Bio-electrochemical synthesis of commodity chemicals by autotrophic acetogens utilizing CO_2 for environmental remediation

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Bio-electrochemical synthesis (BES) is a technique in which electro-autotrophic bacteria such as *Clostridium ljungdahlii* utilize electric currents as an electron source from the cathode to reduce CO_2 to extracellular, multicarbon, exquisite products through autotrophic conversion. The BES of volatile fatty acids and alcohols directly from CO_2 is a sustainable alternative for non-renewable, petroleum-based polymer production. This conversion of CO_2 implies reduction of greenhouse gas emissions. The synthesis of heptanoic acid, heptanol, hexanoic acid and hexanol, for the first time, by *Clostridium ljungdahlii* was a remarkable achievement of BES. In our study, these microorganisms were cultivated on the cathode of a bio-electrochemical cell at −400 mV by a DC power supply at 37°C, pH 6.8, and was studied for both batch and continuous systems. Pre-enrichment of bio-cathode enhanced the electroactivity of cells and resulted in maximizing extracellular products in less time. The main aim of the research was to investigate the impact of low-cost substrate CO_2, and the longer cathode recovery range was due to bacterial reduction of CO_2 to multicarbon chemical commodities with electrons driven from the cathode. Reactor design was simplified for cost-effectiveness and to enhance energy efficiencies. The Columbic recovery of ethanoic acid, ethanol, ethyl butyrate, hexanoic acid, heptanoic acid and hexanol being in excess of 80% proved that BES was a remarkable technology.

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1. Introduction

Microbial electrosynthesis is a form of microbial electro-catalysis, a process where CO2 is reduced via electrons derived through electroactive biofilm on cathode. It totally relies on the innate capability of microbes for electron transport. It can be exploited in a bio-electrochemical system for current generation or to provide electricity to microorganisms for biofuels and biochemical production. A bio-electrochemical cell for all bio-electrochemical systems consists of anode for oxidation and cathode for reduction, and when these are microbially catalysed, they are termed as bio-anode and bio-cathode. Exoelectrogens in the bio-anode oxidize organic matter anaerobically, and discharge electrons, transferred through electron transport chain to the electrode. In the bio-cathode, electrotrophs capture electrons from the cathode for the reduction of CO_2, sulphate or nitrates to multicarbon organic compounds, the precursors of liquid transportation fuel (Fan *et al.* 2012; Sharma *et al.* 2014). Apart from energy generation, technical breakthroughs have extended the BES capability to catalyse the production of volatile fatty acids and alcohols at low cost as compared with other electrochemical processes (Rabaey *et al.* 2010). Conversion of greenhouse gas CO_2 to reduced dense organic molecules like fatty acids, transportation fuel and alcohols within the existing infrastructure is quite attractive (Li *et al.* 2012). Most acetogens are mesophilic bacteria with temperature range from 20 to 45°C, and 37°C is the optimal growth temperature for most of the strains. Production rates are explained by higher solubility of greenhouse gas at this optimal temperature. Production of volatile fatty acids occurs in the acidogenesis phase, and production of alcohols occurs usually when the metabolism shifts from acidogenesis to

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solventogenesis. Bacteria have the ability to reduce CO_2 and H_2 to volatile fatty acids by utilizing the reductive acetyl-CoA pathway and have recently been investigated for the conversion of CO_2 to various organic products in the BES system. Supplying hydrogen was unlikely to be practical because of its energy input and requirement for extensive catalysts. Recently, H_2 has been replaced by a bio-cathode with electron supply as the energy and electron source in a BES system. Electrochemical reduction of CO_2 to a diversity of organics has not been practical because of poor long-term stability of cathodes, sluggishness of CO_2 reduction, non-specificity of desirable products and high cost of cathode. More specific product formation was exercised by incorporating enzyme catalysts on the electrodes, but enzymatic reduction lasted only for matter of hours, which reflected the adsorption of enzymes to the electrode surface and its poor long-term stability (Nevin *et al.* 2010). The establishment of the bio-cathode utilized the set potential approaches along with the addition of hydrogen and chemicals. The first approach was electrical inversion of bio-anode supplied with hydrogen to achieve hydrogen-evolving bio-cathodes based on reversibility of hydrogenases. This approach required ferricyanide as catholyte and ferrocyanide as anolyte. Another inversion method was used for oxygen reduction based on the polarity of BES by utilizing a Potentiostat and by altering the exposure of the bio-film to oxygen and addition of acetate (Cheng *et al.* 2010). A similar method was developed to establish methanogenic bio-cathode by swapping the polarity of the anode and cathode by utilizing a stack of rotatable conductive disks (Cheng *et al.* 2011) which were one-half immersed in wastewater and one-half exposed to headspace gas (Rozendal *et al.* 2008). Recently sediment microbial fuel cell was developed for enrichment of anaerobic exoelectrogenic bacteria that select for electrotrophic bacteria by inversion of anode to bio-cathode (Pisciotta *et al.* 2012). All these previous methods were time-consuming, complicated and based on multistep procedures. A method was developed to use an anaerobic autotrophic bio-cathode centered on heterotrophic pre-augmentation.

The enrichment of bio-cathode for autotrophic electrotrophs for bio-fuel synthesis provides a simplified method to isolate bio-chemicals from different inoculum sources. Bacteria were heterotrophically grown first on glucose, fructose or glycerol, and after pre-enrichment and acclimation of the culture, carbon dioxide was delivered as the sole electron acceptor (Zaybak *et al.* 2013). Bio-cathode catalysis proved to be less expensive as compared to traditional cathodes, which cause toxicity, corrosion and denatured material (Lovley 2011). Microorganisms must be selected based on their ability to switch from heterotrophic to autotrophic metabolism. This mechanism may help comprehend the metabolic activities of various electron donor or acceptor microorganisms during the development of anaerobic specialized bio-cathode. Commercialization of microbial electrosynthesis is a challenge; in order to produce valuable fuels and other organic commodities, pure culture was employed because the diversity of autotrophic acetogens accepts electrons from negatively poised cathode for the reduction of carbon dioxide with high Columbic efficiencies. While mixed cultures primarily produce acetates and other complex metabolites which contribute towards microorganism's metabolism. Pure culture of *Clostridium ljungdahlii* attributed in the development of commercially viable BES system for plethora of products

Another trial was the reactor designing of the BES cell. As BES cells relied totally on the bio-cathode, and its potential was carefully controlled by a potentiostat as employed in initial studies by Zhang *et al.* (2013). Implementation of the potentiostat was to control the cathode potential and to avoid potential fluctuations that could damage the cells. It proved to be impractical because of its limited control in large-scale systems and was energy-intensive for a fixed potential (Rosenbaum and Henrich 2014). A DC power source was utilized to provide a potential difference between electrodes. The potentiostat was used to examine the electroactivity of the bio-film developed at the cathode for both experimental and control systems. These implementations were developed to simplify the reactor and maintenance of energy efficiencies. All modifications in this technique have provided a general approach and additional potential for electro-fuel production.

2. Materials and methods

2.1 *Bacterial strain media and growth conditions*

The bacterial strain used in this research was *Clostridium ljungdahlii* (obtained from DSMZ Deutsche Sammlung Mikroorganismen and Zellkulturen GmbH) endospore-forming motile-shaped gram-positive bacteria cultured in DSMZ medium 879 at 37°C (Nevin *et al.* 2011).

All chemicals used were of highest purity (Sigma Aldrich Chemicals Ltd.) and were used without any further purification. Cells were grown in the medium with composition NH_4Cl 1.0 g, yeast extract 1.0 g, NaCl 0.8 g, $MgSO_4.7H_2O$ 0.2 g, KCl 0.1 g, KH_2PO_4 0.1 g, $CaCl_2.2H_2O$ 0.02 g, $Na_2WO_4.2H_2O$ 0.20 mg and 5.0 g/50 mL fructose solution. The pH of the medium was adjusted to 5.9, and after that followed the addition of trace element, buffers and vitamin solution. All stock solutions were stored at 4°C. The medium was transferred to two serum bottles of 10 mL each, sealed with sterile butyl rubber stopper with 5 mL syringe. These septa are tightly fixed by sealing with aluminum crimp. The seed culture of about 2 mL was used for the inoculation of serum bottles. The serum bottles inoculated with *Clostridium ljungdahlii* were placed

in the incubator inside the anaerobic chamber. Fresh medium was added to serum bottles after every 24 h, and bacterial growth was examined under microscope with proper gram staining. Stock culture of *Clostridium ljungdahlii* was stored at −20°C in falcon tubes with 3:10 mL ratio of liquid broth and glycerol for preservation.

Clostridium ljungdahlii culture growth for all experiments was in modified growth medium by omitting fructose. The sole carbon source was a gas mixture of H_2/CO_2 (10:90). Medium and gas addition was done during regular interval of time, and cell growth was observed by gram staining.

2.2 *Bio-electrochemical reactor operation*

The bio-electrochemical reactor was fabricated with tubular polyacrylate chambers having 2 cm wall thickness. The chamber is partitioned by proton exchange membrane (PEM) with internal dimensions 12×8×12 cm^3 and of capacity 100 cm^3 each. In the cathode, a round carbon cloth and in the anode, a round stainless mesh plate each having 12 cm diameter were connected to the power supply. Inlets and outlets were provided for gas and medium circulation. The scale-up of the process in both batch and continuous system was carried out in explosion-proof anaerobic chamber. Anaerobically prepared sterile basal medium with fructose (80 mL) was added to each chamber. Cathode compartment was inoculated by pre-enriched culture only. The headspace was purged with H_2/CO_2 to help establish a bio-film on the cathode. The media were replaced several times to remove planktonic cells. The periodic removal of planktonic cells enhances the growth of bio-film on the cathode surface. Favorable conditions are provided in heterotrophic biomass growth phase to achieve high cell density. Microbial bio-film was developed on carbon cloth after 1 week of inoculation. The whole reactor operation was conducted in an anaerobic chamber (YQK-11 Jinan Unilab Instrument Co. Ltd). The microbial cell concentration in the cultured media was determined by optical density at 620 nm, based on the calibration curve of a UV spectrophotometer (UV-1602 BMS Biotechnology Medical services). The decrease in the absorbance per day indicated the deposition of bio-film at the cathode (Sharifzadeh *et al*. 2009).

2.3 *Experimental conditions for batch and continuous systems*

After the heterotrophic pre-enrichment and acclimation of bio-film at the cathode, 80 mL of the sterilized medium without fructose was transferred to anode and cathode compartments of the bio-electrochemical reactor. The catholyte consisted of an optimized general growth medium for autotrophic culture of *Clostridium ljungdahlii*. The cathode chamber was spurge with CO2 at regular interval of time, the sole carbon source during autotrophic metabolism. The DC power supply was connected to the bio-electrochemical reactor and the cathode was at −400 mV to provide electrons to bacteria instead of deriving them from hydrogen gas during microbial electrosynthesis process. The anaerobic chamber was continually gassed with N_2/CO_2 mixture to maintain anoxic conditions in the chamber. After one week of cultivation, 5 mL of the samples were collected at time intervals of 24 h and centrifuged for 2 min, and the supernatant was transferred to falcon tubes and freezed for GC-MS analysis (Nevin *et al.* 2010).

The batch system was switched to continuous system with peristaltic pump at the dilution rate of 0.3 mL/s. The current and total applied voltage were measured by a digital voltmeter. Gas phase was switched to CO_2 and consumption of electron was directly from the cathode for the reduction of CO_2, the only carbon source. The samples were collected in sterile falcon tubes for time intervals of 24 h for GC-MS analysis. Samples collected after the definite time intervals were centrifuged, and the supernatant was freezed in the refrigerator.

Control cells were employed with fresh medium and cell-free culture under the same set of conditions utilized for experimental cell. Samples were analysed in the absence of electroactive components by GC-MS.

2.4 *Cyclic voltammetry*

Voltammetry studies were employed to determine the redox potential of redox-active components, the electrochemical activity of microbial strains and to test the performance of cathode material with the potentiostat. Cyclic voltammetry (CV) can also be used to determine the electron shuttles or mediator produced by bacteria too.

The *Clostridium ljungdahlii* acceptance of electrons from the cathode through direct electron transfer was tested by CV with carbon cloth as the working electrode vs. the reference Ag/AgCl electrode. Voltammograms were recorded by applying a potential ramp at a scan rate of 10 mV/s over the range of +0.2 to −0.6 V to the working electrode. *Clostridium ljungdahlii* showed the significant electroactivity with definite redox peaks. When the *Clostridium ljungdahlii* strains were cultivated with graphite electrode at −400 mV, the culture showed the current consumption during the 48 h of cultivation, indicating the development of electroactive bio-film at the cathode (Modestra *et al*. 2015).

CV in the control cell was performed with fresh medium and cell-free suspension, where the supernatant was obtained by centrifuging cells and in the medium without microbes under the same set of conditions applied in the experimental cell. No redox reaction was observed with fresh medium and cell-free culture (Choi *et al.* 2014).

2.5 *Analytical method*

Organic products were analysed by headspace solid-phase micro-extraction (HS-SPME) followed by gas chromatography and mass spectrometry (GC-MS).

2.6 *Preparation of samples for GC-MS analysis*

Liquid broth samples were stored frozen at −16°C and 2 mL was pipetted out into 20 mL screw-cap vials with silicon/PTFE septa with 0.3 [μ]L of internal standard and 0.75 g of NaCl and mixed thoroughly. The pH was adjusted to 6.8 to 7 by 3 M HCl and 0.15 M NaOH.

2.7 *SPME procedure*

SPME device and fiber coating polydimethylsiloxane (100 μm polydimethyl siloxane/carboxene-coated fibre) was utilized for extraction and concentration of sample from liquid broth. For extraction, the solution was placed in 20 mL screw-cap vials with silicon/PTFE septa. 2 mL of sample solution employed for sorption was stirred at 1000 rpm with magnetic stirrer at 37°C for 20 min. After extraction, the fiber was desorbed directly to GC injector for 3 min. This was sufficient time for desorption of all anolyte studied and for reinserting the fiber after the run without any carry-over (Wejnerowska and Gaca 2008).

The quantification of organic products was performed by GC-MS analysis. The gas chromatogram (Agilent Technologies 789A US) was equipped with MS (5975C inert XLEI CI MSD detector), data acquisition system with computer software and capillary column Agilent J&W (HP INNOWAX) with internal diameter 0.32 mm and length 60 m. The column temperature was maintained at 70°C for 2 min, followed by ramping at rate of 4°C/min to 180°C maintained for 2 min and then at the rate of 20°C/min, and after that temperature was increased to 200°C for 3 min. The injector and detector temperatures were 250°C and 280°C respectively. The GC system was operated in split-less mode with helium as carrier gas at flow rate of 1.5 mL/min. The injection volume of the sample for analysis was 1 μL (Feng *et al.* 2008). MS was operated in full scan mode m/z 40–400. Ions for VFA detection were selected using mass spectra of standards generated in SCAN mode Banel and Zygmunt (2011).

2.8 *Calibration*

The calibration of standard solution was performed by GC-MS by following the same set of conditions described above. 2-Ethyl butyric acid was used as an internal standard and volatile acid mixture from (C2 to C7) as an external standard to enhance the accuracy and reproducibility. Calibration curve was made between known concentration of the standard volatile acid mixture and 0.3 mL of 2-ethylbutyric acid as an internal standard in each sample. The concentration of unknown sample was calculated by taking 2 mL of sample with 0.3 mL of internal standard and 0.75 g of NaCl from the calibration graph.

2.9 *Electron recovery against the current consumption*

It was difficult to measure carbon dioxide consumption because of the presence of high concentration of bicarbonates in the medium, but the calculation of electron balance was possible. Electrons appearing in the bio-products accounted for high proportion of electrons consumed by culture. The cultivation method for volatile fatty acids and alcohols formation with CO_2 are according to the following equation. The stoichiometric autotrophic growth of organic products were calculated from the chemical equation and substrate mole concentration by utilizing ideal gas law.

Electron distribution value and final products were calculated by converting each compound to e^- equivalent unit by multiplying e^- equivalent number per mole by mole of each compound.

$$2CO_2 + 8e^- + 8H^+ \rightarrow C_2H_4O_2 + 2H_2O$$

For ethanoic acid, ethyl butyrate, hexanoic acid, heptanoic acid, ethanol, hexanol and heptanol, the e^- equivalent number per mole are 8, 32, 32, 38, 12, 36 and 42 respectively.

Clostridium ljungdahlii captured electrons from the biocathode and reduce carbon dioxide to extracellular volatile fatty acids and alcohols rather than bio-mass formation. Electron recovery was found for both batch and continuous systems by comparing the Coulombic recovery of organic products to the total electrons consumption:

$$n_{CE} = Idt \Big/ xF$$

Where x is the number of moles of electron per mole of compound, F is the Faraday's constant (96485 C mol^{-1}), n_{CE} is the number of electrons recovered in product formation, and I is the current.

The percent cathode recovery was calculated as follows:

$$R_{Cat} = n_{product} \times 100 \Big/ n_{CE}$$

Electrons recovery against the current consumption in continuous system for continuous flow through system is as follows:

$$n_{CE} = Idt \Big/ xFq\Delta S$$

where q is the flow rate of substrate in mL/s and S is the change in the concentration of substrate in g/L (Jaeel *et al.* 2013) and $n_{product}$ is the total moles of product recovered. Electron recovery for both batch and continuous systems were compared after the bio-film development at the cathode (Logan and Bruce 2007; Sleutels *et al.* 2011).

Electrons appearing in the volatile acid and alcohol formation accounted for the high proportion of electrons consumed by the culture. The total amount of the current consumed by system was calculated by integrating the current (A/m^2) against time (s).

The amount of current measured by the potentiostat for *Clostridium ljungdahlii* against −400mV applied potential was 0.0848 A/m^2. Comparative studies of batch and continuous systems were expressed graphically.

3. Results and discussion

3.1 *Gas chromatography mass spectrometry analysis*

The structure and mole fractions of organic acids and other compounds were determined by GC-MS based on peak area of ions. The major compounds were volatile fatty acids and alcohols. The molecular mass, retention time and structural formula of these volatile acids and alcohols are presented in the table 1.

3.2 *Gas chromatograms and mass spectrums*

The structure and mole fractions of various organic acids and alcohols were based on GC-MS peak area of ions. The integrated peaks on gas chromatogram exhibiting the various organic products at m/z ratio of 60, 117, 87, 101 and 83 for ethanoic acid ethyl butyrate, hexanoic acid and heptanoic acid and heptanol with retention time 11.3, 20.31, 21.9, 24.6 and 4.89 respectively (figure 1).

3.3 *GC-MS Results for control cell without microbes*

The GC-MS results of simple medium without cultivation of *Clostridium ljungdahlii* with the entire set of experimental conditions of temperature and pH have shown clearly that no metabolic activities took place in the medium. Mass spectrum without any sharp peaks for organic products revealed that without electroactive cells, no desirable products formed (figure 2).

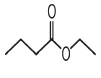
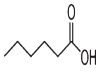
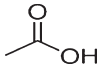
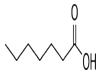
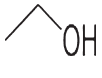
3.4 *Volatile fatty acids and alcohols production in batch and continuous systems*

The quantification of various organic products was analysed by comparing sample peak area with the calibration curve. The major products were volatile fatty acids and alcohols, and their concentration in mM are presented in figure 3 for both batch and continuous systems.

After batch synthesis, the system was switched to continuous system with a peristaltic pump of flow rate of 0.3 mL/s. Samples were screened after regular interval of times in falcon tubes and were treated with 0.3 mL of 2-ethylbutyric acid as an internal standard and 0.75 g of NaCl and were characterized by GC-MS. Concentration of various organic products calibrated by calibration curve are shown in figure 3.

Numerous product formations are responsible for the complexity and non-linearity in the end results. In the batch system, ethanoic, butanoic, hexanoic and heptanoic acids are produced with slight increase in concentrations until 48 h, but at 72 h there is slight decrease in ethyl butyrate concentration; according to Wood–Ljungdahl pathways, two acetyl-CoA converted to acetoacetyl-CoA, which further generated butyryl-CoA, leading to butyrate. But the concentration of butyrate is decreased as butyryl-CoA with one more acetyl-CoA is converted to hexanoyl-CoA for the production of hexanoate and then hexanol in turn shows a slight decrease in hexanoic acid graphically too. As more and more H^+ ions penetrate through the cell membrane, the internal pH increases. To overcome this physiological stress, electroactive catalysts trigger the

Table 1. Chemical composition of volatile fatty acids and alcohols revealed by GC-MS analysis

Compounds	Ethyl butyrate	Hexanoic acid	Acetic acid	Heptanoic acid	Hexanol	Ethanol	Heptanol
RT	20.31	21.9	11.32	24.6	20.9	16.04	4.89
MW	117.1	116.16	60	130.18	102.17	46.06	116.20
MF	$C_6H_{12}O_2$	$C_6H_{12}O_2$	$C_2H_4O_2$	$C_7H14_4O_2$	$C_6H_{14}O$	C_2H_6O	$C_7H_{16}O$
DF							

RT; retention time in minutes, MW; molecular weight, MF; molecular formula, DF; displayed formula.

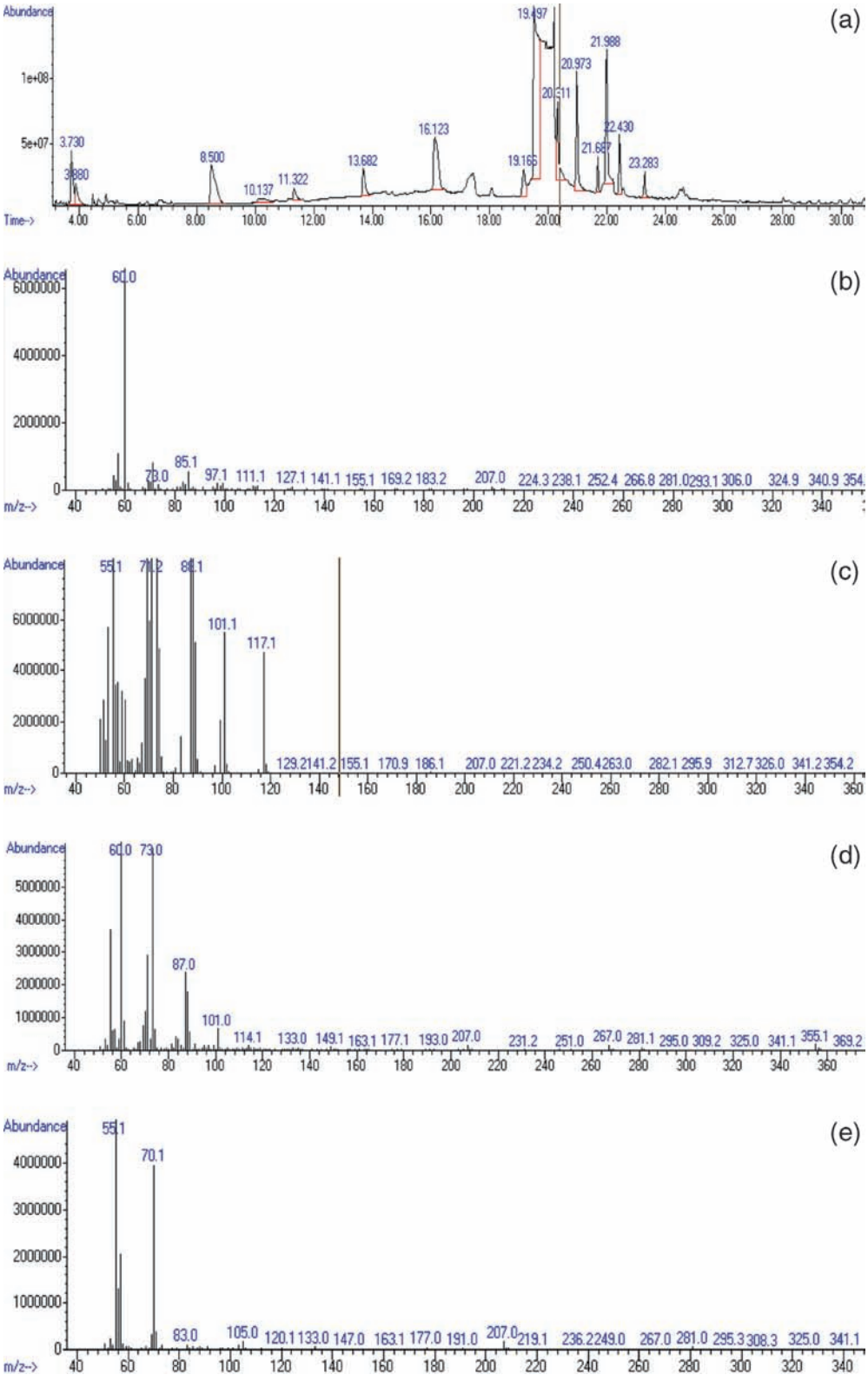


Figure 1. GC-MS analysis for volatile fatty acids and alcohols: (**a**) Gas chromatogram for *Clostridium ljungdahlii*; (**b**) mass spectrum for ethanoic acid, (**c**) ethyl butyrate, (**d**) heptanoic acid and (**e**) heptanol. The occurrence of the ions 60, 117, 101 and 83 for ethanoic acid, ethyl butyrate, heptanoic acid and heptanol.

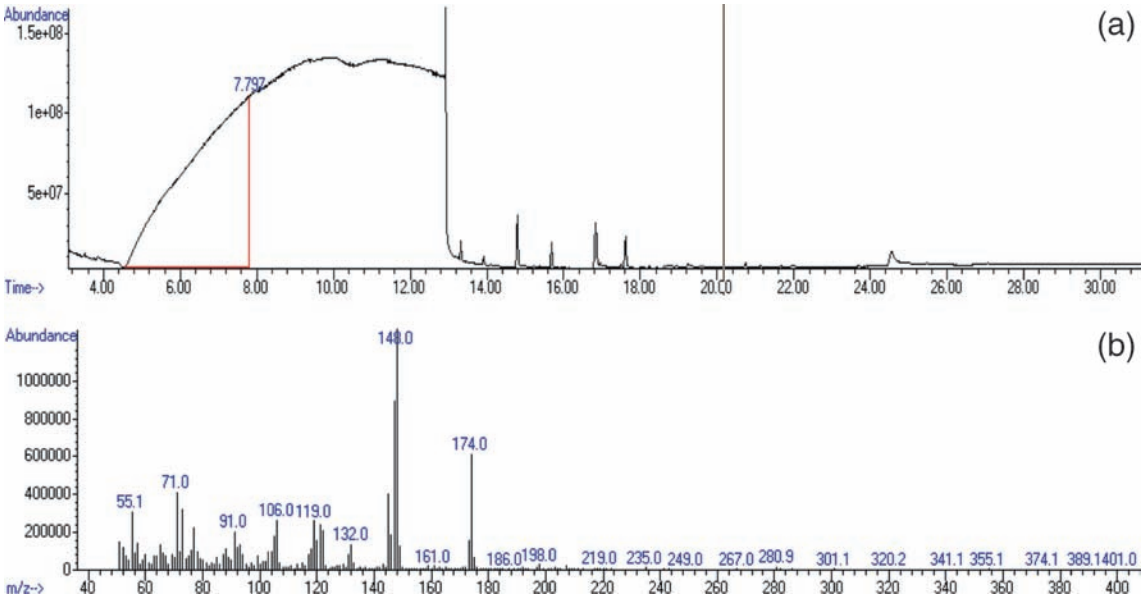


Figure 2. **(a)** Gas chromatogram and (**b**) mass spectrum for control cell without microbes representing no significant peaks for specific products.

solventogenesis for the production of alcohols. The graphical presentation reflects the trend in acidogenesis and solventogenesis when medium is switched from exponential growth phase to non-growth phase under lower pH values. In the batch system, ethanoic acid and ethanol are with maximum concentration, while heptanoic acid and heptanol are at low concentration compared with other products; as the transfer of electrons increase gradually, the reduction of carbon dioxide to organic value-added products decrease slightly, as represented in figure 3b. During the continuous system, as the medium was added uninterruptedly with constant removal of products from the system, this resulted in smooth increase in the concentrations of organic products. The continuous system reveals the same trend of decrease in products concentration with increase in electron transfer, as illustrated in figure 3c and 3d.

The comparative study of batch and continuous BES was found to be the best in the batch system. The concentration of ethanoic acid, ethyl butyrate, hexanoic acid and heptanoic acid were 2.99, 2.66, 2.18 and 2.017 mM respectively in the 120 h of cultivation. Similarly, ethanol, hexanol and heptanol concentrations were 3.19, 2.11 and 0.85 mM respectively. The heptanol and hexanol concentrations were little greater in the continuous system than batch system but this concentration difference is minimum. As *Clostridium ljungdahlii* are gram-positive bacteria, they have greater electrostatic force of attraction to the negative cathode; this trait aids the development of bio-film on the cathode surface for direct capture of electrons. Metabolic reactions follow both acidogenic and solventogenic phase for the production of bio-fuels and bio-commodities. Requirement of electrons were fulfilled by their extraction from water in the anode compartment and were delivered to cathode by external circuit evidenced by cyclic voltammetry.

Microorganisms are able to fix CO_2 as a sole carbon source by the Wood–Ljungdahl (WL) pathway, in which 2 mol of CO_2 are reduced to form 1 mol of acetyl-CoA. The carbonyl or Western branch of WL reduces 1 mol of CO_2 to CO as a part of bi-functional CO dehydrogenase/acetyl-CoA synthase complex (CODH/ACS). The methyl or Eastern branch utilizes formate dehydrogenase (FDH) for the reduction of CO_2 to formate. FDH is subsequently attached to tetrahydrofolate and reduced to the methyl-group. The carbonyl and methyl groups are combined in acetyl-CoA synthase along with a molecule of coenzyme-A to synthesize acetyl-CoA that can either be used for the formation of acetate and thereby regenerate ATP, or for the formation bio-mass via anabolism. In substrate-level phosphorylation, no net ATP is formed in WL pathways. One ATP is formed during the conversion of acetyl-CoA to acetate and one molecule is consumed to form 10-formyltetrahydrofolate via the enzyme 10-formyl-H4folate synthetase. Acetogens must couple reactions within the WL pathway to generate transmembrane ion gradients, from which gradient-driven phosphorylation produces ATP. Two mechanisms have been proposed for gradient-driven phosphorylation. First, membrane-bound Rnf complexes generate proton-gradient as in the case of *C. ljungdahlii* or cytochromes generate proton-gradient in other acetogens. The reduction of 10-methylenetetrahydrofolate to 5-methyltetrahydrofolateis is coupled with proton gradient formation and gradient-driven phosphorylation generates ATP. The second mechanism generates ATP through Na^+-dependent gradient. However,

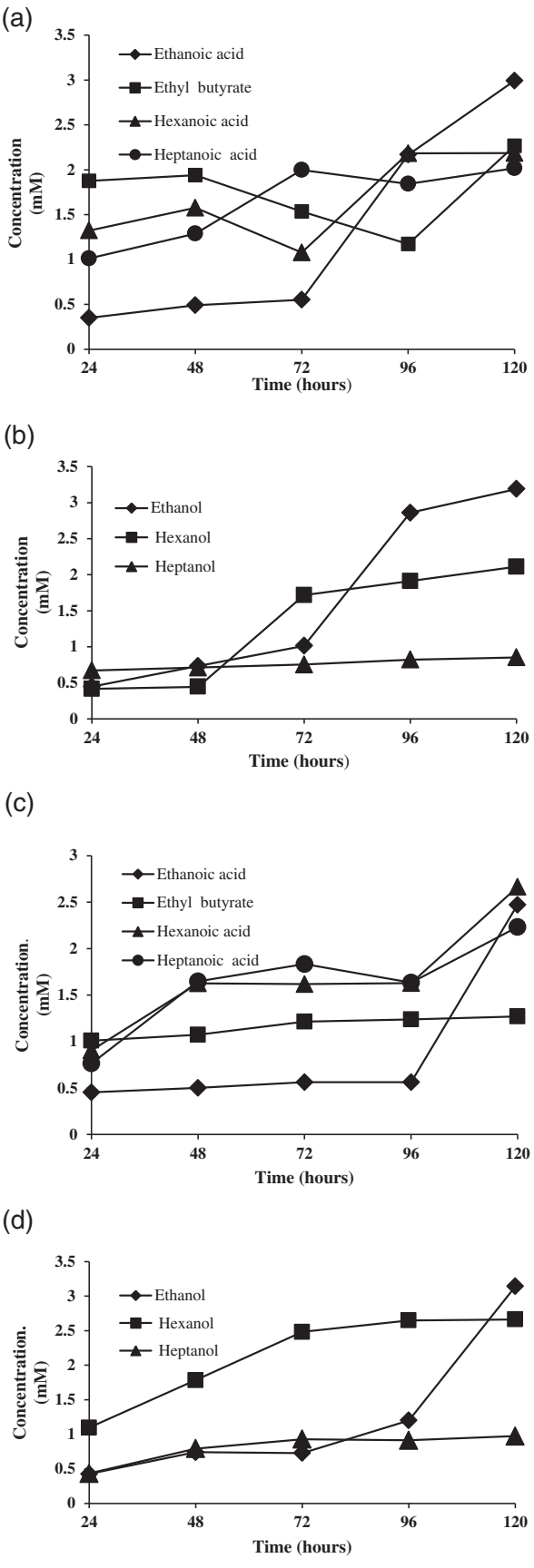


Figure 3. Bio-electrochemical synthesis of volatile fatty acids and alcohols against time duration during Batch and continuous system. (**a**, **b**) Volatile fatty acids and alcohols production in mM during batch system. (**c**, **d**) Volatile fatty acids and alcohols production in mM during continuous system.

the ATP generated are small and limit the growth rate, and these consideration argue that microorganisms culturing should be heterotrophically on fructose first to achieve high cell densities and then switched to gaseous substrate for autotrophic growth (Tracy *et al.* 2012). The adjusted trace metals concentration in medium is helpful for the accumulation of butanol and hexanol, as metal atoms are active sites for the electron transfer and substrate binding in WL pathway. Nickel is an active site for carbon monoxide dehydrogenase (CODH) enzyme to receive electrons from CO in WL pathway. Formate dehydrogenase and aldehyde oxidoreductase (AOR) enzymes are present in alcohol production and are critically tungsten-dependent. Mo is equivalent to W to bind active sites in enzyme AOR which are involved in reduction of organic acids to alcohols. Similarly, Fe is the basic requirement for electron transfer centers in enzymes and cofactors.

Acetyl-CoA undergoes sequential metabolic reactions for the production of organic acids and alcohols that are successively exported from the cell. Volatile fatty acids and alcohol production occur sequentially in acidogenesis and solventogenesis metabolic phases (Ramio *et al.* 2015). Acids are produced during exponential growth phase, and when growth rate slows down and cells enter the stationary phase, alcohol production starts. *Clostridium ljungdahlii* produce alcohols at pH 4 to 4.5 under non-growth condition and volatile fatty acids at pH 6 to 7 (Mohammadi 2014).

Dense bacterial culture was obtained with fructose due to the availability of intracellular enzymes and cofactors. The use of reducing agents triggers the slower growth of bacteria because of reduced ATP formation. This non-growth condition activates alcohol production. Figure 4 reflects the trends in acidogenesis and solventogenesis when the medium switched from exponential growth phase to non-growth phase under lower pH values.

In the recent study, hexanoic acid, hexanol and heptanol were reported along with other compatible liquid fuels. Hexanol synthesis is catalysed by thiolase enzymes which condense two molecule of acetyl-CoA to acetoacetyl-CoA and eventually form butyryl-CoA. For hexanoic acid and hexanol, thiolase condenses butyryl-CoA with acetyl-CoA to form 3-oxo-hexanoyl-CoA, which is converted to hexanoyl-CoA. Hexanoyl-CoA conversion to hexanoate is by the same enzyme as for butyrate. Butanol and hexanol are synthesized by aldehyde and alcohol dehydrogenases from butyryl-CoA and hexanoyl-CoA respectively.

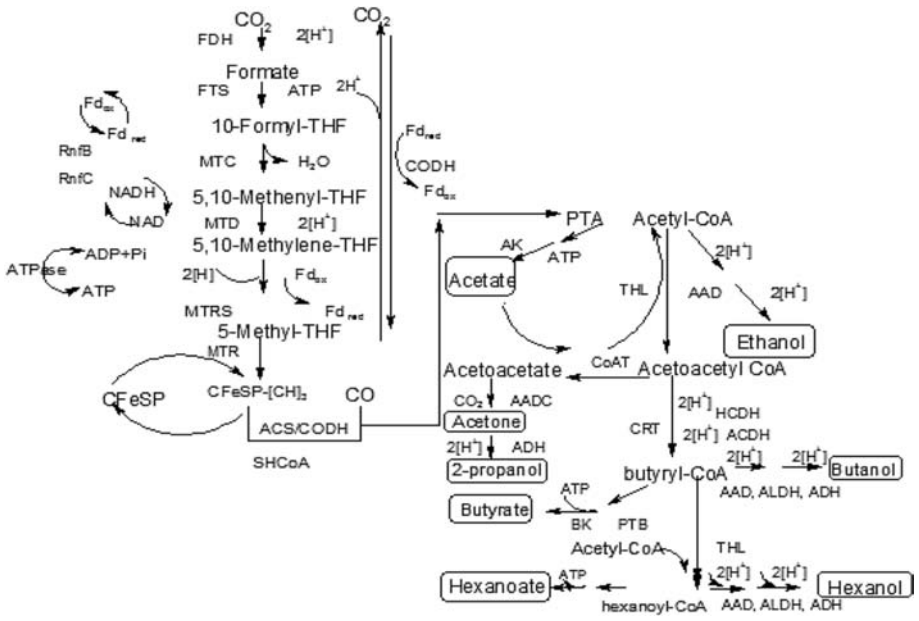


Figure 4. Overview of Wood–Ljungdahl metabolic pathways involved in the production of various metabolites.

3.5 *Cyclic voltammetry*

Cyclic voltammetry of *Clostridium ljungdahlii* was performed with carbon electrode as working electrode against Ag/AgCl reference electrode. The scan rate was 10 mV/s. The culture exhibited the current consumption after 48 h of cultivation. *Clostridium ljungdahlii* showed the significant electroactivity with definite redox peak, which indicates the development of electroactive bio-film development at the cathode.

The reduction and oxidation peaks were observed at (−6.028 E−01 mV, −5.1966 E−05mA/cm^2) and (2.03E−01 mV, 3.90 E−05mA/cm^2) respectively.

The control cell of fresh medium without microbes did not show the preferred redox peaks when CV was conducted against the reference electrode. Due to the absence of electro-active compounds, i.e. microbes, the current transferred through the circuit was limited because the acceptance of electron at cathode was minimum. The current density was increased only

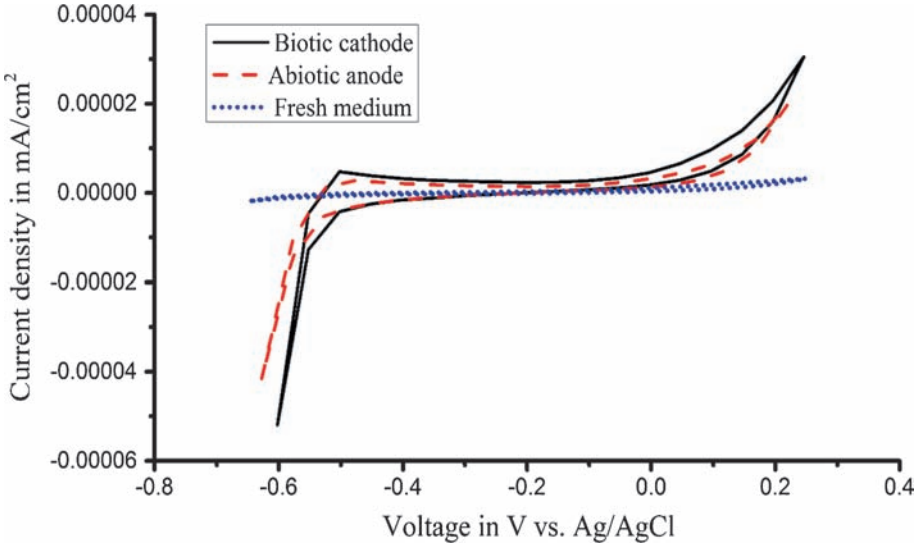


Figure 5. CV of *Clostridium ljungdahlii* for biotic, abiotic cathode and fresh medium against Ag/AgCl reference electrode at scan rate of 10 mV/s.

by increasing the formation of products, which was possible only in the presence of electroactive catalyst (figure 5).

When the fresh medium was re-filled in the cathode compartment, it retained the electrochemical activities due to the development of electroactive catalysts. The current consumption was recovered again when potential was applied, which indicated the transfer of current from cathode to microbes without mediators. Stirring the solution did not substantially affect the current consumption and increase in products, which provides the evidence for the direct electron transfer from the cathode to bio-film. Interruption in the voltage supply affects the activity of microbes and reduce the production.

3.6 *Electrons recovery against the current consumption*

Electron appearing in the volatile acid and alcohol formation accounted for the high proportion of electrons consumed by the culture. The total amount of the current consumed by system was calculated by integrating the current (A/m^2) against time (s).

The amount of current measured by for *Clostridium ljungdahlii* against the −400 mV applied potential was 0.0848 A/m^2. Comparative studies of batch and continuous systems are expressed graphically in figures 6 and 7.

3.7 *Batch system*

Table 2 provides the total current consumed by *Clostridium ljungdahlii* and recovered in volatile fatty acids and alcohols in the batch system.

Table 2. Total current consumed by *Clostridium ljungdahlii* and recovered in volatile fatty acids and alcohols during batch system

Time hours	Total current consumed	Moles Ethanoic acid	Moles Ethyl butyrate	Moles Hexanoic acid	Moles Heptanoic acid	Moles Ethanol	Moles Hexanol	Moles Heptanol
0	0	0	0	0	0	0	0	0
24	0.027164	0.00035029	0.001875	0.0013231	0.0010123	0.0004475	0.000416	0.000671
48	0.054329	0.00048941	0.001939	0.0015772	0.0012885	0.0007316	0.000445	0.000711
72	0.081493	0.00055031	0.001536	0.0010775	0.001998	0.001014	0.001717	0.000753
96	0.108658	0.00218	0.001171	0.0021833	0.0018429	0.002861	0.001911	0.000821
120	0.1358228	0.002993	0.002266	0.002187	0.002017	0.003191	0.002115	0.000853

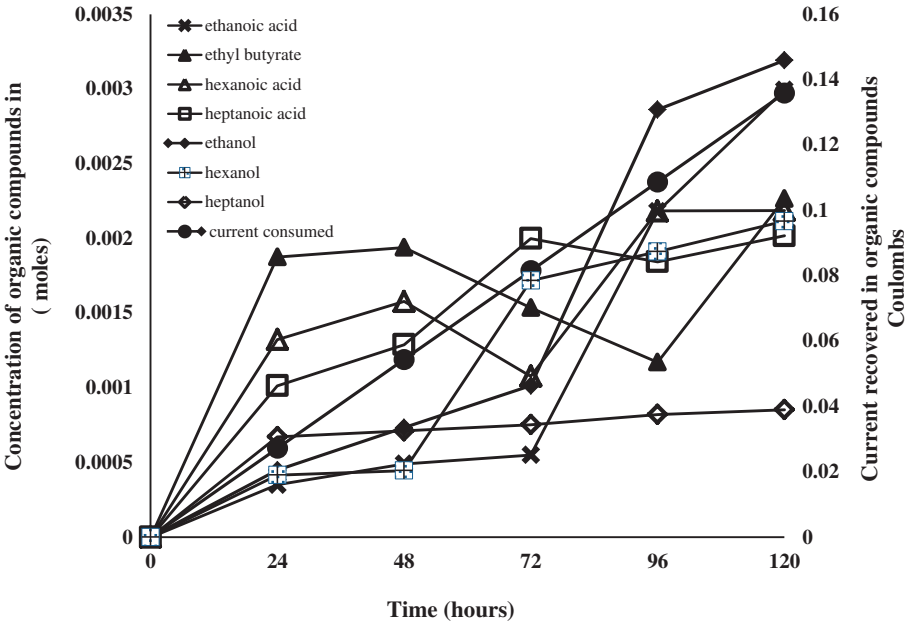


Figure 6. Current recovery in organic acids and alcohols by *Clostridium ljungdahlii* in batch system against the total electrons consumed by biofilm.

3.8 *Percent cathode recovery*

Table 3 gives the percentage of cathode recovery of organic products of *Clostridium ljungdahlii* vs. time duration at constant voltage of −400mV in the batch system.

Table 3. Percent cathode recovery of organic products of *Clostridium ljungdahlii* vs. time duration at constant voltage of −400mV in batch system

Time hours	Percent Ethanoic acid	Percent Ethyl butyrate	Percent Hexanoic acid	Percent Heptanoic acid	Percent Ethanol	Percent Hexanol	Percent Heptanol
0	0	0	0	0	0	0	0
24	3.59	77.027	54.354	49.384	6.893	19.226	36.179
48	5.02	79.656	64.793	62.858	11.270	20.566	38.336
72	5.65	63.100	44.265	97.470	15.621	79.353	40.601
96	22.38	48.106	89.692	89.904	44.075	88.319	44.267
120	30.73	93.090	89.844	98.397	49.158	97.747	45.993

3.9 *Continuous system*

Coulombic recovery of electrons in the continuous system were determined by keeping influent flow rate at 0.3 mL/s. Data exhibited below represent the total current consumed by bio-film and the moles of products recovered from the system. Maximum recovery was observed at time 120 h. Table 4 gives the total current consumed by biofilm and electrons recovered in organic compounds in the continuous system by *Clostridium ljungdahlii*.

Table 4. Total current consumed by biofilm and electrons recovered in organic compounds during continuous system by *Clostridium ljungdahlii*

Time hours	Total current consumed	Moles Ethanoic acid	Moles Ethyl butyrate	Moles Hexanoic acid	Moles Heptanoic acid	Moles Ethanol	Moles Hexanol	Moles Heptanol
0	0	0	0	0	0	0	0	0
24	0.141624982	0.00039025	0.00101	0.0009069	0.0007658	0.000427	0.001096	0.000432
48	0.282476948	0.00050238	0.001074	0.0016255	0.0016473	0.00074	0.001784	0.000793
72	0.423715421	0.00056323	0.0012139	0.001618	0.001832	0.000728	0.002482	0.000929
96	0.564953895	0.00056406	0.0012377	0.0016273	0.0016336	0.001201	0.002048	0.000913
120	0.706192369	0.002471	0.0012692	0.0026638	0.00223	0.003143	0.002063	0.000975

3.10 *Percent cathode recovery*

The Coulombic recovery of volatile fatty acids and alcohols at the applied voltage were observed different for the batch and continuous systems. The maximum recovery was 93%, 89%, 98% and 97% for ethyl butyrate, hexanoic acid, heptanoic acid and hexanol for the batch system. The other products were ethanoic acid, ethanol and heptanol, but their recovery was comparatively less, i.e. 30%, 49% and 45% respectively. The maximum recovery was at 120 h of the cultivation when CO_2 was merely the carbon source (table 5).

In the continuous system, the Coulombic recovery of heptanoic acid, hexanoic acid and hexanol was 94%, 72% and 80%. The utilization of electrons provided by the cathode as a reducing equivalent in bio-fuel production resembles natural photosynthesis, but for CO_2 reduction, there is a need of much higher electron uptake than microbial bio-electrochemical synthesis from glucose, sugar and glycerol. This high demand of electron relatively effects the active reduction pathway and may limit the production of bio-fuel from CO_2.

4. Conclusion

Bio-cathode development via anaerobic reduction reaction requires complex acclimation steps to start, but here

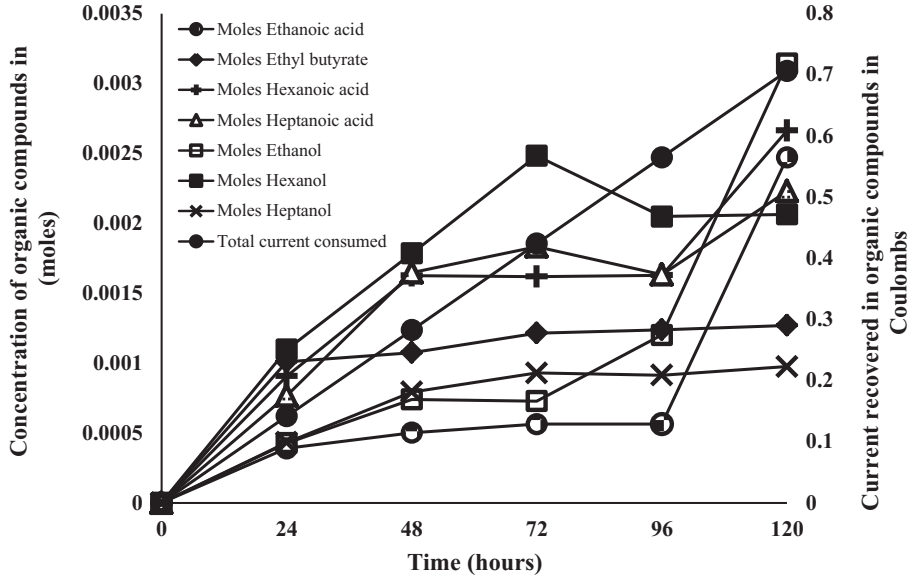


Figure 7. Current recovery in organic acids and alcohols by *Clostridium ljungdahlii* in continuous system against the total electrons consumed by biofilm.

heterotrophic pre-enrichment procedure was employed to enhance the startup bio-cathode to accelerate bio-fuel production. Pre-enrichment method worked in three steps: anaerobic enrichment on fructose to enrich bio-mass on biocathode, inoculation of cathode at −400mV to minimize hydrogen gas evolution, and carbon dioxide provided as sole carbon source to switch from heterotrophic to autotrophic growth of electrotrophs. The bio-electrochemical synthesis by anaerobic autotrophic *Clostridium ljungdahlii* having tendency to accept electrons from cathode has extended the scope of research. The electrosynthesis at bio-cathode is a novel approach for the bio-production by microbial fixation of CO_2. The electricity-driven microbial electrocatalysis of CO_2 and water to intracellular multicarbon compounds offers the possibility to overcome the problems concerning the difference between energy demands and energy supply. BES platform is an attractive alternative CO_2 fixation pathway to photosynthesis. BES coupled with photovoltaic technology is an emerging artificial photosynthesis strategy. In previous studies the cathode was at a fixed potential using a potentiostat; however, such a system would be difficult to control at a large scale. Therefore, the delivery of electrons to cathode was made possible with DC power supply. The cathode at −400 mV was low for the production of hydrogen gas but feasible for the electro-fuel production. Pure culture implementation has demonstrated that a diversity of acetogens accept electrons from negative electrodes for the reduction of carbon dioxide to acetate with high Columbic efficiencies. Previous studies on electrotrophic BES have revealed the production of two or more chemical products, but the present research verified the electrotrophic biosynthesis of an array of various products like heptanoic acid, heptanol, hexanol, ethyl butyrate, ethanol and ethanoic acid. The electro-autotrophic bacteria *Clostridium ljungdahlii* accumulated ethanoic acid 2.993 mM, ethyl butyrate 2.266 mM,

Table 5. Percent cathode recovery of organic products of *Clostridium ljungdahlii* vs. time duration at constant voltage of −400mV in continuous system

Time hours	Percent Ethanoic acid	Percent Ethyl butyrate	Percent Hexanoic acid	Percent Heptanoic acid	Percent Ethanol	Percent Hexanol	Percent Heptanol
0	0	0	0	0	0	0	0
24	0.475	18.671	24.589	32.595	1.065	42.817	8.385
48	0.611	19.854	44.073	70.116	1.846	69.695	15.392
72	0.685	22.440	43.869	77.977	1.8168	96.963	18.032
96	0.686	22.880	44.122	69.533	2.997	80.0089	17.722
120	3.008	23.463	72.225	94.918	7.843	80.594	18.925

hexanoic acid 2.187 mM, heptanoic acid 2.0179 mM, ethanol 3.191 mM, hexanol 2.11 mM and heptanol 0.853 mM during the batch system at 120 h with cathode recovery of 30.73, 93, 89.844, 98.39, 49.15, 97.74 and 45.99% respectively. The results for the continuous system were ethanoic acid 2.47 mM, ethyl butyrate 1.269 mM, hexanoic acid 2.66 mM, heptanoic acid 2.23 mM, ethanol 3.14 mM, hexanol 2.66 mM and heptanol 0.975 mM at 120 h with cathode recovery of 3.008, 23.46, 72.22, 94.91, 7.18, 80.59 and 18.92% respectively. The percentage cathode recovery was greater in the batch system at around 98%. The maximum yield in batch studies was due to the complete reduction of substrate to organic products as compared with that in the continuous system. However, the continuous system has some advantages over batch system for industrial point of view. The continuous system deals with greater amount of substrate concentration than the batch system; all stages of reactions are carried out simultaneously and so overall time required for the process is shortened. The continuous system can be operated in a smaller bio-reactor than the batch reactor.

BES technology proved its worth not only for the production of high-grade multiple compounds but also for its promising environmental and industrial advantages due to its sustainability, renewability and environmental-friendly traits. The concentration and production rates are still low, therefore hampering efficient extraction approaches. This research has proved the feasibility of products derived by CO_2, however, BES still faces numerous operational and technical issues. The lack of fundamental knowledge about the electroactivity of bio-catalysts, the cathode materials, the reactor designing and product recovery require multidisciplinary approaches for sustainable biochemical production. Optimization and scaling of process are strong requirement for commercialization. Reactor design for the next generation should focus on the cathode material, maximizing cathode surface area to support the utmost rates of electrosynthesis.

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