Acetogen biocatalyst *Clostridium* sp. MTEtOH871 engineered with our proprietary electrotransformation technology and equipment: continuous synthesis gas fermentation for selective ethanol production

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Acetogen Clostridum sp. MT683 produced 272 mM acetate (p<0.005) and 263 mM ethanol (p<0.005) fermenting syngas (60% CO + 40% H_2) in a single stage continuous fermentation with zero CO_2 emission. Inactivation of phosphotransacetylase (pta) gene in Clostridium sp. MT683 eliminated acetate production and increased ethanol yield to 363 mM (p<0.005). Ethanol production in Clostridum sp. MT683 was further increased when the synthetic acetaldehyde dehydrogenase (aldh) from Clostridium ljungdahlii was cloned in the multi-copy expression vector in this strain. The resulted biocatalyst increased ethanol yield to 576 mM (p<0.005). Electrotransformation efficiencies were (9.30 $\pm$ 0.15) x 10^6 transformants/µg of the expression vector DNA at cell viability ~15%. The integration frequency of pta inactivation was (2.12 + 0.02) x 10^{-5}. Recorded in real time pulse current oscillations reflected the cell membranes electropermeabilization events. This is the first report on inactivation of pta and expression of synthetic aldh in the acetogen biocatalyst for selective biofuel ethanol production during continuous single step syngas fermentation.

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Introduction

Carbon sources not linked to petroleum or sugars/glycerol has become attractive targets for microbial biocatalysis [1, 2, 3]. Certain microorganisms and Acetogens in particular thrive while fermenting synthesis gas (syngas) as the only carbon and energy source [4, 5, 6]. Syngas components CO and H_2 are reduced by such microorganisms to organic carbon of fuels, chemicals and/or food components.

Syngas can be produced from any carbonaceous material. The preferred carbonaceous materials for syngas production include various types of coal, acid-hydrolysed lignocellulosic biomass waste or natural gas due to availability of such carbon sources under easy logistics solutions and at low costs per 1M BTU of $2.50 in the US and some other countries. Other kinds of carbonaceous material are viable on a local commercial scale such as agricultural or municipal waste.

Syngas components CO and CO_2 can be selectively fermented to specialty, commodity chemicals, food components and fuels using metabolically engineered biocatalysts. A partial gasoline replacement ethanol is the most popular biofuel. Several companies use natural strains of acetogens as biocatalysts for syngas fermentation to fuel ethanol. Such technologies are not commercially viable because of ethanol concentrations in the culture broths not high enough for commercial recovery and also due to low product selectivity with the need to separate ethanol from other carbon-containing liquid products of syngas biocatalysis accompanied by CO_2 emissions in the vent gas [5, 7, 8]. There has not been done any actual metabolic engineering with acetogens before this report. There are no indications that

engineered acetogens-biocatalysts may be available for laboratory or pilot scale testing.

The processes disclosed by some companies describe ethanol concentrations in fermentation broth around 1.5% in continuous fermentations (www.coskata.com/process/; www.ineosbio.com/62-Process_overview.htm) which is below the minimal acceptable in industry level for commercial recovery of this fuel (~4%). One of the objectives of this manuscript is to demonstrate the unique efficiency of metabolic engineering enablement namely the efficient gene delivery technology designed specifically for acetogens with the use of the described approach and proprietary electroporation equipment. Such methodology and hardware opens new frontiers for making any research group efficient in doing the same. With the use of our engineered biocatalyst, we describe a revolutionary new approach of decoupling the process of selective fuel ethanol manufacturing from the petroleum or food-associated carbon sources.

Phosphotransacetylase (*pta*) in *Clostridium* sp. MT683 was inactivated via integration of a construct comprising synthetic Erythromycin resistance gene *erm*(B) flanked with the 300-bp fragment from 122 to 432 bp of *pta* from *C. ljungdahlii* DSM13528 from the promoter side. The resulted *pta*$^-$ recombinants of *Clostridium* sp. MT683 increased ethanol yield from 263 mM ($p<0.005$) to 363 mM ($p<0.005$) in a single stage continuous syngas fermentation due to elimination of acetate production. In addition, about 20 copies of synthetic acetylaldehyde dehydrogenase CL (*aldh*CL) were expressed in the *pta*$^-$ recombinant of *Clostridium* sp. MT683. Expression of *aldh*CL further increased ethanol production from 363 mM ($p<0.005$) to 576 mM ($p<0.005$) in continuous single stage syngas fermentation with zero CO_2 emissions.

Technical aspects and intracellular nano-scale events recorded in real time during optimization of the electrotransformation conditions for gene introduction and inactivation in the Acetogen *Clostridium* sp. MT683 using Syngas Biofuels Energy, Inc. electroporation generator are discussed.

Materials and methods

Bacterial strain and antibiotics

All manipulations with the media, bottles, Petri dishes, buffers, samples and cell were performed under the syngas blend (60% CO + 40% H_2) in customized Anaerobe Chamber AS-580 (Anaerobe Systems, CA) with an extra section added and neoprene sleeves and gloves to create and maintain strictly anaerobic conditions (<1 ppm of Oxygen).

Clostridium sp. MT683 was isolated from the oil well flooding water in Siberia. It is a Gram (+) strictly anaerobic catalase (-) spore-forming rod utilizing CO as the sole carbon source producing only acetate and ethanol (24 various compounds were included in the HPLC standard mixture ranging from C_1 to C_7 in their main carbon chain in the preliminary detection during characterization of the strain – data not shown). The purified culture was grown in liquid (SFB) or solidified with 1.2% Agar medium (SFA) recommended in [10]. We used Wheaton serum bottles with capped full size butyl rubber stoppers for liquid culture work and 100 mm Petri dishes for plating with incubation in vented anaerobic Vacu-Quick Jars (Almore International, EA). The syngas blend in the bottles (25 PSI) and jars (6 PSI) was exchanged every 6 hours to ensure proper feed of the microorganisms. The incubation temperature used was 36^{o}C.

For selection and maintenance of recombinants SFB and SFA contained 35 µg/ml Chloramphenicol (Cm) and/or Erythromycin (Em) (Sigma, St. Louis, MO). *Escherichia coli* JM109 (New England BioLabs, MA) was grown in BHI or on BHA (Becton Dickinson, MD) with 40 µg/ml Cm for cloning and plasmid DNA extraction using Qiagen Miniprep Kit (Valencia, CA).

Continuous syngas fermentation

In preliminary studies we have established the optimal parameters for syngas and liquid flow to maintain steady OD_{600} under that particular bioreactor configuration (data not shown).

Single stage continuous syngas fermentation was performed in BioFlo 2000 (2.5 L) vertical bioreactors (New Brunswick Scientific, CO) with two Rushton impellers 1.4 inch apart from each other and 1 inch from the bottom. We installed flat disk four inch in diameter Pall spargers with pore size 0.5 µm (Pall Corporation, TX) covering the bottom of each vessel. Sterile filtered syngas blend (60% CO + 40% H_2) was used in all continuous fermentation experiments. The impeller rotation speed was set at 500 revolutions per minute (RPM). The fermentation broth volume was maintained at 1.8 L and the pH set at 6.4 $\pm$ 0.1 adjusting it with autoclaved 20% NaOH. Each bioreactor initially filled with 1.5 L of the pre-reduced sterile SBF was purged with the syngas for 24 hours with the pH and temperature controls set P-I-D until resazurin changed color to purple indicating the state of anaerobiosis.

Each bioreactor was inoculated with 250 ml of the overnight seed cultures at the seed OD_{600} 4.65 $\pm$ 0.15 either of *Clostridium* sp. MT683 (with no antibiotic), *Clostridium* sp. MT683 (pMT*cat*EtOH32) (SFB with Cm) or *Clostridium* sp. MTEtOH871 (SFB with Cm and Em). The seed cultures were obtained in similar BioFlo 2000 bioreactors as above in a batch mode. The inoculated bioreactors for continuous syngas fermentations were kept running with no liquid flow and the syngas flow 25 ml/min until the OD_{600} reached 6.60 $\pm$ 0.15. At that point the liquid flow was gradually increased from 0 to 15 ml/min while maintaining the OD_{600} 6.65 $\pm$ 0.32 and the gas flow rate constant. Each bioreactor was gravity fed with the sterile pre-reduced SFB in sterile 10 gallon vented bottles (two 60% filled bottles connected in parallel per each bioreactor). The waste culture broths were gravity flown to two sterile vented 10 Gallon bottles. Vents of all bottles were connected to the reservoir with oxygen-free Nitrogen maintained at normal pressure to balance the change in liquid volumes. Each of the continuous fermentations was run for 25 day. Waste culture broths were sampled to monitor the OD_{600}, detection of ethanol, acetate and n-butanol using HPLC and also for collecting fresh cells for the DNA extraction and electro-transformation experiments.

Headspace vent tube of each bioreactor was connected through a sterile filter to the carousel mechanism distributing samples from various bioreactors to the Portable NDIR Syngas Analyzer (Wuhan Cubic Optoelectronics CO., Ltd, China) via the 0.25" copper tube line. The copper line and the carousel mechanism were purged with oxygen-free sterile nitrogen after each sampling. The analyzed syngas components were CO, CO_2 and H_2 for real-time analysis with sampling every 15 min for each bioreactor.

Multi-copy expression vector pMT*cat*MCS46 for *Clostridium* sp. MT683

The expression vector (Figure 1) was engineered by adding the MCS (*Vei*I (306) to *Pvu*II (628)) of pUC19 (New England BioLabs, MA) to an insert to engineer a multi-copy vector pMVT*cat*MCS46 for heterogeneous gene expression in *Clostridium* sp. MT683. The insert comprised the synthetic Cm-resistance gene and the origin of replication from *Clostridium* sp. MT351 suitable for plasmid replication in both *Clostridium* sp. MT683 and *E. coli* JM109. The synthetic Cm-resistance gene comprised the promoter and terminator sequences of *Clostridium ljungdahlii* DSM13528 (NC014328, region 1010564…1012444) flanking *cat* gene (FM201786) CDS. Promoter and terminator sequences were identified using Softberry Bacterial Promoter, Operon and Gene finding tool (http://linux1.softberry.com/). We used the origin of replication of a ~35-copy number 1.8 kb cryptic plasmid pMT351 we have isolated previously from the Acetogen *Clostridium* sp. MT351 [9]. The identification of the origin in pMT351 was performed using the tool for

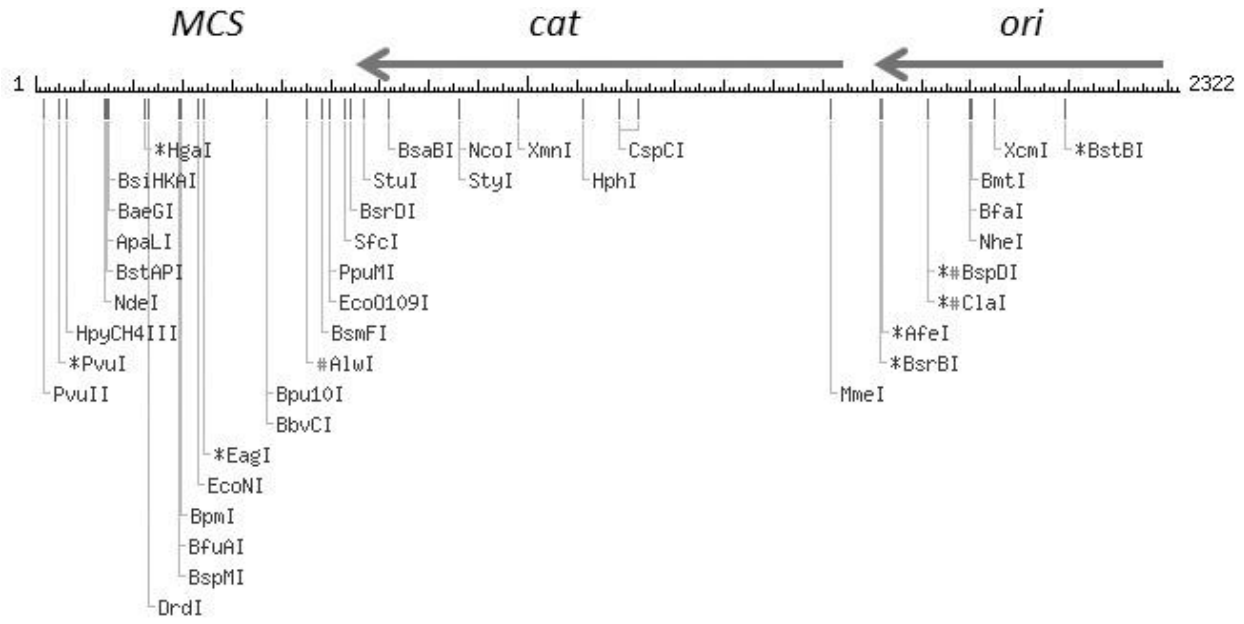


Figure 1. Vector pMTcatMCS46.

plasmid origin detection proposed in [11]. The sequence of the origin of replication used was: 5'-aaaaacgatt ttttattaaa acgtctcaaa atcgtttctg agacgtttta gcgtttattt cgtttagtta tcggcataat cgttaaaaca ggcgttatcg tagcgtaaaa gcccttgagc gtagcgtggc tttgcagcga agatgttgtc tgttagatta tgaaagccga tgactgaatg aaataataag cgcagcgccc ttctatttcg gttggaggag gctcaaggga gtatgaggga atgaaattcc ctcatgggtt tgattttaaa aattgcttgc aattttgccg agcggtagcg ctggaaaatt tttgaaaaaa atttggaatt tggaaaaaat ttggtttgat gttgcgatta atagcaatac aattgcaata aacaaaatga tcgatgctgt ttggcaaaaa aagaaaaagt gattaattta tattttattt atggcgctaa tttattacgg ctttttttgt tgtcggctag ccgattctga tacatttttt taagcacaaa aaccacccaa ttttggagtg gtgtgtaagt gcgcattgtc atgaaaaaat ggcacgcaat ttcatcactt tttaaagtga tgtgtaagtg cgcattgtca tgaaaaaatg gcacgcaatt tcatcacttt ttaaagtgat gtgtaagtgc gcattgttcg aaaaatcgaa ctatgattta tttttgctgt tgtatttatt tttcatcttt tgggttttgg ttttgttttt tgttgctatc gtagtttatt tgctttttaa gggctctatt tttcgttcta cggcattttt ataatttgcc aatataattt ataaaaa-3'. The detection of synthetic *cat* was performed using (I) 5'-tccctcatgg gtttgatttt-3' and (II) 5'-aatgcgcact tacacaccac-3' primers with the expected product size 300 bp.

Vector pMT*cat*EtOH32 for expression of synthetic *aldh*CL of *C. ljungdahlii* DSM13528 in *Clostridium* sp. MT683

The construct was synthesized with insertion of synthetic *aldh*CL gene into *Bam*HI site of the MCS of pMVT*cat*MCS46 to form pMT*cat*EtOH32. The synthetic *aldh*CL comprised promoter and terminator sequences of *C. ljungdahlii* DSM13528 (NC014328, region 1010564…1012444) flanking *C. ljungdahlii aldh* (NC014328, region 1295679…1297232) CDS. Promoter and terminator sequences were identified using Softberry Bacterial Promoter, Operon and Gene finding tool (http://linux1.softberry.com/). The vector with *aldh*CL maintained 20 copies per genome in the recombinants. Detection of synthetic *aldh*CL was performed using (III) 5'-acaaggcgga gttggtaatg-3' and (IV) 5'-tgtgcttgca ccagtacctc-3' primers with the expected product size 247 bp.

Integration vector pMT*erm*(B)*pta* for *pta* inactivation in *Clostridium* sp. MT683

The suicidal integration vector pMT*erm*(B)*pta* was engineered using pUC19 (New England BioLabs, MA) as the backbone with the insert comprising synthetic Em-resistance gene flanked from the promoter side with the 300-bp

fragment from 122 to 422 bp of *pta* from *C. ljungdahlii* DSM13528 (NC014328, region 1376316…1377317) for integration to inactivate the *pta* gene in *Clostridium* sp. MT683. The synthetic Em-resistance gene had the promoter and terminator sequences for the DNA fragment of *Moorella thermoacetica* ATCC39073 (CP000232, region 2626906…2627526) flanking the CDS of *erm*(B) (AY334073). The 300 bp fragment of the *pta* was: 5'-aaatttaatc cttgtaggga atgaaaaggt aataaaagaa aaagcgtcaa aattaggtgt aagtttaaat ggagcagaaa tagtagatcc agagacttca gataaactaa aggcatatgc agatgctttt tatgaattga gaaagaagaa gggaataacg ccagaaaaag cggataaaat agtaagagat ccaatatact ttgctacaat gatggttaaa cttggagatg cagatggatt ggtttcaggt gcggttcata ctacaggtga tcttttgaga ccaggacttc aaatagtaaa-3'. Detection of synthetic *erm*(B) was performed using (V) 5'-ccagaacctc gtccagactt-3' and (VI) 5'-aatgccctttt acctgttcca-3' primers with the expected product size 379 bp.

DNA Isolation, PCR, and DNA synthesis / sequencing

Cell collection, cold washing with 0.1 M Sucrose, treatment with 5 mg/ml lysozyme and SDS lysis were performed in the anaerobic chamber to preserve the DNA preps from the damage with the products of membrane oxidation. All subsequent steps were performed on the bench: Due to the high endonuclease activity in the DNA preparations we had to use the complete range of approaches to decrease the self-digestion of DNA over the extraction time [12]. We used RNA digestion, Proteinase K treatment to decrease the viscosity of the aqueous phase and deactivate endo- and exo-nuclease activities, deproteinization by Tris-equilibrated (pH 8.0) Phenol:Chloroform:Iso-amyl Alcohol mixture (25:24:1 vol/vol/vol) in the presence of 1 M NaCl to enhance the Phenol extraction, and precipitation of the DNA with 3 volumes of ethanol in 0.1 M Sodium Acetate (pH 5.2). Plasmid DNA was extracted in a similar way with 0.2 N NaON was to the SDS. Enzymes and chemicals were purchased from Sigma (St. Louis, MO). Upon the extraction, DNA from *Clostridium* sp. MT683 and its recombinants was additionally purified with Qiagen Gel Extraction Kit (Valencia, CA) to further reduce DNA damage with the residual amounts of the endonucleases.

Phusion® High-Fidelity PCR Kit (New England BioLabs, MA) was used for all PCR reactions.

DNA synthesis of designed *in silico* constructs with mandatory verification of sequences and as well as the synthesis of all primers were performed by Invitrogen (Carlsbad, CA).

Thermal conditions for cell samples during electrotransformation

Preliminary testing was performed using micro thermo resistor-sensor as a part of the TTL-powered Wheatstone bridge with the response time 5 μs coupled with Tektronix TDS220 digital storage oscilloscope (Agilent Technologies, CA). The thermo sensor along with the electrodes shown in Figure 1 was immersed into the cell samples. We used *E. coli* cell samples containing ~10^9 cells in 0.1 ml of purified sterile HPLC grade water placed in disposable polypropylene tubes (cuvettes) imbedded in the ice block prepared as described [13, 14] to precisely fit the wells for the tubes.

Equipment design and electric treatment optimization for electrotransformation

To ensure the cells were collected under anaerobic conditions, we placed EBA™ 21 Benchtop Centrifuge (Cardinal Health, OH) inside the anaerobic chamber. The cells were collected from the waste culture broth flow tube using sterile pre-reduced syringe to preserve sterile and anoxic conditions of the samples. The 10 ml samples were immediately injected into purged with nitrogen sealed sterile serum bottles on ice and immediately transferred to the anaerobic chamber. Then each bottle was opened and the cells transferred to the pre-reduced 15 ml disposable polypropylene tubes. The tubes were chilled on ice for 5 min before collecting via centrifugation for 10 min at 4,140 g. Cells collected from each

originally 10 ml sample of waste culture broth were washed once with sterile cold 0.1 M pre-reduced sucrose and resuspended in 0.1 ml to form a single sample for electrotransformation.

Sterile disposable 0.5 ml polypropylene tubes were used as the electrotransformation cuvettes. The cuvette with the set of re-usable electrodes made of titanium alloy VK-2 with the inter-electrode gat 2 mm is shown in [9, 13, 14].

The Syngas Biofuels Energy, Inc. electroporation generator and their electrode assembly [9, 13] were used to optimize electric pulse parameters to achieve electrotransformation with expression and integration vectors for metabolic engineering. To optimize the pulse voltage at a chosen single square pulse duration 6 ms we prepared 16 identical 0.1 ml samples of cells of *Clostridium* sp. MT683 in sterile cold 0.1 M Sucrose with no DNA added. The samples were subjected to electric treatment with single 6 ms square pulses each at the range of voltages from 2,000 V to 5,250 V with the 250 V increment. A twenty Ω precision thick carbon film low inductance resistor served as the current sensor connecting the negatively charged plate of the power capacitor with the circuit Ground. Tektronix TDS220 digital storage oscilloscope (Agilent Technologies, CA) recorded the real time images of the pulse current. Images on the oscilloscope screen were photographed to document the authenticity of the recordings. The current pulse forms were recorded at the voltages applied to the sample: 2,000; 2,250; 2,500; 2,750; 3,000; 3,250; 3,500; 3,750; 4,000; 4,250; 4,500; 4,750; 5,000; 5,200 and 5,250 V. The recorded images were used to determine the optimal voltage range where the electropermeabilization events took place.

Recorded electropermeabilization events lead to efficient electrotransformation if transforming DNA was added as described in [13]. The rationale of the optimization method was based on identifying the voltage range when the pulse current through the sample suddenly changed with generation of current elements not present in the triggering pulse (Figure 2a). Such changes continued to appear through the voltage magnitude when the cell viability decreased below 10% (the highest voltage level for the optimal pulse voltage range) (Figure 2b). To determine the electrotransformation efficiency and post-pulse cell viability, decimal dilutions of the samples were plated on selective and non-selective SFA, respectively, for each sample treated.

HPLC Analysis

For HPLC analysis a standard mixture containing formate, acetate, ethanol, ethylacetate, butyrate and n-butanol was used on Aminex 87H column (Bio-Rad Laboratories, Inc., Hercules, CA) at 55^{o}C. The mobile phase consisted of the 5 mM sulfuric acid at a flow rate 0.6 ml/min. Detections were performed via refractive index using Waters 2414 RI Detector (Milford, MA). The minimal detection level was 0.3 μM. The samples were prepared by mixing of 0.5 ml 0.1 μM filtered fermentation broth with 0.5 ml 10% H_3PO_3 and filtering again the resulted solution through a 0.1 μM filter to HPLC vial immediately prior the HPLC detection procedure [15].

Results and discussion

Electrotransformation

Selected pulse current form records are shown in Figure 2a and 2b to illustrate the beginning of the membrane electropermeabilization event at 3,000 V and above that voltage through the voltage at which the highest electrotransformation efficiency was achieved. The maximal electrotransformation efficiency was achieved after treatment with the 6 ms input single unipolar square pulse 26.5 kV/cm. Such pulse correlated with the cell viability of 12.5 $\pm$ 0.3% of the original number of the recipient cells prior to electric treatment. With the transforming DNA added for the electrotransformation under the identified optimal electric treatment conditions, the electrotransformation efficiencies were (9.30 $\pm$ 0.15) x 10^6

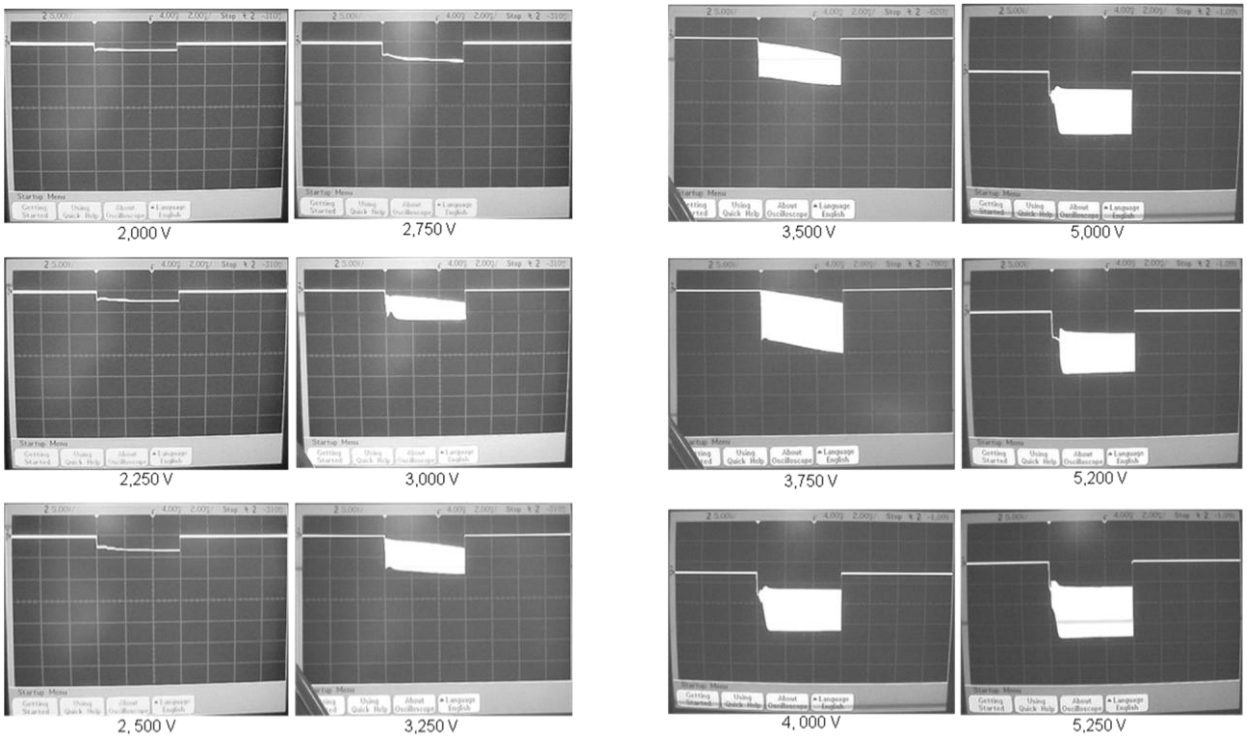


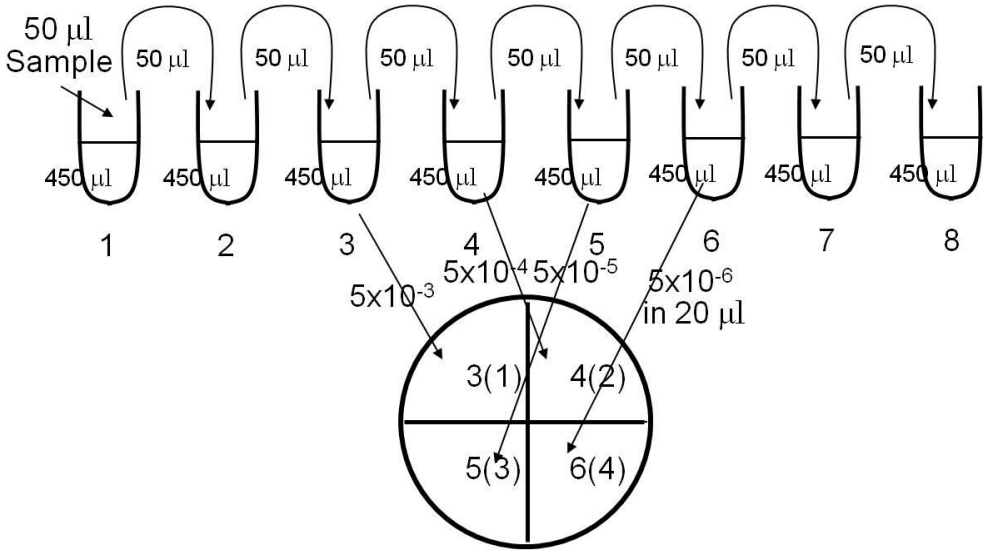
Figure 2. Pulse current images recorded by digital storage oscilloscope at the pulse current sensor and used to identify the range of the voltage which caused electric membrane breakdown essential for electrotransformation. The vertical cell division value was 5 V, the horizontal cell division value was 2 ms. The diagram of the main high voltage/high current circuit relevant to these images was described in [13, 14]

transformants per µg of transforming DNA of the expression vector pMVT*cat*MCS46 at cell viability of 11.6 $\pm$ 0.2%. The scheme of decimal dilutions used to calculate the electrotransformation efficiency is shown in Figure 3a. An example of the selective plate with transformants obtained using Syngas Biofuels Energy, Inc. electrotransformation generator is shown in Figure 3b.

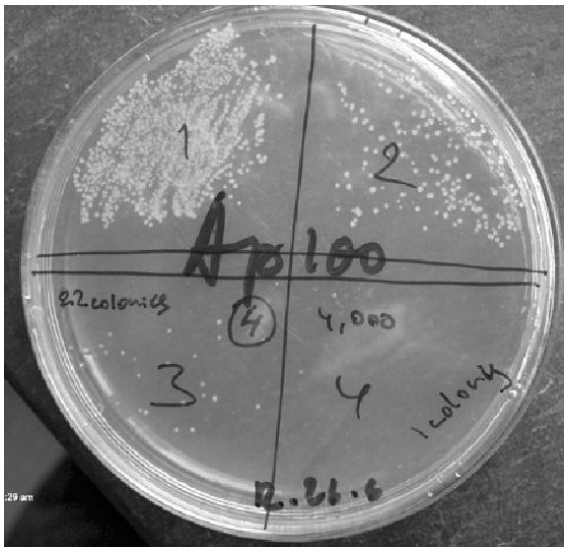
Comparative experiments were performed using BTX-Harvard Apparatus ECM 830 Square Wave Electroporator and the shock chamber (Bio-Rad, CA) along with the 0.1 mm plastic disposable cuvettes (Bio-Rad, CA). We did not detect any colonies on the plates with Em incubated during comparable times under the same conditions as above. At the output voltage set at 3 kV, we sometimes observed electric sparks in the cuvette areas right above the samples at least partially destroying our samples due to reaching limit of the electric field strength of the gas atmosphere surrounding the electrodes with applied high voltage. An example with no transformants on a selective plate when ECM 830 was used is shown in Figure 3c.

Solventogenic microorganisms are stuffed with multi-layer membrane structures. Such structures serve as mechanical barriers preventing transforming DNA molecules from entering the cytosol. To eliminate the barrier function, each of the membrane layers has to be electrically broken. This process is somewhat similar to that of the capacitor insulator breakdown caused by excessive voltage. Transforming DNA molecules are then transported by the pulse electrophoretic force to the cytosol allowing for the DNA replication and transcription [13]. In the circuit design used by Syngas Biofuels Energy, Inc., the pulse

(a)



(b)



(c)

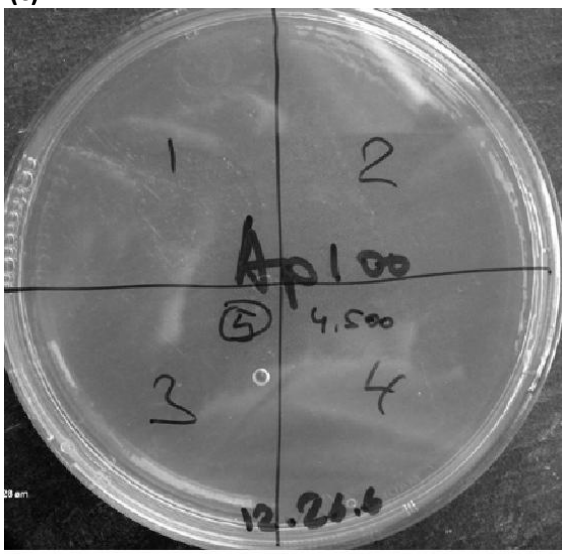


Figure 3. (a) The dilution scheme used for enumeration of transformants to calculate the electrotransformation efficiency on selective agar and the cell viability on non-selective agar. **(b)** Plate with Cm^r clones of *Clostridium* sp. MT683 obtained in electrotransformation experiments with Syngas Biofuels Energy, Inc. electrotransformation generator and the multi-copy expression vector pMVTcatMCS46 as the transforming DNA (series AP 100:4,000; single square 6 ms pulse with amplitude 3,000V; the calculated electrotransformation efficiency was about 10^4 transformants per 1 µg of pMVTcatMCS46 DNA). **(c)** Plate with no Cm^r clones of *Clostridium* sp. MT683 obtained in electrotransformation experiments with ECM 830 electrotransformation generator and the multi-copy expression vector pMVTcatMCS46 as the transforming DNA (series AP 100:4,500; train of ten 600 µs pulses with amplitude 3,000V; the electrotransformation efficiency calculated was 0 transformants per 1 µg of pMVTcatMCS46 DNA).

electric field penetrates a single membrane layer and creates electric pores in it with the extra pulse current going through such pores. The tetrode amplifies this added current flux. Then next membrane layer is subsequently penetrated by the electric field in a similar way via the same mechanism and effect, and so on. The process of subsequent electric breakdown of membrane pockets located next to each other inside the cells happens again and again until all the membrane layers in the way of DNA to cytosol are punctured by the electric

breakdown process. The moment of electropermeabilization of multiple membrane layers is recorded in real time to indicate the optimal voltage for electroporation for the particular type of recipient.

The illustration of our proprietary electroporation approach for achieving time- and cost-efficient electrotranfromation is provided in Figure 2a and 2b. Figure 2a demonstrates the moment when the applied electric field caused massive intracellular membrane breakdown at 3,000 V applied to the sample (beginning of the electropermeabilization event). Figure 2b shows still optimal electroporation conditions range at higher voltages when the electrotransformation efficiency is maximal for a particular type of the recipient cells.

We have to note that there are no commercial generators, except the design we have used for this project with enabled real time monitoring and recording of the actual pulse current and voltage. Such monitoring is essential for identifying the optimal electrotransformation conditions for each particular new strain. The methodology of identifying is simple and quick and does not require adding transforming DNA to the cell samples, selection of the transformants and thus any plating. The methodology is based on the recording of dynamics in the pulse current at various voltages applied to almost identical samples prepared from freshly grown recipient cells [13, 14]. Each positive pulse current flux recorded at the subsequent increase of the pulse voltage in the form of pulse current oscillations as in Figure 2a and 2b corresponds to a new cell membrane electroporation/electric membrane breakdown event for the cells with complex cellular morphology. If cells with simple morphology like *E. coli*, or Lactococci are used, then there is a non-linear sharp pulse current increase at a certain pulse voltage with no pulse current oscillations and further increase of the pulse voltage leads to the destroying of such samples with the over current [13].

The records collected in pulse current monitoring at different voltages are then used in the experiments with the transforming DNA added to the samples and the respective selection/plating procedures to select the desired recombinants. This is the first report on electrofusion of untreated cells of Gram (+) bacteria.

Detection of *adh*LE, *cat*, or *erm*(B) in recombinants

In the *pta* inactivation experiments, the integration frequency was (2.12 $\pm$ 0.02) x 10^{-5} per the number of the recipient cells. The obtained recombinants were analyzed for the presence of *adh*LE, *cat*, or *erm*(B). Figure 4 shows the specific PCR products for the templates containing *aldh*CL, *cat*, or *erm*(B) genes in the respective recombinant cells.

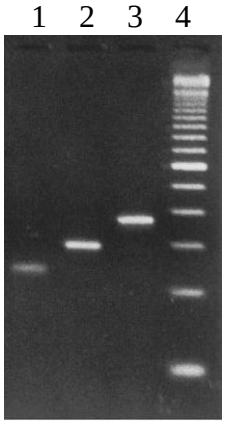


Figure 4. PCR products specific for the templates containing *adh*LE, *cat*, or *erm*(B). Lane 1 - 250 bp product specific for *adh*CL and primers (III) and (IV), lane 2 – 300 bp product specific for *cat* and primers (I) and (II), lane 3 – 397 bp product specific for *erm*(B) and the primers (V) and (VI), lane - 4 - Qiagen 100 bp Ladder.

Single stage continuous fermentation

Figure 5a shows single stage continuous syngas fermentation using *Clostridium* sp. MT683 which represents the original levels of 256 mM acetate ($p<0.005$) and 263 mM ethanol ($p<0.005$) produced by this strain. Recombinant of *Clostridium* sp. MT683 containing the multi-copy expression vector pMVTcatMCS46 produced similar amounts of ethanol and acetate (Figure 5b). There was no significant

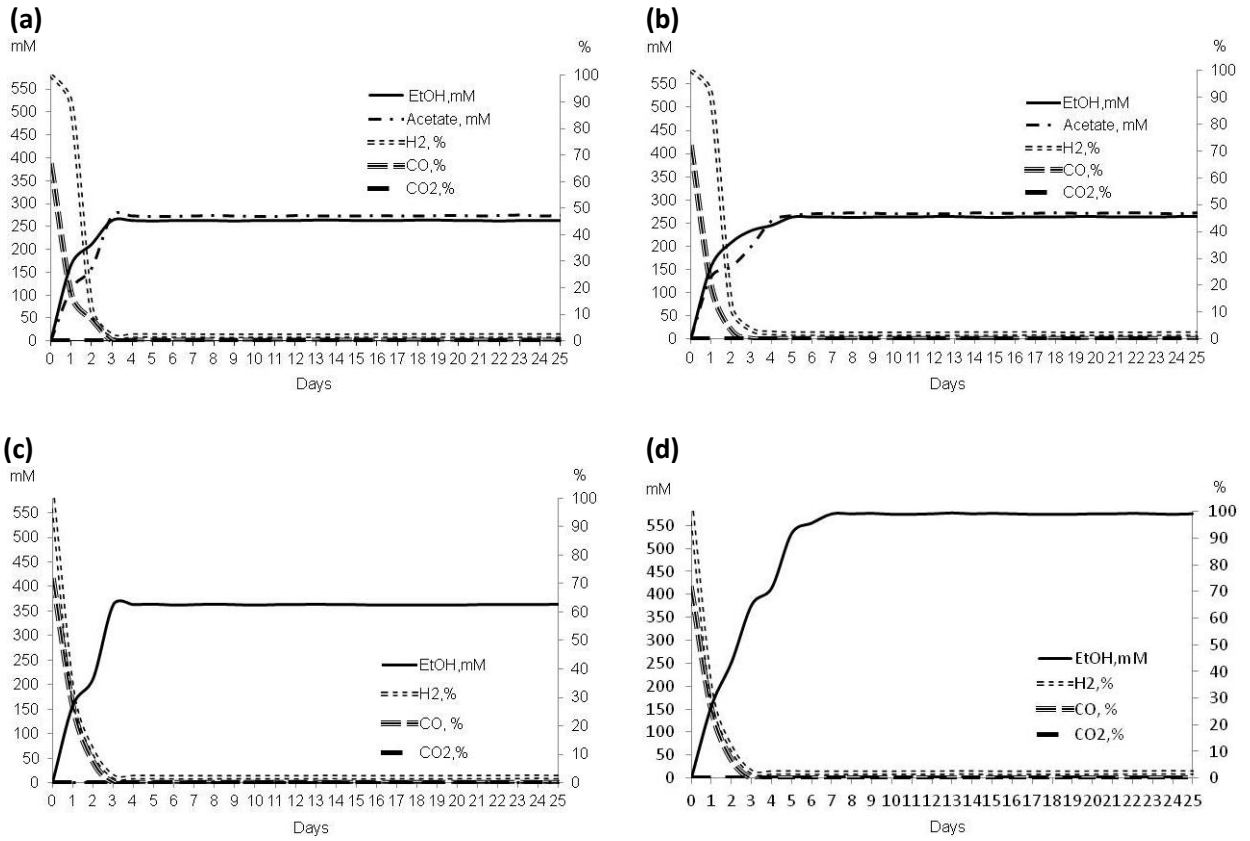


Figure 5. Plates with transformants of the acetogen with **(a)** Continuous syngas fermentation of *Clostridium* sp. MT683; **(b)** Continuous syngas fermentation of *Clostridium* sp. MT683 (pMVTcatMCS46); **(c)** Continuous syngas fermentation of *Clostridium* sp. MT683pta-; **(d)** Continuous syngas fermentation of *Clostridium* sp. MTEtOH871.

delay in the culture performance when the SFB additionally contained Cm for vector maintenance. Inactivation of *pta* in *Clostridium* sp. MT683 resulted in elimination of acetate production by this recombinant at the increased ethanol production reaching ~363 mM ($p<0.005$). We noticed the delay in establishing the stable performance of that biocatalyst (Figure 5c) which we explain by the growth inhibiting effect of the two antibiotics added to SFB for the maintenance of the constructs. The ethanol yield was increased even more when the synthetic *aldh*CL encoding alcohol dehydrogenase from *C. ljungdahlii* DSM13528 was expressed in *Clostridium* sp. MT683 (strain *Clostridium* sp. MTEtOH871). Expression of *aldh*CL increased ethanol production in continuous syngas fermentation to 576 mM ($p<0.005$) (Figure 5d).

The described ethanol yields 2.61% ($p<0.005$) were maintained during continuous syngas fermentation with zero CO_2 emissions. Such yields are about twice of that ever achieved with single- and multi-stage continuous syngas fermentations [5]. Recovery of ethanol becomes economical at its concentration above 4% in water solutions. Therefore the future efforts will be directed 1) to increase ethanol concentrations in the fermentation broth by the means of metabolic engineering, 2) to ensure that the genes encoding ethanol production are stabilized with no need to use selective antibiotics, and 3) to make the biocatalyst strain theft-proof using some additional metabolic engineering approaches. This is the first report on elimination of acetate production and expression of the synthetic *aldh* gene in Acetogen biocatalyst for selective biofuel

ethanol production during single stage continuous syngas fermentation.

Conclusions

Ethanol production was increased due to elimination of acetate production via inactivation of *pta*. Further increase of ethanol yield was achieved when heterogeneous synthetic acetaldehyde dehydrogenase was expressed on the multi-copy number expression vector. Even the production rates achieved in this report make the process economical if another continuous fermentation stage is added to bring the ethanol concentration level to above 4.5% (Tyurin M – unpublished data 2001).

Metabolic engineering of the biocatalysts fermenting syngas has become possible after we have developed the time- and cost-efficient ways to introduce DNA into cells of acetogens and achieve its expression due to the rational *in silico* design of the constructs used.

Continuous single stage syngas fermentation rendered stable day-to-day performance of the engineered biocatalyst strain with up to 2.61% ($p<0.005$) ethanol in fermentation broth in 25 day-long runs. Continuous fermentation has obvious advantage as compared to batch fermentations reported for ABE using solventogenic clostridia [16] due to reducing of overall time for maintenance of the bioreactors about twice in the bioreactor maintenance cycles between subsequent runs.

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