Electronic Supplementary Information for

Co-production of bioelectricity and butanol by engineered *Escherichia coli* fed organic wastes in anodic fermentation

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Supplementary Materials and Methods

Chemicals and Reagents

The following chemicals and reagents were purchased from Sigma Aldrich (New South Wales, Australia): 3, 3', 5, 5'-tetramethylbenzidine (TMBZ), 3-methyl butanol (3MB), ampicillin, calcium chloride dihydrate, chloramphenicol, D-glucose, disodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), glycerol, iron (II) sulphate heptahydrate, isobutanol, isobutyraldehyde, isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin sulphate, M9 minimal salts 5 X powder, magnesium chloride, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium acetate, sodium chloride, streptomycin sulphate, thiamine hydrochloride, titanium wire (0.81 mm diameter), tris(hydroxymethyl)aminomethane (Tris), Triton X and Volatile free acid mix (*Trace*Cert®). Yeast extract was purchased from Millipore (Victoria, Australia). Graphite felt and Nafion 117 membrane were purchased from the Fuel Cell Store (Texas, USA). A reference electrode for samples with suspended solids (double junction Ag/AgCl, 3.5 M KCl) was purchased from Hanna instruments (Victoria, Australia). Coomassie Brilliant Blue R-250 staining and destaining solutions were purchased from Bio-Rad Laboratories (California, USA).

Bacterial strains and plasmids

A list of gene accession numbers, expression plasmids and insertion sites for cloning used and resulting strains used in the studies are given in Supplementary Methods Table 1. Genes were codon optimised for *Escherichia coli* expression, synthesised, and cloned into the corresponding expression plasmids by Genscript (Singapore). Expression of the inserted genes was controlled by a T7 promoter. The cytochrome maturation plasmid, pEC86, was obtained from the Culture Collection of Switzerland (CCOS). *E. coli* BL21 (DE3), purchased from New England Biolabs was transformed with the expression plasmids via the standard heat shock protocol.

Bacterial culturing conditions

E. coli strains were cultured in M9Y medium (per litre: yeast extract, 5 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NH_4Cl, 1 g; NaCl, 0.5 g; $CaCl_2{\cdot}2H_2O$, 0.0147 g; $MgSO_4{\cdot}7H_2O$, 0.246 g; $FeSO_4{\cdot}7H_2O$, 0.00278 g; thiamine hydrochloride, 0.01 g) with the required antibiotic selections chloramphenicol (25 μg/mL), and/or streptomycin (100 μg/mL) and/or kanamycin (50 μg/mL) or ampicillin (50 μg/mL) added. A single colony from a freshly transformed strain or a scraping from the glycerol stocks of the engineered strains, were cultivated aerobically in 5-10 mL of M9Y media in 50 mL tubes at 37°C, 250 rpm for 16 h. The seed cultures were then used to inoculate 100 mL of fresh M9Y media in 250 mL baffled flasks, at dilution of 2% v/v (2 mL of the seed culture) and again incubated aerobically at 37°C, 250 rpm. When the cell density, as measured by OD_{600} reached between 0.6-0.8, 100 μL of 0.1 M IPTG, at a final concentration of 0.1 mM, was added to induce recombinant protein expression. The cultures were then maintained aerobically at 30°C, 200 rpm for the required cultivation period as stated in the following sections. Cultures prepared for anodic electrofermentation followed the prescribed conditions and were cultivated for 16 h following induction (addition of IPTG).

Recombinant genes, Expression plasmids, genes and insertion sites for cloning used in the studies

Gene	Native Organism	Accession number
mtrA	*S. oneidensis*	AAN54830.1
mtrB	*S. oneidensis*	AAN54829.1
mtrC	*S. oneidensis*	AAN54831.1
cymA	*S. oneidensis*	AAN57551.1
kivd	*L. lactis*	Noda et al (2019)
adhA	*L. lactis*	Noda et al (2019)
alsS	*B. subtilis*	WP_251188357.1

Name	Details	Cloning site	Source
pUC19	Amp^r		New England Biolabs
pEC86	*ccmABCDEFGH*		CCOS
pMtr	pCDFDuet-1, *mtrCAB*	NocI/HindIII	This study
pMtrCymA	pCDFDuet-1, *mtrCAB*, *cymA*	NocI/KnpI, NdeI/KnpI	This study
pBUT	pET-28a (+), *kivd, adhA*	HindIII/NotI	This study
pBUTA	pRSFDuet-1, *kivd, adhA, alsS*	NdeI/KpnI, HindIII/NotI	This study

Strains	Description	Selection
BL21(DE3)		none
EC86	BL21(DE3), pEC86	ampicillin
MtrCAB	EC86, pMtr	chloramphenicol
MtrCAB-CymA	EC86, pMtrCymA	Chloramphenicol, streptomycin
MtrCAB-CymA-Kivd-AdhA	MtrCAB-CymA, pBUT	chloramphenicol, streptomycin and kanamycin
MtrCAB-CymA-AlsS-Kivd-AdhA	MtrCAB-CymA, pBUTA	chloramphenicol, streptomycin and kanamycin

Recombinant protein detection

Cells were harvested 24 h after induction, collected by centrifugation at 10,000 rpm, 15°C for 15 min. Approximately 0.8 g of the wet cell mass was resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA and 0.05% v/v Triton X, pH 7.5) and placed in an ice bath during sonication (Q125, Qsonica). Following cell lysis, the suspension was centrifuged at 10,000 rpm, 15°C for 15 min and the supernatant, and separately, the cell debris, were analysed by SDS-PAGE on a 4-12% Bis-Tris Gel (Genscript, New Jersey) at 100V for 60 min. Protein bands were visualised by Coomassie Brilliant Blue R-250 stain and approximate molecular weight determined by molecular weight markers. Separately, SDS-PAGE was treated with the haem-specific dye TMBZA and agitated at low speed for 2 h. Blue-coloured bands were revealed upon hydrogen peroxide (30 mM) addition and washed with 100 mL of deionised H_2O.

Design of the bioelectrochemical system

Anodic electrofermentations were performed in an H-type reactor with two 100 mL chambers (Adam and Chittenden, USA) separated by a Nafion 117 proton exchange membrane and clamped to secure a seal between the chambers. Both the counter electrode (2.5 cm x 2.5 cm, thickness 3.2 mm) and working electrode (2.0 cm x 2.0 cm, thickness 3.2 mm) were constructed from treated graphite felt, and titanium wire (total length 10 cm) securely threaded through each electrode. The large working electrode had the dimensions 10.0 cm x 2.0 cm, thickness 3.2 mm. The projected surface areas of the working electrodes were 8 and 40 cm^2, respectively. To improve the wettability of the graphite felt, the electrode materials were treated with sequential 24 h submersions in 1 M HCl, followed by 1 M NaOH. After each submersion, the graphite felt was rinsed thoroughly with distilled water and air dried. All glassware was autoclaved before use. Control of electrical parameters was achieved through connection to a VMP3 Multichannel Potentiostat (Biologic, USA) via alligator clips securing the titanium wire portion of the electrodes and to a double junction Ag/AgCl, 3.5 M KCl reference electrode (Hanna Instruments, Australia), placed in the anodic chamber.

Operation of the anodic electrofermentation system

Cultures grown for 16 h (as above) were pelleted by centrifugation at 5,000 rpm, 15°C for 10 min. The cell pellet was washed with approximately 15 mL PBS and centrifuged again at 5,000 rpm, 15°C for 10 min. The supernatant was discarded, and the cell pellet resuspended in 100 mL of M9 or M9Y (containing yeast extract) media in the anodic chamber at a final cell density of OD_{600} ~1. The induction agent for recombinant protein expression, 0.1 mM IPTG, was added to the media and the chamber was stirred 200 rpm at 30°C. The counter chamber was filled with 100 mL of PBS and the anodic chamber was sealed to maintain the system in anoxia. The potentiostat was attached to the working electrode polarised at a potential of +0.65 $V_{Ag/AgCl}$ and the current stabilised over 2 h when the respective carbon sources were added by a syringe through a side sample port: glucose (12.5 mM), glycerol (12.5, 25, 50, 100 mM) and acetate (37.5 mM as sodium acetate). Carbon sources were dissolved in MilliQ H_2O and filtered with Supor membrane filters (0.2 μm, Pall Corporation). Reactor liquid (1.5 mL) was sampled at timed intervals by syringe through a side sample port sealed by a rubber stopper to minimise oxygen ingress into the system.

The number of electrons transferred to the working electrode was calculated with the following formula, where $n_{electron}$ is the number of electrons in mole, I is current in amps, t is time in seconds and F is Faraday's constant, 96, 485 C mol^{-1}:

$$n_{electron} = \frac{\int_{t_0}^{t} Idt}{F}$$

Quantitative analysis of carbon feedstocks and extracellular metabolites

Samples were removed at timed intervals from the anodic electrofermentation reactors by syringe and centrifuged at 10,000 rpm for 15 min. The supernatant was removed and filtered with 0.2 μm, (25 mm , Supor membrane filters) and then acidified with 1 M H_3PO_4 (35 μL per mL supernatant) prior to analysis. Volatile metabolites ethanol and short chain organic acids, were quantified by gas chromatography using an Agilent 7890A gas chromatograph (GC, Agilent Technologies) equipped with a DB FATWAX column (30 m x 0.25 mm x 0.25 μm, Agilent Technologies) and a flame ionisation detector (FID). The GC was operated at a split ratio of 15:1 with injection volumes of 1 μL. The injector and detector were maintained at 250°C. The oven was initially held at 40°C for 2 min, and then increased to 45°C at a rate of 5°C min^{-1} and held at 45°C for 4 min and then increased to 230°C at a rate of 15°C min^{-1}, which was then maintained for a final 4 min.

Short chain organic acid metabolites, such as formate and lactate, as well as the carbon sources, glucose and glycerol, were quantified via high performance liquid chromatography using an Agilent 1220 Infinity HPLC (Agilent Technologies) fitted with a Hi-Plex H column (4.6 mm x 250 mm, Agilent Technologies). The column compartment was maintained at 50°C and samples were analysed with both a refractive index detector (RID) and a UV-Vis detector set at a wavelength of 210 nm with mobile phase of 0.1 M sulfuric acid at a flow rate of 0.3 mL min^{-1}.

Standard solutions of acetate, ethanol, isobutanol, 3MB, short chain organic acids, volatile fatty acids, glucose and glycerol, were prepared between 0.1 mM and 50 mM, acidified with 1 M H_3PO_4 and analysed using the GC (acetate, ethanol, isobutanol, 3MB, volatile fatty acids) or HPLC (short chain organic acids, glucose, glycerol) protocols described above. Calibration curves, equations and R^2 values for standards are listed in Suppl Methods Table 2.

Standard curve and correlation data for all analytical standards.

Standard and range of concentrations (mM)	Line of best fit	R^2
Isobutanol	Area = 68.622 x Conc. (mM)	0.999
3MB	Area = 72.18 x Conc. (mM)	0.995
Acetate (0.1 – 1.0)	Area = 18.801 x Conc. (mM)	0.999
Acetate (10 – 40)	Area = 19.745 x Conc. (mM)	0.999
Glucose (0.1 – 2.5)	Area = 57521 x Conc. (mM)	0.999
Glucose (10 – 40)	Area = 55437 x Conc. (mM)	0.999
Glycerol (0.1 – 2.5)	Area = 31009 x Conc. (mM)	0.999
Glycerol (10 – 40)	Area = 30019 x Conc. (mM)	0.999

Peptide mass sequencing

Gel bands of approximate molecular weight to MtrCAB and cymA were excised from SDS-PAGE of lysed supernatants from *E coli* expressing *MtrCAB* and *cymA*. The recombinant proteins were released from the polyacrylamide gel and then trypsin-digested before analysis using the Dionex Ultimate 3000 RSLCnano coupled with a Fusion mass spectrometer (Thermo Scientific). The LC was fitted with column Acclaim PepMap RSLC (75 μm x 50 cm,

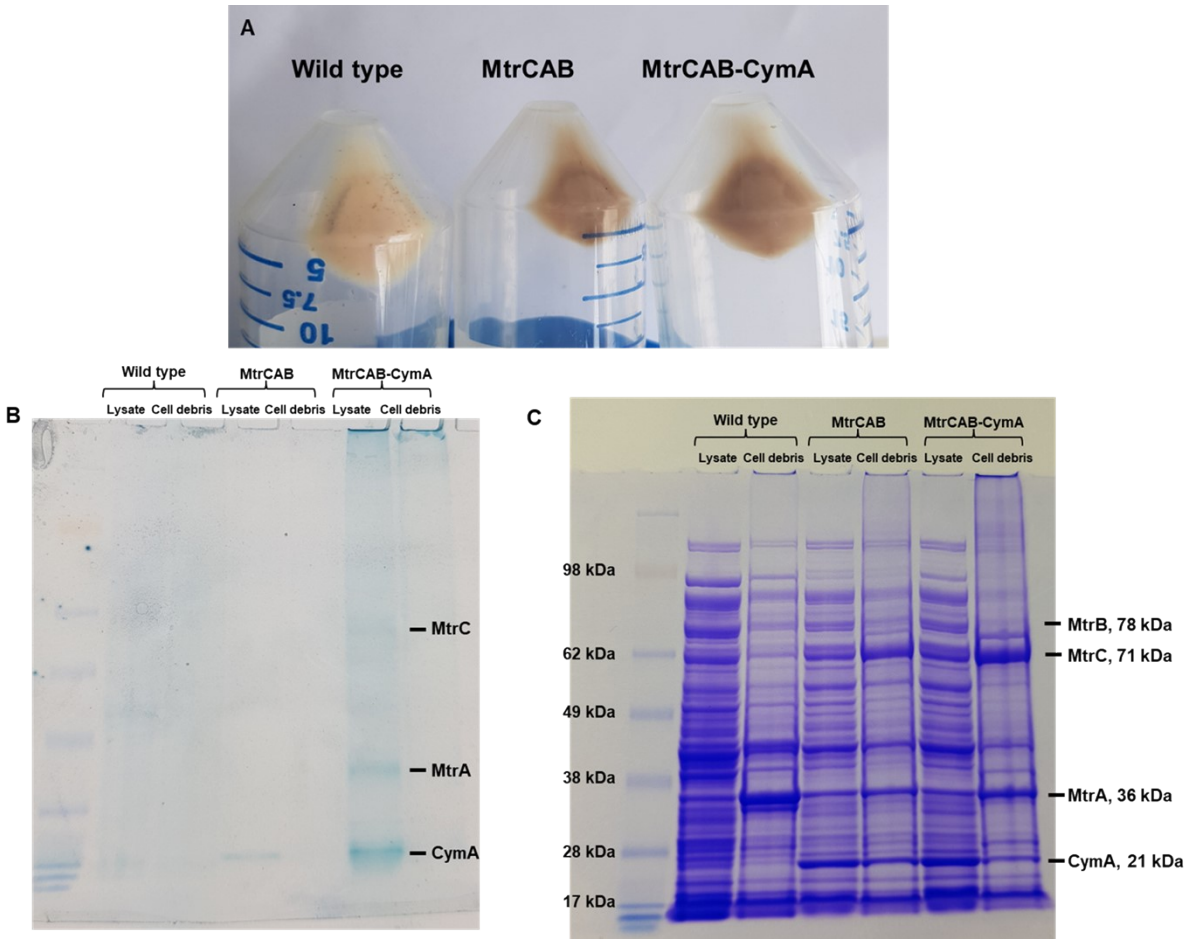
nanoViper, C18, 2 µm, 100Å; Thermo Scientific) and precolumn Acclaim PepMap 100 (100 µm x 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific). The acquisition method was 68_30_DDA_LowComplex using the Byonic (ProteinMetrics) search engine and searched against Uniprot *E. coli* (host) and supplied MtrCAB and cymA sequences. The Protein FDR cutoff was 1%, fixed modification Carbamidomethylation and variable modification Oxidation.

Data analysis and statistical test

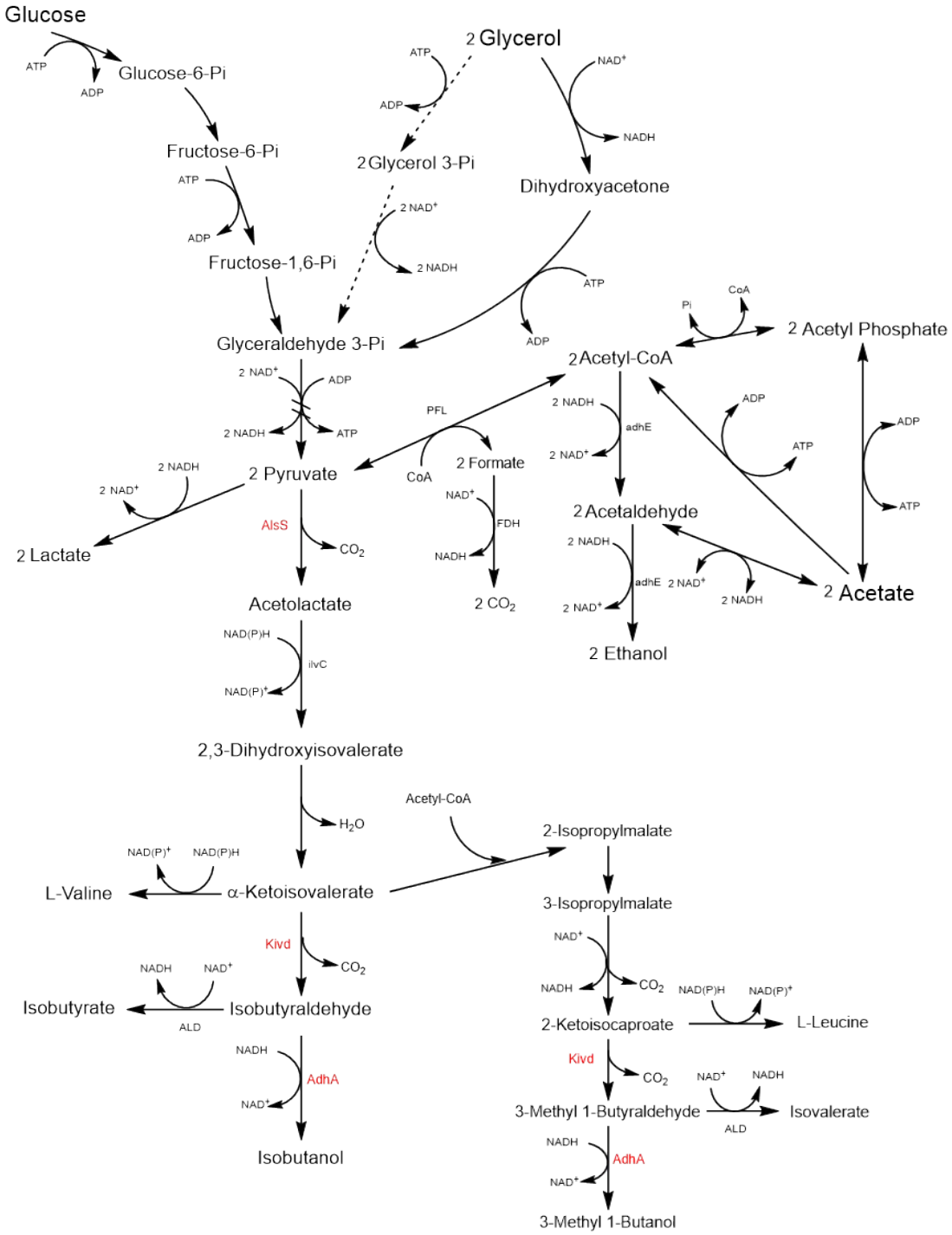
For statistical comparison of two datasets, a two-tailed *t*-test was performed and the significance level was set as □= 0.05. For statistical comparison of three or more datasets, a single factor ANOVA was first performed with the significance level also set as 0.05 with significant comparisons then subsequently analysed by *t*-test.

The current density shown in chronoamperometry figures was calculated through dividing the current output of each system recorded by the potentiostat, by the projected surface area of the working electrode employed. The maximum current density was taken from the maximum value of the curve divided by the projected surface area of the electrode.

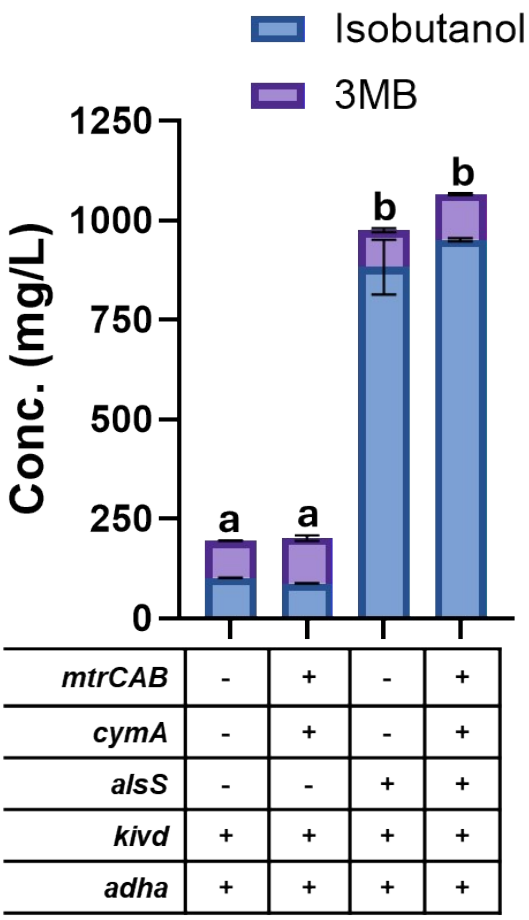
Supplementary Data



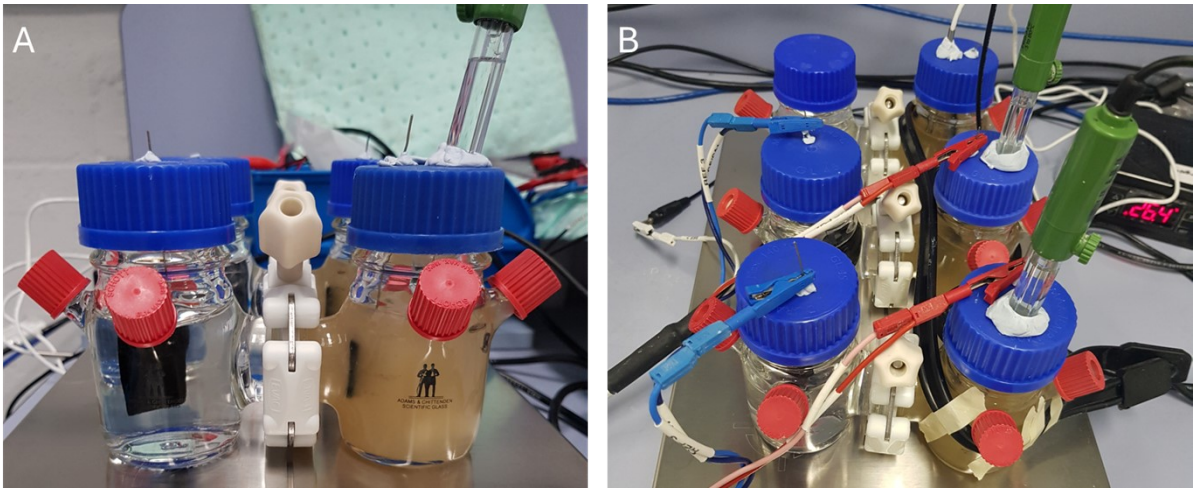
Suppl Fig 1. *E. coli* expressing *MtrCAB* and *cymA* genes to enable electroactivity were induced with 0.1 mM IPTG and cultured for 16 h at 30°C, 200 rpm before chamber addition. A. Red colouration (darker cell pellet) of the recombinant cell pellets compared with wild type cells. SDS-PAGE gels of *E. coli* expressed MtrCAB and cymA proteins stained with B. haem-specific TMBZ dye and C. Coomassie Brilliant Blue solution and protein locations as indicated.



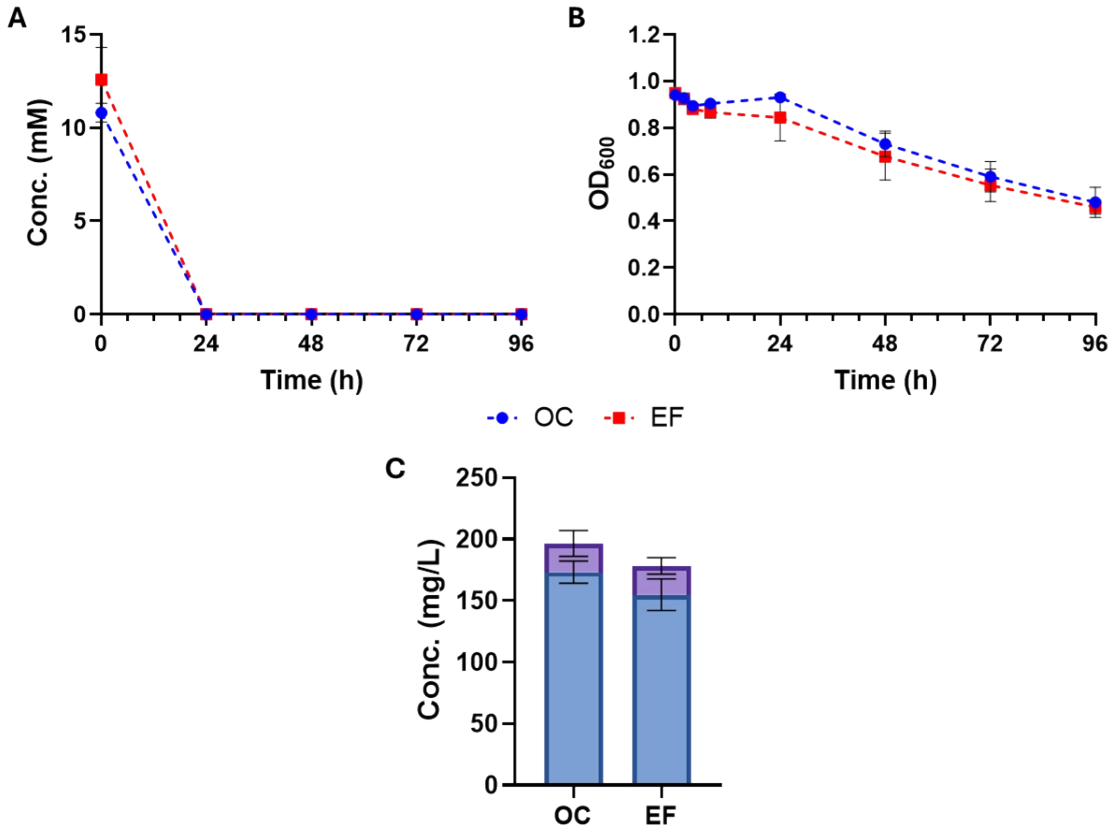
Suppl Fig 2. Metabolic pathway in engineered *E. coli* for the production of isobutanol and 3MB from different carbon substrates analysed in this study: glucose, glycerol, and acetate. Heterologous enzymes expressed in the engineered *E. coli* are indicated in red text. Note: the conversion of glyceraldehyde 3-Pi to pyruvate is shown as a truncated pathway. The conversion indicated by dashed lines occur only under aerobic conditions.



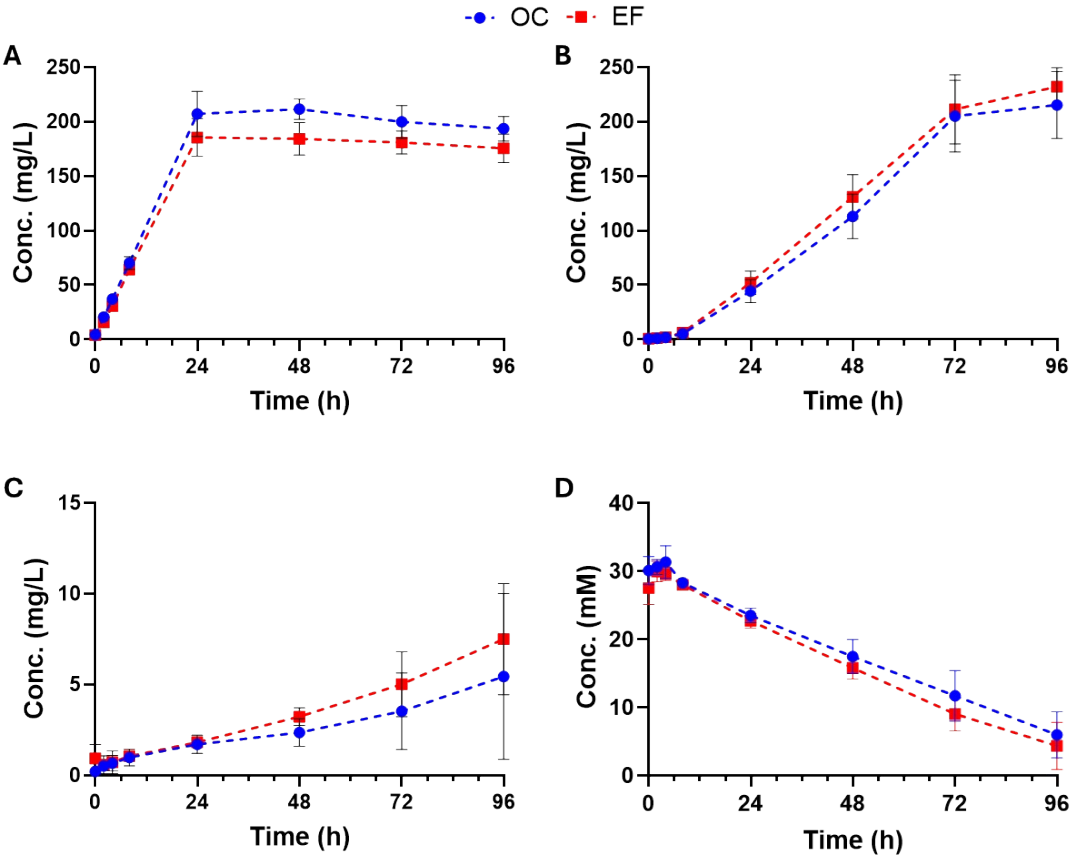
Suppl Fig 3. Extracellular concentrations of isobutanol and 3MB, in aerobic flask cultures of *E. coli* after 96 h: Engineered strains Kivd-AdhA, MtrCAB-CymA-Kivd-AdhA, AlsS-Kivd-AdhA and MtrCAB-CymA-AlsS-Kivd-Adh. Conditions 30°C, 200 rpm, 50 mM glucose added as substrate. Different superscripted letters in the same category indicate significant differences ($p < 0.05$, t-test) after ANOVA comparison. (Data are mean values and error bars are std. deviation (n = 3 for each engineered strain)



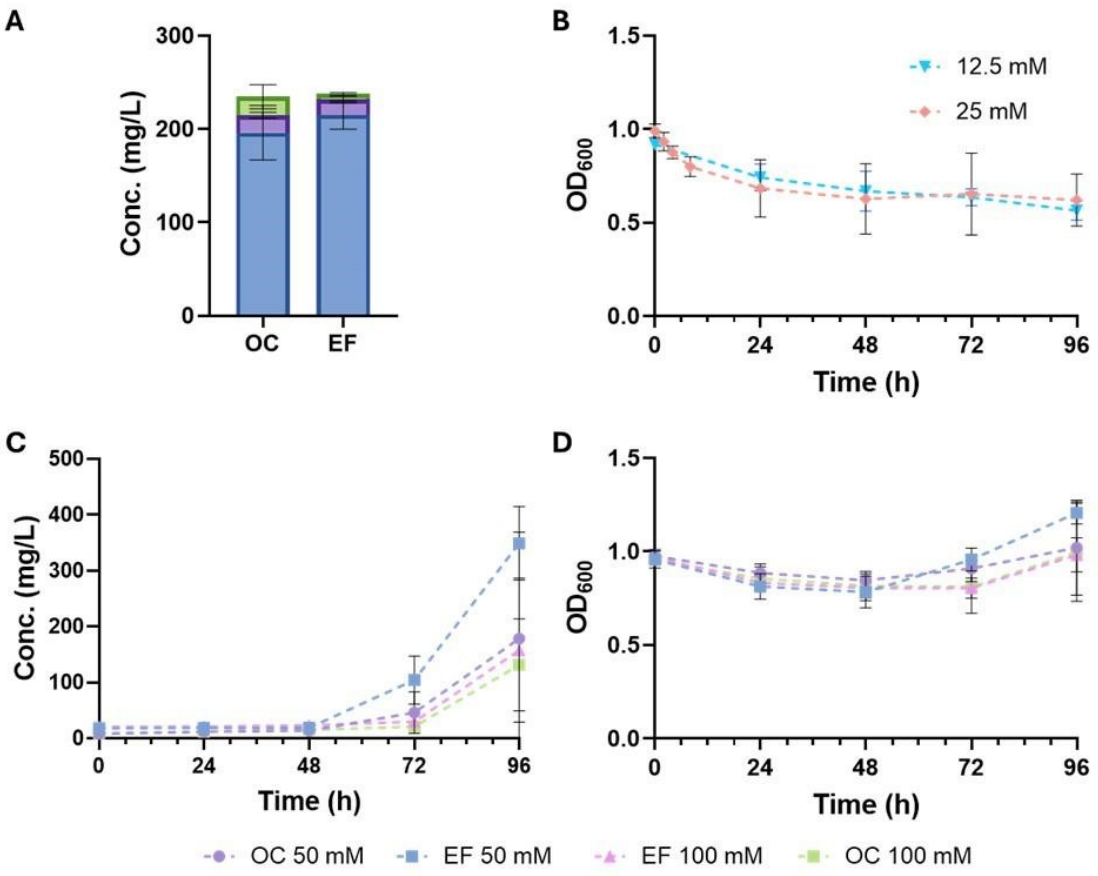
Suppl Fig 4 Construction of anodic electrofermentation systems employed. A. Side view of system setup with M9Y media in the working chamber. B. Top down view of anodic electrofermentation systems with electrodes connected to Biologic potentiostat via crocodile clips.



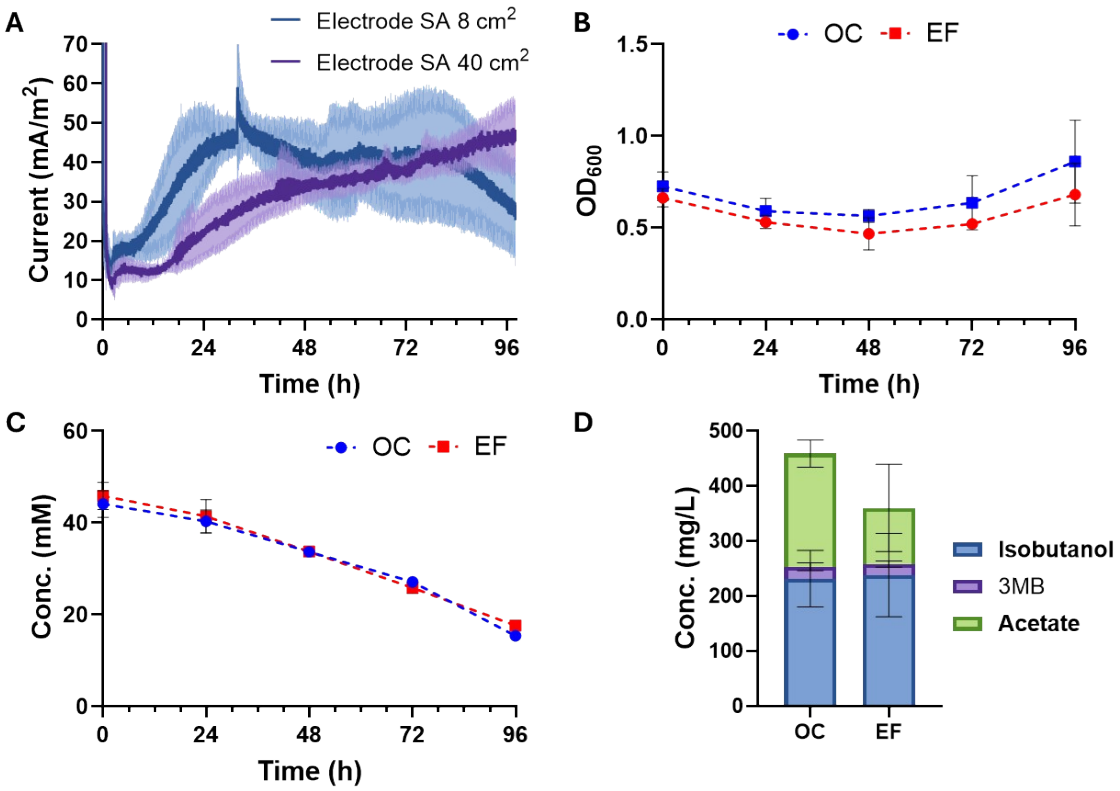
Suppl Fig 5 A. Rapid consumption of glucose (12.5 mM) feedstock over 96 h in anodic electrofermentation and open circuit systems. Glucose concentration was negligible after 24 h. B. Cell density (OD_{600}) of the engineered *E. coli*. The initial decrease observed was attributed to the adhesion of cells to the graphite felt electrodes. C. Maximum production of metabolites, isobutanol and 3MB, in the open circuit and electrofermentation systems. Cultures of MtrCAB-CymA-AlsS-Kivd-AdhA were maintained in M9 (without yeast extract) media for 96 h at 30°C, 200 rpm with the cathode polarised at +0.65 $V_{Ag/AgCl}$. (Data are mean values and error bars are std. deviation (n = 3 for each of EF and OC systems)).



Suppl Fig 6. The production of total butanol metabolites over 96 h with different carbon sources A. glucose (12.5 mM) B. glycerol (25 mM) or C. Acetate (37.5 mM) in anodic electrofermentations or equivalent open circuit systems. D. The consumption of 37.5 mM acetate in the anodic electrofermentation and open circuit systems over 96 h. Cultures of MtrCAB-CymA-AlsS-Kivd-AdhA were maintained in M9 (without yeast extract) media for 96 h at 30°C, 200 rpm with the cathode polarised at +0.65 $V_{Ag/AgCl}$. (Data are mean values and error bars are std. deviation (n = 3 for each of EF and OC systems for each carbon source)).



Suppl Fig 7 A. Maximum production of isobutanol (blue), 3-methylbutanol (purple) and acetate (green) products from 12.5 mM glycerol in anodic electrofermentation and open circuit systems after 96 h fermentation. B. Cell densities of engineered *E. coli* over 96 h in anodic electrofermentation with either 12.5 or 25 mM glycerol added as the carbon feedstock. C. Production of acetate as a metabolite over 96 in anodic electrofermentation and open circuit systems with either 50 or 100 mM glycerol as the carbon feedstock. D. Changes in cell density of engineered *E. coli* over 96 h in anodic electrofermentation and open circuit systems with either 50 or 100 mM glycerol added. Cultures of E. coli strain MtrCAB-CymA-AlsS-Kivd-AdhA were maintained in M9 (without yeast extract) media for 96 h at 30°C, 200 rpm with the cathode polarised at +0.65 $V_{Ag/AgCl}$. Data are mean values and error bars are std. deviation (n = 3 for each of EF systems for each carbon source) and (n=2 for each OC system for each carbon source).



Suppl Fig 8. A. Production of current (normalised to surface area) in the anodic electrofermentation system using working electrodes with projected surface areas of 8 and 40 cm^2. Shading represents standard deviation of replicate data. B. Changes in cell density of engineered *E. coli* over 96 h in anodic electrofermentation and open circuit systems with working electrodes of surface area of 40 cm^2. C. Consumption of glycerol over 96 h in EF and OC systems using a working electrode with surface area of 40 cm^2. D. Maximum production of metabolites in EF and OC systems using a working electrode with surface area of 40 cm^2. Cultures of *E. coli* strain MtrCAB-CymA-AlsS-Kivd-AdhA were maintained in M9 (without yeast extract) media with 50 mM glycerol for 96 h at 30°C, 200 rpm with the cathode polarised at +0.65 $V_{Ag/AgCl}$. Data are mean values and error bars are std. deviation (n = 3 for each of EF and OC systems).

Suppl Table 1. Peptide mass sequencing of excised gel bands indicated in Suppl Fig 1 matched against the entire *E. coli* transcribed protein databank and Mtr and cymA supplied sequences.

SDS-PAGE Band	# AA's in protein	Log Probability	Best score	Total Intensity	# of spectra	# of unique peptides	Sequence Coverage %
MtrA	333	318.19	896.80	168350517.1	95	28	54.65
MtrB	697	309.85	973.30	71956765.7	87	32	42.47
MtrC	671	2433.66	1558.50	4481759264.5	1990	226	95.68
cymA	187	57.85	728.70	9463483.0	12	8	26.74