditions. Tygon tubing was used for all gas connections. Inlet and outlet tubing were connected to autoclavable filters for sterilized gas flow.

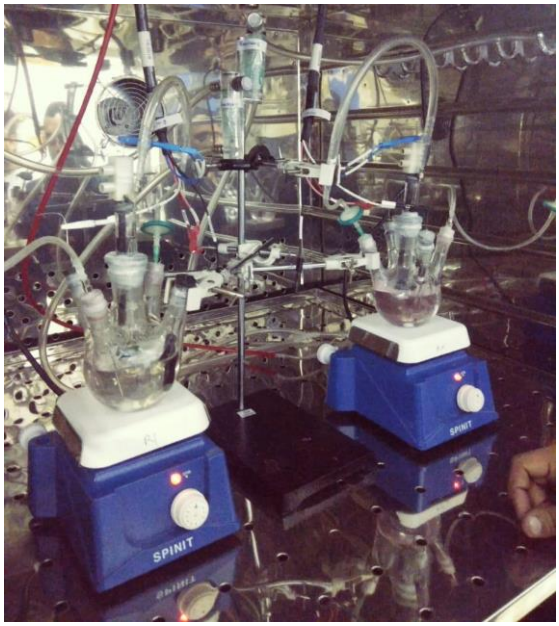


Photo 1: Actual Reactor setup for MES experiments

2.3 MES Experiments

In MES reactors the DSMZ 879 medium lacking cysteine and any other carbon sources was used as the catholyte or growth cum production medium. The anolyte was 50 mM Na_2SO_4 with pH 2, adjusted by H_2SO_4. All MES experiments were conducted with the aid of potentiostat (VMP3, Biologic Science Instruments, France). After setting up of reactors, they were polarized at an applied potential of -0.8 V vs. Ag/AgCl for 24 h before starting the MES experiments. Cyclic Voltammetry (CV) was recorded before inoculation, after inoculation, and after the third batch cycle to understand the role of microorganisms in bioelectrochemical processes. It is an electrochemical technique in which the potential of the working electrode ramps linearly versus time once to the set potential, after which it ramps in the opposite direction and returns to the initial potential. The developed current is measured and plotted against the applied potential. The CVs were recorded in a potential window from -1.4 V to -0.2 V vs. Ag/AgCl at a scan rate of 1 mV/s. A fixed potential of -1.2 V vs. Ag/AgCl was applied at the cathode in order to facilitate the H_2 based production from CO_2 and reduction current was monitored as a function of time by using chronoamperometry technique. Chronoamperometry is the electrochemical technique in which a fixed potential is applied at the working electrode,

and the resulting current is monitored as a function of time. These experiments were conducted in duplicate (i.e., two reactors 1 and 2 or R1 and R2).

Three control experiments were set up along with the main experiments (Figure 11). The three controls were - (i) Abiotic connected - the potential was applied at the cathode, but the reactor was not inoculated, (ii) Biotic unconnected - No applied potential but inoculated, and (iii) Biotic control with external H_2 as the energy source.

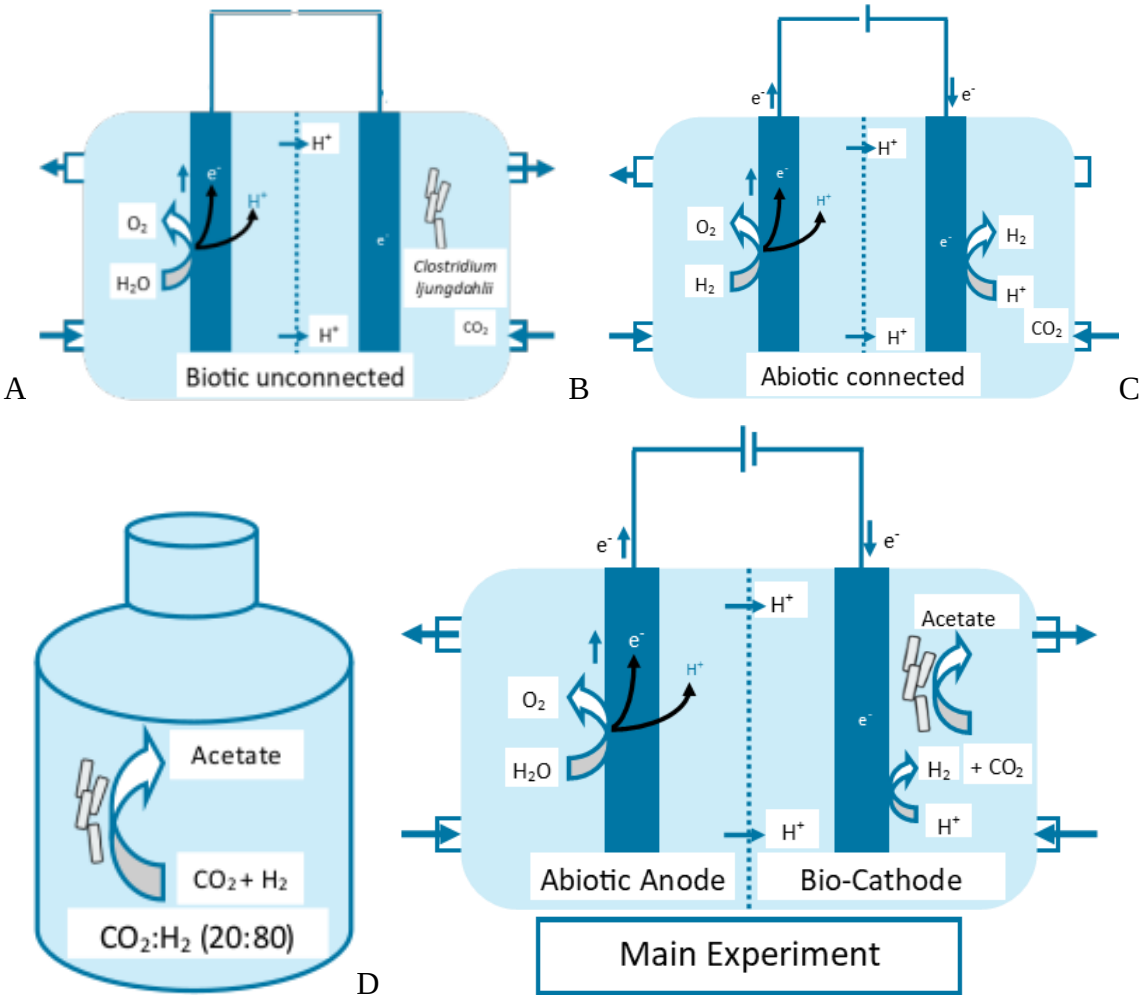


Fig 11: MES Experiments - A) Inoculated unconnected B) Uninoculated connected C) Biotic control with external H_2, and D) Main Experiment

CO_2 was used as the sole carbon source in all cases. Abiotic connected and Biotic unconnected control tests were performed with the same setup as that of the main

experiment while biotic control with pure H_2 test was performed in the serum bottle filled with H_2:CO_2 (80:20) in the headspace. Samples were taken daily for the analysis of pH, OD_{600nm} and soluble organics. If the samples were not analyzed on the day of sampling, they were stored at 4^{o}C and were analyzed within a week.

3. Yeast Experiments with *S. cerevisiae* -

3.1 Cell Culture and Inoculum Preparation

S. cerevisiae was grown on SD media (Table 2) for the inoculum preparation. To check the growth of *S. cerevisiae* on acetate, glucose (20g/L) was replaced with acetate (20 g/L) in the SD medium (Table 3). DSMZ medium 879 was modified for the growth of *S. cerevisiae* by increasing the nitrogen source in the medium to 5g/L (ammonium chloride) and by removing Cysteine which was inhibiting the growth of the yeast. Although the main goal was to evaluate the production of sclareol, additional yeast cultivation experiments were conducted for the production of carotenoids. HIS, LEU and URA were added in the medium for the growth of Sclareol producing strain, and LYS, TRP and MET were added in the medium for the growth of Carotenoid producing strain. Spent media from the MES was supplemented with the Vitamins, Trace Metals, HIS, LEU, URA and additional 5 g/L of ammonium chloride.

Table 2: SD medium composition

Components	Concentration (g/L)
Yeast Nitrogen Base	1.7
Ammonium Sulphate	5
Glucose	20
Supplements (HIS, LEU, URA for Sclareol strain, LYS, TRP, MET for Carotenoid strain)	0.080 each

Table 3: SD media with Acetate as Carbon and Energy Source used to check Terpenoid production

Components	Concentration (g/L)

Yeast Nitrogen Base	1.7
Ammonium Sulphate	5
Acetate	20
Supplements (LYS, TRP, MET)	0.080 each

3.2 *S. cerevisiae* cultivation Experiments for Sclareol production

Four different sets of experiments were conducted. (shown in Fig 12)

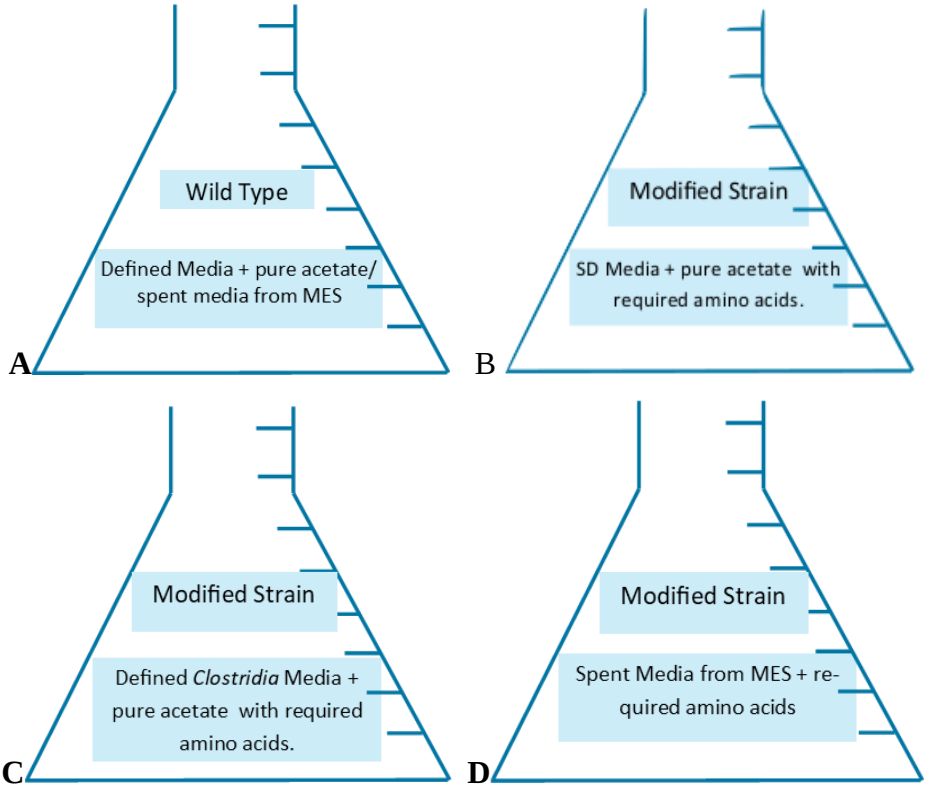


Fig 12: Experiments for *S. cerevisiae* A) Wild type strain grown on SD medium with acetate B) Carotenoids producing strain grown on SD medium with acetate C) Carotenoids and sclareol producing strains grown on modified DSMZ 879 medium D) Sclareol producing strain grown on the spent medium from the MES reactors.

First one was to check the growth of wild type *S. cerevisiae* using acetate as the sole carbon and energy source. The second was to check the possibility of Terpenes synthesis by modified strain while growing on acetate. The third was to check the growth of modified strain and production of Sclareol on modified DSMZ 879 medium. The fourth

was to check the growth of and Sclareol production by the modified strain *S. cerevisiae* on organics (acetate) containing spent media from MES reactors.

4. Measurements and Analysis –

In addition to the electrochemical parameters such as the reduction current production at the set cathode potential in MES experiments, the following parameters were monitored for all experiments.

4.1 pH Measurement

The pH of the growth and production media was measured using the Jenway pH meter.

4.2 Growth Analysis

Microbial growth was monitored by measuring the optical density at 600 nm wavelength (OD_{600nm}) using spectrophotometer (PhotoLab 7600 UV-VIS). Deionized water was used as blank and all the samples were measured in a 10 mm plastic cuvette.

4.3 Microscopy

Bright Field Light Microscopy (Nikon Eclipse E200) was done after staining (Appendix 3) the cultures to confirm the growth of *C. ljungdahlii* after revival and inoculation in the MES reactors.

4.4 Soluble Organics Analysis

The CO2 fixation products in MES reactors were monitored by analyzing the soluble fraction of the COD (chemical oxygen demand) in the catholyte samples. The catholyte samples from the MES reactors were filtered using a 0.22 μM syringe filter. Analysis of the filtered samples, i.e., soluble organics was done using the standard dichromate oxidation and spectrophotometry protocol commonly used for COD analysis (APHA, 2012). A standard/calibration curve for different concentrations of COD is shown in Fig. 11. The soluble fraction analysis provides an accurate estimation of organic compounds in a sample. In the case of *C. ljungdahlii*, acetate is the major or almost exclusive organic compound produced at the neutral pH condition.

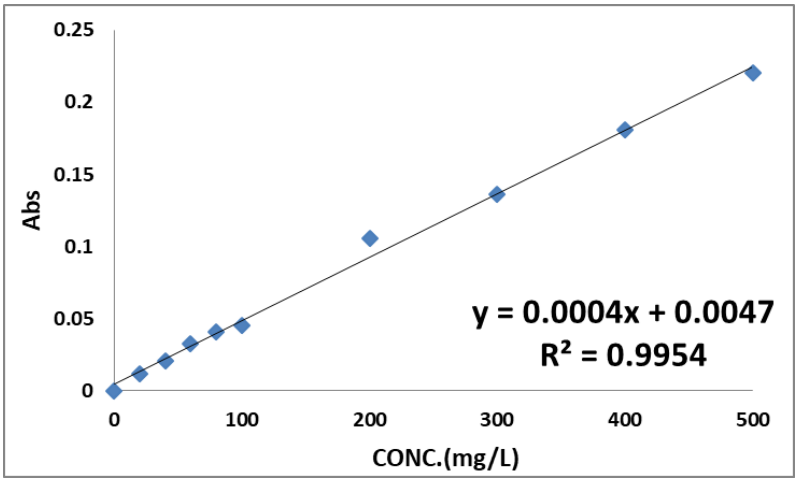


Fig 13 Standard curve for Soluble Organics Analysis (in terms of COD) at 600 nm

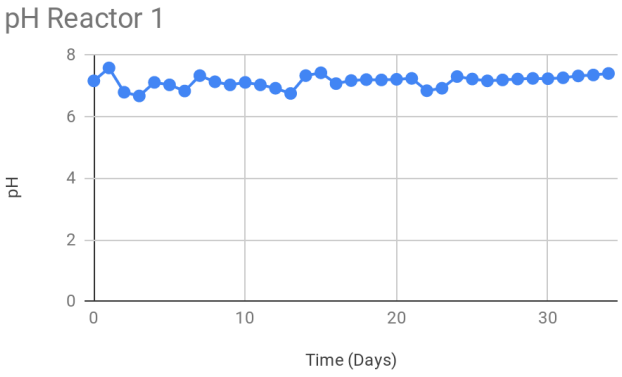
4.5 Gas Chromatography

The headspace Gas Composition of the MES reactors was analyzed using Agilent 490 Micro GC. O_2, N_2, H_2 were separated through Molsieve 5A column and CO_2 through PoraPLOT column, and analyzed via a Thermal Conductivity Detector (TCD). Sclareol produced by *S. cerevisiae* was analyzed on Shimadzu GC equipped with Flame Ionization Detector (FID). GC method for Sclareol detection was followed from Schalk et al. with slight modification. The working conditions were as follows: injector: 300°C; detector: 300°C; oven temperature, start at 50°C, hold for 0.3 min, programmed from 50°C to 150°C at 20°C/min, hold for 0.45 min and from 150°C to 300°C at 5°C/min, hold for 1 min; carrier gas flow (N_2), 25.0 mL/min; injection mode: 1µL injection volume with a 10.0 split.[26]

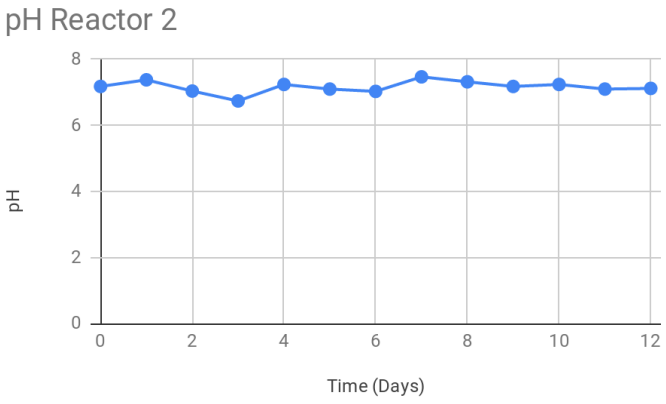
Chapter 3: Results and Discussion

1. Production of organics from CO_2 in MES reactors

The initial pH of the catholyte in each batch production cycle was set to 6.8 which is optimum for the growth of *C. ljungdahlii*. The drop in pH can lead to a slower growth rate and also transition the acidogenesis growth phase of such acetogens to a solventogenesis one. In both MES reactors the pH almost remained constant throughout the experimental run (Fig 14).



A.



B.

Fig 14: pH of the reactors **A.** Reactor 1, **B.** Reactor 2 (data is for 12 days because the reactor failed due to technical reasons)

1.1 Growth and Soluble Organics

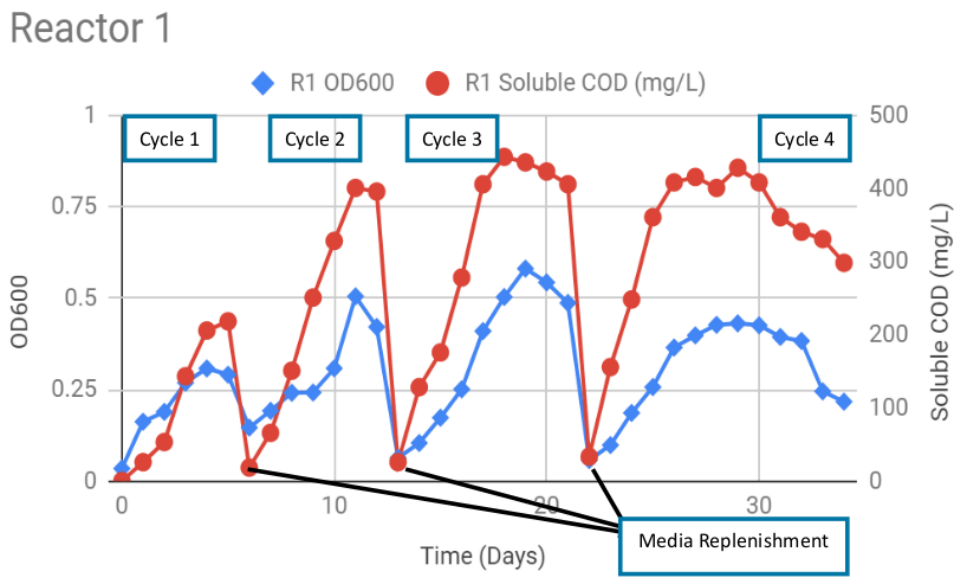


Fig 15: Growth and soluble COD (mg/L) analysis of Reactor 1

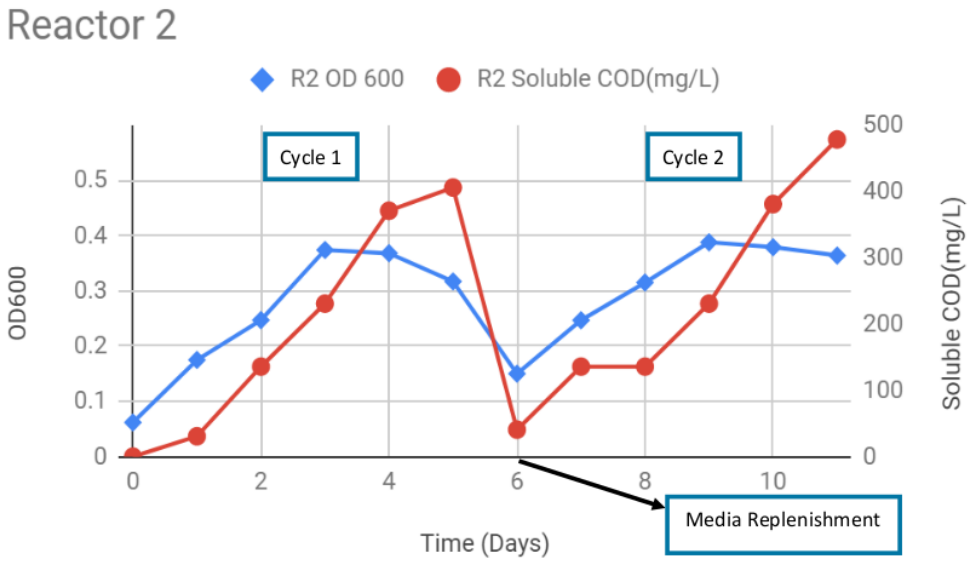


Fig 16: Growth and soluble COD (mg/L) analysis of Reactor 2

In both the reactors, a relation between the growth and soluble organics (acetate) production was observed (Figs 15 and 16). Organics production is coupled to the growth, which becomes stable when the stationary phase is reached. After reaching the stationary phase or at the start of the decline growth phase, the spent medium in the cathode chamber was replenished by the fresh medium and the production cycles were further

continued. The growth curves show that stationary phase is reached after a $4^{th}/5^{th}$ day in these both reactors. R1 was operated for four batch cycles to check the reproducibility of the bioproduction data. R2 failed at the end of the 2^{nd} batch cycle due to the technical reasons, and therefore its further operation was terminated. The maximum organics production achieved in Reactors 1 and 2 was 443.25 mg/L and 478.25 mg/L, respectively.

1.2 Growth and Soluble Organics production in MES Control Experiments

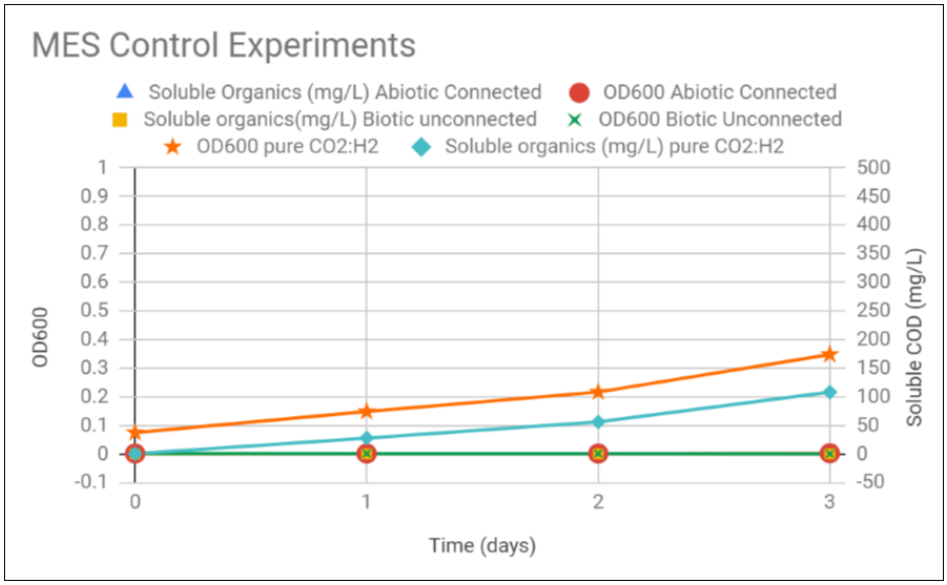


Fig 17: Growth and Soluble organics production in MES Control Experiments

Three different control experiments were performed to confirm that the production of organics (Acetate) from CO_2 is by *C. ljungdahlii* and is electricity-driven H_2-based (Fig 17).

Control 1: Abiotic Connected - There was no growth and organics production observed, which confirms that the inoculated microbes are required for the production of acetate from CO_2.

Control 2: Biotic Unconnected - No growth and production was observed because there was no H_2 production as the system was operated without the applied potential at the cathode. H_2 is used by *C. ljungdahlii* as an energy source to fix CO_2 via Wood Ljungdahl pathway under auto-lithotrophic conditions.

Control 3: Biotic with external H_2 source - In this control, serum bottle was filled with H_2 (80%) in the headspace. This was done to confirm that CO_2 and H_2 are required for acetate production by *C. ljungdahlii*. Both growth and organics production were observed in this case.

1.3 Cyclic Voltammetry (CV) (Reactor 1)

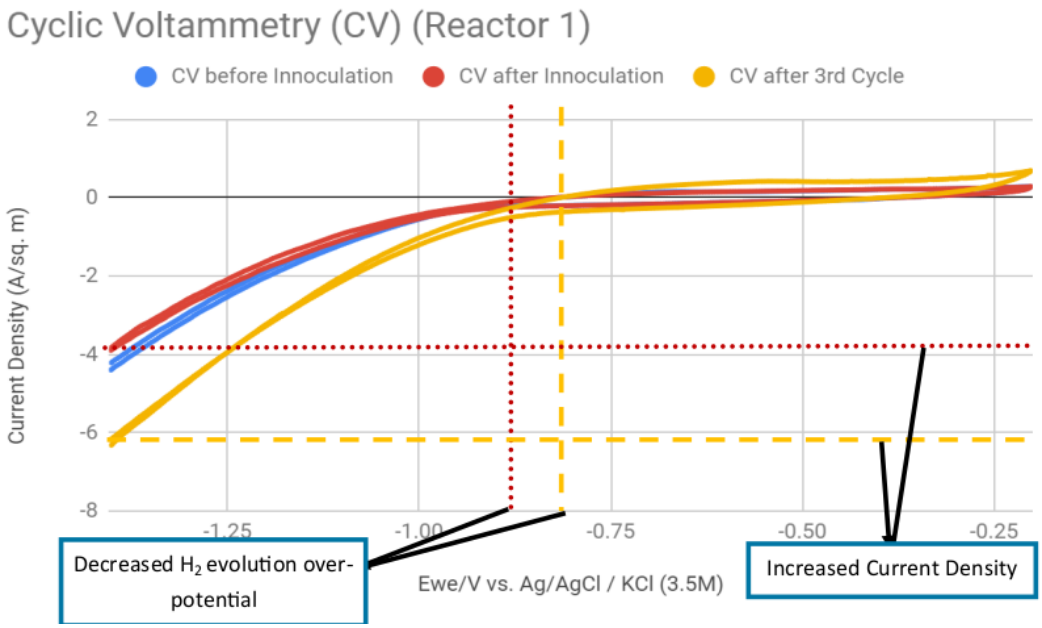


Fig 18: Cyclic voltammetry of Reactor 1 before inoculation, after inoculation and after 3rd batch cycle

CV tests of the cathode recorded before and after inoculation reveal a similar behavior (Fig 18). No redox peaks are seen in these CVs, which suggests the absence of any redox mediators in the catholyte and at the cathode surface. Importantly, a considerable decrease in the H_2 evolution over-potential (about 100 mV) and an increase in the current density is observed in the CVs recorded after the third batch cycle. The decrease in over-potential could be because of the attachment of *C. ljungdahlii* to the electrode surface and the activity of hydrogenase enzyme. Hydrogenase enzyme could be acting as a catalyst for facilitating H_2 production in this case. The increased current density also signifies the enhanced electrocatalysis most likely due to the activity of biotic components at the cathode surface.

1.4 Organics Production Rate and H_2 production Analysis

The organics production rate was calculated by taking the maximum organics produced value divided by the number of days taken to achieve the same and then normalizing it by the catholyte volume (0.2 L). The maximum production rates achieved were **0.37±0.102 g/L/day** and **0.442±0.0512 g/L/day** for Reactors 1 and 2, respectively (Table 4). The difference in the amount of H_2 detected in abiotic connected and the MES reactors suggests the utilization of H_2 by the microbes as the energy source in the MES experiments (Table 4).

Table 4: Organics production rate and H_2 production in different experiments

Analysis	Reactor 1	Reactor 2	Abiotic connected	Biotic Unconnected	External H_2
H2 (%)	2.37±0.18	2.19±0.23	4.76±0.75	0	78.56±0.14
Organic Production Rate (g/L/day)	0.37±0.10	0.44±0.051	0	0	0.179

2. *S. cerevisiae* Cultivation Experiments

2.1 Growth of Carotenoid producing *S. cerevisiae* strain on Acetate

Photo 2 shows the growth of Carotenoid producing strain on 20 g/L acetate as a carbon and energy source in SD media. This confirmed that yeast could grow on acetate and can undergo terpenoid biosynthesis using acetate as the sole carbon and energy source.

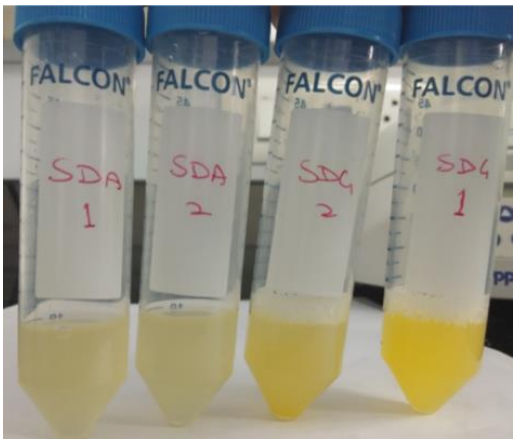


Photo 2: Carotenoid producing *S. cerevisiae* strain growing on 20 g/L acetate in SD media - SDA 1 and SDA 2 while SDG 1 and SDG 2 are controls grown on 20 g/L glucose

2.2 Growth of *S. cerevisiae* on different acetate concentrations in modified defined DSMZ medium 879

Growth analysis was done on various concentrations of Acetate to check the minimum and maximum concentrations up to which *S. cerevisiae* can grow (Fig 19). It was observed that *S. cerevisiae* could grow on as low as a concentration of 1 g/L and a maximum of up to 28 g/L without much effect on growth. However, a clear prolonged lag phase was observed with the acetate concentrations above 1g/L. Compared to glucose, both the lag phase as well the maximum growth was lower in the case of acetate.

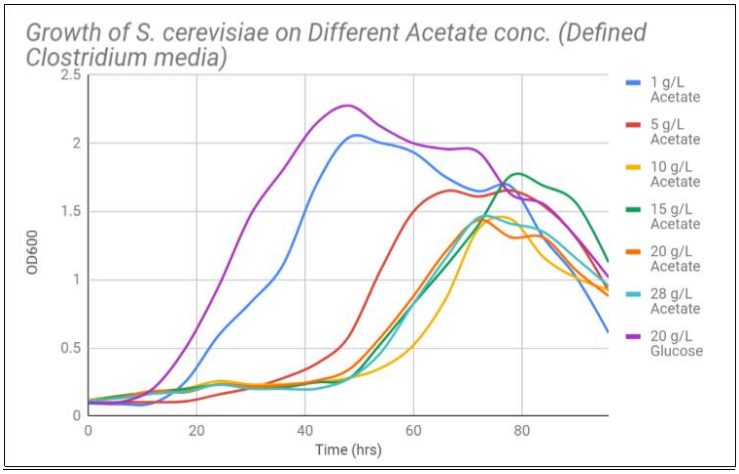


Fig 19: *S. cerevisiae* cultivation on different acetate concentrations

2.3 Analysis of Sclareol Production using GC with FID detector

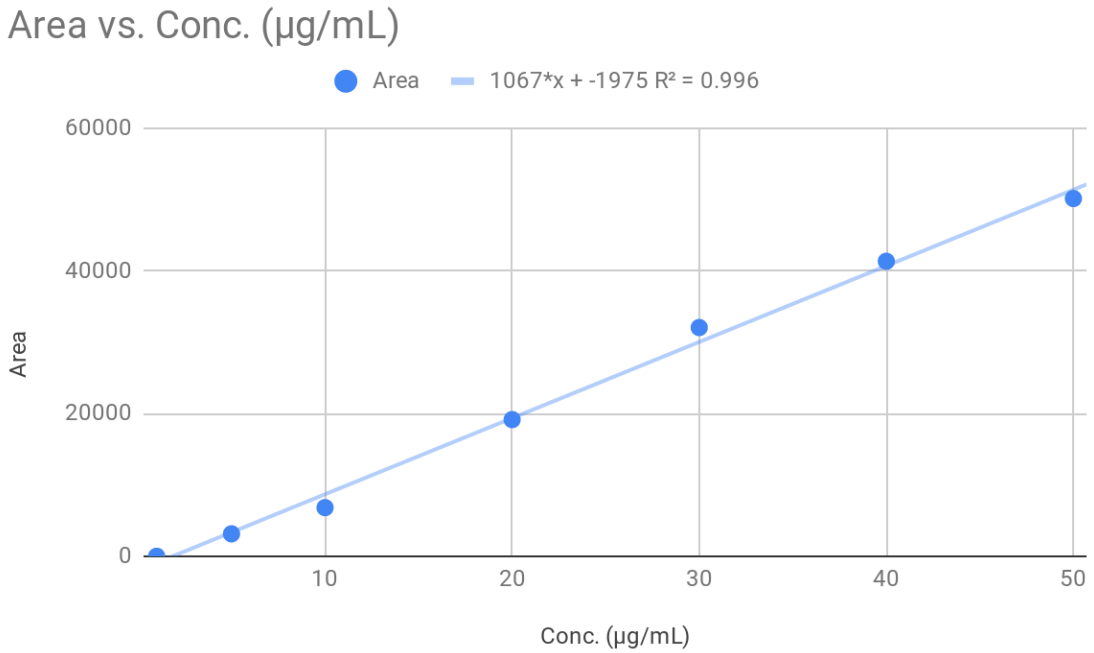


Fig 20: Internal Standard curve for Sclareol Analysis

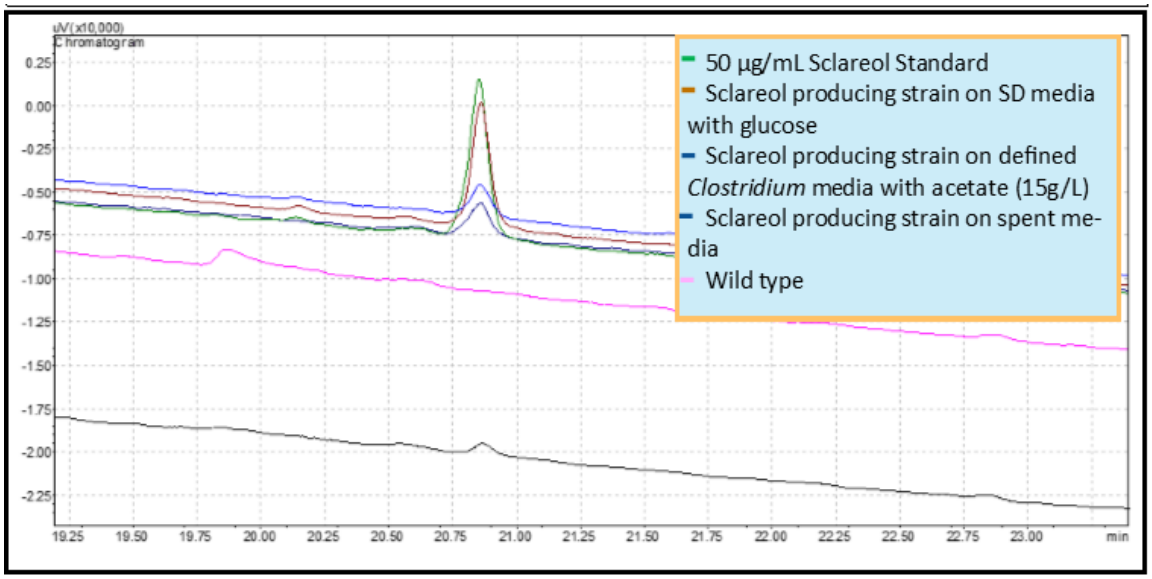


Fig 21: GC-FID chromatogram of Sclareol and peak comparison with standard. Further quantitative estimation of Carotenoids and Sclareol is under progress

Fig 20 shows the internal standard curve, for sclareol, i.e., concentration vs. area of the peak in GC chromatogram. The internal standard method increases the chances of error in quantification depending on the amount of injection as well due to the unavailability of chemical required for external standard preparation this method was used. In the chromatogram (Fig 21), it can be seen that there is a peak at the same retention time as that of the standard, Sclareol producing strain grown on SD media, defined *Clostridium* medium (DSMZ Medium 879) and spent medium from MES, while it is absent in the wild type strain. It confirms the Sclareol production by the modified strain grown on acetate containing spent medium from MES. Quantification based on the prepared internal standard curve is shown in Table 5. Wild type strain did not show any sclareol production. Sclareol strain, when grown on SD media with 20g/L Glucose, shows 35.819 mg/L of sclareol production. When grown on defined *Clostridium media* with 1g/L acetate it achieved 9.463 mg/L production, whereas on spent media from MES reactor it achieved 6.364 mg/L sclareol production.

Table 5: Sclareol Production quantified using GC with FID detector

Sclareol Production Data	
Experiment	**Concentration of Sclareol (mg/L)**
Wild type - SD media	0
Sclareol strain - SD media (Glucose)	35.819
Sclareol strain - defined clostridium media	9.463
Sclareol strain - spent media	6.364

Conclusion and Future Prospects

In this proof-of-principle study, interlinking of Microbial Electrosynthesis (MES) and Yeast Cultivation processes was demonstrated for Sclareol (C_{20}) production. Maximum production of soluble organics up to **478.25 mg/L** at the rate of **0.442±0.0512 g/L/day** from CO_2 was achieved in the MES reactors. Sclareol production up to **6.364 mg/L** in a spent medium containing acetate was achieved. Further quantification of Sclareol and reproducibility tests are under progress. A similar approach could be further tested on a wide range of chemicals in order to add value to the acetate produced from CO_2 via MES. Metabolic Engineering can be done with *S. cerevisiae*, and the integrated process can be further developed. Since acetate is a platform chemical that can be easily produced from CO_2 via MES, further work can be done on developing better acetate utilizing *S. cerevisiae* and other microbial strains.

Appendix 1

879. CLOSTRIDIUM LJUNGDAHLII MEDIUM

NH_4Cl	1.00	g
KCl	0.10	g
$MgSO_4 \times 7\ H_2O$	0.20	g
NaCl	0.80	g
KH_2PO_4	0.10	g
$CaCl_2 \times 2\ H_2O$	0.02	g
Yeast extract	1.00	g
Trace element solution (see medium 141)	10.00	ml
Na-resazurin solution (0.1% w/v)	0.50	ml
$NaHCO_3$	1.00	g
D-Fructose	5.00	g
Vitamin solution (see medium 141)	10.00	ml
L-Cysteine-HCl x H_2O	0.30	g
$Na_2S \times 9\ H_2O$	0.30	g
Distilled water	1000.00	ml

Dissolve ingredients (except bicarbonate, fructose, vitamins and reducing agents), sparge medium with 80% N 2 and 20% CO 2 gas mixture for 30 – 45 min to make it anoxic and adjust pH to 5.5. Dispense medium under same gas atmosphere into anoxic Hungate-type tubes or serum vials and autoclave at 121°C for 15 min. Add fructose, vitamins (sterilized by filtration), cysteine and sulfide from sterile stock solutions prepared under 100% N 2 gas atmosphere and bicarbonate from a sterile stock solution prepared under 80% N 2 and 20% CO_2 gas atmosphere. Adjust pH of complete medium to 5.9! For DSM 13641 replace fructose with 5.00 g/l sucrose added to the autoclaved medium from a sterile anoxic stock solution and increase amount of $NaHCO_3$ to 2.50 g/l. Adjust pH of complete
medium to 6.9

Trace element solution:

Nitrilotriacetic acid	1.50	g
$MgSO_4$ x 7 H_2O	3.00	g
$MnSO_4$ x H_2O	0.50	g
NaCl	1.00	g
$FeSO_4$ x 7 H_2O	0.10	g
$CoSO_4$ x 7 H_2O	0.18	g
$CaCl_2$ x 2 H_2O	0.10	g
$ZnSO_4$ x 7 H_2O	0.18	g
$CuSO_4$ x 5 H_2O	0.01	g
$KAl(SO_4)_2$ x 12 H_2O	0.02	g
H_3BO_3	0.01	g
Na_2MoO_4 x 2 H_2O	0.01	g
$NiCl_2$ x 6 H_2O	0.03	g
Na_2SeO_3 x 5 H_2O	0.30	mg
Na_2WO_4 x 2 H_2O	0.40	mg
Distilled water	1000.00	ml

First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals. Adjust final to pH 7.0 with KOH.

Vitamin solution:

Biotin	2.00	mg
Folic acid	2.00	mg
Pyridoxine-HCl	10.00	mg
Thiamine-HCl x 2 H_2O	5.00	mg
Riboflavin	5.00	mg
Nicotinic acid	5.00	mg
D-Ca-pantothenate	5.00	mg
Vitamin B_{12}	0.10	mg
p-Aminobenzoic acid	5.00	mg
Lipoic acid	5.00	mg
Distilled water	1000.00	ml

Appendix 2

Electrode Treatment

- Keep the electrodes dipped in 0.1 N HCl solution for 2 hours.
- Rinse and dip in distilled water for 1 hour.
- Then dip in 0.1 N NaOH for 2 hours.
- Again rinse and dip in distilled water for 2 hours.

Membrane Treatment (Nafion Membrane)

- Cut into the required dimension.
- 1 hour in lightly boiling (~80C) 3% H_2O_2
- Rinse in DI water
- 2 hours in lightly boiling H_2O
- 1 hour in lightly boiling 0.5M H_2SO_4
- 2-3x rinse in lightly boiling (80-90C) DI water
- Store in DI water

Appendix 3

Directions:

Prepare a Slide Smear:

- Transfer a drop of the suspended culture to be examined on a slide with an inoculation loop. If the culture is to be taken from a Petri dish or a slant culture tube, first add a drop or a few loopful of water on the slide and aseptically transfer a bit of the colony. It should only be a very small amount of culture. A visual detection of the culture on an inoculation loop already indicates that too much is taken.
- Spread the culture with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter. It is possible to put 3 to 4 small smears on a slide, if more than one culture is to be examined.
- Hold the slide with a clothespin. Allow to air dry and fix it over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear with water without a significant loss of the culture.

Gram Staining:

1. Flood the fixed smear with Gram's crystal violet Solution. Let stand for 60 seconds.
2. Pour off the stain and gently wash with tape water from a faucet or a plastic water bottle.
3. Flood with Gram's iodine Solution. Allow it to remain for 60 seconds.
4. Pour off the iodine solution and gently wash with tape water. Shake off the excess water from the surface.
5. 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.
6. Gently wash the smear with tape water.
7. Counterstain with Gram's safranin Solution for 60 seconds.

8. Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shaken to remove most of the water and air-dried.
9. Examine the finished slide under a microscope (oil immersion objective).

Attention: Wash off any spilled stain immediately with water to avoid leaving permanent marks in the sink, lab bench, or glassware.

Result:

- Gram-positive organisms are bluish purple
- Gram-negative organisms are pinkish red

Bibliography

1) Qiancheng Ma, “Greenhouse Gases: Refining the Role of Carbon Dioxide”, NASA Science Briefs, NASA, 1998, NASA, www.giss.nasa.gov/research/briefs/ma_01/.

2) Ritchie, Hannah, and Max Roser. “CO_2 and Other Greenhouse Gas Emissions.” Our World in Data, 11 May 2017, ourworldindata.org/co2-and-other-greenhouse-gas-emissions.

3) Dr. Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) and Dr. Ralph Keeling, Scripps Institution of Oceanography (scrippsco2.ucsd.edu/).

4) Martens, Johan A., et al. “The Chemical Route to a Carbon Dioxide Neutral World.” ChemSusChem, vol. 10, no. 6, 2017, pp. 1039–1055., doi:10.1002/cssc.201601051.

5) Budzianowski, Wojciech M. “Implementing Carbon Capture, Utilisation and Storage in the Circular Economy.” International Journal of Global Warming, vol. 12, no. 2, 2017, p. 272., doi:10.1504/ijgw.2017.084510.

6) Cuéllar-Franca, Rosa M., and Adisa Azapagic. “Carbon Capture, Storage and Utilisation Technologies: A Critical Analysis and Comparison of Their Life Cycle Environmental Impacts.” Journal of CO2 Utilization, vol. 9, 2015, pp. 82–102., doi:10.1016/j.jcou.2014.12.001.

7) “Chemical Utilization of CO2 into Chemicals and Fuels” National Academies of Sciences, Engineering, and Medicine. Gaseous Carbon Waste Streams Utilization: Status and Research Needs. Washington, DC: The National Academies Press. 2019, p.63-96, doi: https://doi.org/10.17226/25232.

8) Bushuyev, Oleksandr S., et al. “What Should We Make with CO2 and How Can We Make It?” Joule, vol. 2, no. 5, 2018, pp. 825–832., doi:10.1016/j.joule.2017.09.003.

9) “Biological Utilization of CO2 into Chemicals and Fuels” National Academies of Sciences, Engineering, and Medicine. Gaseous Carbon Waste Streams Utilization: Status and Research Needs. Washington, DC: The National Academies Press. 2019, p.97-136, doi: https://doi.org/10.17226/25232.

10) Potter, M. C. “Electrical Effects Accompanying the Decomposition of Organic Compounds.” Proceedings of the Royal Society B: Biological Sciences, vol. 84, no. 571, 1911, pp. 260–276., doi:10.1098/rspb.1911.0073.
11) Bajracharya, Suman, et al. “An Overview on Emerging Bioelectrochemical Systems (BESs): Technology for Sustainable Electricity, Waste Remediation, Resource Recovery, Chemical Production and Beyond.” Renewable Energy, vol. 98, 2016, pp. 153–170., doi:10.1016/j.renene.2016.03.002.
12) Patil, Sunil A., et al. “Electron Transfer Mechanisms between Microorganisms and Electrodes in Bioelectrochemical Systems.” Advances in Chemical Bioanalysis, 2012, pp. 71–129., doi:10.1007/11663_2013_2.
13) Rabaey, Korneel, and René A. Rozendal. “Microbial Electrosynthesis — Revisiting the Electrical Route for Microbial Production.” Nature Reviews Microbiology, vol. 8, no. 10, 2010, pp. 706–716., doi:10.1038/nrmicro2422.
14) Kato, Souichiro. “Biotechnological Aspects of Microbial Extracellular Electron Transfer.” Microbes and Environments Microbes and Environments, vol. 30, no. 2, 2015, pp. 133–139., doi:10.1264/jsme2.me15028.
15) Nevin, Kelly P., et al. “Microbial Electrosynthesis: Feeding Microbes Electricity To Convert Carbon Dioxide and Water to Multicarbon Extracellular Organic Compounds.” MBio, vol. 1, no. 2, 2010, doi:10.1128/mbio.00103-10.
16) Nevin, Kelly P., et al. “Electrosynthesis of Organic Compounds from Carbon Dioxide Is Catalyzed by a Diversity of Acetogenic Microorganisms.” Applied and Environmental Microbiology, vol. 77, no. 9, 2011, pp. 2882–2886., doi:10.1128/aem.02642-10.
17) Patil, Sunil A., et al. “Selective Enrichment Establishes a Stable Performing Community for Microbial Electrosynthesis of Acetate from CO2.” Environmental Science & Technology, vol. 49, no. 14, 2015, pp. 8833–8843., doi:10.1021/es506149d.
18) Banerjee, Areen, et al. “Lactose-Inducible System for Metabolic Engineering of Clostridium Ljungdahlii.” Applied and Environmental Microbiology, vol. 80, no. 8, 2014, pp. 2410–2416., doi:10.1128/aem.03666-13.
19) Ljungdahl, Lars G. “A Life with Acetogens, Thermophiles, and Cellulolytic Anaerobes.” Annual Review of Microbiology, vol. 63, no. 1, 2009, pp. 1–25., doi:10.1146/annurev.micro.09120

20) Batlle-Vilanova, Pau, et al. “Microbial Electrosynthesis of Butyrate from Carbon Dioxide: Production and Extraction.” Bioelectrochemistry, vol. 117, 2017, pp. 57–64., doi:10.1016/j.bioelechem.2017.06.

21) Min, Fung, et al. “Gas Fermentation for Commercial Biofuels Production.” Liquid, Gaseous and Solid Biofuels - Conversion Techniques, 2013, doi:10.5772/52164.

22) Paramasivan, Kalaivani, and Sarma Mutturi. “Progress in Terpene Synthesis Strategies through Engineering of Saccharomyces Cerevisiae.” Critical Reviews in Biotechnology, vol. 37, no. 8, 2017, pp. 974–989., doi:10.1080/07388551.2017.1299679.

23) NxtGen Reports. “2018-2023 Global Sclareol Consumption Market Report.” NxtGen Reports, NxtGen Reports, 15 Aug. 2018, www.nxtgenreports.com/market-research-reports/2018-2023-global-sclareol-consumption-market.

24) Liu, Xiaonan, et al. “Engineering Microbial Cell Factories for the Production of Plant Natural Products: from Design Principles to Industrial-Scale Production.” Microbial Cell Factories, vol. 16, no. 1, 2017, doi:10.1186/s12934-017-0732-7.

25) Caniard, Anne, et al. “Discovery and Functional Characterization of Two Diterpene Synthases for Sclareol Biosynthesis in Salvia Sclarea (L.) and Their Relevance for Perfume Manufacture.” BMC Plant Biology, vol. 12, no. 1, 2012, p. 119., doi:10.1186/1471-2229-12-119.

26) Schalk, M., Pastore, L., Mirata, M. A., Khim, S., Schouwey, M., Deguerry, F., Daviet, L. (2012). “Toward a Biosynthetic Route to Sclareol and Amber Odorants.” Journal of the American Chemical Society, 134(46), 18900–18903. doi:10.1021/ja307404u