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Metabolic Shift of *Clostridium butyricum* by External Electron Supply in Raw Glycerol Electrofermentation

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Crude glycerol can serve as a low-cost substrate in biotechnological applications. Electrofermentation is a bioelectrochemical technology in which it is possible to improve and control microbial fermentation increasing the specificity of metabolic pathways. This paper evaluates the effect of external electron supply on the production of metabolites of commercial interest with a metabolic network of *C. butyricum*. The results obtained from a model simulation show that electrofermentation could improve the yields of 1,3-propanediol (23%) and hydrogen (45%) using glycerol as substrate. Also, it was established experimentally that the native strain *Clostridium sp.* IBUN158B, related to *C. butyricum,* is electroactive and can increase the product/substrate yield values of 1,3-propanediol (16%) when culture in a cathodic electrofermentation process with the electron carrier Neutral Red in the culture medium. In conclusion, the crude glycerol electrofermentation with *C. butyricum* has potential as an alternative process to traditional fermentation to control the redox state during the synthesis of biochemicals and increase the production of metabolites of commercial interest, but more basic and applied research is needed to elucidate the mechanisms of electron transfer, to reveal the underlying regulatory mechanisms and give to the process real economic and environmental advantages.

1. Introduction

Crude glycerol from the biodiesel industry is an attractive raw material because it is renewable, available in massive quantities, and with low price (Kumar and Verma, 2020). *C. butyricum* has been reported as a good hydrogen producer with lactate, butyrate, acetate, ethanol, 1,3-propanediol and CO2 as the main by-products from glycerol traditional fermentation (TF) (Wang *et al.*, 2021). Recently, Electrofermentation (EF) technology has received much attention (Utesch *et al.*, 2019). EF combines microbiology and electrochemistry, opening the possibility of manipulating the intracellular cofactors' regeneration rates to achieve a desired cellular physiology. In this technology, the external electrons from a power source are accepted by electroactive bacteria directly or indirectly, changing the intracellular redox balance and promoting the formation of reductive products (Zhou *et al.*, 2016). Experimental research on cathodic electrofermentation (CEF) in the *Clostridium* genus has been focused only on some species like *C. pasteurianum* (Rosenbaum *et al.*, 2019), *C. acetobutylicum* (Kim and Kim, 1988), *C. tyrobutyricum* (Choi, Um and Sang, 2012), and *C. beijerinckii* (Zhang *et al.*, 2021). Studies about the CEF process with a *C. butyricum* metabolic network have not been developed. Thus, this research aims to evaluate *in silico* and experimentally the *C. butyricum* metabolic network response to a cathode electrons flux supply and its effect on the metabolic products yields using crude glycerol as substrate.

2. Methods

2.1 Core metabolic model construction

The model is developed in the DOE System Biology Knowledge Base (KBASE) following the recommended procedure (Edirisinghe *et al.*, 2018). Initially, the genome version published in the NCBI database of *Clostridium sp* IBUN13A (GenBank accession NZ_JZWG00000000.1), was imported to KBASE. It is a Colombian soil isolated *Clostridium sp*. strain, closely related to *Clostridium butyricum*, identified as suitable solvent and acid producers, including acetic acid, butyric acid, ethanol, butanol, acetone, and hydrogen from glucose or 1,3-PD

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from glycerol (Montoya *et al.*, 2001).Then, it was annotated and the first draft model was built using the Build Metabolic Model application based on the ModelSEED algorithm (Henry *et al.*, 2010). Later, the draft model was curated based on literature data (Tracy *et al.*, 2012), KEGG database (Kanehisa and Goto, 2000), and a genomic metabolic model for *C. butyricum* iCbu641 (Serrano-Bermúdez, González Barrios and Montoya, 2018). The model reconstruction is in the KBASE narrative interface (https://narrative.kbase.us/narrative/91426/). Additionally, it included electron utilization pathways and electron transfer mechanisms. However, the molecular details of electron transfer mechanisms in clostridia microorganisms are not yet well understood (Shi *et al.*, 2016). So, the mechanisms used in this study are based on the generic transfer mechanism proposed by Kracke and collaborators (Kracke and Krömer, 2014). The first (ET1) increases the reduced NADH amount coupled with ATP production (Eq. 1).

$$(3)NAD^{+}_{[c]} + (2)ADP_{[c]} + (2)P_{[c]} + (4)H^{+}_{[c]} + (6)e^{-}_{[c]} \rightarrow (2)H_2O_{[c]} + (2)ATP_{[c]} + (3)NADH_{[c]} \quad (1)$$

where the letter e^{-} represents electrons. This electron pathway includes a mediator and a membrane-bound complex cascade as cytochromes with simultaneous ATP generation (Kracke and Krömer, 2014). The second electron utilization pathway (ET2) evaluated, only increases the NADH amount by direct NAD^{+} reduction with electrons and protons by membrane-bound enzymes like hydrogenases or mediator molecule diffusion (Eq. 2)

$$(1)NAD^{+}_{[c]} + (2)H^{+}_{[c]} + (2)e^{-}_{[c]} \rightarrow (1)NADH_{[c]} \quad (2)$$

The third reaction (Eq. 3) represents the exogenous electrons supplied to the cell cytoplasm. Here [e] and [c] indicate whether the metabolite is extracellular or cytoplasmic, respectively. Similar electron transfer mechanisms are evaluated in other microorganisms like *E. coli* and *C. acetobutylicum* (Arbter *et al.*, 2019).

$$(1)e^{-}_{[e]} \rightarrow (1)e^{-}_{[c]} \quad (3)$$

The final microbial model network includes 114 reactions, 20 external metabolites, and 71 internal metabolites. This study assessed an objective biomass production function developed especially for *Clostridium sp.* All essential metabolites are part of the core network. Similar biomass functions have been used with models for *C. autoethanogenum* (Kracke *et al.*, 2016) and *C. kluyveri* (Koch *et al.*, 2017).

2.2 Elementary Mode Analysis (EMA)

The Elementary Mode Analysis (EMA) methodology is the calculation of all solutions rather than only one best solution. This analysis does not consider factors such as thermodynamics, enzymatic kinetics, gene regulation, or product toxicity (Kracke and Krömer, 2014). The elementary modes analysis was done in MATLAB R2019a (Matlab, 2012) with the CellNetAnalyzer software tool, Version 2019.3 (von Kamp *et al.*, 2017). It evaluated the EF effect on the theoretical product yields (Eq. 4). It was independently compared two different generic electron transfer mechanisms (ET1 and ET2), evaluating the percentage increase (PI) or decrease of the product yield average (Eq. 5). The product yield is defined as:

$$Yield_{product} = \frac{Flux_{product} * Carbon_{product}}{Flux_{substrate} * Carbon_{substrate}} \quad (4)$$

where $Flux_{product}$ is the reaction rate for product leaving, $Flux_{substrate}$ is the reaction rate for substrate uptake, $Carbon_{product}$ is the carbon atoms number in the product, and $Carbon_{substrate}$ is the carbon atoms number in the substrate. The percentage increase (PI) or decrease in the product yield average value is defined as:

$$PI_{product} = \frac{Yield_{product:ET1 or ET2} - Yield_{product:NoET}}{Yield_{product:NoET}} * 100 \quad (5)$$

where $Yield_{product\ ET1\ or\ ET2}$ is the product yield average value obtained when ET1 or ET2 is activated in the metabolic network. $Yield_{product\ NoET}$ is the product yield average value obtained when no electron transfer mechanism is active in the metabolic network.

2.3 Bacterial strain, fermentation media, bioelectrochemical reactors, culture, and product analysis.

The Colombian native strain IBUN158B used in this study belongs to the family *Bacillaceae* of the genus *Clostridia* and its species is closely related to *C. butyricum*. This strain was obtained from the Strain and Gene Bank of the Institute of Biotechnology of the National University of Colombia (IBUN) and this study is framed in the Access Contract to genetic resources and derived products No. 162 of the Ministry of Environment and Sustainable Development of the Republic of Colombia. It is Gram-positive, classified by their nutritional requirements as proteolytic and saccharolytic. This native strain has been characterized biochemically (Serrano-Bermúdez, González Barrios and Montoya, 2018) and molecularly and does not exhibit pathogenicity (Comba-González *et al.*, 2013). All chemicals were purchased at the highest grade available and used without further

purification. The Reinforced Clostridial Medium (RCM) was used to culture the pre-inoculum and inoculum. The medium with glycerol (Medium 1) contained the following ingredients (per liter of distilled water): 0.004 g of Biotin, 0.003 g of PABA, 1.8 g of Na_2HPO_4, 3.0 g of Yeast Extract, 50 g of Glycerol, 0.5 g of L-cysteine chloride and 4 mL of mineral solution. In this study crude glycerol (87% w/w) from biodiesel production were used for Medium 1 (Cárdenas *et al.*, 2006). Glycerol medium additionally included 0.1 mM of neutral red (NR) (CAS: 553-24-2, Sigma) to serve as a redox mediator to deliver electrons to growing cells, which was added separately from a stock solution after sterile-filtering. An H-type reactor with a double chamber was used as a bio-electrochemical system. The volume of each chamber was 300 mL; the chambers were physically separated by a Nafion 117 cation exchange membrane. The cathode and anode were a graphite felt electrode (6 cm^2). The anodic solution was PBS 1x buffer and the cathode compartment was filled with sterilized glycerol medium (300 ml). The reference electrode was Ag/AgCl, NaCl 1 M; It was submerged in the cathode compartment. To remove residual oxygen, each compartment was completely purged with filtered nitrogen gas using a ventilation filter (0.3 mm pore size). The reactor temperature was kept using a heating band. A constant voltage of -0.4 V versus Ag/AgCl was set to the cathode (working electrode) using a potentiostat (DY2311, DigiIvy brand) to work with the electron carrier NR (Choi, Um and Sang, 2012). Initially, the microorganism was kept in sporulated form in a vial stock with medium RCM. For each fermentation, bacteria were grown in 50 mL serum bottles sealed by butyl rubber stoppers and an aluminum crimp containing 40 mL of RCM medium under N_2 (99.9%). A volume of 3 mL (O.D. 0.5) was taken from the stock. The strain was activated by thermal shock (70 °C for 10 minutes). Then, the vials were placed in an orbital stirrer at 200 rpm and 37°C for 12 hours to have the pre-inoculum. Then, 3 mL of the pre-inoculum vials (O.D. 1.8) were injected into new vials for the inoculum. These were stirred for 7 hours until O.D. 1.0 was reached (exponential phase). The bacterium was inoculated in 3 mL into the cathode compartment filled with Medium 1. The electric current was measured using the potentiostat and continuously controlled at 4 Hz by an interconnected computer. The fermentation conditions were started at a temperature of 37 °C and a stirring speed of 50 rpm per 20 hours of fermentation. Samples of 1 ml were taken every 4 hours. All fermentations were conducted in duplicate. The pH, gas and temperature control system belong to the BIOSTAT® A system. The growth of the microorganism was checked by optical density (O.D.) at a wavelength of 680 nm, in a BioRad ® spectrophotometer. The metabolites 1,3-PD, glycerol, butyric acid, acetic acid, and lactic acid, were quantified through a methodology developed within the research group using ultrafast liquid chromatography (UFLC Shimadzu ® Prominence LC-20AD) with a refractive index detector (Shimadzu ® RID 10A) at a temperature of 60 °C and an AMINEX HPX–87H column (Biorad ®) at 63 °C with mobile phase of sulfuric acid 3 mM and a flow of 0.5 mL / min. The running time was 50 minutes.

3. Results and discussion

3.1 EF simulation effect on product yield.

In the base case, 1,3-propanediol (1,3-PD) was produced in the highest yield from glycerol, followed by carbon dioxide (CO2), butyrate, lactic acid, acetate, and ethanol. (Figure 1). There are changes in the value of the yields for the products obtained, applying the different electron transfer mechanisms. The ET1 mechanism with the glycerol substrate in the network increased the yields of 1,3-PD (23%) and CO2 (3%). The product yields that decreased were acetate (-6%), butyrate (-63%), ethanol (-52%) and lactic (-24%).

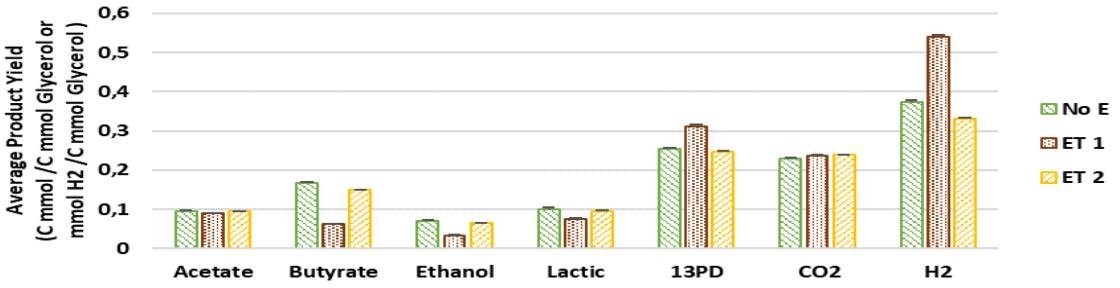

Figure 1. Product yields using different electron transfer mechanisms. No E: Non-electron transfer, ET1: Cathodic electron supply coupled to ATP production, ET2: Only cathodic electron supply increases NADH production.

The ET2 mechanism also affected the metabolic network but in a separate way than the ET1 mechanism. For glycerol, ET2 caused an CO2 average yield increase by (4%) and a decrease in the values of acetate (-1%),

butyrate (-11%), ethanol (-8%), lactate (-6%) and 1,3-PD (-3%). The results obtained for hydrogen showed an increase in yield with ET1 (45%) for glycerol and ET2 shows an 11% decrease. From the previous result, it is observed that only two metabolites present a potential increase in the product yield when the metabolic network is altered by the addition of electrons from an external source such as a cathode. 1,3-PD increases when the ET1 mechanism is active with glycerol as a substrate and hydrogen also. The simulation performed shows that the supply of cathode electrons in the metabolic network of *C. butyricum* can modify the values of internal flows and redox balance, modifying the product/substrate yields of all metabolites and promoting the production of reduced substances as hydrogen and 1,3-PD. It is also observed that the increases in the average yields obtained with the simulation are of the same order as other previously developed simulations performed for other microorganisms of interest under similar conditions of glycerol CEF (Kracke and Krömer, 2014).

3.2 IBUN158B CEF with crude glycerol as substrate.

The glycerol CEF with NR by the IBUN158B strain presented changes in the product profile and current consumption with respect to TF These results show that applying a voltage to perform a CEF with controlled pH and 0.1 mM of NR added to the culture has different effects on the product - substrate yield for 1,3-PD, lactate, acetic and butyric acids with the IBUN158B strain and crude glycerol as substrate (Table 1). Specifically, there is an increase in yield of 1,3-PD when compared to the TF process (16,4%). Butyric acid and lactate yield decreased with CEF while the acetic acid increased its yield. The electrical current increases after inoculation, then a decrease in the growth phase and finally another increase when the culture goes into the stationary phase. Also, it was found that with the electrofermentation a lower biomass value is obtained (26% decrease).

Table 1. Stoichiometric results comparison for products and electrons supply.

Metabolite	Control (TF) (mM)	Standard deviation	CEF (mM)	Standard deviation	T-statistic	P-value	Control (TF) yield (%)	CEF yield (%)	Change (%)
Glycerol	-434.9	0.7	-434.3	0.2	0.07	0.48			
1,3-PD	234.3	11.8	272.4	27.6	3.42	0.09	53.9	62.7	16.4
Butyrate	41.6	1.6	17.5	1.8	118.13	0.003	9.6	4.0	-57.9
Acetate	19.5	1.0	59.2	0.6	110.16	0.003	4.5	13.6	203.4
Lactate	15.9	0.6	7.5	0.6	227.41	0.001	3.7	1.7	-53.1
Total soluble metabolites	311.4		356.5						
Electron delivered (µM)	0.0		123.1						

There were also products that had a titer reduction such as butyrate (57%) and lactate (53%). Table 1 shows the standard deviation of two biological replicates for the metabolite data and Student's t-test values, as well as the performance changes for each metabolite. Significant differences ($P < 0.05$) were found in the final concentrations of various metabolites between control and electrofermentation conditions. The results obtained seem consistent with the hypothesis that the CEF process together with the presence of NR in the culture medium leads to a redirection of the NADH/NAD balance together with the carbon flows through different pathways including the biomass production. A higher NADH/NAD+ ratio resulted in an increase of the flux of glycerol to the 1,3-PD branch and increasing its yield compared to the control. Also, the integration of the current consumption profile showed that, during the 18 h electrofermentation (Figure 2), approximately 123.1 µM of equivalent electrons were delivered to *Clostridium sp.* IBUN158B culture. This value does not correspond to the observed changes in the concentration of reduced products such as 1,3-PD. The electrons delivered to the culture were several orders of magnitude lower than the concentration increase observed for *Clostridium sp.* IBUN158B. This behavior could indicate that the supply of electrons to a TF not only affects the NADH/NAD+ balance, but also generates a regulatory response that affects the expression of other metabolic pathways. When comparing these results with the values reported by other strains in the state of the art, it is seen that the IBUN158B strain is within the ranges published in the literature to produce 1,3-PD by CEF and the other effects observed on metabolites and biomass. For example, experimental evidence on the growth dynamics of *Clostridium* species in CEF cultures (Choi, Um and Sang, 2012; Rosenbaum *et al.*, 2019; Zhang *et al.*, 2021) have shown a decrease in the biomass growth rate indicating that the central metabolism branching points of these bacteria are influenced by the NADH/NAD+ ratio, which could drive a carbon fluxes redistribution producing higher yields of some metabolites and a biomass decrease (Harrington *et al.*, 2015). This phenomenon is also observed with *C. pasteurianum* DSM 525 that simultaneously used both cathode and substrate (glucose and glycerol) as electron donors to enhance reducing metabolites (butanol and 1,3-PD) via direct electron transfer. Here *C. pasteurianum* has an increase in butanol from glucose (20% change) and 1,3-

PD from glycerol (21% change).(Choi *et al.*, 2014). Also with the same strain, the use of two transporters NR and redox mediator Bright Blue (BB) was evaluated. In this system the carriers increased product/substrate yields by up to 33% for butanol in NR fermentations and 21% for 1,3-PD in BB fermentations compared to the respective product controls. (Utesch *et al.*, 2019). Also, it could be considered that the native strain still has a range of process improvement since the theoretical calculation of the central metabolic model, conducted in this work indicates that the increase in yield can reach up to 23%. So, the production of 1,3-PD by EF from crude glycerol with a native strain is possible but needs further technological development to make it a scalable reality.

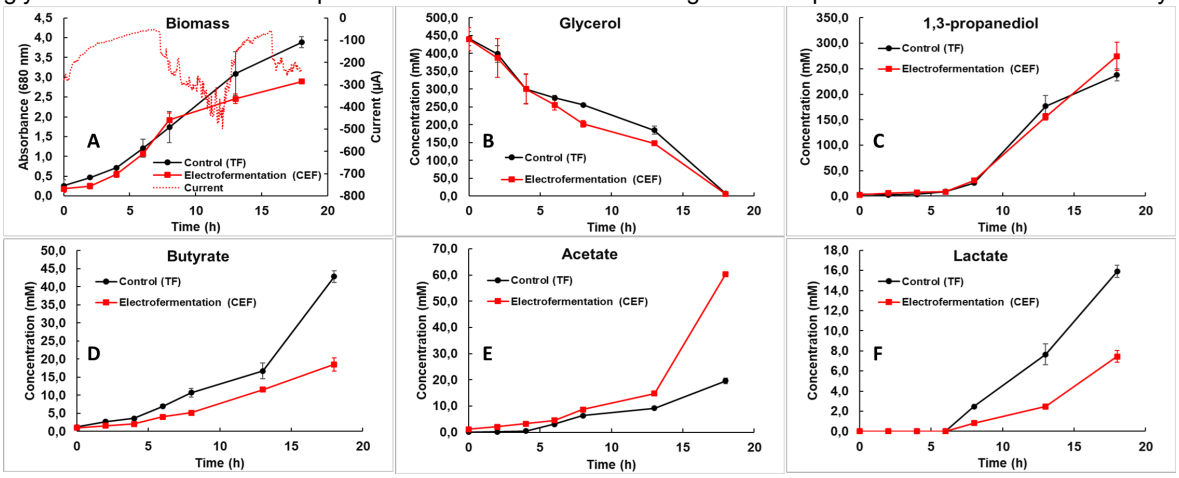

Figure 2. H-reactor batch profiles of control traditional fermentation (TF) and cathodic electrofermentation (CEF) for Clostridium sp. IBUN 158B. (A) Single representative Chronoamperometry plot of current delivered to electrofermentation media plotted together with cell growth tracked by OD680. Substrate and product metabolite profiles are shown for (B) glycerol, (C) 1,3-PD, (D) butyrate, (E) acetate, and (F) lactate. All data points are averages of two replicate experiments, with error bars for standard deviation.

4. Conclusion

The results suggest that an increase in 1,3-PD production and regulation of the cellular metabolic pathways are viable by electrode-driven control in CEF with *Clostridium butyricum* compared to TF. However, the implementation of this technology in its current state requires a significant investment in technical infrastructure, control systems, electrodes, electron carriers and exchange membranes, which impacts the capital and operating costs of the process. This is highlighted by different techno-economic analyses that have indicated that currently EF technology, under the technical conditions in which it is studied at laboratory scale, is not viable. (Gadkari, Gu and Sadhukhan, 2018; Jourdin *et al.*, 2020) and requires further research in various aspects to make the technology competitive in the market. Among them are, development of low-cost and environmentally friendly raw material pretreatments, creation of low-cost electrodes with high electron conductivity and biofilm formation promotion, design of new bioreactors that integrate exchange processes to minimize operating costs, discover the molecular details of electron transfer mechanisms in microorganisms such as clostridia (Shi *et al.*, 2016), improvement of the biocatalyst to increase the final concentration and transfer of electrons and purification processes with lower cost, energy use and environmental impact.

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