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Genome tailoring powered production of isobutanol in continuous CO_2/H_2 blend fermentation using engineered acetogen biocatalyst

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Abstract The cell energy fraction that powered maintenance and expression of genes encoding pro-phage elements, *pta-ack* cluster, early sporulation, sugar ABC transporter periplasmic proteins, 6-phosphofructokinase, pyruvate kinase, and fructose-1,6-disphosphatase in acetogen *Clostridium* sp. MT871 was re-directed to power synthetic operon encoding isobutanol biosynthesis at the expense of these genes achieved via their elimination. Genome tailoring decreased cell duplication time by 7.0 ± 0.1 min ($p < 0.05$) compared to the parental strain, with intact genome and cell duplication time of 68 ± 1 min ($p < 0.05$). *Clostridium* sp. MT871 with tailored genome was UVC-mutated to withstand 6.1 % isobutanol in fermentation broth to prevent product inhibition in an engineered commercial biocatalyst producing 5 % (674.5 mM) isobutanol during two-step continuous fermentation of CO_2/H_2 gas blend. Biocatalyst *Clostridium* sp. MT871RG-$_{11}$IB^{R}6 was engineered to express six copies of synthetic operon comprising optimized synthetic format dehydrogenase, pyruvate formate lyase, acetolactate synthase, acetohydroxyacid reductoisomerase, 2,3-dihydroxy-isovalerate dehydratase, branched-chain alpha-ketoacid decarboxylase gene, aldehyde dehydrogenase, and alcohol dehydrogenase, regaining cell duplication time of 68 ± 1 min ($p < 0.05$) for the parental strain. This is the first report on isobutanol production by an engineered acetogen biocatalyst suitable for commercial manufacturing of this chemical/fuel using continuous fermentation of CO_2/H_2 blend thus contributing to the reversal of global warming.

Keywords Acetogens · Isobutanol · Genome tailoring · *Tn*7-based gene integration · CO_2 continuous fermentation · Syngas continuous fermentation

Introduction

Energy production from fossil fuels, manufacture of food and chemicals using biocatalysis, and breathing of living beings oxidize carbohydrate carbon to CO_2 with oxygen as the end electron acceptor. The age of reservoir hydrocarbons ranges from over 1 million to more than 600 million years ago (http://theseventhfold.com/peak-oil-101/oil-field-formation/). Most crude oil produced today is between 10 and 270 million years old (http://prodigyoilandgas.com/oil-and-gas-science.html). Nearly 85 % of discovered petroleum reserves, accumulated over millions of years, was almost depleted within just about 200 years. Resulting CO_2 spikes significantly add to the exponentially increasing air CO_2. Doubling of the Earth's population every 35 years from 7 billion in 2011 supports exponentially progressing global warming (http://geography.about.com/od/geographyglossaryd/g/ggdoublingtime.htm) after commercialization of antibiotics (http://www.yale.edu/ynhti/curriculum/units/1998/7/98.07.02.x.html) and modern vaccines (http://www.who.int/bulletin/volumes/86/2/07-040089/en/). In the air gas blend, CO_2 is the heaviest gas with density 1.977 g m^{-3} (air is 1.205 g m^{-3}) (http://www.engineeringtoolbox.com/gas-density-d_158.html). With extra CO_2 on the surface,

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planet surface temperature continues to rise causing more water vapors leaving Earth's gravity field into outer space. NASA's ice discovery on the Moon suggests that Earth actively loses fresh water to the outer space vacuum (http://nssdc.gsfc.nasa.gov/planetary/ice/ice_moon.html; http://www.universetoday.com/95054/earth-has-less-water-than-you-think/). The existence of Moon ice means that Earth's water vapors are trapped on the cold surface while still evaporating from ice (http://www.atmos-chem-phys.net/3/1131/2003/acp-3-1131-2003.pdf). Therefore, global warming will bring a substantial fresh water shortage by 2070 ± 20 years thus causing dramatic changes to the world's economy. Fresh water reserves comprise <3 % of the total planet water reserves (http://ga.water.usgs.gov/edu/earthwherewater.html; http://ngdc.noaa.gov/mgg/global/etopo1_ocean_volumes.html) and most likely will become currency at that time.

Market analysis

Fuel isobutanol is similar to high octane gasoline for high performance engines but with no detonation, no need to modify ignition chamber or to adjust ignition cycle with up to 60 % *n*-butanol in gasoline blends [6]. Despite the long anticipated availability of this perfect next generation fuel extender [24], there is no actual tradeable isobutanol on the fuel market as of yet despite the efforts since 2006 of Gevo (http://gevo.com/) and ButaMax (http://www.butamax.com/). The US$550 billion fraction of the gasoline market is determined by a 60 % gasoline replacement rate with isobutanol fuel extednder. Such an attractive replacement rate has inspired outstanding scientists to develop synthetic expression systems encoding isobutanol from sugars [1, 25]. In addition to \$0.60 gallon^{-1} Blender's Credit, the 2005 EPA Act allows each *n*-butanol gallon to count as 1.3 gallons (http://www.netl.doe.gov/publications/proceedings/01/carbon_seq/7b1.pdf) for the blender's tax incentive purpose. Isobutanol chemical market size is \$560 M year^{-1} at the manufacturing cost from corn starch of \$2.28 gallon^{-1}, while there are no data on the manufacturing costs from petrochemical feedstock (http://www.olade.org/biocombustibles/Documents/Ponencias%20Chile/Sesion%205_C%20Machado_Enbrapa_Brasil.pdf). Isobutanol may be sold at a wholesale price comparable to the gasoline wholesale price of \$2.85 gallon^{-1} (http://www.eia.gov/dnav/pet/pet_pri_refmg_dcu_nus_m.htm) at a stable manufacturing cost with \$0.60 gallon^{-1} blender tax credit applied. Therefore, lowering the isobutanol manufacturing costs will help to occupy isobutanol fuel and chemical market and generate revenue streams manufacturing renewable fuel using our disruptive sustainable technology with negative carbon footprint. When air CO_2 is available at \$15.00 ton^{-1} liquid (http://www.netl.doe.gov/publications/proceedings/01/carbon_seq/7b1.pdf), raw material carbon cost will be about \$0.19 gallon^{-1}, affecting isobutanol manufacturing costs substantially. For commercialization, the established goal might include reaching 8.5 % isobutanol at the second stage of continuous air CO_2 fermentation using engineered acetogen-biocatalyst fermenting air CO_2 directly and selectively to isobutanol with up to 97 % raw material carbon recovery as carbon of isobutanol. Isobutanol from air CO_2 process with negative CO_2 footprint, commercialized at the scale of a \$550 billion fraction of the \$900 billion gasoline market would consume 2.45262×10^9 tons of air CO_2 annually. This is equivalent to 4.809058×10^{10} vehicles taken off the road; alternatively, each 1.2 tons of isobutanol manufactured from air CO_2 will be equivalent to one vehicle taken off the road for a year (http://www.epa.gov/otaq/models/moves/index.htm; http://www.epa.gov/otaq/climate/documents/420f11041.pdf).

An additional \$25 billion market is for isobutene produced from isobutanol via dehydration. Isobutene is a feedstock for the manufacture of polybutene or butyl rubber. Synthetic rubber is made from monomers of isoprene, butadiene, chloroprene, or isobutene via polymerization, with cross-linking using isoprene addition (http://www.icis.com/Articles/2011/07/01/9481223/rubber+industry+seeks+bio-based+chemicals+potential.html). Isobutene can be easily obtained from isobutanol using commercially available alumina dehydration catalysts (http://www.fischer–tropsch.org/DOE/_conf_proc/USDOE-COALLIQ&GAS/94008962/de94008962_rpt3.pdf).

Elimination of genes not used by a particular processes versus gene inactivation or "silencing" has been proven to render more energy to power synthetic pathways in engineered acetogen biocatalysts where elimination of *pta* compared to just inactivation of this gene boosted ethanol production by >20 % [19, 32]. The difference in robustness was reported for a plasmid-carrying versus a plasmid-cured acetogen strain in favor of the plasmid-cured strain [7]. These observations, when combined led to the idea that efficient powering of amplified synthetic operon is likely possible if the host strain genome is tailored by elimination of certain genes unnecessary for the particular process [2–7, 15, 31, 33–36]. In addition, elimination of particular genes encoding glycolysis enzymes adds the value of a theft-proof feature to the biocatalyst preventing unauthorized propagation in the absence of syngas or CO/H_2 blend as a feed (this report) if the set of eliminated genes was unknown to the educated thief.

Genome tailoring releases fractions of the cell energy pool to power maintenance and expression of the amplified isobutanol biosynthesis operon via elimination of 11 genes in this report. Eliminated genes encoded: pro-phage(s) elements *phi*LC3$_i$ and *phi*LC3$_p$; *pta-ack* cluster; early sporulation genes *spo*0A and *spo*0J; sugar ABC transporter

periplasmic proteins (*stp*ABC); 6-phosphofructokinase (*pfk*); *pfk*2 gene encoding 6-phosphokinase-2 (*pfk*2); pyruvate kinase (*pyvk*); and fructose-1,6-disphosphatase (*fdp*) in *Clostridium* sp. MT871. Genome tailoring decreased cell duplication time by 7.5 ± 0.1 min ($p < 0.05$), compared to the parental strain with intact genome and cell duplication time of 68 ± 1 min ($p < 0.05$).

To prevent product inhibition, the authors overcame isobutanol toxicity for eubacteria reported at the level of 0.169 mM in fermentation broth [18]. *Clostridium* sp. MT871 with tailored genome was mutated to withstand elevated concentrations of isobutanol in fermentation broth without increase of cell duplication time, thus with no anticipated product inhibition of the biocatalyst to-be-engineered to produce isobutanol. UVC mutagenesis, offering multiple simultaneous mutations at high frequency [140,] was chosen to perform multi-step mutagenesis to select mutants of *Clostridium* sp. MT871 with tailored genome resistant to 823 mM of isobutanol (6.1 %). Such isobutanol concentration exceeds the industry standard for isobutanol minimum concentration in fermentation broth of 4 % for a commercially sound manufacturing cost [13].

The resulting mutant with reduced genome *Clostridium* sp. MT871RG$_{11}$IsoBut^{R} was engineered to express six copies of the synthetic operon encoding isobutanol biosynthesis and comprising, optimized for this strain, synthetic format dehydrogenase (*fdh*), pyruvate formate lyase (*pfl*), synthetic acetolactate synthase (*als*), acetohydroxy-acid reductoisomerase (*ilv*C), 2,3-dihydroxy-isovalerate dehydratase (*ilv*D), branched-chain alpha-ketoacid decarboxylase gene (*kdc*A), and bi-functional aldehyde/alcohol dehydrogenase (*al-adh*) to enable isobutanol production by the resulting biocatalyst strain *Clostridium* sp. MT871RG$_{11}$IB^{R}6. The amplified synthetic isobutanol operon was integrated into the *att*Tn*7* integration site created during the genome tailoring step and was not affected by the UVC mutagenesis.

This is the first detailed report on engineering of isobutanol producing acetogen biocatalyst suitable for commercial manufacturing of this chemical/fuel using continuous fermentation of CO_2/H_2 blend. Further increase of the produced isobutanol concentration will be desirable to make manufacturing costs outmost competitive.

Materials and methods

Bacterial strain and antibiotics

All manipulations with cell, media/solutions, and glass and plastic labware for microbial cultivation were performed under syngas blend (60 % CO + 40 % H_2 v/v) in a customized Anaerobe Chamber AS-580 (Anaerobe Systems, CA, USA) with added section. Neoprene sleeves and gloves were used to maintain anaerobic conditions with <0.1 ppm of oxygen. The syngas blend composition was elaborated, based on reported earlier empirical stoichiometry of the syngas fermentation process using various engineered biocatalysts to prevent CO starving, causing quick initiation of sporulation event [2, 4–6]. The gas blend 20 % CO_2 + 80 % H_2 (v/v) was used for continuous fermentations.

Parental strain *Clostridium* sp. MT871 was isolated from oil well flooding water in Saratov region (Russian Federation) in 2006 and characterized in detail in 2009. The purified culture was grown in syngas fermentation broth (SFB) or on solidified with 1.2 % syngas fermentation agar (SFA) [28]. The gram (+) strictly anaerobic catalase (−) spore-forming rod utilizes CO/CO_2 as the carbon source to make up 377 mM acetate only. Wheaton serum bottles with capped full size butyl rubber stoppers were used for liquid cultures and 100 mm Petri dishes for plating. Plates were incubated in vented anaerobic Vacu-Quick Jars (Almore International, Beaverton, OR, USA). Syngas in bottles (1.76 kg cm^{-2}) and in jars (0.42 kg cm^{-2}) was exchanged every 6 h to ensure proper microbial feed. Inoculated anaerobic chamber bottles were incubated in IncuShaker 10 L (Southwest Science, Bordentown, NJ, USA). The incubation temperature used was 36 °C. *Clostridium* sp. MT871 was UVC-mutated to tolerate 0.668 mM isobutanol in SFB during single/double step continuous CO_2/H_2 blend fermentation.

Recombinant acetogens were grown in SFB or on SFA containing 35 μg ml^{-1} Chloramphenicol (Cm) (Sigma, St. Louis, MO, USA), or without antibiotic. Accumulation of certain recombinant DNA was performed in *E. coli* JM109 (New England BioLabs, Ipswich, MA, USA) using brain heart infusion broth (Becton–Dickinson, Laurel, MD, USA) in the presence of 40 μg ml^{-1} Cm. Bromocresol purple, the pH indicator added at 0.2 mg ml^{-1} rendering SFA cherry red color at neutral pH and turning color of the respective colonies to yellow at pH below 6.5, allowed detection of colonies producing acids; the non-transformed parental strain, and recombinants producing formic or mevalonic acid at respective stages of the biocatalyst strain engineering.

Spo (+) phenotype detection

Cells exposure to 50 % ethanol for 1 h at 37 °C under anaerobic conditions decreases cell viability of *spo*$^{-}$ recombinants to zero [15, 31, 33, 34, 36] in contrast to 100 % survival of the *spo*$^{+}$ parental strain. Validation of the *spo*$^{-}$ genotype was performed using RT-PCR [15, 31, 33, 34, 36] with respective primers (Table 1).

Table 1 Primers for RT-PCR or qPCR

Gene	Product size (bp)	Primers
cat	180	5′-aacaatttccccgaaccatt-3′; 5′-tggttacaatagcgacggaga-3′
pta	355	5′-gggaagtgcaaaacacgaat-3′; 5′-agcttgtgcttgaactgctg-3′
ack	385	5′-tgggaactagatgcggtgat-3′; 5′-agtgcctctccccttttctt-3′
*spo*0A$_{425\cdot1}$	134	5′-ataataatgcctcaccttga-3′; 5′-cctaatgttatagcccttg-3′
*spo*0A$_{425.2}$	156	5′-aagttttcaagagatgatcc-3′; 5′-ttttgtaactgccgataata-3′
*spo*0J$_{640}$	151	5′-attctctgcaaatcttcttg-3′; 5′-aaggcagctaagaatgataa-3′
*spo*0J$_{460}$	157	5′-tgctcttgtattgactgaga-3′; 5′-gagcattgatacctgaaagt-3′
*phi*LC3$_i$	231	5′-aaaagttggaatatataatgacctgga-3′; 5′-tagtggccatggttttaccc-3′
*phi*LC3$_p$	193	5′-cacagtgcgatctctatgtcaa-3′; 5′-aagtggatttcagattgaggataa-3′
*stp*ABC	231	5′- ggctcctcaaacagaagacg-3′; 5′-gctcctgcttgtttttggtc-3′
pfk	250	5′- ttgcagaaggtattggtgga-3′; 5′-ggctcacttagtgcctcgtc-3′
*pfk*2	162	5′-taagttctttcgccccttga-3′; 5′-gcattgcagaaaaaggagga-3′
pyvk	223	5′-ggggcgtattcccaatactt-3′; 5′-taacccggtctagtccctga-3′
fdp	194	5′-tgaatcctctccacaccaca-3′; 5′-accacgggtgtattccattg-3′
*fdh*λ	921	5′-ctccttctgtagccggtctg-3′; 5′-cccaagacgtggaaggtaaa-3′
pfl	914	5′-gaaggaagcaatggaagcag-3′; 5′-atccttccgtctttccacct-3′
als	865	5′-tgcagtgttgccagtctttc-3′; 5′-cccagatgaacgaccgtact-3′
*ilv*C	229	5′-agcaggttcagcacttggtt-3′; 5′-agcacaaccaattcctttgg-3′
*ilv*D	179	5′-cgcttaaatcgtccgtcaat-3′; 5′-caagcaccttgtcctggaat-3′
*kdc*A	243	5′-gaagccaaattgcggataaa-3′; 5′-cacgatcttctgttgctcca-3′
adh	208	5′-tgggtgaacaacaaccttca-3′; 5′-cgactcgaacctcatccatt-3′

Determination of cell duplication time

A single 36- to 48-h-old colony with approximately 2×10^5 CFU was inoculated in 20 ml of SFB in a 100-ml serum bottle, the bottle was capped and pressurized with CO_2/H_2 blend, then incubated in a shaker at 300 rpm for 17 h and 36 °C outside the anaerobic chamber. The bottle was then placed in an anaerobic chamber, and 1 ml of the culture was taken to prepare and plate decimal dilutions as described [15, 33, 34]. The cell duplication times were calculated for *Clostridium* sp. MT1874 and its recombinants according to the equation: $N = 2^n$, where N is the number of cells in the sample, and n is the cell duplication time in min.

This equation is based on assumptions that (1) the studied acetogen strains have a simple binary cell division cycle, (2) are not triggered to sporulate due to the test performance [15, 33, 34], and (3) a single cell forms a colony.

Continuous fermentation of CO_2/H_2 blend

A system comprising BioFlo 2000 (2.5 l) and BioFlo 310 (5.0 l) vertical bioreactors (New Brunswick Scientific, Edison, NJ, USA), each with two Rushton impellers 3.5 cm apart and 2.5 cm from the bottom of the vessels were used for two-step continuous CO_2/H_2 blend fermentation. Ten-cm flat disk Pall spargers with pore size 0.5 μm (Pall, Jersey Village, TX, USA) covered the bottoms of each vessel. A sterile filtered CO_2/H_2 blend as above was used in all experiments. The impeller speed was set at 200 rpm. The fermentation broth volume was maintained at 1. 8 and 3.8 l, respectively, at pH 6.4 ± 0.1 adjusting it with 0.1 μm filtered pre-reduced 5 M NH_4OH (if necessary). Each bioreactor with 1.5 and 3.5 l, respectively, of pre-reduced sterile SFB [28] was purged with CO_2/H_2 blend for 24 h with the pH and temperature controls set P-I-D until the resazurin became colorless indicating anaerobiosis. BioFlo 2000 was inoculated with 250 ml of overnight seed cultures with OD_{600} 3.65 ± 0.15 of either cultures inoculated. Inoculated bioreactors for continuous CO_2/H_2 blend fermentations were kept running with no liquid flow and CO_2/H_2 blend flow 25 ml min^{-1} until the OD_{600} reached 6.60 ± 0.15. At that point, the liquid flow was gradually increased from 0 to 0.25 ml min^{-1}, maintaining the OD_{600} 6.60 ± 0.16 and adjusting the culture volume to 1.8 l, keeping the gas blend flow constant for the 25-day-long runs. The waste culture broth was gravity flowed to BioFlo 301 with the originally preset liquid volume of 2.3 l then adjusted to 3.8 l with the waste flow from BioFlo 2000. Thus, the total two-bioreactor culture volume was 5.6 l at the constant gas blend flow rate of 25 ml min^{-1}. Each BioFlo 2000 was fed using a peristaltic pump with sterile pre-reduced SFB from two connected in-parallel 38-l vented bottles per each bioreactor. The waste culture broths were gravity flowed to two similar sterile bottles kept under strict anaerobiosis. Vents of all bottles were connected to the reservoir with

oxygen-free sterile nitrogen connected to a nitrogen bottle via ultra precision/low flow regulator QPV (Equilibar, Fletcher, NC, USA) to equilibrate the changes in liquid volumes. Waste culture broths passing to waste culture 38-l bottles through sterile flasks with extra ports were sampled aseptically under anaerobic conditions every 15 min (96 data points daily) to monitor the OD_{600} detection of acetate using HPLC, and also for collecting fresh cells for DNA extraction, electrotransformation, and dry cell weight determination experiments. The vent of each BioFlo 310 was connected via a sterile filter to the carousel mechanism distributing samples from various bioreactors to the Portable NDIR Syngas Analyzer Gasboard 3100P (Wuhan Cubic Optoelectronics, China) (http://www.gassensor.com.cn/English/Product/108155822.html) via a 6.3-mm copper line. The line and the carousel mechanism were automatically purged with sterile oxygen-free nitrogen after each sampling to prevent cross-contamination during sampling. Vent gas components CO, CO_2 and H_2 were analyzed in real-time every 15 min (96 data points daily) for each bioreactor. The data were collected in five independent 25-day-long continuous CO_2/H_2 blend fermentation runs for five independent clones of the above strains each and are presented based on the significance of differences between means. Selected waste culture broth and vent gas samples were also analyzed to compare the performance of continuous CO_2/H_2 blend fermentation by cultures in BioFlo2000 versus the respective BioFlo 310.

Operation of custom bench-top multi-fermentor modules (36 vessels, 50 ml liquid volume each)

Seed cultures for BioFlo2000 inoculations were obtained in custom bench-top multi-fermentor modules (36 vessels, 50 ml liquid volume each) manufactured by the sister company Biocatalyst Gurus, Houston, TX, USA (www.syngasbiofuelsenergy.com). Each vessel was inoculated with cells from a single colony suspended in 2 ml of SFB and kept running with CO_2/H_2 blend flow 0.55 ml min^{-1} using a peristaltic pump to the OD_{600} 1.95 ± 0.06 at pH 6.4 ± 0.1 adjusting it with 0.1 μm filtered pre-reduced 5 M NH_4OH if necessary. The vent of each vessel was connected to 6.3-mm copper line with a steady flow of sterile oxygen-free nitrogen set at 1 ml min^{-1} to mitigate any sudden vent pressure shifts compromising strict anaerobiosis fermentation conditions.

Dry cell weight determination

Dry cell weight was determined in a Gravity Convection Oven WU-05012-13 (http://www.coleparmer.com/) at 65 °C in plastic trays filled with 10 ml of waste cultures grown in BioFlo 2000 until their stable residual moisture ~1.5 %, and the data was normalized for analysis as described [7, 32, 34].

Electrotransformation cuvettes, electrodes and electrotransformation

A Benchtop Centrifuge EBA™ 21 (Cardinal Health, OH, USA) was placed inside the anaerobic chamber to concentrate cells under anaerobic conditions from the waste culture broth (see continuous fermentation section) collected aseptically and to preserve anoxic conditions for the samples and the fermentation set-ups. Samples (10 ml each) were collected as sterile, sealed purged with nitrogen, chilled on ice serum bottles, and transferred to the anaerobic chamber. There the cells were transferred to 15-ml disposable polypropylene centrifuge tubes, chilled on ice and concentrated via centrifugation. Resulting pellets were washed once with sterile cold 100 mM pre-reduced sucrose and re-suspended in the same solution to make 0.1 ml from each original 10 ml sample of the waste culture broth. Additional tests were performed to ensure the correct condition of cell samples prepared for electrotransformation. Special care was taken to prevent dramatic loss in cell viability when taken away from the syngas-fed bioreactor. In no case were cells starving in CO for longer than 20 min in total while on ice. To monitor good cell sample quality, a B & K Precision Model 830B Capacitance Meter (http://www.mcmelectronics.com/product/B-K-PRECISION-830B-/73-640) was used to make sure cell sample capacitance did not decrease below 5 μF which is the state of good cell health [2–7, 15, 29, 31–34] to ensure reproducibility and quality of the results. A typical 0.1- to 0.2-ml cell sample in a 0.5-ml sterile polypropylene tube set in an ice block measures 5–8 μF for the custom electrode assembly [15]. A Dr. Tyurin electrotransformation generator, disposable cuvettes (sterile 0.5 ml PCR tubes imbedded into ice block) and their proprietary nickel–titanium alloy flat parallel electrodes manufactured by the sister company, Biocatalyst Gurus (Houston, TX, USA) (www.syngasbiofuelsenergy.com) were used for electric treatment with single 6-ms square pulses at 6,150 V with digital recording of the pulse current as described [2–7, 15, 31–35]. Cell viability was monitored by counting colonies grown on SFA inoculated using 0.1-ml aliquots of decimal sample dilutions [2–7, 15, 30, 32–35].

DNA isolation

DNA isolation was performed under anoxic conditions through A clear lysate stage to preserve DNA preps from the damage with the products of membrane oxidation [2–7, 15, 29, 31–34] with modifications. Cell collection, cold wash with sterile 100 mM Sucrose in HPLC-grade water,

treatment with 5 mg ml^{-1} 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: RNA digestion, proteinase K treatment to decrease viscosity of the aqueous phase and deactivate endo- and exo-nuclease activities, de-proteinization by tris-equilibrated (pH 8.0) phenol/chloroform/isoamyl alcohol mixture (25:24:1 vol/vol/vol) in the presence of 1 M NaCl, and precipitation of DNA with three volumes of ethanol in 100 mM sodium acetate (pH 5.2). All enzymes and chemicals were purchased from Sigma, St. Louis, MO, USA. Upon the extraction, the DNA was additionally purified with Qiagen Gel Extraction Kit (Valencia, CA, USA) to decrease degradation with endogenous endo- and exo-nucleases.

HPLC analysis

For HPLC analysis which was performed as described [2–7, 15, 31–35] standard positive control mixture containing formate, acetate, ethanol, acetaldehyde, acetone, acetoacetate, ethyl acetoacetate, butyraldehyde, butyrate, *n*-butanol, and mevalonic acid were used on Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at 55 °C with 5 mM sulfuric acid as the mobile phase at 0.6 ml min^{-1} flow rate. Detections were performed via refractive index using Waters 2414 Infra Red Detector (Waters, Milford, MA, USA). The minimal detection limit was set at 0.1 mM. The samples were prepared by mixing of 0.5 ml of 0.1 μm filtered fermentation broth with 0.5 ml 1.76 M H_3PO_3 and filtering the resultant solution through a 0.1-μM filter to HPLC vials, both done no later than 10 min prior to the HPLC detection.

Fatty acid analysis

Standards and reagents

Lauric (C12:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), isopropylstearate (C20), monounsaturated fatty acid (C20:1) and cyclopropane (C21) fatty acids (HPLC grade for GC analysis), oleic acid sodium salt (HPLC grade for GC analysis) and dichloromethane (DCM) (HPLC grade for GC analysis stabilized with amylene) were purchased from Fluka (Buchs, Switzerland). Pure Sodium oleate powder (assay of fatty acids min 92 %), hydrochloric acid solution (37 %) and 1-propanol (p.a.-ACS) were purchased from Sigma.

Calibration

Calibration curves were produced from a series of standard solutions (25, 50, 100, 250, 500, and 1,000 mg l^{-1}) prepared with the following acids (above): C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20, C20:1, and C21 in a DCM solution. Pentadecanoic acid (C15:0) was used as the LCFA internal standard (IS).

Sample processing

The standards (DCM solution), and experimental samples in DCM liquid were subjected to a similar procedure, ensuring that the organic phase and the aqueous phase always comprised the ratio 0.7:0.3 ml in a total volume of 1 ml. For the standards and experimental samples, once resuspended in DCM, 0.7 ml were transferred into glass vials. Afterwards, 0.01 ml of the IS solution (1,000 mg l^{-1}), 0.19 ml of HCl:1-propanol (25 % v/v) and 0.1 ml of HPLC grade water were added. For fatty acid analysis, 10-ml samples of collected anaerobically bioreactor waste flow (2.2 g l^{-1} DCW) were transferred to the anaerobic chamber. Cells of each sample were pelleted by centrifugation, resuspended in 0.5 ml of HPLC grade H_2O and transferred to Agilent Technologies 5183-2067 Clear Vials with screw caps (Agilent Technologies, Santa Clara, CA, USA) and after cap tightening were frozen in dry ice–ethanol mix. Frozen samples with subsequently loosened caps were transferred to pre-chilled to −70 °C chamber of a 2.5-l Console Cascade Freeze Dry System, −84 °C with PTFE interior (Cole Palmer, Vernon Hills, IL, USA) and dried up until the water vapor pressure became steady at 1 mbar. The content of the vials was weighed (22.000 ± 0.003 mg sample weight; $p < 0.05$) and DCM (0.7 ml), IS (0.01 ml), HCl:1-propanol (0.19 ml), and ultra-pure water (0.1 ml) were added. The mixtures were then vortex-mixed and incubated in sealed vials at 100 °C for 3.5 h in Phoenix Dry Rod Oven High Temperature Type 300 (http://www.airgas.com/content/details.aspx?id=7000000000387). In parallel experiments, desiccated cell pellets were extracted by DCM to measure total fatty acid content in the extract. Sample volumes of 5 μl were analyzed by GC where every five experimental samples were separated with the standard, in five independent repeats.

Gas chromatography

This analysis was carried out in a GC system (CP-9001 Chrompack) (Agilent Technologies) equipped with a flame ionization detector. LCFA were separated using an eq. CP-Sil 52 CB 30 m × 0.32 mm × 0.25 mm column (Teknokroma, Tr-wax), with carrier gas helium at 1.0 ml min^{-1}. Temperatures of the injection port and detector were 220 and 250 °C, respectively. Initial oven temperature was set 50 °C for 2 min, with a 10 °C min^{-1} increase to 225 °C, and then a final isothermal plateau for 10 min.

Promoter and terminator sequences for the components of all vectors

Promoter and terminator sequences for the components of all vectors were identified using the SoftBerry Bacterial Promoter, Operon and Gene Finding tool (http://linux1.softberry.com/).

RT-PCR

Qiagen OneStep RT-PCR Kit and mRNAs isolated from recombinants of *Clostridium* sp. MT871 with subsequently more and more reduced genome until its complete tailoring as planned was used as described [15, 30, 32–34]. Primers specifically to detect the expression of genes targeted and eliminated in recombinants are listed in Table 1.

PCR

PCR to detect presence of *att*Tn*7* was performed using PCR Kit (Qiagen, Valencia, CA, USA) DNA templates isolated from *Clostridium* sp. MT871 with tailored genome (named *Clostridium* sp. MT871TG) using *ack::aat*Tn*7* primers: 5′-gcctgccaacgaaatgtat-3′; 5′-ttgttcttggtgacttgccta-3′.

DNA sequencing and synthesis

DNA synthesis along with DNA sequencing (both DNA strands) for all constructs used here including all primers, and DNA inserts (for pUC19 backbone), were performed by Integrated DNA Technologies, San Diego, CA, USA).

Genome tailoring

Genome tailoring comprised elimination of *phi*LC3$_i$ and *phi*LC3$_p$, *pta, ack*, *spo*0A, *spo*0 J, *stp*ABC, *pfk*, *pfk*2, *pyvk*, and *fdp*.

Integration vector for *pta* elimination in *Clostridium* sp. MT871

Vector pMTloxApta

Integration vector pMT*lox*1*pta* for elimination of *pta* in *Clostridium* sp. MT871 comprised a cassette inside a multiple cloning site of pUC19 (New England BioLabs) serving as a backbone for DNA accumulation in *E. coli*. The first component of the cassette was terminator 1 (region 3491…3541 of NC_014328) flanked by a 450-bp fragment (1375865…1376315) just upstream of the *pta* (NC_014328, region 1376316…1377317). The second component was *lox66* [5] upstream of a synthetic Cm-resistance gene comprising *cat* (region 13456…14106 of FM201786) under the promoter sequence (region 2156–2233 of NC_014328) flanked with *lox71* [5] downstream. The third component of the cassette was a 456-bp fragment (1377318…1377774) just downstream of the *pta* (NC_014328, region 1376316…1377317) flanked by terminator 2 (NC_014328, region 8965…9061).

For electrotransformation, the amount of transforming DNA (pMT*lox*A*pta*) was 10 μg per 0.2-ml samples with selection of recombinants using resistance to Cm.

Elimination of *pta* from chromosome of *Clostridium* sp. MT871

Cm^r recombinant cells were prepared as for the regular electrotransformation, suspended in Cre reaction buffer (New England BioLabs). Each sample was electrotransformed in the presence of 15 U of Cre-recombinase (New England BioLabs) using parameters as for the DNA electrotransformation [2–7, 15, 30, 32–35]. Decimal dilutions of the samples were plated on non-selective SFA to obtain single colonies as illustrated in Fig. 2. Only colonies sensitive to Cm were further tested for the absence of *pta* expression using RT-PCR with the primers listed in the Table 1 (*Clostridium* sp. MT871*pta*$^-$).

Integration vector for *ack* elimination and creation of *att*Tn*7* insertion site

Vector pMT1loxAack.attTn7

Integration vector pMT1*lox*A*ack.att*Tn*7* for elimination *ack* in *Clostridium* sp. MT1243 comprised a cassette inside multiple cloning site of pUC19 serving as a backbone for DNA accumulation in *E. coli* as described [29]. The first component of the cassette was the 61-bp recognition site for Tn*7* (*att*Tn*7*): 5′-gcggcgggagtaccgctcggcgcaccgatccggccttcggatcgatgcgcctgccaacgaa-3′ as per [36] with added downstream terminator 1 (region 3491…3541 of NC_014328) flanked by a 323-bp fragment (NC_014328, region 1376994…1377317) just upstream of the *ack* (NC_014328, region 1377362…1378558). The second component was *lox66* [5] upstream of a synthetic Cm-resistance gene comprising *cat* (region 13456…14106 of FM201786) under the promoter sequence (region 2156–2233 of NC_014328) flanked with *lox71* [5] downstream. The third component of the cassette was a 456-bp fragment (1378559…1379015) just downstream of the *ack* (NC_014328, region 1377362…1378558) flanked by terminator 2 (NC_014328, region 8965…9061). Resistant to Cm colonies were further tested for the presence of *att*Tn*7* using PCR with the primers for the presence of region comprising *att*Tn*7*: 5′-gcctgccaacgaaatgtat-3′; 5′- ttgttcttggtgacttgccta-3′ with the expected product size of 224 bp.

The same colonies were rtPCR tested for expression of synthetic *cat* using primers indicated in Table 1. For electrotransformation, 10 μg of pMT1*lox*A*ack.att*Tn*7* DNA per 0.2 ml sample was used with selection of recombinants using resistance to Cm.

Elimination of *ack* from chromosome of *Clostridium* sp. MT871

Cells of recombinants resistant to Cm were prepared as for regular electrotransformation, suspended in Cre reaction buffer. Each sample was electrotransformed in the presence of 15 U of Cre-recombinase using parameters as for DNA electrotransformation. Decimal dilutions of samples were plated on non-selective SFA to obtain single colonies. Only colonies sensitive to Cm were further RT-PCR tested for the absence of *ack* expression using respective primers (Table 1).

Elimination of *spo*0A gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *spo*0A gene from the chromosome of *Clostridium* sp. MT871 was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *spo*0A. For that purpose, integration vector pMT*lox*A*spo*0A similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 640-bp fragment (region 1223000…1223640) upstream of *spo*0A from *C. ljungdahlii* DSM13528 (region 1223641…1224456 of NC_014328) and the 460-bp (1224457…1224917) fragment downstream of *spo*0A (NC_014328, region 1223641…1224456) was used to eliminate the ORFs of this early sporulation gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *spo*0A expression using primers listed in Table 1.

Elimination of *spo*0J gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *spo*0J gene from the chromosome of *Clostridium* sp. MT871 was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of the *spo*0J gene. For that purpose, integration vector pMT*lox*A*spo*0J similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 425-bp fragment (4620133…4620558) upstream of *spo*0J (region 4620559…4621419 of NC_014328) and the 125-bp fragment downstream of *spo*0J (4621420…4621545) from *C. ljungdahlii* DSM13528 (NC_014328, region 4620559…4621419) were used to remove this early sporulation gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *spo0J* expression using respective primers (Table 1).

Elimination of genes encoding *phi*LC3 and *phi*LC3 integrase from the chromosome *Clostridium* sp. MT871

Elimination of genes encoding *phi*LC3 and its integrase from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *phi*LC3 and *phi*LC3 genes. For that purpose, integration vector pMT*lox*A1 *phi*LC3 similar to pMT*lox*A*pta*37 was engineered with the only difference that, instead of *pta* fragments, the 505-bp fragment (22285…22790 of NC_014328) upstream of the region 22791…23966 of NC_014328 and the 506-bp fragment downstream of 24696…25259 of NC_014328 (NC_014328, region 25260…22766) were used to remove the ORFs of these genes. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of expression of genes encoding *phi*LC3 and its integrase using primers listed in Table 1.

Elimination of *stp*ABC gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *stp*ABC gene from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *stp*ABC gene. For that purpose, integration vector pMT*lox*A*stp*ABC similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 344-bp fragment upstream of *stp*ABC (region 126001…126345 of NC_014328) and the 348-bp fragment downstream of *stp*ABC from *C. ljungdahlii* DSM13528 (NC_014328, region 127333…127681) were used to remove this sugar transport gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *stp*ABC expression using respective primers (Table 1).

Elimination of gene encoding *pyvk* from the chromosome of *Clostridium* sp. MT871

Elimination of gene encoding *pyvk* from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *pyvk* gene. For that purpose, integration vector pMT*lox*A1*pyvk* similar to pMT*lox*A*pta*37 was engineered with the only difference that, instead of *pta* fragments, the 586the bp fragment upstream of *pyvk* (region 337021…337607 of NC_014328) and the 595-bp fragment downstream of *pyvk* (339366…339961 of NC_014328) were used to remove the ORFs of these genes. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of expression of genes encoding *pyvk* using primers listed in Table 1.

Elimination of *pfk* gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *pfk* gene from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *pfk* gene. For that purpose, integration vector pMT*lox*A *pfk* similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 467the bp fragment upstream of *pfk* (region 2855281…2855748 of NC_014328) and the 402-bp fragment downstream of *pfk* from *C. ljungdahlii* DSM13528 (NC_014328, region 2856799…2857201) were used to remove this gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *pfk* expression using respective primers (Table 1).

Elimination of *pfk2* gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *pfk2* gene from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *pfk*-2 gene. For that purpose, integration vector pMT*lox*A*pfk*-2 similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 559-bp fragment upstream of *pfk*-2 (region 336061…336620 of NC_014328) and the 664bp fragment downstream of *pfk*-2 from *C. ljungdahlii* DSM13528 (NC_014328, region 337577…338221) were used to remove this gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *pfk*-2 expression using respective primers (Table 1).

Elimination of *fdp* gene from the chromosome of *Clostridium* sp. MT871

Elimination of targeted *fdp* gene from the chromosome of *Clostridium* sp. MT871was followed by the gene removal event similar to that described in detail above for elimination of *pta* but aimed at elimination of *fdp* gene. For that purpose, integration vector pMT*lox*A*fdp* similar to pMT*lox*A*pta* was engineered with the only difference that, instead of *pta* fragments, the 453-bp fragment upstream of *fdp* (region 3204601…3205054 of NC_014328) and the 547-bp fragment downstream of *fdp* from *C. ljungdahlii* DSM13528 (NC_014328, region 3207054…3207601) were used to remove this gene. Only colonies sensitive to Cm were further tested using RT-PCR for the absence of *fdp* expression using respective primers (Table 1).

UV mutagenesis using MT254UVnator

Custom made apparatus MT254UVnator manufactured by the sister company Biocatalyst Gurus, Inc. (Houston, TX) (www.syngasbiofuelsenergy.com) rendering 18 Joules of UV-C (254 nm) was used for generating UV-induced mutations in acetogens. The apparatus comprises a polypropylene tray covered inside with aluminum reflecting screen and two 9-W UV-C 254 nm powered with independent switching power supplies connected to a precision timer allowing power from the regular ~120-V outlet with 5 s exposure time increments. The bulbs are parallel to the surface of bench or anaerobic chamber and are located 1¼″ (3.175 cm) apart from it. Its liquid-proof design allows sterilization of the inner surfaces and the UV bulbs via wiping with sterile cotton ball soaked in 99.9 % isopropanol outside anaerobic chamber in laminar cabinet in addition to 254 nm UV light sterilizing for 15 min each time prior to experiments after the MT254UVnator was transferred back to anaerobic chamber.

The mutagenesis procedure was performed in anaerobic chamber aseptically

UVC-mutagenesis was performed in six independent steps each time using samples from the previous step, increasing the isobutanol concentration from 0 to 823 mM with 137 mM increments. For each step, ~2 × 10^{11} cells of *Clostridium* sp. MT871TG were collected using centrifugation from 100 ml of culture broth (DCW ~2.2 g l^{-1}) pushed out of the BioFlo 2000 fermentor with an equal volume of pre-reduced sterile nutrient medium, and re-suspended in 30 ml of SFB to layer 15-ml samples evenly in two 100-mm Petri dishes. After placing the MT254UVnator over the dish, the UVC light (254 nm, 18 W) was lit for 35 s (preliminary optimization data not shown). Collected by centrifugation, UVC-irradiated cells (two 15-ml polypropylene centrifugation tubes) were then re-suspended in 1 ml of SFB for each pellet, and plated by 0.1 ml on 20 100-mm Petri dishes with SFA containing the next, in the specified range of six, concentration of isobutanol for resistance to which resistant isobutanol mutants were to be selected. Three 72-h-old colonies obtained at each selection step were re-suspended in 0.5 ml of SFB to inoculate two vessels (2 × 50 ml SFB per each selected clone of three) to run fermentation in a custom bench-top multi-fermentor module (Biocatalyst Gurus) with isobutanol concentration used for selection on Petri dishes. Enriched cultures were then used to make ~2 × 10^{11} cell samples for the next step of the UV-mutagenesis with selection of new mutants resistant to the next higher concentration of the respective chemical. The procedure was repeated six times for each chemical

until the resistance threshold was reached by the mutated strain named *Clostridium* sp. MT871TG_IsoButanol^R.

Integration of synthetic isobutanol pathway

Rationale for recombinants selection

Recombinant colonies producing isobutanol would have higher cell membrane permeability for ethidium bromide compared to colonies formed by non-transformed cells. Recombinant colonies glow orange with plates placed above Dark Reader (http://www.clarechemical.com) emitting wavelength 405 nm. Decontaminated with HPV, Dark Reader was placed inside an anaerobic chamber to immediately pick up glowing colonies for further purification of producing isobutanol [6, 33].

Integration vector pMT.IsoButOH.Tn7.6

Synthesized and supplied in circular form, integration vector pMT.IsoButOH.Tn7.6 for integration of isobutanol biosynthesis synthetic genes in isobutanol-resistant mutant comprised the left end of Tn*7* (Tn*7*L) (JQ429758, region 1893…2058) and then all the subsequent components in inverted orientation relative to the Tn*7*L: terminator (NC_014328, region 8965…9061), alcohol dehydrogenase (*adh*) (CP002273, region 54315…55358), 3′-aaa-5′ spacer, branched-chain alpha-ketoacid decarboxylase gene (*kdc*A) (region 100…1826 of AY548760), 3′-aaa-5′ spacer, 2,3-dihydroxy-isovalerate dehydratase (*ilv*D) (region 18011…19792 of U92974), 3′-aaa-5′ spacer, acetohydroxyacid reductoisomerase (*ilv*C) (region 22045…23079 of U92974), 3′-aaa-5′ spacer, acetolactate synthase (*als*) (AY072795, region 1…1436), pyruvate formate lyase (*pfl*) and synthetic formate dehydrogenase (*fdh*): *fdh*λ (region 777256…779385 of NC_014328), *fdh* subunit D (region 781536…781967 of NC_014328), NADH dehydrogenase I subunit E (*dhI*E) (region 782263…782748 of NC_014328), NADH dehydrogenase I subunit F (*dhI*F) (region 782741…784540 of NC_014328), formyl methanofuran dehydrogenase subunit E (*fmdh*E) (region 996986…997510 of NC_014328), all separated by 5′-aaa-3′ spacers, and the promoter sequence (region 2156–2233 of NC_014328), in six identical copies separated by 5′-aaa-3′ spacers, and then the right end of Tn*7* (Tn*7*R) (JQ429758, region 189…387).

Statistical analysis

The significance of differences between means for all fermentation experiments was evaluated based on a one-sided *t* test as described in [23]. Daily sampling comprised 96 points for the listed liquid and gas components of the process for each vessel in operation for 25 days of continuous fermentation runs in BioFlo2000, in five independent replicas using syngas or CO_2/H_2 blends.

Results

Parental strain *Clostridium* sp. MT871 produced spores

Clostridium sp. MT871 produced 377 mM acetate ($p < 0.005$) establishing steady production level in culture broth by the seventh day of continuous CO_2/H_2 blend fermentation. Figure 1 shows the metabolic profile of *Clostridium* sp. MT871.

Cell duplication time for the parental strains was 68 ±1 min ($p < 0.05$) at dry cell weight 2.15 ± 0.01.

As Table 2 shows, elimination of specified genes led to shortening of the cell duplication time. Elimination of both *pta* and *ack* caused cells to duplicate every 66 min. When all 11 genes were not participating in inorganic carbon

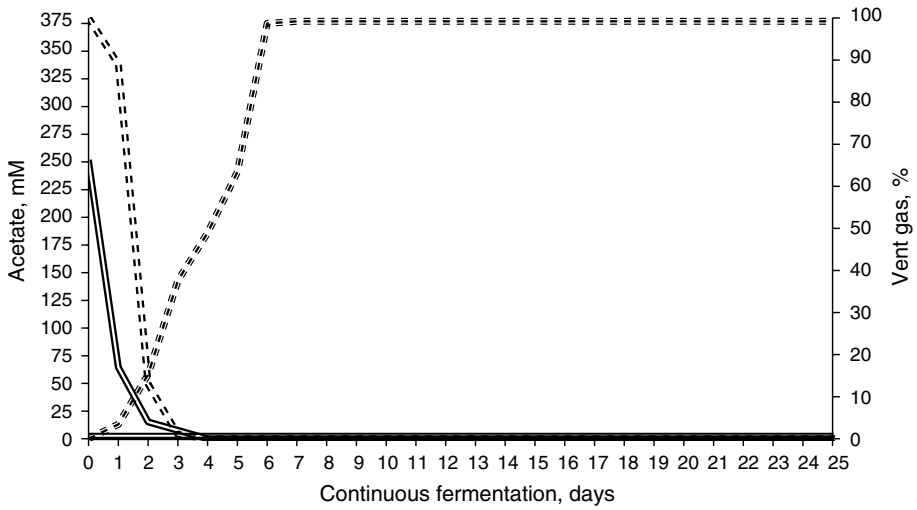

Fig. 1 Continuous CO_2/H_2 blend fermentation using parental strain *Clostridium* sp. MT871. Acetate *triple square dotted line*. Vent gas composition: H_2 *double broken line*, CO_2 *solid double line*, CO *thin–thick solid line*

Table 2 Cell duplication time (*DT*, min) and dry cell weight (*DCW*, g l^{-1}) for parental strain, strain derivatives at various stages of genome reduction and isobutanol-resistant mutant with reduced genome

Strain	Genotype	DT	DCW
Clostridium sp. MT871	Parental strain	68 ± 1	2.15 ± 0.01
Clostridium sp. $MT871RG_1$	*pta*$^-$	67 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_2$	*pta*$^-$ *ack*$^-$ *aat*Tn*7*	66 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_3$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$	66 ± 1	2.15 ± 0.01
Clostridium sp. $MT871RG_4$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$*spo*0J$^-$	65 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_5$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$ *spo*0J$^-$ *phi*$LC3_i^-$	64 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_6$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$ *spo*0J$^-$ *phi*$LC3_i^-$ *phi*$LC3_p^-$	64 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_7$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$ *spo*0J$^-$ *phi*$LC3_i^-$*phi*$LC3_p^-$ *stp*ABC$^-$	63 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_8$	*pta*$^-$ *ack*$^-$ *aat*Tn*7* *spo*0A$^-$ *spo*0J$^-$ *phi*$LC3_i^-$*phi*$LC3_p^-$ *stp*ABC$^-$ *pfk*$^-$	63 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_9$	*pta*$^-$ *ack*$^-$ *spo*0A$^-$ *spo*0J$^-$*phi*$LC3_i^-$ *phi*$LC3_p^-$ *aat*Tn*7* *stp*ABC$^-$ *pfk*$^-$ *pfk2*$^-$	62 ± 1	2.16 ± 0.01
Clostridium sp. $MT871RG_{10}$	*pta*$^-$ *ack*$^-$ *spo*0A$^-$ *spo*0J$^-$*phi*$LC3_i^-$ *phi*$LC3_p^-$ *aat*Tn*7* *stp*ABC$^-$ *pfk*$^-$ *pfk2*$^-$ *pyvk*$^-$	62 ± 1	2.15 ± 0.01
Clostridium sp. $MT871RG_{11}$	*pta*$^-$ *ack*$^-$ *spo*0A$^-$ *spo*0J$^-$*phi*$LC3_i^-$ *phi*$LC3_p^-$ *aat*Tn*7* *stp*ABC$^-$ *pfk*$^-$ *pfk2*$^-$ *pyvk*$^-$ *fdp*$^-$	61 ± 1	2.15 ± 0.01
Clostridium sp. $MT871RG_{11}IsoBut^R$	*pta*$^-$ *ack*$^-$ *spo*0A$^-$ *spo*0J$^-$*phi*$LC3_i^-$ *phi*$LC3_p^-$ *aat*Tn*7* *stp*ABC$^-$ *pfk*$^-$ *pfk2*$^-$ *pyvk*$^-$ *fdp*$^-$	61 ± 1	2.16 ± 0.01

Table 3 Recombination frequency of gene removal tools for each particular gene eliminated per the number of recipient cells ($p < 0.05$)

Strain gene	Clostridium sp. MT871	*Clostridium* sp. $MT871RG_{12}$	*Clostridium* sp. $MT871RG_{12}IsoBut^R$
pta	$8.2 \pm 0.2 \times 10^{-3}$	$7.8 \pm 0.3 \times 10^{-3}$	$8.6 \pm 0.2 \times 10^{-3}$
ack	$6.3 \pm 0.2 \times 10^{-3}$	$3.8 \pm 0.1 \times 10^{-3}$	$3.9 \pm 0.1 \times 10^{-3}$
spo0A	$7.1 \pm 0.2 \times 10^{-3}$	$3.6 \pm 0.1 \times 10^{-3}$	$5.8 \pm 0.3 \times 10^{-3}$
spo0J	$5.2 \pm 0.1 \times 10^{-3}$	$3.8 \pm 0.1 \times 10^{-3}$	$6.5 \pm 0.2 \times 10^{-3}$
phi$LC3_i$	$4.5 \pm 0.2 \times 10^{-3}$	$4.4 \pm 0.1 \times 10^{-3}$	$6.3 \pm 0.2 \times 10^{-3}$
phi$LC3_p$	$7.8 \pm 0.1 \times 10^{-3}$	$9.6 \pm 0.3 \times 10^{-3}$	$8.9 \pm 0.3 \times 10^{-3}$
*stp*ABC	$7.5 \pm 0.3 \times 10^{-3}$	$5.5 \pm 0.2 \times 10^{-3}$	$3.5 \pm 0.1 \times 10^{-3}$
pfk	$7.3 \pm 0.2 \times 10^{-3}$	$3.6 \pm 0.1 \times 10^{-3}$	$6.3 \pm 0.2 \times 10^{-3}$
*pfk*2	$7.8 \pm 0.1 \times 10^{-3}$	$6.3 \pm 0.2 \times 10^{-3}$	$7.1 \pm 0.2 \times 10^{-3}$
pyvk	$6.3 \pm 0.2 \times 10^{-3}$	$6.8 \pm 0.2 \times 10^{-3}$	$9.8 \pm 0.3 \times 10^{-3}$
fdp	$6.2 \pm 0.2 \times 10^{-3}$	$9.2 \pm 0.3 \times 10^{-3}$	$8.1 \pm 0.2 \times 10^{-3}$

The cat integration frequency was the same as for the particular integration construct used for gene removal as indicated in Table 3

of CO_2 reduction to organic carbon of cell mass, the cell duplication time was shortened by 7.0 ± 0.1 min compared to that of the parental strain.

Frequencies of integration for integration vectors used for the elimination of each particular gene are shown in Table 3.

Elimination of respective genes was confirmed by the absence of expression using RT-PCR with primers indicated in Table 1. Integration of *aat*Tn*7* was confirmed by PCR and primers 5′-gcctgccaacgaaatgtat-3′; 5′-ttgttcttggtgacttgccta-3′.

HPLC data

Elimination of *pta* caused loss of acetate production with no other changes to the metabolic profiles of the reduced genome derivatives of the parental strain, also including the isobutanol resistant mutant lacking 11 genes of the original parental genotype. No other liquid organic carbon-containing products were detected in the culture broths of *Clostridium* sp. $MT871RG_{11}$ and *Clostridium* sp. $MT871RG_{11}IsoBut^R$ during continuous fermentation of the CO_2/H_2 blend. *Clostridium* sp. $MT871RG_{11}$ and *Clostridium* sp. $MT871RG_{11}IsoBut^R$ reached stable DCW and gas blend consumption patterns on the third day of continuous fermentation of the CO_2/H_2 blend as compared to the parental strain, where the same growth parameters became stable only on the sixth day of continuous CO_2/H_2 blend fermentation. The engineered biocatalyst strain *Clostridium* sp. $MT871RG_{11}IB^R6$ started producing 674.5 mM isobutanol under the same fermentation conditions (Fig. 2). Selected data on isobutanol accumulation in BioFlo 2000 are presented in Table 4 to help better understand the

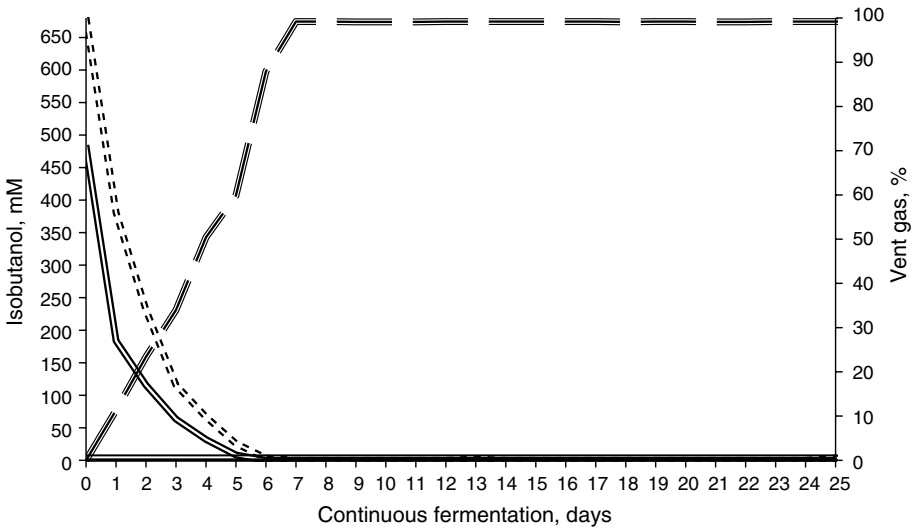

Fig. 2 Continuous CO_2/H_2 blend fermentation using engineered biocatalyst strain *Clostridium* sp. MT871RG-$_{11}$IB^{R}6. Isobutanol *triple square long dashed line*. Vent gas composition: H_2 *double broken line*, CO_2 *solid double line*, CO *thin–thick solid line*

Table 4 Isobutanol (*IB*) accumulation in BioFlo 2000 (1st step of the two-step continuous gas blend fermentation design)

Day	0	1	2	3	4	5	6	7	8	10	15	20	25
IB (mM)	0	12	86	112	156	198	223	276	289	289	289	289	289

The data were collected in five independent 25-day-long continuous CO_2/H_2 blend fermentation runs for five independent clones of the above strains each and presented based on the significance of differences between means

stoichiometry of the process at the first step of continuous CO_2/H_2 blend fermentation along with the total isobutanol content at the second fermentation step (Fig. 2).

UV mutagenesis using MT254UVnator

At each UVC-mutagenesis step, 35 s exposure to the UVC light (18 J) reduced cell viability by ~10^{10} ml^{-1} ($p < 0.05$) rendering 5–16 colonies on 20 agar plates with isobutanol added at concentrations chosen from the noted range (0–823 mM in six equal increments) for the respective mutagenesis step. Three such isobutanol-resistant colonies picked at each mutagenesis step of the same strain were further enriched at the same concentration of isobutanol in SFB in the multi-fermentor vessels containing 50 ml SFB + isobutanol, each. During enrichment, there was no detectable change in cell duplication time (61 ± 1 min) at each mutagenesis step ($p < 0.05$). The UV-mutant was named *Clostridium* sp. MT871RG$_{11}$IsoBut^R.

Fatty acid composition

Total cell fatty acid content and particular fatty acid distribution data for parental strain, mutant with reduced genome, and isobutanol producing recombinant are presented in Table 5.

With no regard to the particular strain, total fatty acid content was found to be similar in all tested samples, 215.03 ± 0.05 mg ($p < 0.05$). Clostridium sp. MT871RG$_{11}$ had a slightly increased share of stearic acid (C 18) compared to the parental strain *Clostridium* sp. MT871. UVC mutagenesis changes the mutant cell fatty acid profile with the additional presence of isopropylstearate (C20:0), monounsaturated fatty acid (C20:1) and cyclopropane fatty acid (C21:0). Isobutanol-resistant recombinant *Clostridium* sp. MT871RG$_{11}$IsoBut^R had essentially the same composition of cell fatty acids compared to the mutant used for metabolic engineering, except that isopropylstearate (C 20:0) and cyclopropane fatty acid (C21:0) were detected at 1 % excess each. Cell fatty acid composition of the engineered biocatalyst strain *Clostridium* sp. MT871RG$_{11}$IB^{R}6 was similar to isobutanol-resistant recombinant *Clostridium* sp. MT871RG$_{11}$IsoBut^R, except that there was a further decrease of palmitic acid content in favor of increasing by 1 % isopropylstearate (C20:0) and cyclopropane fatty acid (C21:0), each (Table 5).

Integration of synthetic isobutanol pathway

Integration of vector pMT.IsoButOH.Tn*7*.6 was detected at a frequency of 9.2 ± 0.3 × 10^{-6} per number of recipient cells. In three independent experiments, 6,235 Petri dishes inoculated with 0.1 ml each of the third dilution of electroporated cell samples were tested to detect five recombinants glowing on Petri dishes with EtBr placed above the Dark Reader (405 nm wave length). Each 72-h-old Petri

Table 5 Average total cell fatty acid content and particular fatty acid composition of parental strain, recombinant with reduced genome, and isobutanol-resistant mutant of that recombinant

Strain	Total fatty acid (mg)	Fatty acids	%
Clostridium sp. MT871	215.02	Palmitic (C16:0)	49
		Palmitoleic (C16:1)	21
		Stearic (C18:0)	17
		Oleic (C18:1)	12
		Isopropylstearate (C20:0)	1
Clostridium sp. MT871RG$_{11}$	215.01	Palmitic (C16:0)	49
		Palmitoleic (C16:1)	21
		Stearic (C18:0)	18
		Oleic (C18:1)	11
		Isopropylstearate (C20:0)	1
Clostridium sp. MT871RG$_{11}$IsoBut^{R}	215.05	Palmitic (C16:0)	25
		Palmitoleic (C16:1)	20
		Stearic (C18:0)	23
		Oleic (C18:1)	19
		Isopropylstearate (C20:0)	6
		Monounsaturated fatty acid (C20:1)	5
		Cyclopropane fatty acid (C21:0)	2
Clostridium sp. MT871RG$_{11}$IB^{R}6	215.02	Palmitic (C16:0)	23
		Palmitoleic (C16:1)	20
		Stearic (C18:0)	23
		Oleic (C18:1)	19
		Isopropylstearate (C20:0)	6
		Monounsaturated fatty acid (C20:1)	6
		Cyclopropane fatty acid (C21:0)	3

dish had about $1.15 \pm 0.05 \times 10^3$ colonies ($p < 0.05$) of the size suitable for further analysis. One of the purified glowing colonies was named *Clostridium* sp. MT871RG$_{11}$IB^{R}6.

RT-PCR revealed expression of the synthetic genes comprising structural parts of *fdh*λ, *pfl*, *als*, *ilv*C, *ilv*D, *kdc*A, and *adh* (primers are from Table 1). Detected expression of the synthetic operon encoding production of isobutanol in optimized isoleucine–valine–phenylalanine pathway (Fig. 3) in *Clostridium* sp. MT871RG$_{11}$IB^{R}6 rendered stable isobutanol production in continuous CO_2/H_2 blend fermentation, shown in Fig. 2.

Discussion

One of the tools for the reversal of global warming, commercial biocatalysis of air CO_2, may be used for direct and selective manufacturing of chemicals, fuels, and food components. The use of the acetyl-CoA pathway is a sound alternative to photosynthesis to reduce atmospheric CO_2 [2–7, 15, 30, 32–35]. Acetogens are known for the highest ratio of cell surface area-to-cell volume to render the shortest paths from enzymes to the extracellular medium, and from extracellular gas nano-bubbles to the intracellular enzymes, at almost 97 % of CO_2 carbon recovered as the carbon of the target product [2–7, 15, 30, 32–35]. With cell doubling time ~65 min under optimal growth conditions, acetogens are the best platform for commercial biocatalysis compared to higher plants and algae, which also have complex cell architecture, and split CO_2 carbon flow significantly with only a small fraction of that recoverable as enzymatically digestible carbohydrates in addition to ~21 h cell doubling time [2–7, 15, 30, 32–35]. Acetogen-based biocatalysis does not require excessive land and USP-grade water reserves for the batch fermentation process in plastic bags where CO_2 is pumped in and oxygen out (algae, cyanobacteria), while offering benefits of selective commercial biotransformation of air CO_2 using engineered biocatalysts [2–7, 15, 30, 32–35]. *In lieu* of that, biocatalysis of vent gas of >100 MW IGCC power plants (100 % CO_2) to carbohydrates is gaining increasing interest along with direct and selective fermentation of CO_2 extracted from air using engineered biocatalysts [2–7, 15, 29, 31–34] with one of the most optimal cell volume to cell surface ratios [39]. There is an indication that CO_2 extracted from air may be available at \$15.00 ton^{-1} liquid (http://www.netl.doe.gov/publications/proceedings/01/carbon_seq/7b1.pdf), thus creating the route to reverse global warming via

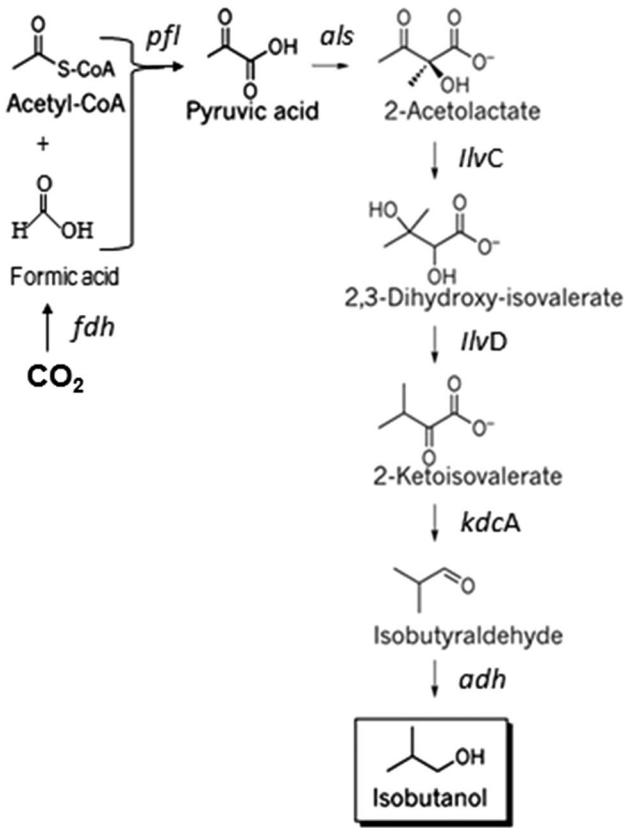

Fig. 3 The engineered acetyl-CoA → to isobutanol pathway in *Clostridium* sp. MT871RG$_{11}$IB^{R}6

direct and selective air CO_2 biocatalysis to target carbohydrates based on the commercialization scale. Another important component to make carbohydrates via direct CO_2 reduction to carbohydrate carbon, H_2, was suggested for economically sound production via water hydrolysis powered by modern solar panels covering 20,000 gallon bioreactor roofs, where solar panels recovering 1 kW m^{-2} (http://www.kyocerasolar.com/assets/001/5154.pdf) render enough electric power with molecular O_2 as the electrolysis by-product [15, 33, 34, 37].

Wide use of acetogen fermentations of syngas or CO_2/H_2 blends is impeded by two misconceptions in the state of the art. There is a concern that the efficiency of gas blend fermentations is limited by low rates of gas-to-liquid mass transfer [12]. For instance, water solubility of H_2, CO, and CO_2 is only 1, 10, and 1,450 mg l^{-1}, respectively. At liquid and gas blend flow rates of 1 and 25 ml min^{-1}, stable dry biocatalyst cell weight (DCW) was >2.1 g l^{-1} in a 2.5-l bioreactor or a million times above hydrogen solubility in water at ambient temperature. Calculations show that, under such conditions, ~26 g of CO_2 was pumped through such a bioreactor over a 24-h period [2–7, 15, 30, 32–35].

For the experimental set-up used with total volume 5.6 l at the liquid flow rate 0.25 ml min^{-1}, the residential time was 22,400 min, while with the actual CO_2 share in the gas blend 20 % (20 % CO_2 + 80 % H_2), corresponding to 5 ml min^{-1} of pure CO_2, it rendered 197.9 g CO_2 l^{-1} for the same time. Therefore, the reported concentration of the continuous fermentation product isobutanol around 5 % suggests rather inefficient use of the introduced source of inorganic carbon due to the low efficiency of the vertical mixing gas bioreactor design for use in continuous gas blend fermentation, but not the presence of the additional carbon source in the medium. The medium included fructose, yeast extract, and tryptone not to mention vitamins, at the total organic carbon supply is quite insufficient to be mentioned as part of the fermentation product carbon.

In addition to that, wild and engineered acetogens, while consuming the CO_2/H_2 blend both performed the so-called gas–water shift reaction in the cells with hard-to-measure carbon stoichiometry, while the fermentation product and comparative fermentation efficiency data might be easily extrapolated to predict the CO and CO_2 contribution to the fermentation process stoichiometry.

Indeed, wild and engineered acetogens both perform intracellularly the so-called gas–water shift reaction with hard-to-measure stoichiometry, while the fermentation product and comparative fermentation efficiency data might be easily extrapolated based on the known efficiency of CO and CO_2 reduction [9–11, 18, 21, 30–36] contributing to the fermentation stoichiometry:

$$CO + H_2O \leftrightarrow CO_2 + H_2 \quad (1)$$

$$6\,CO + 3H_2O = CO_2 + 0.85\ \text{isobutanol} + 0.03\ \text{Cell Carbon} \quad (2)$$

$$2.4\,CO_2 + 7\,H_2 = 0.26\ \text{isobutanol} + 0.008\ \text{Cell Carbon} + 2\,H_2O \quad (3)$$

Equations (1)–(3) clearly indicate that the efficiency of CO used as the substrate by the engineered biocatalyst was higher compared to that of CO_2. In other words, the amount of energy produced during CO reduction exceeds the comparable number if CO_2 was the only substrate, correlating with observations that only about 70 % of biocatalyst productivity was reported when syngas blend (CO + H_2) was replaced with the CO_2 + H_2 blend. Based on (1), the volumetric bioreactor productivity dropped, but in carbon stayed essentially the same if only CO was the inorganic carbon source. In addition to that, CO_2 builds a substantial reserve in liquid due to its high water solubility, preventing biocatalyst cells from rapid starving should the gas feed and a sudden decline in gas blend supply occur. Therefore, while higher gas blend volumetric efficiencies of syngas (CO + H_2) direct and selective fermentation were reported for similar processes [2–7, 15, 30, 32–35], the manufacture of syngas requires burning of fossil fuels thus contributing

to global warming. The objective of the authors' work was to create a new potentially disruptive technology thus enabling the creation of a new line of chemical/fuel industry not competing with the existing technologies rendering the same, but at the expense of air CO_2 as the carbon source for the reported technological process to manufacture isobutanol. Research is underway aimed at in-house manufacture of inexpensive CO_2 from air to make it available at about \$1.50 ton^{-1} raw to feed the process (current CO_2 retail prices vary from \$5 to 20 ton^{-1} raw in-house from the natural gas manufacturer or at a refinery to \$90–150 ton^{-1} compressed as the retail price (http://ethanolproducer.com/articles/7674/carbon-dioxide-apps-are-key-in-ethanol-project-developments; Dr. Henri Chevrel, L'Air Liquide S.A., personal communication, 2013).

Passive diffusion of nano-size gas blend bubbles directly into cells via lipid windows in cell envelopes explained the absence of correlation between gas solubility in the water phase and the detected DCWs [2–7, 15, 30, 32–35]. Cell lipid infrastructure allows the building and maintaining of dense biocatalyst suspensions in culture broths for efficient gas blend conversion [27]. Solubility of gases in cell lipids is higher compared to solubility in water [3, 7, 9, 15, 27], with the reported molar partition of CO_2 and egg lecithin in liposomes as 0.95 [2–7, 15, 30, 32–35]. Passive diffusion of gas into acetogen biocatalyst cells means no cell energy is needed for transport of gases rapidly consumed inside the cells, thus offering no cell energy drain for the biocatalyst feed process. In contrast to inorganic carbon reduction in acetogens causing energy release and preservation as ATP, use of sugar carbon for subsequent oxidation to fuels and chemicals consumes energy for substrate cellular intake [15]. The second misconception links the acetate pathway in acetogens to major routes of energy conservation [10–12]. Elimination of the acetate pathway in engineered acetogen biocataslysts to render the process ultimately selective did not compromise their robust performance and dry cell weight numbers for selective production of methanol, formate, ethanol, acetone, *n*-butanol, 2,3-butanediol, or mevalonate from syngas [2–7, 15, 30, 32–35]. These facts correlated with the detailed analysis of energy conservation in the acetyl-CoA pathway: each mol of ATP generated in the acetate pathway is immediately consumed by the methyl branch [18]. Therefore, the acetate pathway does not serve as the major sink for the reduced carbon. Elimination of the acetate pathway improved biocatalyst robustness when the excess of acetyl-CoA was fitted to the particular process needs via metabolic engineering [2–7, 15, 30, 32–35]. Powering of acetogen cells was explained via $\Delta\mu Na^+$-driven phosphorylation of ADP with CO and H_2 as the energy source and CO_2 as the electron acceptor. In the Wood–Ljungdahl pathway, ATP synthesis is directly powered by $\Delta\mu Na^+$ [2–7, 9, 15, 30, 32–35]. Acetogens produce Gibb's free energy: 95 kJ mol^{-1} reducing 2 mol of CO_2 to 1 mol of acetyl-CoA [2–7, 10, 11, 15, 30, 32–35].

In the traceable 3.5-billion-year-long past, acetogens have acquired glycolysis millions of years ago via wthe ell-studied mechanism of horizontal evolution [22]. Glycolysis allows for cell survival and propagation when sugars are available as the carbon source for subsequent oxidation. Alternatives in mechanism carbon flow paths require complex intracellular maintenance similar to that if, for instance, an eight-cylinder pickup track is driven with only four cylinders actively combusting gasoline. That means that either gycolysis or the acetyl-CoA pathway may operate at a given moment. The objective of the genome tailoring approach is to make the genome as metabolically fit as possible. The resulting biocatalyst pays an enormous price for that. It loses the capability to survive in the environment but gains a nearly theoretical performance specific to the particular chemical process. The sudden added value of that is that recombinants with a tailored genome are hard to steal if the educated thief does not know the exact composition of the supportive nutrient medium to maintain the stolen culture. Inactivation of several glycolysis genes was the objective of this project aimed at unleashing the cell energy fraction to power an integrated then amplified synthetic isobutanol path. The genome tailoring approach is new. Genome reduction approach is well known. Scarab genomics perfectioned a genome reduction approach aimed at the reduction of cell duplication time and increasing recombinant robustness (http://www.scarabgenomics.com/). Scarab genomics offers reduced genome enterobacterium which can be easily modified back to any of the known enterobacteria genera by inserting specific genes for that genus to make *Shigela*, *Salmonella*, *E. coli*, or any other specific genus member [9]. Surprisingly, Scarab genomic efforts have led to reduced genome recombinants which have reduced cell duplication times, clearly proving the author's concept that extra gene "burden" in a particular genome affect strain performance and robustness just like a group of adopted foster children affects the overall economic performance of a family on a fixed budget.

To prevent product inhibition, the authors overcame isobutanol toxicity for eubacteria reported at the level 0.169 mM in the fermentation broth [17], *Clostridium* sp. MT871 with tailored genome was mutated to withstand elevated concentrations of isobutanol in fermentation broth without increase of cell duplication time, thus with no anticipated product inhibition of the biocatalyst to be engineered to produce isobutanol. UVC mutagenesis offering multiple simultaneous mutations at high frequency [14, 38] was chosen to perform multi-step mutagenesis to select mutants of *Clostridium* sp. MT871 with a tailored genome resistant to 688 mM of isobutanol (6.1 %). Such an isobutanol concentration exceeds the industry minimal standard

for minimal isobutanol concentration in fermentation broth (4 %) [13].

The UVC exposure causes UV signature and non-UV signature mutations. UV signature mutations comprise the replacement of C to T at pyrimidin–pyrimidin locations, error-free translesion DNA synthesis, and the formation of cyclobutane pyrimidine dimers. UV signature mutations also comprise error-prone translesion DNA synthesis (TLS) mediated by TLS polymerarses which perform TLS by relaxing or ignoring the conventional Watson–Crick base-pairing rule between an incoming dNTP and the template base of DNA (5′-NTC-3″ to TTT, G to T, T to G). Non-UV signature mutations comprise triplet mutation (5′-NTC-3″ to TTT) and error-prone TLS [14]. Often such mutations have pleiotropic effects [26].

Indeed, the isobutanol-resistant mutant had a content of cell fatty acids different from that of the parental strain. Multiple changes in composition and content of the fatty acid cellular pool in mutants were obtained in six independent mutation steps with selection using isobutanol in concentrations increased at each subsequent mutation step. Therefore, structural genome changes would require the whole genome re-sequencing to obtain detailed maps of the mutations resulted in increased tolerance to isobutanol. At the time the project was done (2006–2009), the cost of single bp sequencing was $0.15 (http://www.axeq.com/axeq.html), thus bringing sequencing of the whole genome with the size 4.9×10^6 bp to a cost >$700,000. Modern DNA sequencing technologies and companies offer the whole genome sequencing price below $4,000 [40]. Therefore, the authors of this manuscript prepared three years back would leave solving the mystery of isobutanol resistance in acetogens to academic experts relying on grant support, and move forward with commercialization of the developed technology first. The industrial approach will be to proceed forward with the engineering of respective commercial grade biocatalysts resistant to the commercially relevant concentrations of the target chemicals, in order to render near theoretical biocatalyst performance for the industrial process when multiple copies of respective synthetic operons encoding the target products would be integrated at the created *att*Tn*7* sites.

Gene elimination as opposed to gene inactivation via knocking out or "silencing" with anti-sense RNA was proven to render more cell energy to power synthetic pathways in engineered acetogen biocatalysts where elimination of *pta* compared to just inactivation of this gene boosted ethanol production by >20 % [19, 32]. The difference in robustness was reported for a plasmid-carrying versus a plasmid-cured acetogen strain in favor of the plasmid-cured strain [7]. These observations when combined led to the idea that efficient powering of amplified synthetic operon is likely possible if the host strain genome is tailored by elimination of certain genes unnecessary for the particular process [2–7, 15, 31, 33–36]. Scarab genomics has reported a similar tendency of increased robustness in enterobacteria recombinants with reduced genome (http://www.scarabgenomics.com/skins/skin_1/pdf/reports/E%20%20coli%20Host%20Case%20Study%20ScarabXpress%C2%AE-1%20%28T7LAC%29%20Yields%2012X%20More%20Protein%20Than%20BL21%28DE3%29.pdf).

Industrial applications of biocatalysts engineered in steps combining genome reduction, mutation to the target product to ensure biocatalyst robustness, and integration of multiple copies of the optimized synthetic operon encoding synthesis of the target chemical or fuel have been discussed [2–7, 15, 31, 33–36]. The feed for the industrial process is syngas or a CO_2/H_2 blend where H_2 is the product of solar panel-powered water electrolysis in situ at the manufacturing site with Kyocera solar panels rendering up to 1 kW m^{-2} (http://www.kyocerasolar.com/assets/001/5154.pdf) depending on the geographical location of the manufacturing. However, despite the obviousness that reduction of inorganic carbon of CO/CO_2 to carbohydrates requires H_2 as the carbohydrate component, there are reports claiming substitution of molecular H_2 in the feed gas blend with just free low energy electrons emitted by cathode immersed in the reaction volume. Therefore, there is a need to address the authors' concern that such a misconception would not lead to an industrial application.

There are reports on the use of acetogens for the proof-of-concept production of chemicals from CO_2 where an electrode-emitting cathode is a part of the bioreactor system to replace H_2 in the feed gas blend [20, 21, 28]. The process comprises water electrolysis producing molecular oxygen which heavily affects activity of CODH and other oxygen-sensitive enzymes in acetogens during reduction of CO_2 ($2CO_2 + 2H_2O \rightarrow CH_3COOH + 2O_2$), thus stopping biocatalysis and triggering cells to sporulate [2–7, 15, 31–35]. It has to be emphasized that on-going research on electron feed to the reaction volume with acetogens is associated with water electrolysis to release protons, which then react with electrons to render molecular hydrogen incorporated further into carbohydrate molecules, which acetogens make with reducing CO_2. Therefore, the amount of carbohydrate produced is proportional to the amount of electric energy in the form of low energy electrons emitted by the cathode into the reaction volume. The bad news is that the reduction of inorganic carbon by acetogens requires strictly anoxic conditions at redox potentials below −450 mV. Such anoxic conditions are in opposition to O_2 accumulation during electron feed by the cathode as a part of the process. Interpretation of the fact that cathode-mediated electron flow renders energy to support ATP accumulation

in acetogen cells is another misconception ignoring the fact that ATP synthesis in acetogens is directly powered by $\Delta\mu Na^+$ as described [9–11, 15, 18, 32]. Low cathode current densities with simultaneous oxygenation of the process render low product yields preventing this approach from commercial applications, in addition to the need to process tremendous amounts of liquid to recover the diluted target product (<1 M) under aseptic conditions. The approach does not seem realistic or economic for commercial applications.

It was demonstrated that multi-step UVC mutants of the parental strain with genome reduced by 11 genes led to selection of stable mutants with reduced cell duplication time as compared to the parental strain. The combination of resistance to the product with the expression of amplified synthetic operon encoding isobutanol biosynthesis in acetogen rendered isobutanol production stable in a continuous fermentation of CO_2/H_2 blend at the product level suitable for commercial manufacture of isobutanol. More experimental work will be needed to engineer a better and commercially sound biocatalyst strain with near theoretical performance to make isobutanol manufacturing technology, using CO_2 as the carbon source, competitive (<$0.70 gallon^{-1}) at 99 % isobutanol and isobutanol concentration in fermentation broth above its water solubility level at ambient temperature.

This isobutanol from the CO_2 report is right at the margin between purely academic observations and ready for commercialization of a biocatalyst strain producing isobutanol at the 5 % level in continuous fermentations. Nobody yet has described steady continuous fermentations of the CO_2/H_2 gas blend rendering 5 % isobutanol in fermentation broth. In fact, there are no sugar-fermenting technologies, model or commercial, rendering a continuous fermentation process with isobutanol yield near 5 %. The most important is the environmental impact of the results described, possible only if this technology is commercialized to fill the $550 billion market of isobutanol; thousands of new jobs will be created in a few years to come and it should also drop air CO_2 levels by at least two times just 250 years from now, thus reducing global warming [37]. The aim of this report is purely educational to bring new light on the old phenomenon of inorganic carbon reduction directly and selectively to organic carbon of target carbohydrates, with the anticipation of the growing popularity of this approach through its acceptance worldwide.

Conclusion

This report describes the development and application of standard molecular biology tools for engineering of industrial grade strain biocatalysts suitable for CO_2 reduction to valuable chemicals such as isobutanol, food components, with solid processed waste (used biocatalyst cells). The detailed approach is provided for targeted gene elimination to secure fractions of the cell energy pool to power a customized synthetic isobutanol biosynthesis pathway. The optimized acetogen synthetic isobutanol pathway was integrated into a designed integration site in the chromosome. The biocatalyst *Clostridium* sp. MT871RG$_{11}$IB^{R}6 was engineered to express six copies of synthetic operon comprising optimized synthetic format dehydrogenase, pyruvate formate lyase, acetolactate synthase, acetohydroxyacid reductoisomerase, 2,3-dihydroxy-isovalerate dehydratase, branched-chain alpha-ketoacid decarboxylase gene, aldehyde dehydrogenase, and alcohol dehydrogenase, regaining a cell duplication time of 68 ± 1 min ($p < 0.05$) of the parental strain. This is the first report on isobutanol production by an engineered acetogen-biocatalyst suitable for commercial manufacturing of this chemical/fuel using continuous fermentation of a CO_2/H_2 blend thus contributing to the reversal of global warming. The technology might become the crucial global technological approach to CO_2 reduction in the atmosphere in the immediate future, since the planet is already experiencing global warming getting out of control. Making carbohydrates from inorganic gaseous carbon may become crucial for "dry" fermentations with litte need for a cGMP QA/QC process and associated personnel/overhead expenses for future space travel/settling applications where it is often possible to find ideally sterile and energy-rich environments.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Atsumi S, Li Z, Liao JC (2009) Acetolactate synthase from *Bacillus subtilis* serves as a 2-ketoisovalerate decarboxylase for isobutanol biosynthesis in *Escherichia coli*. Appl Environ Microbiol 75(19):6306–6311. doi:10.1128/AEM.01160-09
2. Berzin V, Kiriukhin M, Tyurin M (2012) Selective production of acetone during continuous synthesis gas fermentation by engineered biocatalyst *Clostridium* sp. MAceT113. Lett Appl Microbiol. doi:10.1111/j.1472-765X.2012.03272.x
3. Berzin V, Tyurin M (2012) Acetogen biocatalyst *Clostridium* sp. MTEtOH871 engineered with our proprietary electrotransformation technology and equipment: continuous synthesis gas fermentation for selective ethanol production. J Biotech Res 4:54–64
4. Berzin V, Kiriukhin M, Tyurin M (2012) Elimination of acetate production to improve ethanol yield during continuous synthesis gas fermentation by engineered biocatalyst *Clostridium*

sp. MTEtOH550. Appl Biochem Biotechnol 167(2):338–347. doi:10.1007/s12010-012-9697-5
5. Berzin V, Kiriukhin M, Tyurin M (2013) Cre-*lox*66/*lox*71-based elimination of phosphotransacetylase or acetaldehyde dehydrogenase shifted carbon flux in acetogen rendering selective overproduction of ethanol or acetate. Appl Biochem Biotechnol 195(3):181–188. doi:10.1007/s12010-012-9864-8
6. Berzin V, Kiriukhin M, Tyurin M (2013) Selective n-butanol production by *Clostridium* sp. MTButOH1365 during continuous synthesis gas fermentation due to expression of synthetic thiolase, 3-hydroxy butyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase and NAD-dependent butanol dehydrogenase. Appl Biochem Biotechnol 169(3):950–959. doi:10.1007/s12010-012-0060-7
7. Berzin V, Kiriukhin M, Tyurin M (2013) "Curing" of plasmid DNA in acetogen using microwave or applying an electric pulse improves cell growth and metabolite production as compared to the plasmid-harboring strain. Arch Microbiol 195(3):181–188. doi:10.1007/s00203-012-0862-6
8. Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453–1462. doi:10.1126/science.277.5331.1453
9. Brock TD (1989) Evolutionary relationships of the autotrophic bacteria. In: Schlegel HG, Bowien B (eds) Autotrophic bacteria. Science Tech, Madison, pp 499–512
10. Drake HL (1994) Acetogenesis. Chapman & Hall, London
11. Müller V (2003) Energy conservation in acetogenic bacteria. Applied and Environmental Microbiology 69(11):6345–6353. doi :10.1128/AEM.69.11.6345- 6353.2003
12. Gaddy JL, Arora DD, Ko C-W, Phillips JR, Basu R, Wikstrom CV, Clausen EC (2001) Methods for increasing the production of ethanol from microbial fermentation. US Patent 7285402
13. Grady MC, Jahic M, Patnalk R (2009) Method for producing butanol using two-phase extractive fermentation. Patent publication No.: US 2009/0305370A1
14. Ikehata H, Ono T (2011) The mechanisms of UV mutagenesis. J Radiat Res 52:115–125
15. Kiriukhin M, Tyurin M (2013) Mevalonate production by engineered acetogen biocatalyst during continuous fermentation of syngas or CO_2/H_2 blend. Bioprocess Biosyst Eng. doi:10.1007/s00449-013-0991-6
16. Li SY, Srivastava R, Suib SL, Li Y, Parnas RS (2011) Performance of batch, fed-batch, and continuous A-B-E fermentation with pH-control. Bioresour Technol 102(5):4241–4250
17. Minty JJ, Lesnefsky AA, Lin F, Chen Y, Zaroff TA, Veloso AB, Xie B, McConnell CA, Ward RJ, Schwartz DR, Rouillard J-M, Gao Y, Gulari E, Lin XN (2011) Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*. Microb Cell Fact 10:18. doi:10.1186/1475-2859-10-18
18. Müller V (2003) Energy conservation in acetogenic bacteria. Appl Environ Microbiol 69(11):6345–6353. doi:10.1128/AEM.69.11.6345-6353.2003
19. Nakashima N, Goh S, Good L, Tamura T (2012) Multiple-gene silencing using antisense RNAs in *Escherichia coli*. Methods Mol Biol 815:307–319. doi:10.1007/978-1-61779-424-7_23
20. Nevin KP, Hensley SA, Franks AE, Summers ZM, Ou J, Woodard TL, Snoeyenbos-West OL, Lovley DR (2011) Electrosynthesis of organic compounds from carbon dioxide is catalyzed by a diversity of acetogenic microorganisms. Appl Environ Microbiol 77(9):2882–2886. doi:10.1128/AEM.02642-10
21. Nie H, Zhang T, Cui M, Lu H, Lovley DR, Russell TP (2013) Improved cathode for high efficient microbial-catalyzed reduction in microbial electrosynthesis cells. Phys Chem Chem Phys 15(34):14290–14294. doi:10.1039/c3cp52697f
22. Polz MF, Alm EJ, Hanage WP (2013) Horizontal gene transfer and the evolution of bacterial and archaeal population structure. Trends Genet 29(3):170–175. doi:10.1016/j.tig.2012.12.006
23. Ross SM (2000) Introduction to probability and statistics for engineers and scientists, 2nd edn. Academic, San Diego
24. Savage N (2011) Fuel options: the ideal biofuel. Nature 474(7352):S9–S11. doi:10.1038/474S09a
25. Savrasova EA, Kivero AD, Shakulov RS, Stoynova NV (2011) Use of the valine biosynthetic pathway to convert glucose into isobutanol. J Ind Microbiol Biotechnol 38(9):1287–1294. doi:10.1007/s10295-010-0907-2
26. Seaton SC, Silby MW, Levy SB (2013) Pleiotropic effects of GacA on *Pseudomonas fluorescens* Pf0-1 in vitro and in soil. Appl Environ Microbiol 79(17):5405–5410. doi:10.1128/AEM.00819-13
27. Simon SA, Gutknecht J (1980) Solubility of carbon dioxide in lipid bilayer membranes and organic solvents. Biochim Biophys Acta 596(3):352–358
28. Tanner RS, Miller LM, Yang D (1993) *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I. Int J Syst Bacteriol 43(2):232–236
29. Tremblay PL, Zhang T, Dar SA, Leang C, Lovley DR (2012) The Rnf complex of *Clostridium ljungdahlii* is a proton-translocating ferredoxin:NAD+ oxidoreductase essential for autotrophic growth. MBio 4(1):e00406–e00412. doi:10.1128/mBio.00406-12
30. Tyurin MV, Doroshenko VG, Oparina NYu (1997) Electrofusion of *Escherichia coli* cells. Membr Cell Biol 11(1):121–129
31. Tyurin M, Kiryukhin M, Berzin V (2012) Electrofusion of untreated cells of the newly isolated acetogen *Clostridium* sp. MT351 with integrated in the chromosome *erm*(B) or cat leading to the combined presence of these antibiotic resistance genes in the chromosome of the electrofusion products. J Biotech Res 4:1–12
32. Tyurin M, Kiriukhin M (2013) Ethanol overproduction during continuous syngas fermentation due to expression of amplified ethanol biosynthesis pathway integrated using *Tn*7-tool and powered at the expense of eliminated acetic acid production and spore formation. J Appl Microbiol. doi:10.1111/jam.12123
33. Tyurin M, Kiriukhin M (2013) 2,3-Butanediol production by engineered acetogen biocatalyst during continuous fermentation of syngas or CO_2/H_2 blend. Appl Biochem Biotechnol 170(6):1503–1524. doi:10.1007/s12010-013-0285-0
34. Tyurin M, Kiriukhin M (2013) Selective methanol or formate production during continuous CO_2 fermentation by the acetogen biocatalysts engineered via integration of synthetic pathways using Tn7-tool. World J Microbiol Biotechnol 29(9):1611–1623. doi:10.1007/s11274-013-1324-2
35. Tyurin M (2013) Gene replacement and elimination using λRed- and FLP-based tool to re-direct carbon flux in acetogen biocatalyst during continuous CO_2/H_2 blend fermentation. J Ind Microbiol Biotechnol 40(7):749–758. doi:10.1007/s10295-013-1279-1
36. Tyurin M, Kiriukhin M (2013) Expression of amplified synthetic ethanol pathway integrated using Tn7-tool and powered at the expense of eliminated pta, ack, spo0A and spo0J during continuous syngas or CO_2/H_2 blend fermentation. J Appl Microbiol 114(4):1033–1045. doi:10.1111/jam.12123
37. Tyurin M (2013) Reversal of global warming using $3 trillion market force: chemicals and fuels produced directly and selectively in continuous fermentations of gas blends comprising CO and CO_2. In: Carter B, Keyes and Olivia C, Lucero (eds) Environmental aspects of global warming. Nova Science, New Developments in Global Warming Research. https://www.novapublishers.com/catalog/product_info.php?products_id=42716

38. Gak E, Tyurin M, Kiriukhin M (2014) UV-induced mutagenesis in acetogens: resistance to methanol, ethanol, acetone, or n-butanol in recombinants with reduced genomes during continuous CO_2/H_2 gas blend fermentation. World J Microbiol Biotechnol. doi:10.1007/s11274-013-1579-7
39. Young KD (2006) The selective value of bacterial shape. Microbiol Mol Biol Rev 70(3):660–703. doi:10.1128/MMBR.00001-06
40. Zimmerman E @CNNMoneyTech June 25, 2013. http://money.cnn.com/2013/06/25/technology/enterprise/low-cost-genome-sequencing/