Journal of CO_2 Utilization 20 (2017) 190–199

Contents lists available at ScienceDirect

Journal of CO_2 Utilization

journal homepage: www.elsevier.com/locate/jcou

Microbial electrosynthesis of carboxylic acids through CO_2 reduction with selectively enriched biocatalyst: Microbial dynamics

J. Annie Modestra[1], S. Venkata Mohan[1,*]

Bioengineering and Environmental Sciences Lab, EEFF Department, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad 500 007, India

ARTICLE INFO

Keywords:
CO_2 sequestration
Chronoamperometry
Volatile fatty acids (VFA)
Heat pretreatment
Microbial electrochemical technology

ABSTRACT

Microbial electrosynthesis is an emerging strategy of transforming CO_2 into valuable chemicals by exploiting the capabilities of bacteria. As biocatalyst is considered as prime factor in bioelectrochemical CO_2 reduction, we used an enrichment technique comprising of heat pretreatment of parent inoculum followed by enrichment under gas mixture of H_2 and CO_2. Here, we use a double chambered bioelectrochemical system (BES_H) wherein cathode chamber was inoculated with enriched acetogenic (homoacetogenic) bacteria, subsequently polarized with voltage of −0.8 V vs Ag/AgCl (s). BES_H operation resulted in synthesis of carboxylic acids/volatile fatty acids (VFA), with major proportion confined to production of acetic acid (12.57 mM) with a maximum current density of 650 ± 50 mA/m^2 generated in response to applied voltage. While in the previous study, BES_C (untreated) depicted comparatively low yield of acetic acid (3.19 mM). A maximum of 67% reduction in bicarbonate concentration and columbic efficiency of 38% was noticed signifying the utilization of inorganic carbon for bio-electrochemical synthesis of acetic acid. Efficiency of BES was also discussed in terms of energy discharge. Few signals were obtained on voltammetric signature at various potentials which might correspond to the redox potential of cytochromes, flavo proteins and Fe-S proteins suggesting their involvement as electron carriers during microbial electrosynthesis. Phylogenetic analysis was comparatively made between untreated and heat treated enriched bacteria which revealed members belonging to *Clostridiaceae* dominant in heat treated inoculum, while methanogens appeared in untreated inoculum. Selective enrichment of biocatalyst proves to be advantageous for CO_2 reduction in BES which yielded high productivity of carboxylic acids in comparison to untreated biocatalyst.

1. Introduction

Although CO_2 is considered as a green house gas, the potential benefits offered by CO_2 are driving the research towards using it as a valuable resource [1–3]. Microbial electrosynthesis (MES) research finds the utilization of CO_2 as substrate to synthesize multi carbon organic chemicals [4]. MES or bioelectrochemical system (BES) uses biologically driven electrode potential as a source of reducing equivalents replacing the external supply of hydrogen [1,5–8]. BES comprises of various components viz., biocatalyst, electrode materials, reactor configuration, membrane etc. which play a key role in determining its performance. Among all these components, microbial community/biocatalyst can be considered as corner stone in BES, as they promote biochemical reactions for utilizing CO_2 to synthesize multi-carbon organic chemicals.

Studies were carried out using pure/single strain as well as mixed microbial population in BES [6,9–21]. However, adequate knowledge is required in understanding the mixed microbial community and its interactions towards electro-synthesizing green chemicals. A special group of bacteria is able to sequester or uptake CO_2 anaerobically and chemolithoautotrophically which thereby synthesizes value added chemicals/products with hydrogen as an electron donor [5]. Enrichment of specific group of bacteria for utilizing CO_2 as substrate for high yield of desired products is therefore of high importance in microbial electrosynthesis. Homoacetogenic bacteria are the notable bacteria to date in diverse MES experiments for their ability in fixing CO_2 [9,11,19,22,23].

Pretreatment of microbial population is a key step in developing a homoacetogenic bacterial community. Various methods of pretreatment generally suppress the growth of methanogenic bacteria that essentially utilize H_2 and CO_2 for their growth and synthesize methane [24,25]. Choice of pretreatment method need to be optimized to validate the sustenance of methanogenic inhibitory effect on mixed bacterial community. The bacterial population resulted after pretreat-

* Corresponding author.
E-mail address: vmohan_s@yahoo.com (S.V. Mohan).
[1] Academy of Scientific and Innovative Research (AcSIR).

http://dx.doi.org/10.1016/j.jcou.2017.05.011
Received 9 March 2017; Received in revised form 8 May 2017; Accepted 12 May 2017
Available online 01 June 2017

ment will be selectively enriched where various procedures exist to grow and develop a homoacetogenic bacterial community by subjecting to a gas mixture of H_2 and CO_2 [26–30]. After successful growth in anaerobic conditions, the enriched culture will be used as biocatalyst at cathode. The most studied metabolic pathway for CO_2 utilization is the Wood-Ljungdahl/acetyl-CoA pathway where homoacetogens (acetogens) would synthesize acetate through a cascade of steps [31,32]. Members of *Clostridiaceae* are popular acetogens that aid in the synthesis of carboxylic acids, in which acetate production is dominated. Acetogens are versatile and are capable of growing at mesophilic, thermophilic and psychrophilic range of temperature conditions [29]. Some of them have flagella, spore formation capability and can be either Gram negative or Gram positive. Exploration of various bacteria is needed which would bring a dramatic change in the performance of BES/MES in fixing CO_2 for the synthesis of multi-organic chemicals.

Keeping in view about the role of microbiome in BES system, we designed the present study by choosing heat pretreatment for enriching acetogens and suppressing methanogenic bacteria for bioelectrochemical reduction of CO_2 towards synthesis of multi-carbon organic acids. Bacterial community structure is identified for pre and post enrichment inoculum and is comparatively analyzed with control BES operation (previous study, [9]). Bio-electrochemical circuitry responsible for electron transfer and potential of BES in terms of energy discharge is also studied and discussed.

2. Materials and methods

2.1. Biocatalyst

2.1.1. Selective enrichment of homoacetogenic bacteria (biocathode)

Selective enrichment of homoacetogenic bacteria was carried out in two stages. During stage-I, anaerobic consortia acquired originally from full scale anaerobic bioreactor treating wastewater was used as parent inoculum. This was subjected to heat pretreatment by heating at 80 °C for 2 h to enrich acidogenic bacteria and to suppress non-spore forming hydrogen consuming methanogenic bacteria coexisting in bacterial consortia [25,33]. Methanogenic bacteria, if present would redirect the metabolic pathway towards methane production upon consuming most of the available reducing equivalents. During stage-II, enrichment of homoacetogens was conducted by growing the bacteria resulted from stage-I in serum bottles (200 ml) filled with 120 ml of designed synthetic wastewater (DSW: KH_2PO_4: 0.25 g/l, K_2HPO_4: 0.25 g/l, NH_4Cl: 0.5 g/l, $MgCl_2$: 0.3 g/l, $CoCl_2$: 25 mg/l, $ZnCl_2$: 11.5 mg/l, $CuCl_2$: 10.5 mg/l, $CaCl_2$: 5 mg/l, $MnCl_2$: 15 mg/l, $NiSO_4$: 0.16 g/l, $FeCl_3$: 0.03 g/l, yeast extract: 0.5 g/l) and subjecting to a head space gas mixture of CO_2:H_2 (20:80) [9]. Addition of yeast extract exerts a stimulatory effect on the growth of autotrophic acetogens subjected to CO_2 and H_2 environment [28]. Bacteria were grown by incubating at 30 °C in rotary shakers maintained at 100 rpm.

2.1.2. Anodic biocatalyst

Anode chamber of BES was inoculated with anaerobic untreated parent inoculum as biocatalyst enriched in DSW excluding yeast extract. As a source of carbon, 1.5 g/l of glucose was supplemented for the growth and metabolism of bacteria.

2.2. Design of bioelectrochemical system

A double chambered BES was designed and fabricated using Schott-Duran glass bottles with a total/working volume of 2.5/2.0 l [9]. BES consisted of an anode and a cathode chamber separated by a proton exchange membrane (PEM: Nafion 117, Sigma–Aldrich). Prior to use, NAFION 117 was pretreated by boiling sequentially in 30% H_2O_2, followed by deionized water (pH 7.0), 0.5 M H_2SO_4 and deionized water each for 1 h to increase the porosity and activate the sulfonated groups responsible for proton transfer. Non-catalyzed graphite plates were used as electrodes at both anode and cathode chambers to aid surface catalyzed microbial electron transfer reactions. Prior to use, graphite electrodes were pretreated using 0.1 N HCl to eliminate impurities and enable the active reactive sites on electrode surface. Copper wires were used to establish contact with electrodes as current collectors. The electrodes were completely submerged in anolyte and catholyte respectively and mixing was enabled with the aid of magnetic stirrers. Proper leak proof sealing was employed to ensure anaerobic environment at both the chambers.

2.3. Operation

A two phase experimental methodology was employed for the bioelectrochemical reduction of CO_2 to carboxylic acids using BES. During phase-I, selective enrichment of homoacetogenic bacteria was carried out and was subsequently used as cathodic biocatalyst. Experimental operation at phase-II was carried out in BES using enriched biocatalyst at cathode and parent inoculum at anode chamber respectively. BES was operated chronoamperometrically with a polarized potential of −0.8 V (vs Ag/AgCl (S)) using a potentiostat-galvanostat system (Biologic VMP3). pH of catholyte was set to 8.5 using 1 N HCl/NaOH. Uniform distribution of carbon source was ensured by allowing mixing of catholyte with the aid of magnetic stirrer. CO_2 as sole carbon source was supplemented in the form of sodium bicarbonate to the catholyte consisting of DSW. In the case of anode chamber, untreated parent inoculum was used as biocatalyst with glucose as carbon source. Initially, pH of the anolyte was set to 6 using 1 N HCl/NaOH. The reducing equivalents generated through the metabolic activity of anodic bacteria will be driven towards cathode with the aid of applied potential for the reduction of CO_2 into carboxylic acids. Drop in carboxylic acids concentration (acetic acid) and reductive catalytic currents were used as indicators for feed change. Experiment was carried out in fed batch mode with each cycle operated for a retention time of 72 h. The experimental results of BES_H (BES operated with heat treated and enriched inoculum) in present study is compared with BES_C (BES operated with untreated and enriched inoculum) reported during previous study [9]. Results during enrichment and phylogenetic analysis were discussed for BES_H, BES_B (BES operated with BESA treated and enriched inoculum), BES_C along with C_{En} (control inoculum which is untreated but enriched) and H_{En} (heat treated and enriched inoculum).

2.4. Bio-electrochemical analysis

The biochemical behavior of biocatalyst was studied at respective time intervals. Change in pH was monitored by pH meter. Carboxylic acids profile was analyzed using high performance liquid chromatography (HPLC; Shimadzu LC10A) employing UV-Vis detector (210 nm) and C18 reverse phase column (250 × 4.6 mm diameter; 5 µm particle size; flow-rate: 0.5 ml/h) with mobile phase of 40% acetonitrile in 1 mN H_2SO_4 (pH, 2.5–3.0). Twenty µl of filtered sample (0.22 µ porosity) was injected to column for analysis. Gas consumption during enrichment stage of experiments was monitored using Gas Chromatography (NUCON 5765) with thermal conductivity detector (TCD) and Heysep Q column (1/8" × 2 m) employing Argon as carrier gas. The injector and detector were maintained at 60 °C each and the oven was operated at 40 °C isothermally. Reduction in bicarbonate concentration was estimated according to standard methods [34]. Bio-electrochemical behavior of BES system in terms of reductive catalytic currents, energy discharge and charge in coulombs was analyzed through chronoamperometry (CA) technique using a potentiostat-galvanostat system (Biologic VMP3 model). In addition, variation in redox catalytic currents, electron discharge phenomenon and involvement of redox mediators as electron carriers was studied by performing cyclic voltammetry (CV) and derivative cyclic voltammetry (DCV) at a scan rate of 10 mV/s vs Ag/AgCl (S).

2.5. Microbial inventory

Bacterial dynamics of biocathodes along with selectively enriched bacteria with respect to parent inoculum was analyzed through PCR-DGGE by collecting the samples prior and post operation of BES reactors. Total genomic DNA was extracted using Soil Kit (Macherey-Nagel) following the manufacturer's protocol, and the V3 region of 16s rRNA coding gene [primer 341F, 50-CCTACGGGAGGCAGCAG-30; primer 517R, 50-ATTACCGCGGCTGCTGG-30; GC clamp added to primer is 40 bp clamp 50-GCCCGGGGCGCGC CCC GGGCGGGGCGG GGGCACGGGGGG-30] was amplified using 341F, 517R and 341 FGC primers, with 40 bp of GC repeats added to 5′ end of 341 FGC primer. PCR product was purified using Qiagen PCR purification kit and subjected to denaturing gradient analysis to evaluate the shifts/diversity in microbial community. A denaturing gradient of 40–60% was executed to separate the amplified 16S rDNA fragments by carrying out electrophoresis at a constant voltage of 80 V for 8 h at 60 °C. The gel images were captured using a Molecular Imager G: BOX EF System (Syngene International Ltd.). Selected bands from all the samples were excised with a sterile pipette tip. The excised gel was incubated over night at 4 °C in 50 µl sterile distilled water. DNA from these overnight incubated samples was used as template for PCR amplification using 341F and 517R primers. Amplified PCR products after purification (Fermentas) were sent for sequencing. All the 16S rDNA partial sequences were subjected to BLASTN to identify the nearest taxa. These sequences were further aligned with closest matches found in the GenBank database (http://www.ncbi.nlm.nih.gov/) using CLUSTAL X. Phylogenetic trees were constructed using neighbor-joining methods using MEGA6 software.

3. Results and discussion

3.1. Phase I- *Enrichment of homoacetogenic bacteria*

Fig. 1 illustrates the decrement in headspace gas represented in percentage as an average for ten cycles with each cycle comprising a total retention time of 60 h. A maximum of 57% H_2 and 62% CO_2 reduction was observed in H_{En} during enrichment which depicts the consumption of headspace gas for their metabolism towards acetogenesis (homoacetogenesis). Decrement in headspace gas was presumed to be an indicator for the enrichment of homoacetogens and the growth was determined by monitoring the gas consumption profiles with respect to time analyzed through gas chromatography. This enriched culture was subsequently used for microbial electro synthesis reaction

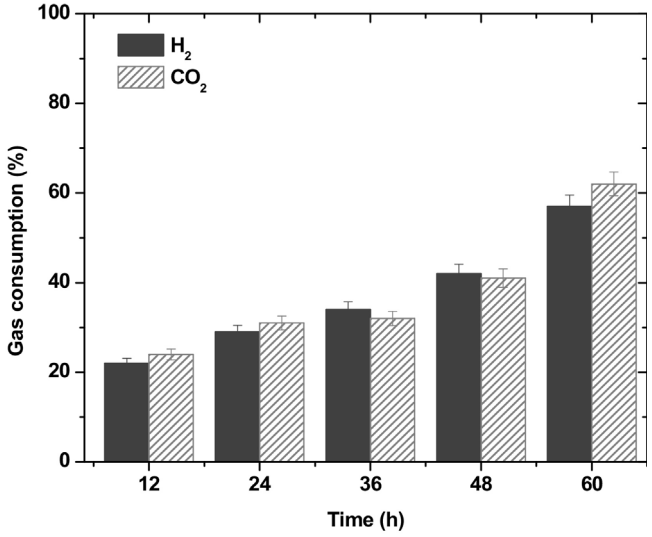

Fig. 1. Enrichment of homoacetogens depicted in terms of gas consumption.

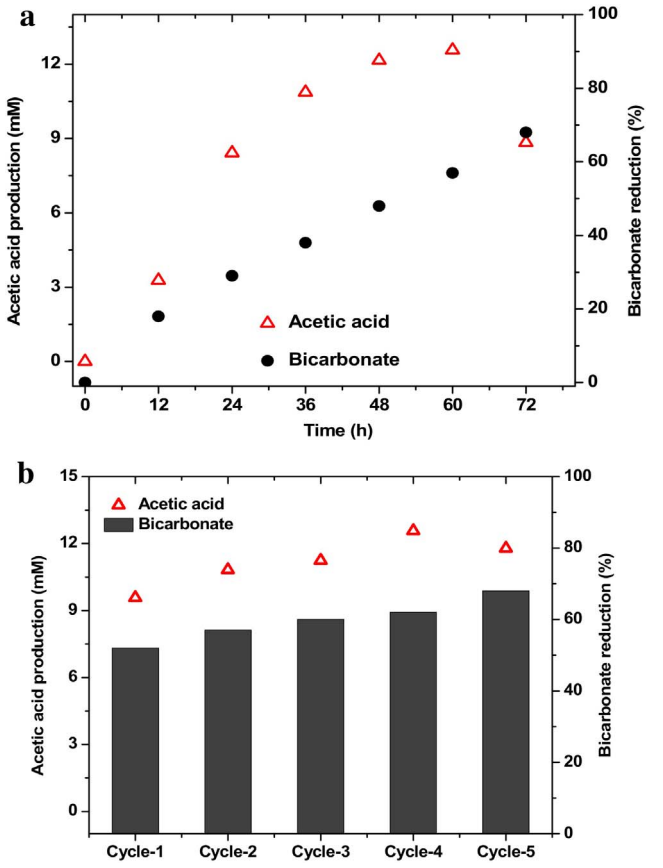

Fig. 2. (a) Bicarbonate reduction in percentage and acetic acid production represented in mM for single cycle operation (b) Maximum bicarbonate removal and acetic acid production represented for each fed batch cycle.

in cathode chamber. In the case of C_{En}, a maximum consumption of 58% H_2 and 49% CO_2 was noticed [9]. Marginal variation observed in gas consumption profiles of H_{En} and C_{En} might be attributed to the favorable conditions for growth of methanogens and homoacetogens in the presence of H_2 and CO_2. The same was depicted in phylogenetic analysis, which showed the members related to homoacetogens in H_{En}, while C_{En} showed the presence of diverse bacteria along with methanogenic bacteria.

3.2. Phase II-microbial electrosynthesis

3.2.1. Acetic acid synthesis

Fig. 2 depicts maximum acetic acid productivity for five cycles of operation represented post optimization. BES_H resulted in a maximum acetic acid production of 12.57 mM (3.01 g/l) during microbial electrosynthesis reaction catalyzed by enriched biocatalyst. While in the case of BES_C (0.9 g/l), comparatively low productivity of acetic acid was noticed than BES_H [9]. This might be due to the effective enrichment of biocatalyst (homoacetogens), which direct the reducing equivalents towards acetic acid production rather than methane production. Opting for alkaline pH and inoculum pretreatment lead to the production of desirable acetic acid and not methane [35]. Fig. 2a represents the acetic acid productivity with respect to time for one cycle (taken as average for 5 cycles). Acetic acid production was initiated from 12 h of operation and was observed to increase gradually till 60 h followed by a decrement at 72 h of operation. This might be ascribed to the bio-electrochemical reaction aided by biocatalyst towards utilization of bicarbonate for synthesis of acetic acid. A decrement observed in acetic acid concentration can be attributed to the consumption of acetic acid as carbon source by other bacteria co-existing in mixed population

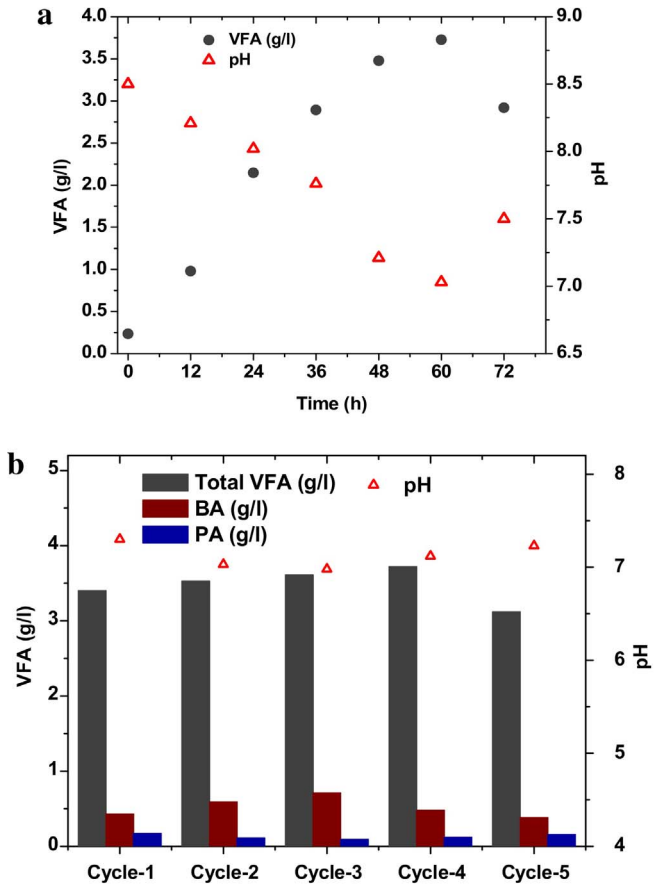

Fig. 3. (a) Change in pH and volatile fatty acid (VFA)/carboxylic acids generation during single cycle operation (b) Maximum total VFA production, pH drop and other VFA production represented for each fed batch cycle.

along with the enriched homoacetogenic bacteria.

3.2.2. Total carboxylic acids

Although the biocatalyst used at biocathode is enriched homoacetogenic bacteria, study depicted the bio-electrochemical synthesis of a mixture of carboxylic acids upon CO_2/bicarbonate reduction. Fig. 3 illustrates the synthesis of a mixture of carboxylic acids during BES_H operation. A gradual increment in concentration of carboxylic acids was noticed till 60 h followed by a decrement at 72 h. The decrement in carboxylic acids concentration might be due to the utilization of C2–C4 compounds as carbon source by other bacteria co-existing in mixed population or utilization for the synthesis of other compounds viz., alcohols (not analyzed during the study). A total of 3.72 g/l of carboxylic acids were bioelectrochemically synthesized by utilizing bicarbonate as sole carbon source along with an applied potential acting as driving force for directing the reducing equivalents. On the contrary, BES_C (control) synthesized a total of 1.2 g/l carboxylic acids which is comparatively lower than BES_H, depicting the role of enriched biocatalyst as an effective inducer towards high productivity of carboxylic acids. Among the mixture of carboxylic acids synthesized in BES_H, acetic acid concentration was high followed by butyric and propionic acids. This is in congruence with the enrichment adopted during the study, wherein the major population of bacteria was homoacetogens which would specifically direct the metabolism towards acetic acid synthesis by utilizing CO_2/bicarbonate as carbon source. A total of 0.62 g/l of butyric acid and 0.09 g/l of propionic acid were synthesized during BES_H operation. Production of butyric (C4) and propionic acids (C3) might have been either due to the activity of other bacteria capable of synthesizing butyrate/propionate or the elongation of C2 compounds (acetic acid) towards C3 and C4 compounds. It is also to be noted that the presence of bicarbonate enhances VFA synthesis during anaerobic conditions by maintaining optimal pH, as bicarbonate acts as a buffering agent as well [36]. Synthesis of C2–C4 compounds through C1 (CO_2/bicarbonate) reduction seemed to be advantageous which would aid in reducing CO_2 concentration as well as synthesizing high value chemicals in a bio-electrochemical route.

3.2.3. Bicarbonate reduction

The sole carbon source used during the study is sodium bicarbonate which will be utilized chemoautotrophically by the enriched homoacetogenic bacteria [26]. Initially 20 mM of bicarbonate was supplemented and the concentration was observed to decrease with the increase in time for each cycle operation. Samples were collected at regular time intervals for each cycle operation to monitor the changes in bicarbonate concentration. Fig. 2 represents the increase in bicarbonate reduction percentage for five cycles along with the decrement in concentration at regular time intervals during one cycle operation. A maximum of 68% reduction in bicarbonate concentration was depicted during BES_H operation which signifies the utilization of bicarbonate as inorganic carbon substrate by biocatalyst towards microbial electrosynthesis of carboxylic acids. While in the case of BES_C (control), bicarbonate reduction was only 28% which is relatively lower than BES_H. This might be attributed to the well enriched chemolithoautotrophic homoacetogenic bacteria in utilizing bicarbonate in BES_H. Besides this, bicarbonate reduction was also monitored with respect to time in BES_H, which was observed to increase gradually till the end of cycle. Prevalence of alkaline range of pH also favored dissolution of bicarbonate in catholyte which in turn aided in the utilization of bicarbonate for carboxylic acids synthesis [9]. Bicarbonate also acts as electron acceptor which seemed to be reduced bioelectrochemically towards the targeted carboxylic acids synthesis in the present study.

3.2.4. pH

pH determines the redox behavior of system, since the metabolism of biocatalyst is regulated by variation in pH. Initially BES_H was operated at alkaline pH of 8.5 and the variation in pH was monitored at regular time intervals (Fig. 3). pH was observed to decrease gradually with time till 60 h. Thereafter, an increment in pH was noticed at 72 h. This variation in pH is in congruence with the bio-electrochemical synthesis of carboxylic acids. Increment in pH noticed after 60 h is attributed to the utilization of carboxylic acids as substrate by bacteria, which causes a rise in extracellular pH in BES_H system environment [35]. A maximum drop in pH was observed in BES_H during cycle-3 which is noticed to be 6.98, However, variation in pH is not below 6.98 (nearly 7) in BES_H maintaining a buffered environment for the production of carboxylic acids. Optimal pH regulation in the presence of bicarbonate, which acts as a buffering agent might have contributed for bio-electrosynthesis of carboxylic acids maintaining a buffered environment. On the contrary, less pH drop (7.82) was observed in BES_C, which is in correlation with low yield of carboxylic acids and less reduction in bicarbonate concentration.

3.3. Chronoamperometric currents generation

A polarized voltage of −0.8 V (vs Ag/AgCl (S)) was chronoamperometrically employed on working electrode (biocathode) of BES to evaluate the generation of reductive catalytic currents (RC) in response to applied potential. RC were recorded for every 300 s during the polarized conditions. Fig. 4a represents the evolution of current density (CD) which is observed to increase with each additional cycle operation. The current density profiles reported in Fig. 4 are drawn post optimization of BES_H operated for atleast 10 cycles. Initially, CD was observed to be $-250\ mA/m^2$ which successively progressed to $-600\ mA/m^2$ by second cycle and remained more or less similar for the next consecutive cycles. This shows the acclimatized nature of the enriched biocatalyst in synthesizing carboxylic acids inclined with the

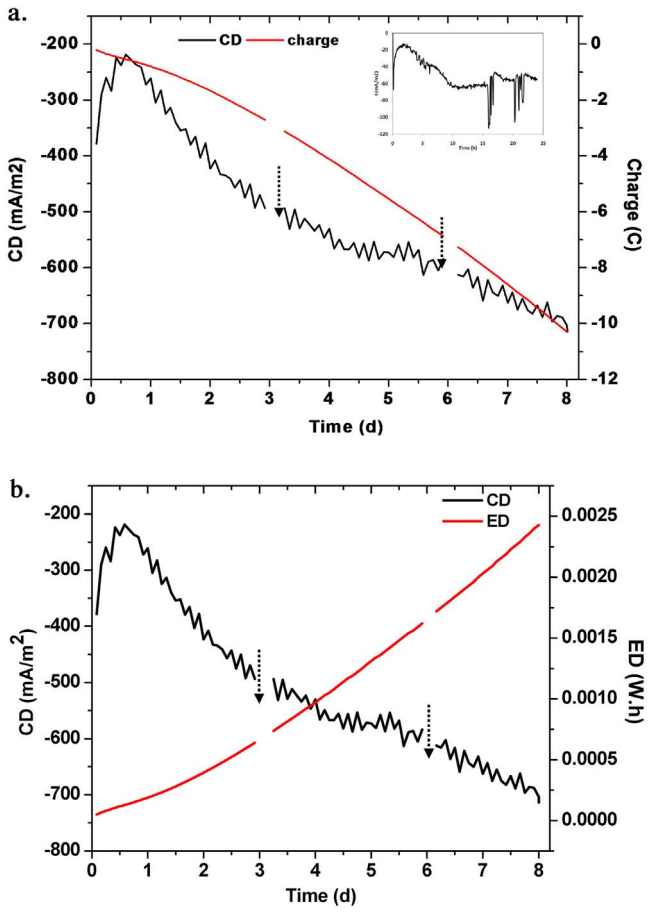

Fig. 4. (a) Chronoamperometry profiles illustrating the response current generation and charge in coulombs derived at an applied voltage of −0.8 V vs Ag/AgCl (s). Inset figure indicates the current generation profile over a period of 24 h (Arrow mark indicates feed change) (b) Chronoamperometry profiles illustrating the current generation and energy generation in the form of energy discharge (ED) in W.h derived at an applied voltage of −0.8 V vs Ag/AgCl (s).

polarized potential, nutrient medium and pH conditions. Thereafter, a maximum CD of -720 mA/m^2 was noticed during third cycle. Successive increment in current density during BES operation is an indicator of increased biofilm formation on electrode surface that catalyzes the bioelectrochemical CO_2 reduction towards more yield of carboxylic acids electrotrophically. Whereas operation of $BES_{C,}$ was able to generate a maximum of -250 ± 30 mA/m^2 of RC which is comparatively lower than BES_H. This is in congruence with the enriched bacterial community in BES_H that would accept the reducing equivalents via surface catalyzed bioelectrochemical CO_2 reduction. While in the case of $BES_{C,}$ microbiome community was different which might utilize the reducing equivalents towards undesirable metabolites synthesis or for interspecies electron transfer among the diverse microbiome. Besides CA analysis, columbic efficiency (CE) was calculated for BES_H and was found to be 38% higher than BES_C (15%). This is in congruence with bicarbonate reduction and utilization of reducing equivalents towards the synthesis of acetic acid.

3.3.1. Electron storage

In addition to CD evolution, the amount of charge which depicts the number of electrons passed during bioelectrochemical reduction reactions was monitored [37]. The observed charge profiles depict the amount of coulombs utilized during bio-electrochemical CO_2 reduction towards carboxylic acids synthesis. A progressive increment in the amount of electrons utilized was observed in BES_H which was −3 C during first cycle, which increased up to −10 C during third cycle. On the contrary, BES_C depicted a maximum charge of −3 C during the complete bioelectrochemical reduction reaction. The observed results are in congruence with bicarbonate reduction and acetic acid productivity, which indicates the bacterial community of biofilm consisting of enriched biocatalyst in BES_H in utilizing the reducing equivalents.

3.3.2. Energy discharge

More traditionally, BES was always discussed in terms of current density evolution and the number of electrons supplied during bioelectrochemical reduction reactions. However, BES operated either potentiostatically/galvanostatically can also generate power portraying the potential of bioelectrochemical circuitry. In terms of a chemical fuel cell/battery, energy discharge represents the amount of energy that can be discharged under polarized potential/current conditions [38]. Generally, the factor energy discharge is dependent on two parameters which are applied voltage and responsive current density [38]. In the case of BES, which involves bio-electrochemical circuitry, the effective capabilities of biocatalyst in addition to the aforementioned parameters will be considered to determine the efficiency of BES. Fig. 4b represents the energy discharge obtained during BES_H operation potentiostatically, which generates current in response to an applied voltage of −0.8 V (vs Ag/AgCl (S)). In addition, the energy discharge can also be evaluated by considering the input voltage (polarized reduction potential) and the response current generation. With the increase in current density in response to applied voltage, the energy discharge also increased progressively. A maximum of 0.0025 W h (equivalent to 9000 mJ/energy) was recorded during BES_H operation, while it was 0.0009 W h for BES_C. This shows the efficiency of BES in not only sequestering CO_2, but also in generating energy under an applied voltage of −0.8 V with a response current density. The efficiency of generating the bio-electrochemically derived power proves that BES posses inherent advantageous over conventional electrochemical techniques, which does not need regeneration of catalysts.

3.4. Bio-electrochemistry

Cyclic voltammetry (CV) analysis was performed for BES_H to elucidate the redox catalytic currents generation as well as the involvement of electron carriers in a scan window of −1.0 to +1.0 V at a scan rate of 10 mV/s. CV depicted simultaneous oxidation and reduction behavior where the redox catalytic currents were appeared to be −3.9 mA and 3.89 mA (Fig. 5). This might be due to the regulated transfer and acceptance of electrons at working electrode from counter electrode under the polarized environment. Capacitance noticed in the

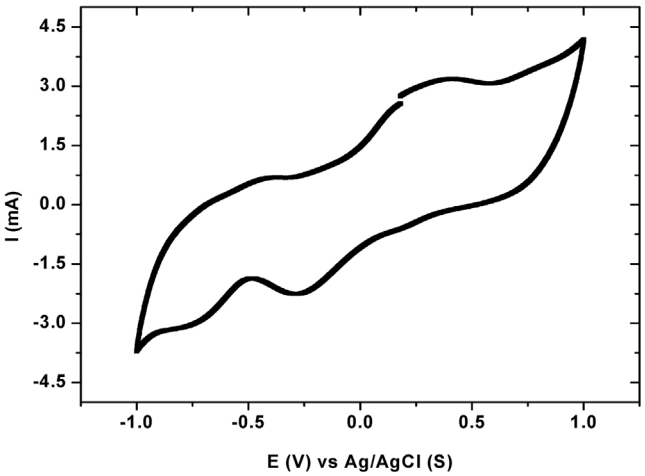

Fig. 5. Cyclic Voltammogram at a scan rate of 10 mV/s depicting the presence of electron carriers in the form of peaks in a scan window of $E_i = -1.0$ V and $E_f = 1.0$ V vs Ag/AgCl (S).

voltammogram might be attributed to the transient currents generated due to the electrochemical activity of electrode in addition to faradic activity by microbial catalyst. In addition, biofilm formed on electrode surface holds biochemical and electrochemical charge carriers within, that might contribute for capacitance and might be have functional advantageous as capacitive biocathode. A maximum exchange current density (ECD) of 0.2 A/m^2 per electrode surface was observed throughout oxidation and reduction reactions observed during CV analysis. This represents the number of electrons exchanged for carrying out oxidation and reduction reactions for the microbial electrosynthesis of carboxylic acids through CO_2 reduction. Two reversible peaks and a quasi reversible peak were observed during both oxidation and reduction scans depicting their role in electron transfer during oxidation and reduction. The peaks were observed at −0.261 V, −0.696 V, 0.29 V, −0.459 V, 0.59 V vs Ag/AgCl (S). The obtained peak potentials were tailored against standard redox potentials which will result in the identification of electron carriers participated during the bioelectrochemical reduction. The redox mediators detected at these potentials are identified to be cytochromes, flavo proteins, nitrates and cytochrome complexes. Cytochromes are the membrane bound proteins which are commonly associated with most kind of bacteria. These help in pumping the electrons out of the membrane to the outside of cell. Flavo proteins are generally flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) that involve in the electron transport. This is also often termed as electron transfer flavoprotein (ETF) which functions as a specific electron acceptor for dehydrogenases in transferring the electrons. The flavo proteins detected during CV analysis might have been responsible in shuttling electrons towards dehydrogenases involved during bioelectrochemical reduction reactions. In addition nitrates, an inorganic electron acceptor has been observed during CV, which might be attributed to the involvement of one of the simulated wastewater components that contains nitrogen/nitrates source within it. The observed redox peaks during voltammetric analysis elucidates the role of these complexes/molecules as electron carriers during the bioelectrochemical reduction reactions towards the synthesis of carboxylic acids. On the other hand, a quasi reversible peak was obtained for BES_C which might be ascribed to the presence of cytochrome, a membrane bound protein that acts as electron carrier.

3.4.1. Redox mediators

In order to unravel the unidentified/unnoticed peaks during voltammetric analysis, derivative voltammetry analysis was carried out [39]. Derivative cyclic voltammetry (DCV) depicted the presence of additional peaks apart from the redox mediators identified from CV analysis (Fig. 6). Most of the peaks were observed at negative potentials

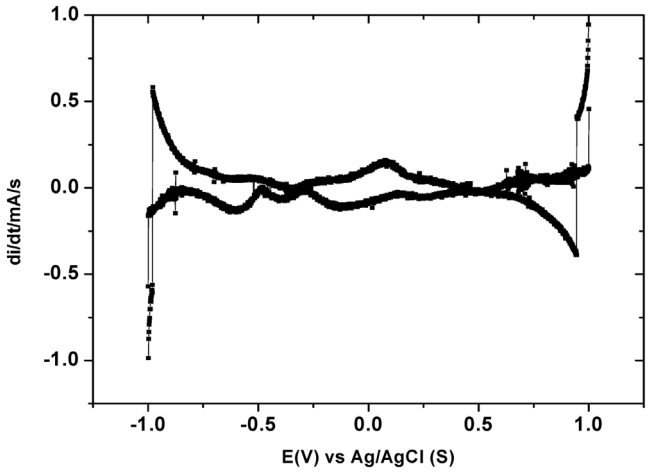

Fig. 6. First derivative voltammetry deduced from CV (scan rate: 10 mV/s) portraying the peaks as electron carriers in a scan window of E_i = −1.0 V and E_f = 1.0 V vs Ag/AgCl (S).

which represent the electron donating capacity, while the other peaks identified at positive potentials represent the electron acceptors. The fate of appearance/participation of any electron carrier depends on the difference in potential between the electron donor and electron acceptor. Redox mediators were observed at 0.075 V, 0.0166 V, − 0.520 V, −0.339 V and −0.373 V vs Ag/AgCl (S). These potentials were tailored with respect to standard hydrogen electrode (SHE) and are discussed. Peak potentials might correspond to the presence of cytochromes-C, NAD+/NADH, Ubiquinone and iron-sulfur proteins (Fe-S). Since the bacteria used is mixed culture and contains diversified population, detection of electron mediators cannot be confined to specific bacteria. The identified redox mediators were appeared similar to proteins/molecules/complexes that are being involved during electron transport. NAD+/NADH is the most widely known electron couple that donates two electrons during the bio-electrochemical reduction. In addition, ubiquinone is also a complex that transfers two electrons during the process by donating the electrons. Iron-sulfur proteins are the surface associated proteins characterized by iron-sulfur clusters that majorly participate in oxidation and reduction reactions during electron transport. Most of the Fe-S proteins act as subunits for many enzymes/metallo proteins viz., hydrogenases, dehydrogenases, oxido-reductases etc. which will aid in the electron transport and also regulation their expression. The Fe-S proteins detected during DCV also might have played a pivotal role in assisting NAD+/NADH couple, a class of dehydrognases responsible for reduction reactions.

3.5. Phylogenetic analysis

To elucidate the shifts/changes in bacterial community prior to and after operation in BES, phylogenetic analysis was performed for parent inoculum (UN C) along with enriched control inoculum (UN C_{En}) and enriched control inoculum operated in BES (UN BES_C). This was compared with heat treated enriched inoculum (H_{En}) and heat treated enriched inoculum operated in BES (BES_H) (Fig. 7). Parent inoculum which was untreated and acquired from anaerobic wastewater treatment plant showed members belonging to Phyla *Proteobacteria, Bacteroides*, *Firmicutes* and other uncultured bacteria. Bacteria belonging to family *Bacilleacea, Clostridieacea*were dominant in phylum Firmicutes, while *methylobacterium* belonging to phylum Proteobacteria was also present. Since the culture was sampled from a wastewater treatment plant, mixed diversity was observed owing to the conditions prevailing in the treatment plant [40]. All these bacteria have diverse functions but only a few aid in the synthesis of carboxylic acids . This population was enriched under the headspace gas mixture of H_2 and CO_2. This enriched culture demonstrated the presence of bacteria belonging to *Methylobacterium* sp., uncultured type-II methylotrophs, uncultured bacteria and a few *Firmicutes* in UN C_{En}. Major portion of bacterial distribution was dominated by the presence of methanogens and uncultured bacteria. This is attributed to the prevalence of favorable conditions (H_2 and CO_2) for the enrichment of hydrogenotrophic methanogens that can readily utilize CO_2 in the presence of hydrogen as electron donor for methane formation [41–43]. As observed in UN C_{En}operation, acetic acid/carboxylic acids productivity was less in comparison to pretreated and enriched bacteria (H_{En}). This might be because of the presence of methanogenic population which tend to form methane rather than carboxylic acids synthesis [9]. These methanogens can also be considered as hydrogenotrophic methanogens which will utilize CO_2 in the presence of H_2 and synthesize methane.

In the case of UN BES_C which is untreated, enriched and operated at cathode in BES, bacterial community remained more or less similar to what was present during the enrichment (stable microbial community). However, a few shifts in population were identified showing the enrichment of members belonging to *Proteobacteria* and *Clostridiaecea* in UN BES_C. Presence of other methanogenic bacterial population remained similar as during enrichment. Members belonging to *Proteobacteria* and *Clostridiaecea* that were present in parent inoculum

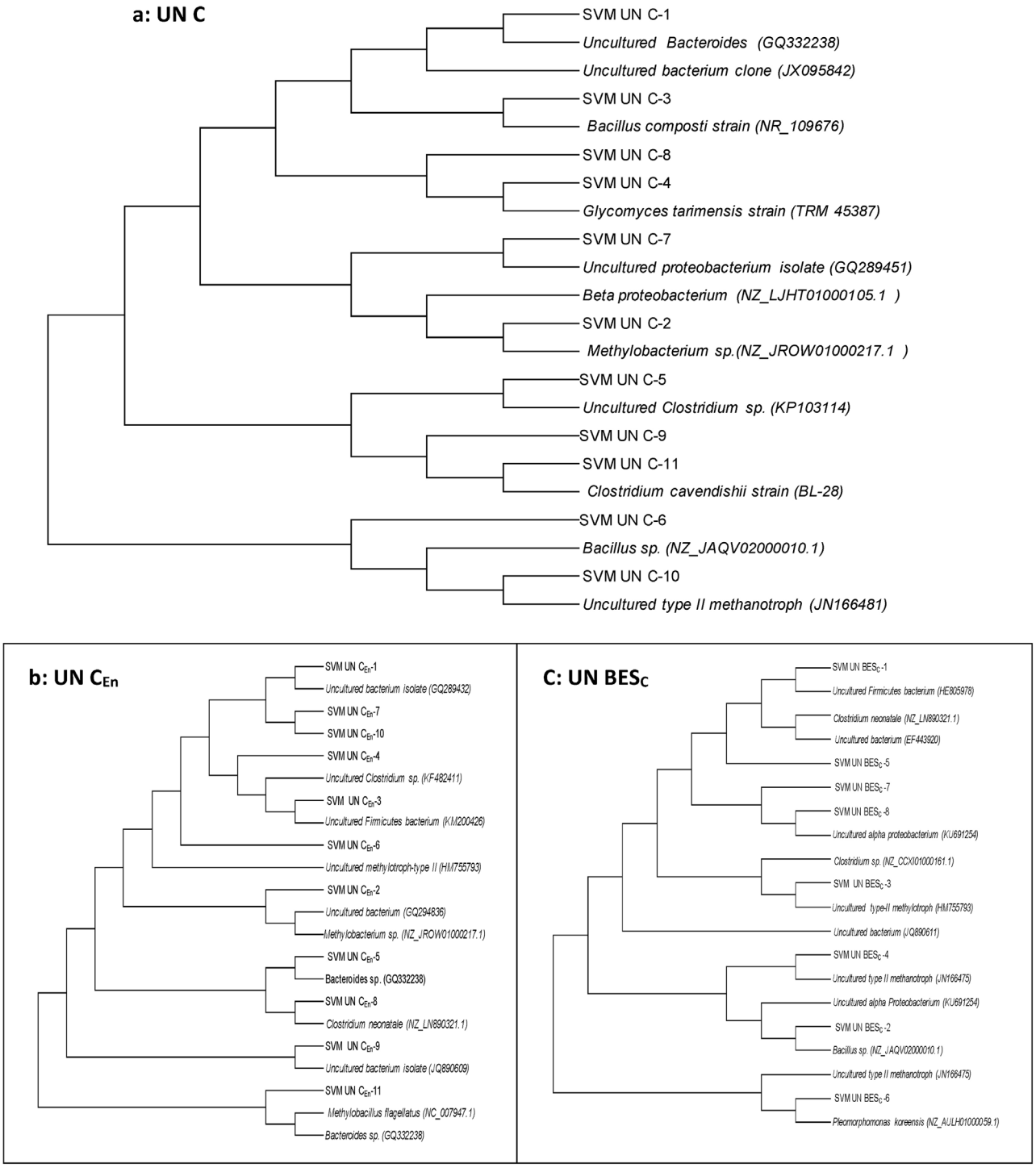

Fig. 7. Microbial community structure depicted in the form of Phylogenetic trees based on boot-strap neighbor joining method (a) UN C: Parent inoculum (b) UN C_{En}: Untreated control and enriched under H_2 and CO_2 atmosphere (c) UN BES_C: Control, enriched and operated in BES (d) H_{En}: Heat treated and enriched under H_2 and CO_2 atmosphere (e) BES_H: Heat treated, enriched and operated in BES.

and also UN BES_C, did not appear during enrichment. This shift in microbial population might be due to the prevalence of experimental conditions that would stimulate the enrichment of few bacteria and suppress other bacteria [44]. Since bacterial metabolism favor the pathway towards methane formation rather than acetic acid synthesis in the presence of H_2 and CO_2 (according to Gibbs free energy), the same was depicted during UN BES_C with major portion of reducing equivalents directed towards methane [45]. Carboxylic acids, more specifically acetic acid productivity was less in UN BES_C, which is in congruence with the microbial community structure where methanogens were found to be dominant that are responsible for utilizing electrons for methane production (Table 1).

On the contrary, phylogenetic analysis performed for heat pretreated culture (H_{En} and BES_H) demonstrated shifts in microbial community. Heat pretreatment suppress the growth of methane forming methanogens and enrich the acidogenic and acetogenic bacteria. $H_{En,}$ which depicts the heat treated and enriched bacteria under gas mixture of H_2 and CO_2, revealed the presence of members belonging to *Firmicutes,Proteobacteria* and uncultured bacteria. Various species of *Clostridiaecea* and *Bacilleacea* were enriched among the phylum

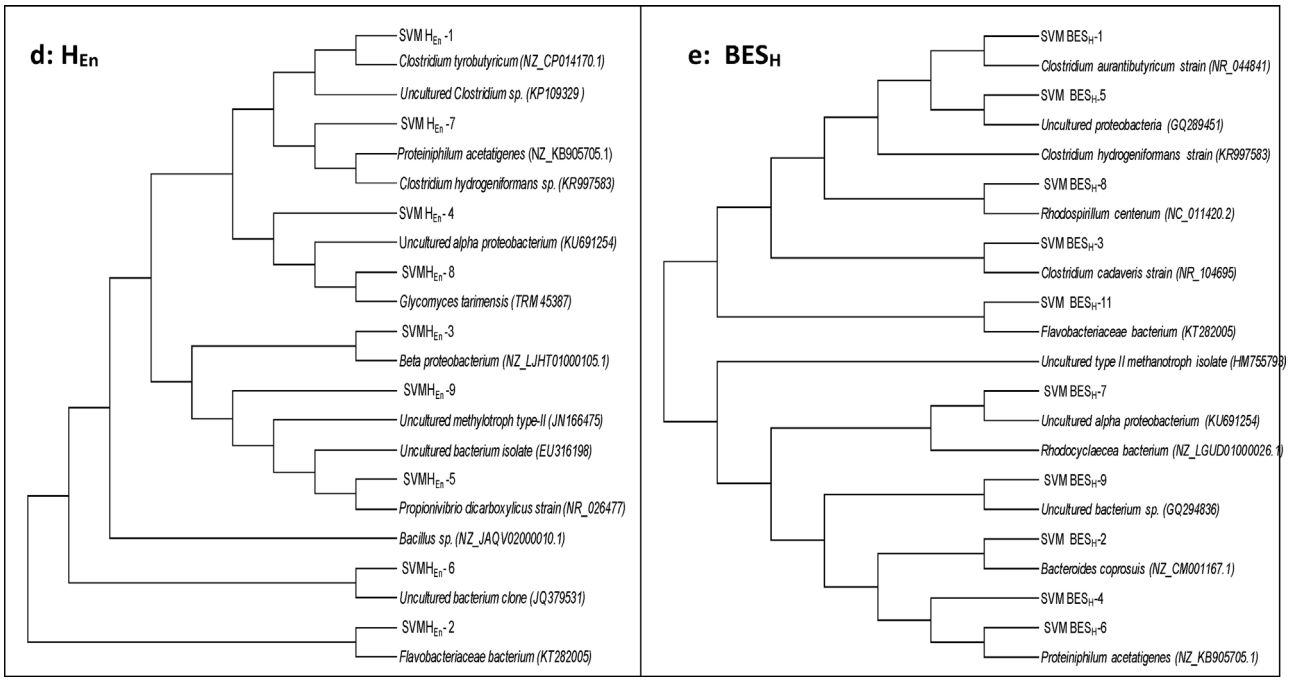

Fig. 7. (*continued*)

Table 1
Comparative data of BES_C, BES_B and BES_H representing the variation in acetic acid production with respect to other parameters.

Biocatalyst in BES operation ([a])	Maximum drop in pH	Maximum current density (mA/m^2)	Acetic acid productivity (mM)/(g/l)	Total carboxylic acids (g/l)	Other carboxylic acids (g/l)	Columbic efficiency (CE%)	Bicarbonate reduction (%)
BES_C	7.82	250 ± 30	3.19/0.9	1.2	0.3	15	28
BES_B	8.1	340 ± 20	8.01/2.1	3.5	1.4	24	35
BES_H	6.98	650 ± 50	12.57/3.01	3.72	0.71	38	67

[a] BES_C: Control operation where BES was operated without any pretreatment of inoculum. BES_B: BES operated with BESA treated inoculum [9]. BES_H: BES operated with heat treated inoculum.

Proteobacteria. Major proportion of bacteria was related to *Clostridiaecea* family comprising of *Clostridium hydrogeniformans*, *Clostridium tyrobutyricum* and *uncultured Clostridium sp*. These are known to be potential acetogens which would synthesize the carboxylic acids with acetic acid being the major fraction [46]. Besides *Clostridiaecea*, bacteria belonging to Phylum *Proteobacteria* viz., *Propionivibrio dicarboxylicus* was also identified which is a strict anaerobe capable of synthesizing propionic acid. A small fraction of propionic acid detected during BES operation, might have been due to the presence of this potential strain. *Proteiniphilum acetatigenes*, belonging to Phylum *Bacteroidetes* was also identified which can grow at pH 6.0-9.7 in the presence of yeast extract and anaerobic conditions [47]. The prevalence of favorable conditions for growth of *P. acetatigens* might have contributed for the enrichment and synthesize acetic acid. In the case of BES_H, members belonging to *Clostridium* were dominant followed by the presence of *Proteobacteria*, *Bacteroides* and *Flavobacteriaecea*. Members of *Clostridiaecea* are known to have a significant role in carboxylic acids synthesis, specifically acetate. The aforementioned microbial community composition was more or less similar to the bacterial population present during H_{En}. Interestingly, members of *Rhodospirillum* and *Rhodocycleacea* were enriched during BES_H operation. *Rhodospirillum* sp. is known to grow at diverse conditions viz., aerobic and anaerobic with CO_2 as carbon source [48].

Presence of applied potential, anaerobic conditions and CO_2 as carbon source might have stimulated the enrichment of *Rhodospirillum centenum*, a member belonging to photosynthetic group of organisms. Phylogenetic analysis revealed the enrichment of *Clostridiacea* members, which are known potential acetogens in the heat pretreated cultures responsible for the biosynthesis of acetic acid through bioelectrochemical CO_2 reduction. High carboxylic acids productivity and columbic efficiency are in congruence with the microbial population observed in BES_H. On the contrary, in control operation (untreated and enriched under headspace gas mixture of H_2 and CO_2) methylotrophs, also called hydrogenotrophic methanogens were the dominant population which would utilize the reducing powers more towards methane formation than carboxylic acids. This is also well correlated with the low columbic efficiency and carboxylic acids production in control operation.

3.6. Interphasial Chemistry of H_2, H_2O and CO_2

CO_2 containing a single carbon atom is electron poor with an oxidation state of IV. Also, oxygen of water molecule is electron rich and will donate an electron pair to carbon. Carbonic acid is formed upon transfer of proton from water to oxygen of CO_2 unit which will be in equilibrium with bicarbonate anion (Eq. 1–5). Reaction between water and dissolved carbon dioxide is reversible and rapid. CO_2, when dissolved in H_2O can exist in any of the three forms viz., carbonates (CO_3^{2-}), bicarbonates (HCO_3^-), carbonic acid (H_2CO_3) or CO_2 in gaseous or soluble form along with hydroxyl ions and other ions based on the pH range (Eq. 6–8) (Fig. 8).

$$CO_2\ (g) \leftrightarrow CO_2\ (l) \quad (1)$$

$$CO_2\ (l) + H_2O\ (l) \leftrightarrow H_2CO_3\ (l) \quad (2)$$

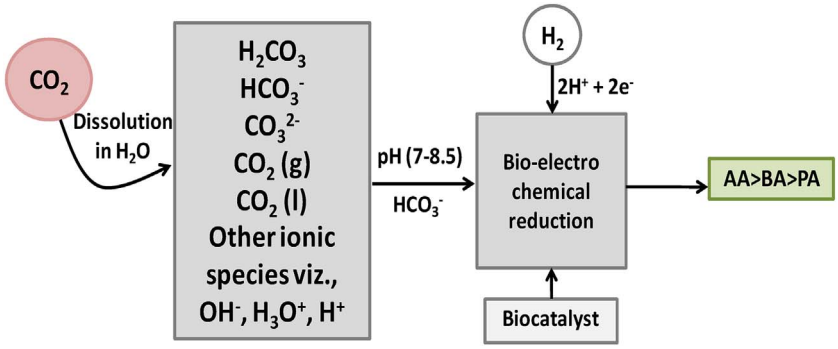

Fig. 8. The possible presence of various ionic species and the bioelectrochemical CO_2 reduction towards carboxylic acids synthesis.

$$H_2CO_3 + H_2O \leftrightarrow HCO^{3-} + H_3O^+ \quad (3)$$

$$HCO^{3-} + H_2O \leftrightarrow CO_3^{2-} + H_3O^+ \quad (4)$$

$$CO_3^{2-} + H_2O \leftrightarrow H_3O + + CO_2 \uparrow \quad (5)$$

More generally, the aforementioned species of CO_2 exists based on the pH range

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \text{ (acidic/low pH)} \quad (6)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \text{ (neutral to low alkaline pH)} \quad (7)$$

$$HCO_3 - \leftrightarrow CO_3^{2-} + H^+ \text{ (alkaline/high pH)} \quad (8)$$

In the present study, as near neutral to alkaline pH range was opted, bicarbonate is the most readily available form of CO_2, which acts as electron acceptor by accepting the electrons directly from electrode in BES.

H_2 can be represented as H_2 (g) or $2e^-$ and $2H^+$. In BES, H_2 is more commonly represented in the form of reducing equivalents (e− and H +). In a study on enzymatic electrosynthesis, CO_2 in carbonates form was found to be utilized as substrate for methanol synthesis at low acidic pH [49]. However, homoacetogenic bacteria, which can grow at diverse range of pH from slightly acidic to alkaline range, bicarbonate form of CO_2 is the predominantly available form as a substrate. Therefore, $HCO^{3-} + 8e^- + 8H^+ \rightarrow CH_3COOH$ is the required step for microbial electrosynthesis of acetic acid from CO_2.

Homoacetogenic bacteria employs Wood-Ljungdahl pathway, wherein CO_2/bicarbonate gets reduced to carbon monoxide (CO), by accepting two electrons/two protons [26]. The pathway initiates with the formation of CO, which follows Eastern/methyl branch and Western/carbonyl branch to finally synthesize 1 mole of acetic acid from 2 moles of CO_2/HCO_3^- [50]. Enzymes are involved at each step for the conversion of CO_2 to acetate via formate and other reduced intermediates.

4. Conclusions

This study demonstrates the potential of enriched microbial community to utilize CO_2 as substrate electrolithoautotrophically by using bioelectrochemical system. Heat pretreatment used as a strategy for selectively enriching homoacetogenic bacteria was proved to be efficient in the development of a microbial community dominantly comprising of members belonging to *Clostridiaecea*. BES_H resulted in the synthesis of mixture of carboxylic acids, with acetic acid (12.57 mM) being the major fraction, while it was comparatively lower in BES_C (3.19 mM), depicting the role of enriched bacterial community towards high yield of carboxylic acids in BES_H. A minor proportion of butyric (C4) and propionic acids (C3) were also observed during BES_H operation, which might be attributed to the existence of other bacterial population that are capable of synthesizing these C3 and C4 chemicals as depicted in phylogenetic analysis. Voltammetric analysis of BES_H depicted the involvement of NAD + /NADH, membrane bound proteins, Fe-S proteins, flavins etc. as electron carriers during bio-electrochemical reduction reactions. Energy discharge of 9000 mJ/energy was noticed in BES_H in response to chronoamperometrically polarized voltage of −0.8 V vs Ag/AgCl (S). Study unraveled the potential of BES in not only synthesizing the multi-carbon organic chemicals but also in generating energy by simultaneously minimizing the gaseous waste.

Acknowledgements

The authors wish to thank the Director, CSIR-IICT, Hyderabad for the support and encouragement in carrying out this work. Research was supported by Department of Biotechnology (DBT), Government of India as National Bioscience Award (BT/HRD/NBA/34/01/2012(vi)) and CSIR-XII network project (SETCA, CSC-0113). J.A.M duly acknowledge CSIR for providing fellowship.

References

[1] S.V. Mohan, J.A. Modestra, K. Amulya, S.K. Butti, G. Velvizhi, Trends Biotechnol. 34 (2016) 506–519.
[2] R.C. Franca, A. Azapagic, J. CO_2 Util. 9 (2015) 82–102.
[3] S.V. Mohan, S.K. Butti, K. Amulya, S. Dahiya, J.A. Modestra, Trends Biotechnol. 34 (2016) 852–855.
[4] T. Ueki, K.P. Nevin, T.L. Woodard, D.R. Lovley, mbio.asm.org. 5 (2014) 14–1636.
[5] S.V. Mohan, G. Velvizhi, J.A. Modestra, S. Srikanth Ren, Sustain. Energy Rev. 40 (2013) 779–797.
[6] K.P. Nevin, T.L. Woodard, A.E. Franks, Z.M. Summers, D.R. Lovley, mBio 1 (2010) 00103–00110.
[7] K. Rabaey, R.A. Rozendal, Nat. Rev. Microbiol. 8 (2010) 706–716.
[8] L. Jourdin, Y. Lu, V. Flexer, J. Keller, S. Freguia, Chem. Electron Chem. 3 (2015) 581–591.
[9] J.A. Modestra, B. Navaneeth, S.V. Mohan, J. CO_2 Util. 10 (2015) 78–87.
[10] G. Mohanakrishna, J.S. Seelam, K. Vanbroekhoven, D. Pant, Faraday Discuss. 183 (2015) 445–462.
[11] S.A. Patil, J.B.A. Arends, I. Vanwonterghem, J.V. Meerbergen, K. Guo, G.W. Tyson, K. Rabaey, Environ. Sci. Technol. 49 (2015) 8833–8843.
[12] T. Zhang, H. Nie, T.S. Bain, H. Lu, M. Cui, O.L. Snoeyenbos-West, A.E. Franks, K.P. Nevin, T.P. Russell, D.R. Lovley, Energy Environ. Sci. 6 (2013) 217–224.
[13] C.W. Marshall, D.E. Ross, E.B. Fichot, R.S. Norman, H.D. May, Appl. Environ. Microbiol. 8 (2012) 8412–8420.
[14] C.W. Marshall, E.V. LaBelle, H.D. May, Curr. Opin. Biotechnol. 24 (2013) 391–397.
[15] H. Nie, T. Zhang, M. Cui, H. Lu, D.R. Lovley, T.P. Russell, Phys. Chem. Chem. Phys. 15 (2013) 14290–14294.
[16] S. Bajracharya, A. Heijne, X.D. Benetton, K. Vanbroekhoven, C.J.N. Buisman, D.P.B.T.B. Strik, D. Pant, Bioresour. Technol. 195 (2015) 14–24.
[17] M. Su, Y. Jiang, D.J. Li, Microbiol. Biotechnol. 23 (2013) 1140–1146.
[18] P.L. Tremblay, T. Zhang, Front. Microbiol. 6 (2015), http://dx.doi.org/10.3389/fmicb.2015.00201.
[19] P.L. Tremblay, D. Hoglund, A. Koza, I. Bonde, T. Zhang, Sci. Rep. 5 (2015) 16168, http://dx.doi.org/10.1038/srep16168,2015.
[20] R. Ganigue, S. Puig, P.B. Vilanova, M.D. Balaguer, J. Colprim, Chem. Comm. 51 (2015) 3235–3238.
[21] N. Xafenias, V. Mapelli, Int. J. Hydrogen Energy 39 (2014) 21864–21875.
[22] K.P. Nevin, S.A. Hensley, A.E. Franks, Z.M. Summers, J. Ou, T.L. Woodard,

O.L. Snoeyenbos-West, D.R. Lovley, Appl. Environ. Microbiol. 77 (2011) 2882–2886.
[23] L. Jourdin, T. Grieger, J. Monetti, V. Flexer, S. Freguia, Y. Lu, J. Chen, M. Romano, G.G. Wallace, J. Keller, Environ. Sci. Technol. 49 (2015) 13566–13574.
[24] S.V. Mohan, V.L. Babu, P.N. Sarma, Bioresour. Technol. 99 (2008) 59–67.
[25] S.V. Mohan, Int. J. Hydrogen Energy 34 (2009) 7460–7474.
[26] M. Leclerc, A. Bernalier, G. Donadille, M. Lelait, Anaerobe 3 (1997) 307–315.
[27] S.F. Lajoie, S. Bank, T.L. Miller, M.J. Wolin, App. Environ. Microbiol. (1988) 2723–2727.
[28] H.L. Drake, S.L. Daniel, C. Matthies, K. Kusel, Acetogenesis, in: H.L. Drake (Ed.), Chapman and Hall, New York, 1994, pp. 3–60.
[29] H.L. Drake, K. Kusel, C. Matthies, Prokaryotes 2 (2006) 354–420.
[30] J. Wiege, M. Braun, G. Gotlschalk, Curr. Microbiol. 5 (1981) 255–260.
[31] L.G. Ljungdahl, Annu. Rev. Microbiol. 40 (1986) 415–450.
[32] S.W. Ragsdale, E. Pierce, Biochim.Biophys. Acta Proteins Proteomics 1784 (2008) 1873–1898.
[33] R.K. Goud, O. Sarkar, S.V. Mohan, Int J. of Hyd. Energy 39 (2014) 7572–7586.
[34] APHA, 20th ed., American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA, 1998.
[35] S. Dahiya, O. Sarkar, Y.V. Swamy, S.V. Mohan, Bioresour. Technol. 182 (2015) 103–113.
[36] J.A. Rogers, C.L. Davis, J. Dairy Sci. 65 (1982) 944–952.
[37] K. Chandrasekhar, K. Amulya, S.V. Mohan, Waste Manage. 45 (2015) 57–65.
[38] W.I. Al Sadat, L.A. Archer, App. Sci. Eng. Sci. Adv. 2 (2016) e1600968.
[39] J.A. Modestra, S.V. Mohan, RSC Adv. 4 (2014) 34045–34055.
[40] G. Mohanakrishna, S.K. Butti, R.K. Goud, S.V. Mohan, Bioelectrochemistry 115 (2017) 11–18.
[41] T.L. Miller, M.J. Wolin, Arch. Microbiol. 131 (1982) 14–18.
[42] G.A. Weaver, J.A. Krause, T.L. Miller, M.J. Wolin, Gut 27 (1986) 698–704.
[43] S.D. Molenaar, P. Saha, A.R. Mol, T.H.J.A. Sleutels, A. Heijne, C.J.N. Buisman, Int. J. Mol. Sci. 18 (2017) 204.
[44] K.V. Krishna, S.V. Mohan, Biores. Technol. 213 (2016) 146–154.
[45] O.R. Kotsyurbenko, M.V. Glagolev, A.N. Nozhevnikova, R. Conrad, FEMS Microbiol. Ecol. 38 (2001) 153–159.
[46] K. Sugaya, D. Tuse, J.L. Jones, Biotechnol. Bioeng. 28 (1986) 678–683.
[47] S. Chen, X. Dong, Int. J. Syst. Evol. Microbiol. 55 (2005) 2257–2261.
[48] F.H. Yildiz, H. Gest, C.E. Bauer, J. Bacteriol. 173 (1991) 5502–5506.
[49] F.S. Baskaya, X. Zhao, M.C. Flickinger, P. Wang, Appl. Biochem. Biotechnol. 162 (2010) 391–398.
[50] S.V. Mohan, G.N. Nikhil, P. Chiranjeevi, C.N. Reddy, M.V. Rohit, A. Naresh Kumar, O. Sarkar, Biores. Technol. 215 (2016) 2–12.