1 Microbial Communities Performing Hydrogen Solventogenic Metabolism of Volatile Fatty Acids

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Abstract

This work evaluates four different physicochemical pretreatments (acidic, thermal, acidic-thermal and thermal-acidic) on an anaerobic inoculum used for alcohol production from acetate and butyrate. All experiments were conducted in single batches using acetate and butyrate as substrates at 30 °C and with a pressurized headspace of pure H₂ at 2.15 atm (218.2 MPa). Thermal and acidic-thermal pretreatments lead to higher production of both ethanol and butanol. Mathematical modelling shows that the highest attainable concentrations of ethanol and butanol produced were 122 mg L⁻¹ and 97 mg L⁻¹ for the thermal pretreatment (after 17.5 days) and 87 mg L⁻¹ and 143 mg L⁻¹ for the acidic-thermal pretreatment (after 18.9 days). Acetate was produced in all assays. Thermodynamic data indicated that a high H₂ partial pressure favoured solventogenic metabolic pathways. Finally, sequencing data showed that both thermal and acidic-thermal pretreatments selected mainly the bacterial genera *Pseudomonas*, *Brevundimonas* and *Clostridium*. The acidic-thermal pretreatment selected a bacterial community more adapted to the conversion of acetate and butyrate into ethanol and butanol, respectively. Thermal-acidic pretreatment was unstable, showing significant variability between replicates. Acidic pretreatment showed the lowest alcohol production.

Keywords

Anaerobic digestion, inoculum pretreatment, dark fermentation, alcohols, anaerobic microbiology

Background

The constant increase of prices of fossil fuels and the extensive land requirements for crop cultures targeting ethanol production is forcing the market to consider substitute avenues. The development of bioprocesses using organic residues as raw materials could be an alternative for fuel production. Anaerobic processes can be used to produce alcohol through solventogenic processes [1] and volatile fatty acids (VFAs) and hydrogen (H₂) through acidogenic processes [2]. The conversion of any wastes

into VFAs and H_2 has an immediate commercial interest. Both propionic and butyric acids are raw materials of great interest, with many applications in various sectors, such as pharmaceutical and chemical industries [3]. H_2 can be considered a raw material for subsequent processes and as an energy carrier to feed fuel cells [4].

Although anaerobic acidogenic processes could be used for wastes valorization, downstream processing of the VFAs obtained through those processes, such as bioconversion into their corresponding alcohols using H_2 produced concomitantly, could have an even greater economic interest [5,6]. Ethanol and butanol produced through such processes can be used as drop-in liquid fuels, which have a higher market value per unit of energy. This two-steps approach (acidogenic followed by solventogenic processes) for ethanol and butanol production could both improve solvent production and reduce the toxicity linked to acidogenic processes products [6,7].

The most important parameters influencing anaerobic solventogenic fermentations include pH, organic acids, nutrient limitation, temperature, oxygen, and inoculum source [1,6,8]. Most of the studies performed on ethanol and butanol production through anaerobic solventogenic processes focussed on using sugars as carbon source and on using microbial pure cultures, with the most studied bacterial genera being *Thermoanaerobacter*, *Thermoanaerobacterium* and *Caloramator* under thermophilic conditions [9–13], and *Clostridium* [14–18].

Using mixed microbial cultures rather than pure cultures to produce alcohols from wastes through biological processes is of great interest. Mixed cultures increase process stability, improve the resistance to both toxicity and microbial contaminations, and bring higher substrate flexibility [19,20]. However, to date, there is still a lack of fundamental knowledge on mixed cultures used as inoculum, especially when H_2 is used as an electron donor for the conversion of organic acids through solventogenic processes. Characterization of microbial communities capable of performing such processes is thus preponderant.

Enhancement of microbial communities through pretreatment of the inoculum is an effective way to induce changes in the communities to improve process performance. This approach has already been successfully tested, but mainly for optimizing the acidogenic step in H_2 production through dark fermentation [21–23]. Pretreatments used in those studies consisted of modifications of the pH and temperature applied to the inoculum. Since microbial communities involved in acidogenic processes could use H_2 as an electron donor to shift their metabolism to produce alcohols [1,5,24], the same inoculum pretreatments could be applied to improve solventogenic processes.

The present work evaluated the effects of four pretreatments of a mixed microbial population (acidic, thermal, acidic-thermal and thermal-acidic) on its capacity to convert VFAs into alcohols, using H_2 as electron donor and an equimolar mixture of acetate and butyrate as carbon sources. The composition and dynamics of the mixed microbial community were analyzed.

4 Materials and Methods

4.1 Medium

The carbon source was an equimolar mixture of acetate and butyrate (17 mmol L^{-1} , which correspond to 1,000 and 1,476 mg L^{-1} of acetate and butyrate, respectively). The nutrient medium (micro and macro) composition was prepared considering previous work on anaerobic digestion microbiology [25] and is described in Table 1. The initial pH was adjusted to 6.0.

Table 1 – Nutrients composition of the medium [25].

Nutrient	Concentration	Nutrient	Concentration	Nutrient	Concentration
NH_4Cl	$1 \text{ g}\cdot\text{L}^{-1}$	$\text{MnCl}_2\cdot 4\text{H}_2\text{O}$	$50 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	$50 \text{ }\mu\text{g}\cdot\text{L}^{-1}$
NaCl	$100 \text{ mg}\cdot\text{L}^{-1}$	AlCl_3	$50 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	Pyridoxine Chloride	$10 \text{ }\mu\text{g}\cdot\text{L}^{-1}$
$\text{MgCl}_2\cdot 6\text{H}_2\text{O}$	$100 \text{ mg}\cdot\text{L}^{-1}$	$\text{CoCl}_2\cdot 6\text{H}_2\text{O}$	$50 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	HCl Concentrated	$1 \text{ }\mu\text{L}\cdot\text{L}^{-1}$
$\text{CaCl}_2\cdot 2\text{H}_2\text{O}$	$50 \text{ mg}\cdot\text{L}^{-1}$	$\text{NiCl}_2\cdot 6\text{H}_2\text{O}$	$92 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	$\text{Na}_2\text{SeO}_3\cdot 5\text{H}_2\text{O}$	$100 \text{ }\mu\text{g}\cdot\text{L}^{-1}$

$K_2HPO_4 \cdot 3H_2O$	400 mg·L ⁻¹	EDTA	500 µg·L ⁻¹	Nicotinic acid	5 µg·L ⁻¹
$FeCl_2 \cdot 4H_2O$	2 mg·L ⁻¹	Biotin	2 µg·L ⁻¹	Pantothenic acid	5 µg·L ⁻¹
H_3BO_3	50 µg·L ⁻¹	Riboflavin	5 µg·L ⁻¹	B ₁₂ Vitamin	0.1 µg·L ⁻¹
$ZnCl_2$	50 µg·L ⁻¹	Thiamine	5 µg·L ⁻¹	p-aminobenzoic acid	5 µg·L ⁻¹
$CuCl_2 \cdot 2H_2O$	38 µg·L ⁻¹	Folic acid	2 µg·L ⁻¹	Thioctic acid	5 µg·L ⁻¹

79

80 4.2 Raw Inoculum

81 The sludge used as raw inoculum in all assays was a primary digestate of the Carleton Corner Farms
82 (Marionville, ON, Canada – 45° 11' 14.0" N; 75° 21' 54.1" W) collected in May 2013. The sludge was
83 sieved three times using a 2 mm mesh sieve to eliminate all inert and heterogeneous lignocellulosic
84 materials. The total volatile solids (TVS) content of the sieved sludge was 41 ± 3 mg TVS·L⁻¹. The
85 sludge was centrifuged (Sorval™ RC 6 Plus, Thermo Inc.) for 40 min at 10k min⁻¹ and at 5°C. The
86 supernatant was discarded, and the pellet was resuspended in a phosphate buffer (500 mg·L⁻¹ PO₄³⁻),
87 using a homogenizer and disperser (Ultraturrax™ T25, IKA Inc.) for 10 min at 15k·min⁻¹. The sludge
88 was then sonicated to disaggregate possible granules and biofilms, using a sonicator (Vibra Cell™
89 VC130, Sonics Inc.) with 30W of power. This step was repeated four times, on ice, with a time/volume
90 dependence relation of 4 s·mL⁻¹, with 2 minutes interval between sonications. These steps were carried
91 out to ensure the homogeneity of the inoculum and to wash possible organic dissolved materials
92 present in the sludge, which could be used as an alternative carbon source during the process. The pH
93 was corrected to 6.0 using a 1.0 M solution of HCl under vigorous stirring. This processed sludge was
94 considered as the control inoculum.

95 4.3 Pretreatments

96 Four different physicochemical pretreatments – acidic, thermal, acidic-thermal, and thermal-acidic –
97 were performed on the control inoculum. Pretreated inocula were then compared to each other, using

the control inoculum as reference. All pretreated inocula were submitted to a starvation process to reduce the length of the lag phase before inoculation. This step consisted of incubating the inoculum for 72 hours at 30°C, under stirring of 50 min⁻¹. The control inoculum was submitted to the same starvation process and presented a TVS content of 31 ± 0 mg TVS·L⁻¹.

Acidic pretreatment consisted of decreasing the raw inoculum pH to 3.0 with a 12 M HCl solution under continuous stirring, followed by an incubation of 24 hours at 30°C, with stirring of 50 min⁻¹. pH was then increased to 6.0 using a 2 M NaOH solution under stirring, followed by an incubation of 24 hours at 30°C under stirring of 50 min⁻¹. In both pH decreasing and increasing inoculum, pH was controlled every hour for the five first hours of incubation to assure its stability. The acidic pretreated inoculum had a TVS of 29 ± 1 mg TVS·L⁻¹.

The thermal pretreatment consisted of heating the control inoculum at 90°C for 20 minutes, under stirring and using a water bath. The inoculum was then immediately transferred to an ice bath until it reached room temperature (23°C). The thermally pretreated inoculum had a TVS of 31 ± 0 mg TVS·L⁻¹.

The acidic-thermal and thermal-acidic pretreatments consisted of performing the two pretreatments sequentially, as indicated by the pretreatment name. The second pretreatment was performed immediately after the first one. The acidic-thermal pretreated inoculum had a TVS of 36 ± 3 mg TVS·L⁻¹ and thermal-acidic pretreated inoculum had a TVS of 32 ± 0 mg TVS·L⁻¹.

4.4 Experimental Setup

Initial physicochemical parameters for each experiment (pretreatments and control) and initial concentration of acetate, butyrate (before inoculation) and inoculum are shown in Table 2. Each experiment was carried out in quintuplicates, using 538 ± 3 mL sealed glass bottles, with an initial

working volume of 110 mL. All the bottles were incubated upside down to avoid any diffusion of H₂ through the bottle's cap. All experiments were performed at 30°C under a 150 min⁻¹ stirring. At the beginning of each experiment, the headspace of each bottle was totally replaced by pure H₂ (99.99%) at a pressure of 2.39 ± 0.08 atm (242.2 ± 7.9 MPa). Such headspace replacement was repeated after each sampling to ensure constant pressure and composition all along with the experiment.

Table 2 – Initial conditions (prior to inoculation) for each assay.

Assay	pH	Acetate mg·L ⁻¹	Butyrate mg·L ⁻¹	Inoculum mg TVS·L ⁻¹	Total H ₂ mmol	Dissolved H ₂ [*] mmol·L ⁻¹
Control	5.89	1153	1727	11.3 ± 0.2 (5)	37.9 ± 1.6 (5)	1.69
Acidic	5.94	1139	1708	11.0 ± 0.2 (5)	39.2 ± 0.2 (5)	1.74
Thermal	6.07	1143	1712	12.8 ± 3.7 (5)	40.2 ± 0.1 (5)	1.78
Acidic-thermal	5.84	1233	1846	12.7 ± 0.2 (5)	40.8 ± 0.2 (5)	1.81
Thermal-acidic	5.85	1213	1816	11.3 ± 0.1 (5)	39.7 ± 1.3 (5)	1.77

Values in parenthesis are the number of replicates. * Dissolved H₂ was estimated through Henry's Law [26,27]. Initial analyses of the liquid phase at the beginning of the assay confirmed the absence of significant amounts of alcohols, mono or disaccharides, and fatty acids other than acetate and butyrate.

4.5 Physicochemical Analyses

pH monitoring was performed with a portable potentiometer (AccumetTM AP115, Fisher ScientificTM), with a microprobe electrode (AccumetTM 55500-40, Cole ParmerTM), using the method 4500-H⁺ B, described by APHA [28]. TVS analyses were performed in quintuplicate, following method 2540-E, accordingly to APHA [28]. The pressure inside the bottles was measured using a digital manometer (DM8200, General Tools & InstrumentsTM) with a range pressure of 0 - 6,804 atm (0 – 689.5 MPa). Dissolved CO₂ was measured through alkalinity determination by potentiometric titration [29,30].

Mono, disaccharides, and organic fatty acids were analyzed using a WatersTM HPLC, which consisted of a pump (model 600) and an autosampler (model 717 Plus). The system was equipped with a

refractive index detector (model 2414) for mono and disaccharides analyses. Organics acids were monitored from the same samples using the same equipment through a linked photodiode array detector (model 2996). A TransgenomicTM ICSep IC-ION-300 (300 mm x 7.8 mm outer diameter) column was used to separate all compounds and was operated at 35°C. The mobile phase was 0.01 N H₂SO₄ at 0.4 mL min⁻¹ under an isocratic flow.

Alcohols (methanol, ethanol, acetone, 2-propanol, tert-butanol, n-propanol, sec-butanol, and n-butanol) were measured on an AgilentTM 6890 gas chromatograph (GC) equipped with a flame ionization detector [31].

100 µL gas samples, obtained using a gas-tight syringe (model 1750, HamiltonTM), were used for gas composition (H₂, CO₂, CH₄ and N₂) measurements with a GC (HP 6890, Hewlett PackardTM) equipped with a thermal conductivity detector (TCD) and an 11 m x 3.2 mm 60/80 mesh packed column (ChromosorbTM 102, SupelcoTM). The column temperature was held at 50 °C for the entire run (4 min). The carrier gas was argon. The injector and detector were maintained at 125°C and 150°C, respectively.

4.6 16S rRNA gene Sequencing and microbial characterization

Bacterial 16S rRNA genes (V2 region) were amplified using the set of primers 16S-F343 IonA L01 (343-357; 5' TACGGRAGGCAGCAG 3') and 16S-R533 Ion P1 (516-533; 5' ATTACCGCGGCTGCTGGC 3') [32]. A sample-specific multiplex identifier was added to each forward primer and an Ion Torrent adapter to each primer. DNA extraction was conducted [33]. DNA was then purified [34,35]. Amplification reactions were performed in a final volume of 20 µL, which contained 1 µL of DNA, 0.5 µM of each primer, 7.5 µL of RNase free H₂O and 10 µL of 2X HotStarTaqTM Plus Master Mix (HotStarTaqTM Plus Master Mix Kit, Qiagen, USA). PCR conditions were an initial denaturation of 5 min at 95°C followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and

45 s at 72°C, with a final elongation step of 10 min at 72°C. PCR products were purified and quantified using a QIAquick™ gel extraction kit (Qiagen, USA) and a Quant-iT PicoGreen™ double-stranded DNA quantitation kit (Life Technologies Inc., USA) according to manufacturer's instructions. The pooled amplicons were then sequenced using the Ion Torrent™ (Life Technologies Inc., USA) sequencing platform with a 314 chip. Bacterial 16S rRNA gene sequences generated were and analyzed using the ribosomal database project (RDP) classifier [36], using a bootstrap confidence cutoff of 50%, as recommended by RDP classifier [37] for short sequences (less than 250 bp). Before analysis, sequences shorter than 75 bp and sequences with unidentified bases (N) were removed.

4.7 Kinetics

Kinetic parameters for ethanol and butanol production were calculated using a modified Boltzmann sigmoidal model, as shown by Equation 1. The model was modified to incorporate parameter r_{max} as the maximum rate of the process ($\text{mg L}^{-1} \text{d}^{-1}$).

$$C(t) = C_{max} - \frac{C_{max}}{1 + e^{\left(\frac{4 \cdot r_{max}(t-t_m)}{C_{max}}\right)}} \quad \text{Equation 1}$$

Where $C(t)$ is the function of concentration in respect of time (mg L^{-1}), t is time (d), C_{max} is the maximum concentration reached (mg L^{-1}), and t_m is the value of time when $\frac{dC(t_m)}{dt} = r_{max}$ (d).

From some of the calculated parameters showed in Equation 1, is possible to calculate the length of both lag (t_i , d) and exponential (t_e , d) phases, as depicted in Equation 3 and 4, respectively.

$$t_i = t_m - \frac{C_{max}}{2 \cdot r_{max}} \quad \text{Equation 3}$$

$$t_e = \frac{C_{max}}{r_{max}} \quad \text{Equation 4}$$

178

179 All fittings were performed using the software Microcal Origin ProTM 9.0, using a Levenberg-
180 Marquardt algorithm for fitting and initializing the equations parameters.

181 4.8 *Metabolisms' Gibb's free energy*

182 Gibb's free energy values were calculated for all pathways depicted in Figure 1, except for pathway 1,
183 for both initial (ΔG_r^I) and final (ΔG_r^F) conditions and were compared with the free energy for standard
184 conditions (ΔG_r^o). ΔG_r^o values for each reaction were estimated [38,39], considering 1 mol of each
185 product and reagent at STP. ΔG_r^I and ΔG_r^F values were calculated using the Nernst equation at 30 °C
186 and considered the concentrations of the metabolites presented in Table 4. To make all calculations
187 possible, compounds that were not detected through the experiments, such as butanol, propionate,
188 propanol, and ethanol, were assumed to be present, but at the same concentration of their respective
189 lower detection limit for each methodology. This represents 10^{-3} mmol L⁻¹ for volatile acids and
190 alcohols and 10^{-1} mmol L⁻¹ for CO₂.

191 5 **Results and Discussion**

192 5.1 *Metabolic Model and Molar Balances*

193 For all experiments, gas composition of the headspace was monitored prior to its replacement and H₂
194 pressure was constantly kept at 2.15 ± 0.10 atm. H₂ was the sole gas detected in the headspace in all
195 cases, except for the control experiment, which showed a methane production of 0.24 mmol (5.37 mL
196 at standard temperature and pressure (STP)). Initial and final concentrations of the main metabolites

197 (added or produced) and of dissolved H₂ and CO₂ are presented in Table 3. Dissolved H₂ was not
 198 measured but estimated through Henry's law [26,27], and its concentration was considered constant,
 199 due to its continuous replacement. Dissolved CO₂ was calculated from the alkalinity value measured at
 200 the beginning of each assay. CO₂, O₂ and N₂ were never detected at the headspace. Acetone, lactate,
 201 and methanol were only sporadically detected in the liquid phase, in traces concentrations (below 1 mg
 202 L⁻¹). Neither propionate nor alcohols were detected at the beginning of each experiment.

203

204 Table 3 – Mean concentrations of all metabolites detected and of dissolved H₂ and CO₂.

Metabolite ^a	Concentration (mg L ⁻¹)									
	Control		Acidic		Thermal		Acidic-thermal		Thermal-acidic	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Acetate	1225 ± 1	1575 ± 40	1222 ± 3	1492 ± 24	1206 ± 2	1722 ± 120	1235 ± 3	1645 ± 44	1224 ± 2	1447 ± 26
Butyrate	1831 ± 3	1773 ± 24	1828 ± 1	1935 ± 14	1791 ± 4	1796 ± 17	1840 ± 5	1664 ± 25	1831 ± 4	1738 ± 58
Propionate	-	42 ± 12	-	35 ± 4	-	107 ± 25	-	52.8 ± 9.2	-	40.1 ± 5.4
Ethanol	-	40 ± 6	-	14 ± 2	-	104 ± 7	-	94 ± 9	-	33 ± 6
Propanol	-	-	-	0.3 ± 0.2	-	-	-	1.8 ± 0.1	-	1.0 ± 0.3
Butanol	-	49 ± 8	-	11 ± 2	-	83 ± 3	-	154 ± 14	-	61 ± 25
Dissolved H ₂	1.6 ± 0.1		1.7 ± 0.0		1.6 ± 0.1		1.7 ± 0.1		1.7 ± 0.1	
Dissolved CO ₂	3.07	-	2.40	-	3.30	-	1.56	-	2.19	-
Duration (d)	29.2		29.1		29.1		29.1		29.0	

205 ^aAll values are expressed in mg L⁻¹ except for dissolved H₂ and CO₂ expressed in mmol L⁻¹. Mean concentrations of all volatile acids and
 206 alcohols was calculated considering 5 replicates. The amount of H₂ was kept constant in all experiments, there were no difference in its
 207 initial and final concentrations. Dissolved H₂ mean concentration considered 50 replicates.

208 Acetate and butyrate concentrations along the process demonstrated acetate production in all cases.
 209 Only minor variations of butyrate concentrations (slight decrease for the control and the acidic-thermal
 210 and thermal-acidic pretreatments, low production for the acidic and the thermal pretreatments) were
 211 observed. Such results suggested an alternative organic matter input in the system. This hypothesis is
 212 consistent with the decrease of the concentration of total volatile solids (TVS) that was observed for all
 213 conditions tested, as a direct effect of the different pretreatments on the biomass, which might have
 214 resulted in partial microbial cell death. Such non-living cells constituted non-soluble organic matter
 215 which was probably hydrolyzed and then consumed as a supplementary carbon source. Based on well-
 216 known anaerobic acidogenic and solventogenic metabolisms [1,24], and considering the inoculum as
 217 the only possible alternative source of organic matter, an alternative metabolic model including this
 218 alternative contribution and considering the metabolites (added and produced) presented in Table 3,
 219 was developed, as shown in Figure 1.

220 Figure 1 depicts butanol as the only product from lysed inoculum (pathway 1). Pathway 1 seems the
 221 only way to explain butanol production maintaining the butyric acid concentration constant since it is
 222 thermodynamically unfeasible to form butanol from acetate. Acetate could only be produced from
 223 butyrate, considering the high concentration of hydrogen and lack of CO₂ in all assays, indicating that
 224 acetate was produced through acetogenesis from butyrate (pathway 2) or homoacetogenesis (pathway
 225 7) after initial dissolved CO₂ was consumed.

Metabolic Pathways

- ① Organic matter in the inoculum to butyrate
- ② Acetogenesis from butyrate $\text{But}^- + 4\text{H}_2\text{O} \rightarrow \text{Ac}^- + 2\text{CO}_2 + 6\text{H}_2$
- ③ Solventogenesis from butyrate to butanol $\text{But}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{But(OH)} + \text{H}_2\text{O}$
- ④ Acidogenesis from butyrate to propionate $\text{But}^- + 2\text{H}_2\text{O} \rightarrow \text{Prop}^- + \text{CO}_2 + 3\text{H}_2$
- ⑤ Solventogenesis from propionate to propanol $\text{Prop}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{Prop(OH)} + \text{H}_2\text{O}$
- ⑥ Solventogenesis from acetate to ethanol $\text{Ac}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{Eth(OH)} + \text{H}_2\text{O}$
- ⑦ Homoacetogenesis $2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{Ac}^- + \text{H}^+ + 2\text{H}_2\text{O}$

226

227 Figure 1 – Alternative metabolic model proposed for the solventogenic process using butyrate and
 228 acetate as substrates. Pathways in orange are exergonic; pathways in blue are endergonic (at 30 °C, pH
 229 of 6.0, with a constant CO₂ concentration of 10⁻³ mmol L⁻¹, in initial conditions as depicted in Table 3).

230 The metabolic model depicted in Figure 1 was used to perform a molar balance of all carbon inputs and
 231 outputs during the process (Table 4) and estimates the butyrate input from inoculum. All balances were
 232 performed on a 1 L basis and derived from initial and final experimental values obtained in all

233 conditions (Table 3), except for the organic matter from the inoculum (pathway 1) which was
234 expressed in butyrate equivalent.

235 Table 4 – Molar balance of all metabolites detected in all metabolic pathways of each experiment.

Metabolite	Pathway ^a	Molar Balance (mmol) ^b				
		Control	Acidic	Thermal	Acidic-thermal	Thermal-acidic
Butyrate	Initial [*]	21.0	21.0	20.6	21.1	21.0
	Final [*]	20.4	22.2	20.6	19.1	20.0
	1	+ 3.16	+ 3.56	+ 6.92	+ 4.73	+ 1.96
	2	- 2.53	- 1.73	- 4.34	- 3.92	- 1.57
	3	- 0.66	- 0.15	- 1.12	- 2.08	- 0.82
	4	- 0.57	- 0.48	- 1.46	- 0.75	- 0.57
Butanol	3 [*]	+ 0.66	+ 0.15	+ 1.12	+ 2.08	+ 0.82
Propionate	4 [*]	+ 0.57	+ 0.48	+ 1.46	+ 0.75	+ 0.57
	5	-	-	-	- 0.03	- 0.02
Propanol	5 [*]	-	-	-	+ 0.03	+ 0.02
Acetate	Initial [*]	20.7	20.7	20.4	20.9	20.7
	Final [*]	26.7	25.3	29.2	27.9	24.5
	2	+ 2.53	+ 1.73	+ 4.34	+ 3.92	+ 1.57
	6	- 0.87	- 0.30	- 2.26	- 2.04	- 0.72
	7	+ 4.35	+ 3.17	+ 6.72	+ 5.08	+ 2.95
Ethanol	6 [*]	+ 0.87	+ 0.30	+ 2.26	+ 2.04	+ 0.72
Dissolved CO ₂	Initial [*]	3.07	2.40	3.30	1.56	2.19
	2	+ 5.05	+ 3.46	+ 8.68	+ 7.85	+ 3.14
	4	+ 0.57	+ 0.48	+ 1.46	+ 0.75	+ 0.57
	7	- 8.69	- 6.34	- 13.4	- 10.2	- 5.90

236 ^{*}Values obtained experimentally (derived from initial and final concentrations as indicated in Table 3).

237 ^aMetabolic pathways described in Figure 1.

238 ^bBalance on a 1 L basis; “+” indicates a production; “-” indicates a consumption.

239

240 According to the mass balance shown in Table 4, CO₂ was produced by converting butyrate into
241 acetate (pathway 2) and by acidogenesis from butyrate to propionate (pathway 4). This CO₂ was then
242 consumed to form acetate through a homoacetogenic pathway (pathway 7). This hypothesis was

243 supported by the absence of gaseous CO₂ in all experiments, followed by an increase in acetate
 244 concentration. This CO₂ absence may be linked to microbial communities more adapted to convert
 245 butyrate and CO₂ into acetate (pathways 2 and 7, respectively) rather than to the conversion of acetate
 246 into ethanol (pathway 6). This metabolic model also indicates that butyrate was mainly consumed to
 247 produce acetate (pathway 2) in all studied conditions and the organic matter from the inoculum
 248 (pathway 1) represented an external input since butyrate concentrations were almost constant in all
 249 assays, although consumed to form butanol (pathway 3). Ethanol and butanol were produced (pathways
 250 3 and 6) in all experiments. These pathways were more active in the experiments with thermal and
 251 acidic-thermal pretreatments, as these conditions showed the highest ethanol and butanol production
 252 among all other experiments. Although acidogenesis from butyrate to propionate (pathway 4) was
 253 active in all conditions, conversion of propionate into propanol (pathway 5) was not an important
 254 metabolic pathway. Propanol production was not expressive in neither experiments since it showed the
 255 highest concentration of 1.82 mg L⁻¹ in acidic-thermal pretreatment essay.

256 The successful closure of molar balances shown in Table 4, with a stoichiometrically balanced sum of
 257 all inputs and outputs (considering the contribution of the inoculum), indicates that the metabolic
 258 model proposed in Figure 1 accurately represents the solventogenic processes occurring in all studied
 259 conditions. An energy balance based on the mass balance depicted in Table 4 was calculated to
 260 estimate Gibb's free energy values for each pathway for both initial and final conditions (Figure 2).
 261 Those values provide an overview of the thermodynamic feasibility of each pathway involved in the
 262 solventogenic process and tend to validate the metabolism proposed in Figure 1.

263

Metabolic Pathway	Metabolism Free Energy (kJ mol ⁻¹)										
	ΔG_r^0	Control		Acidic		Thermal		Acidic-thermal		Thermal-acidic	
		ΔG_r^I	ΔG_r^F	ΔG_r^I	ΔG_r^F	ΔG_r^I	ΔG_r^F	ΔG_r^I	ΔG_r^F	ΔG_r^I	ΔG_r^F
2	109	234	-158	221	-162	237	-156	200	-156	217	-160
3	-20,6	-291	-129	-291	-168	-290	-116	-291	-99	-291	-123
4	55,7	-129	-170	-135	-177	-127	-147	-146	-163	-138	-171
5	-24,4	-47	-205	-47	-201	-47	-228	-47	-125	-47	-135
6	-20,5	-290	-129	-290	-154	-290	-107	-291	-109	-290	-131
7	-40	-67	339	-54	337	-70	341	-33	340	-50	337

Figure 2 – Gibbs' free energy for initial (ΔG_r^I), final (ΔG_r^F) and standard (ΔG_r^0) conditions for each pathway (as defined in Figure 1) in each experiment. Greenish values (< 0) are exergonic and reddish values (> 0) are endergonic.

Energetic profiles could help to anticipate changes within metabolic pathways. As shown in Figure 2, the estimated ΔG_r^0 values indicate that acetogenesis from butyrate and acidogenesis from butyrate to propionate (pathways 2 and 4, respectively) are theoretically thermodynamically unfeasible metabolisms. Nevertheless, these pathways became thermodynamically feasible due to the absence (or low concentration) of dissolved CO₂ at the course of each essay. Acetogenesis from butyrate (pathway 2) was likely inhibited at the beginning of each experiment due to the concentration of H₂ and CO₂. During the experiment, CO₂ was progressively consumed through homoacetogenesis (pathway 7), which rendered pathway 2 thermodynamically feasible. At low concentrations of CO₂ as found after the beginning of each essay, the homoacetogenic pathway might have become thermodynamically unfeasible. Gibb's free energy values found for these pathways could explain the production of acetate observed in all studied conditions, all along the process. Metabolic pathways 3 (solventogenesis from butyrate to butanol) and 6 (solventogenesis from acetate to ethanol) were thermodynamically feasible

281 in all studied conditions all along the process, explaining the observed production of such alcohols.
 282 Metabolic pathway 4 (acidogenesis from butyrate to propionate) was unfeasible at the ending of each
 283 condition, showing that propionate production was stationed in a low concentration at some point of the
 284 experiments.

285 Figure 3 – Evolution of the standard Gibbs' free energy (ΔG°_r) in function of the partial pressure of H_2 .
 286 Evolution for each metabolic pathway (as defined in Figure 1), at 30 °C, and with a standard
 287 concentration of 1.0 mol L^{-1} for each reagent other than H_2 . Shaded area indicates
 288 thermodynamically feasible reactions.
 289

290
 291 As described previously, H_2 has a significant role as a co-substrate in the anaerobic process energetics
 292 [40,41]. As shown in Figure 3, solventogenesis of butanol from butyrate (pathway 3) is less sensitive to
 293 low ppH_2 than solventogenesis of ethanol and propanol, respectively from acetate (pathway 6) and
 294 propionate (pathway 5). By extrapolation, equilibrium ($\Delta G^{\circ}_r = 0$) in pathway 3 would be achieved at a

295 p_{H_2} of $4 \cdot 10^{-4}$ atm (43.7 Pa), implying that solventogenesis of butanol could theoretically be carried
 296 out at very low p_{H_2} . It is also possible to infer from Figure 3 that at p_{H_2} higher than 0.62 atm
 297 (62.5 MPa), acidogenesis of propionate (pathway 4) and acetogenesis of acetate (pathway 2) from
 298 butyrate will stop, shutting down the production of acetate, propionate and H_2 . In addition to low CO_2
 299 concentrations, high p_{H_2} thus represents an important factor that renders such process
 300 thermodynamically feasible and favours solventogenesis from VFAs.

301 5.2 Alcohols and VFAs metabolisms

302 As shown in Figure 4, the highest concentrations of alcohols were obtained for both thermal and acidic-
 303 thermal pretreatments, with the best rate observed for the acidic-thermal pretreatment, especially for
 304 butanol production. On the opposite, the lowest alcohol production was observed for the acidic
 305 pretreatment. Acetate and butyrate concentrations along the essays confirmed the contribution of the
 306 inoculum as an important source of organic matter as the substrate for alcohol production, since in all
 307 conditions tested, acetate concentration increased, and butyrate concentration increased or only slightly
 308 decreased. These results reinforced the hypothesis described in Figure 1 and Table 4 of an acetate
 309 production through acetogenesis of butyrate (pathway 2) and homoacetogenesis (pathway 7), and
 310 preferentially through pathway 2 due to low concentrations of CO_2 at the beginning of all assays.

311

Figure 4 – Kinetics of VFAs (acetate and butyrate) and alcohols (ethanol and butanol) productions for each studied condition. Red dotted- and blue dashed-lines represent the modified Boltzmann model fittings for ethanol and butanol concentrations, respectively.

Figure 5 – Behaviour of propionate concentration for each studied condition.

As shown in Figure 5, propionate was produced in all conditions tested. No significant difference was observed in the concentrations produced ($42.4 \pm 10.0 \text{ mg L}^{-1}$), except for the thermal pretreatment essay, for which the level of production was 2.5 times higher than in all other conditions. As presented in Table 3, propionate was then consumed and converted into propanol (pathway 5, Figure 1) for the acidic, acidic-thermal and thermal-acidic pretreatments. Despite the favourable thermodynamics of this reaction (Figure 2), propanol was only produced in trace amounts. Oxidation of propionate into acetate was considered unlikely since such reaction is energetically unfeasible in standard conditions ($\Delta G_r^\circ = 53.3 \text{ kJ mol}^{-1}$), and that, as previously described [39], at high H_2 concentrations ΔG_r values are increased.

Table 5 presents the parameters of the modified Boltzmann model fitting the experimental data from Figure 4. Those parameters are related to the kinetics of alcohol production and compare efficiencies between all pretreatments. A high correspondence was obtained between replicates for all conditions tested, apart from the thermal-acidic pretreatment. The correlation coefficient was very low in that condition, only reaching 0.5 for ethanol and 0.4 for butanol, indicating that the process was

unpredictable and could not be reproduced. Due to its instability and unpredictability, the thermal-acidic pretreatment was thus no longer considered for analyses.

Table 5 – Parameters of the modified Boltzmann model fitting for ethanol and butanol production.

Parameters		Pretreatment of the inoculum				
		Control	Acidic	Thermal	Acidic-thermal	Thermal-acidic
Ethanol	C_{max} (mg L ⁻¹)	38.5 ± 1.5	13.6 ± 0.7	122 ± 10	87.3 ± 1.9	27.6 ± 2.8
	t_m (d)	12.7 ± 0.6	10.4 ± 0.8	21.0 ± 1.1	6.9 ± 0.3	6.1 ± 1.0
	r_{max} (mg L ⁻¹ d ⁻¹)	1.9 ± 0.0	0.7 ± 0.0	7.0 ± 0.48	9.4 ± 0.7	3.1 ± 1.2
	R ²	0.96	0.89	0.94	0.95	0.50
	t_i (d)	2.7	0.9	12	2.3	1.6
	t_e (d)	20	19	18	9.3	9.0
Butanol	C_{max} (mg L ⁻¹)	50.7 ± 2.3	10.9 ± 0.5	96.7 ± 6.8	143 ± 2	53.8 ± 5.4
	t_m (d)	17.6 ± 0.7	14.2 ± 0.8	21.1 ± 1.0	5.3 ± 0.2	5.1 ± 0.8
	r_{max} (mg L ⁻¹ d ⁻¹)	2.4 ± 0.0	0.5 ± 0.0	5.5 ± 0.5	25 ± 2	26 ± 23
	R ²	0.97	0.94	0.96	0.96	0.40
	t_i (d)	7.0	2.9	12	2.4	4.1
	t_e (d)	21	23	18	6	2

C_{max} , maximum concentration; t_m , time when maximum production rate is achieved; r_{max} , maximum production rate; R², correlation coefficient; t_i and t_e , initial and ending time of exponential growth phase.

As shown in Figure 4 and Table 5, both alcohol production and maximum production rates (r_{max}) were improved for thermal and acidic-thermal pretreatments. Although the highest ethanol production was observed for the thermal pretreatment, the best results were obtained for the acidic-thermal pretreatment, which allowed the best butanol production and an increase of 4.5 times of the ethanol and of 10.2 times of the butanol maximum production rate. In opposition, a decrease in alcohol production was observed for the acidic pretreatment. Such results clearly indicate that an acidic-thermal pretreatment of the inoculum is a promising approach for designing more efficient and smaller-sized bioreactors.

Length of the lag phase (t_i) and duration of the bacterial exponential growth phase (t_e) were evaluated for each pretreatment and compared to the control by considering alcohol production curves as growth-associated curves (Table 3). The length of the lag phase is related to the time required for a bioreactor to initiate its process (start-up) and achieve higher rates of alcohol production. For both ethanol and butanol production, the shortest lag phases were observed for the acidic and acidic-thermal pretreatments. In both pretreatments, the lag phase was shorter than found in the control essay. The most extended lag phase was observed for the thermal pretreatment, which was considerably greater for both ethanol and butanol production than observed in control. Such results indicate that the thermal pretreatment had the highest impact on the inoculum, while acidic and acidic-thermal pretreatments selected microbial communities which were the best adapted to solventogenic processes. Variations in the duration of the bacterial exponential growth phases (t_e) were also observed (Table 5). A shorter exponential growth phase is representative of a faster process. However, this parameter must be evaluated concomitantly with the maximum rates of production (r_{max}). For example, a short t_e occurring at low r_{max} indicates a process occurring with low efficiency. Among all the conditions tested, low t_e values and high r_{max} values were obtained for the acidic-thermal pretreatment for ethanol and butanol production. Taken all together, results obtained for all conditions tested indicate that the acidic-thermal pretreatment was the best approach to generate a rapid and efficient process.

Figure 6 – Evolution of pH along the process for each studied condition.

pH was initially set to a value of 5.92 ± 0.09 for all essays, and their value increased during the experiments (Figure 6). This rise in pH values likely reflects the consumption of H_2 as an electron donor to form alcohols since solventogenic pathways (3, 5 and 6) require the consumption of H^+ and alcohols show a low ionization on water. Such a pH increase occurred to a lesser extent in all conditions, including an inoculum acidic pretreatment (acidic, acidic-thermal and thermal-acidic pretreatments). This difference might be explained by a more drastic initial pH drop resulting from the acidic addition on these pretreatments, probably lowering the buffering capacity of the inoculum. This hypothesis is reinforced by the concentrations of dissolved CO_2 , which were higher for both control and thermal pretreatment than acidic and acidic-thermal pretreatments (Table 3).

Figure 7 – Evolution of the total volatile solids (TVS) along the process for each studied condition.

A decrease in total volatile solids (TVS) was also observed for all conditions tested (Figure 7). This decrease occurred probably due to a direct effect of the different pretreatments on the biomass, resulting in partial microbial cell death. Such non-living cells constituted non-soluble organic matter which was probably hydrolyzed and then consumed as a carbon source, contributing to the organic matter input proposed in pathway 1 of Figure 1. The TVS concentration stopped decreasing and started to increase after 6.3 days in the acidic-thermal pretreatment slightly. Such evolution is probably linked to the higher values of μ_{max} observed for this pretreatment (Table 5), which reflect an increase in the biomass growth's rate, and consequently, the TVS concentration.

5.3 Bacterial communities

Based on the results presented above and focusing on alcohol production, both thermal and acidic-thermal pretreatments experiments were submitted to microbial community analyses. Bacterial populations were thus characterized and monitored all along with those two processes. Thirteen phyla were detected, namely *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Synergistetes*, *Cyanobacteria*, *Tenericutes*, *Spirochaetes*, *Deinococcus-Thermus*, *Fibrobacteres*, *Verrucomicrobia*,

392 *Chloroflexi* and *Nitrospirae*. Among those, three (*Firmicutes*, *Proteobacteria* and *Bacteroidetes*)
393 represented up to 94.6% of the total OTUs detected in each sample for both pretreatments (Figure 8).
394 *Firmicutes* was the most abundant phylum detected in the initial non-treated inoculum (corresponding
395 to 66.6% of the detected OTUs). Both pretreatments generated a shift in the bacterial population by
396 stimulating the development of *Proteobacteria* and strongly reducing the number of *Firmicutes*.
397 Bacterial populations evolved differently along the process for the two pretreatments. In the thermal
398 pretreatment experiment (Figure 8A), a decrease of *Proteobacteria* was observed concomitantly with
399 an increase in *Bacteroidetes* and *Firmicutes*, the latest becoming the dominant phylum at the end of the
400 process. This shift started after 200 hours of experiment, which correspond to the early beginning of the
401 bacterial exponential growth phase (Figure 4C and Table 5). In opposition, in the acidic-thermal
402 pretreatment experiment (Figure 8B), relative stability was observed for the bacterial population, with
403 *Proteobacteria* remaining the main phylum all along the process ($72.0 \pm 4.99\%$ of the detected OTUs)
404 and *Firmicutes* constantly being the second phylum of importance ($24.0 \pm 5.10\%$ of the detected
405 OTUs). *Bacteroidetes* increased during the process, before starting to decrease after 500h of
406 experiment, to reach their initial level at the end of the experiment.

Figure 8 – Evolution of the three most representative bacterial phyla for the thermal (A) and acidic-thermal (B) pretreatments. Dashed lines represent OTUs associated to those same phyla found in the inoculum without pretreatment.

Deeper phylogenetic analyses were performed down to the genus level to infer the potential metabolic pathway(s) that could be associated with the enhancement of alcohol production observed for both thermal and acidic-thermal pretreatments (Figure 9). Substantial differences were noticed between the bacterial population from the initial inoculum (before any pretreatment) and those from the thermal and acidic-thermal pretreated inocula.

Figure 9 – Evolution of the bacterial population at the genus taxonomic level for thermal and acidic-thermal pretreatments, and comparison with the initial bacterial population (I) for the 31 most detected genera.

422 A total of 317 genera were detected in all samples tested, but only 31 of them, representing up to 85%
 423 of the bacterial population, were considered for further analysis (Figure 9). One of the most noticeable
 424 changes consisted of an increase of *Pseudomonas* in pretreatment experiments compared to the initial
 425 population. Such increase was observed as soon as the first hours of experiments. *Pseudomonas* then
 426 remained a dominant genus of the bacterial population, despite a slight decrease observed for the
 427 thermal pretreatment in the second half of the process. Several *Pseudomonas* species have been
 428 genetically well-characterized and have been shown to possess the genetic components for both ethanol
 429 (from pyruvate) and butanol (from glycerol and pyruvate) production [42]. The prevalence of
 430 *Pseudomonas* is likely linked with the higher levels of alcohol production observed in thermal and
 431 acidic-thermal pretreatments. Some *Pseudomonas* species also possess genes responsible for the
 432 degradation of ethanol into acetyl-CoA [42]. A pathway related to ethanol degradation might explain
 433 better butanol production compared to ethanol, observed for the acidic-thermal pretreatment. Among
 434 the other noticeable results, two bacterial genera, namely *Acinetobacter* and *Paenibacillus*, which were
 435 not detected in the initial population, appeared to be positively affected by both pretreatments. It is
 436 being reported [43,44] that *Acinetobacter* strains are related to alcohol consumption as they can express
 437 alcohol dehydrogenase (ADH) to convert ethanol into acetate, reverting the solventogenic pathway 6.
 438 As shown in Figure 2, this reversed pathway probably not occurred during the essays since in all
 439 experiments, in their beginning and ending, ΔG_r for pathway 6 were thermodynamically feasible.

440 Conversely, ADH is also a molecule that is strongly related to bacterial quorum sensing with a key role
 441 in biofilm formation [44,45]. The growth of *Acinetobacter* was more stimulated in the thermal
 442 pretreatment, so this quorum sense mechanism could be linked with the best performance of this
 443 pretreatment to produce alcohols, considering that the presence of ethanol could have stimulated the
 444 expression of ADH, although the alcohol degradation metabolism was probably shut down. Acidic-

445 thermal pretreatment preferentially stimulated the growth of *Paenibacillus*, which is directly related to
446 alcohol production [46].

447 In addition to those changes, several other bacterial genera evolved differently depending on the
448 pretreatment applied (Figure 9). The thermal pretreatment appeared to stimulate the growth of
449 *Brevundimonas*, *Bacteroides*, *Butyrivibrio*, *Bacillus*, *Alcaligenes*, *Eubacterium*, *Clavibacter*,
450 *Psychrobacter*, *Serratia* and *Microbulbifer*. The latter three being even exclusively detected in this
451 pretreatment. On the opposite, the acidic-thermal pretreatment appeared to improve the growth of
452 *Tissierella*, *Novosphingobium*, *Sedimentibacter* and *Yersinia*. The latter one being exclusively detected
453 in this pretreatment.

454 The genus *Brevudimonas* started to increase in the thermal pretreatment after 8.3 days, coinciding with
455 the very beginning of the exponential growth phase (Figure 4 and Table 5). Members belonging to this
456 genus have already been shown to possess metabolic pathways related to the acidogenesis of alcohols
457 [47]. High H₂ partial pressures applied during the process might have rendered solventogenesis
458 thermodynamically favourable through the reverse same metabolic pathway (Figure 3). The genus
459 *Yersinia* started to increase in the acidic-thermal pretreatment after approximately 4.2 days, coinciding
460 with the early exponential growth phase (Figure 4 and Table 5). Members belonging to this genus have
461 already been shown to possess metabolic pathways to convert pyruvate into butanol and ethanol [48].
462 The genus *Sedimentibacter* is one of the predominant genus found in the fermentation of Baijiu, and it
463 could be directly related to alcohol production [49]. It is noticeable that the genus *Clostridium*, which is
464 composed of a high number of known alcohol producers [15,50–52], was detected for both
465 pretreatments all along the process, without any significant variations. The genus *Clostridium* usually
466 produces alcohol by converting glycerol and pyruvate into ethanol and butanol. Thus, this genus
467 probably has an essential role in producing ethanol and butanol in both thermal and acidic-thermal

468 pretreatments. There is no reporting on alcohol-producing metabolism for all other genera found within
469 the experiments.

470 Other results were observed at higher taxonomic levels, notably decreasing microorganisms from the
471 *Peptostreptococcaceae* family and the *Epsilonproteobacteria* class, in both pretreatments.
472 *Peptostreptococcaceae* family represented almost half (45% of total bacterial OTUs) found in the
473 initial inoculum (before any pretreatment), strongly decreased its presence to $5.4 \pm 2.2\%$ in thermal and
474 $9.2 \pm 3.1\%$ in acidic-thermal pretreatments. *Epsilonproteobacteria* class represented up to 6% of total
475 bacterial OTUs in the initial inoculum but significantly decreased to less than 0.3% of total bacterial
476 OTUs in both thermal and acidic-thermal pretreatments. Such results indicate that neither bacteria from
477 the *Peptostreptococcaceae* family nor *Epsilonproteobacteria* class played a significant role in the
478 processes studied.

479 **6 Conclusions**

480 Characterization of the initial microbial population showed the presence of bacteria possessing
481 metabolic pathways for alcohol production. Both thermal and acidic-thermal pretreatments were able to
482 select the best adapted bacterial communities to produce ethanol and butanol. The significant decrease
483 of members belonging to the family *Peptostreptococcaceae* coupled to an increase of *Pseudomonas*
484 appeared to be related to the best performance of ethanol and butanol production. The acidic-thermal
485 pretreatment generated the best results, reaching a concentration of 87 mg L^{-1} of ethanol and of 143 mg
486 L^{-1} of butanol after 240 hours of processing. The thermal pretreatment achieved the highest ethanol
487 production (122 mg L^{-1}) but at a much slower rate (after 710 hours). The two other pretreatments
488 studied showed either instability and inconsistency for the thermal-acidic pretreatment or very low
489 alcohol production for the acidic pretreatment. Among the VFAs added to the medium, butyrate was
490 used solely as the substrate, acetate being produced along the process. The mass balance study

491 highlighted another substrate input, which was probably originating from the inoculum.
 492 Thermodynamic data indicated that homoacetogenesis was a critical pathway to produce acetate from
 493 dissolved CO₂ and H₂. Finally, H₂ partial pressure was a preponderant factor for solventogenesis,
 494 enabling alcohol production and inhibiting its consumption.

7 List of Abbreviations Used

STP	Standard temperature and pressure (0° C and 1 atm);
ppH ₂	Partial H ₂ pressure (atm);
R ²	Levenberg-Marquardt algorithm correlation coefficient;
TVS	Total volatile solids (mg L ⁻¹);
OTU	Operational taxonomic unit;
HPLC	High performance liquid chromatography;
PCR	Polymerase chain reaction;
RDP	Ribosomal data project;
VFA	Volatile fatty acid;
But ⁻	Butyrate;
Prop ⁻	Propionate;
Ac ⁻	Acetate;
But(OH)	Butanol;
Prop(OH)	Propanol;
Eth(OH)	Ethanol;
$C(t)$	Concentration in respect of time (mg L ⁻¹);
t	Time (h);
t_i	Initial time for exponential growth phase (h);
t_e	Ending time for exponential growth phase (h);
t_m	Time in which maximum production rate (r_{max}) is achieved (h);
r_{max}	Maximum production rate (mg L ⁻¹ h ⁻¹);
C_{max}	Maximum concentration (mg L ⁻¹);
ΔG_r	Variation of Gibbs' free energy at a given condition (kJ mol ⁻¹);
ΔG_r°	Variation of Gibbs' free energy at standard condition (kJ mol ⁻¹);
ΔG_r^F	Variation of Gibbs' free energy at final experiment condition (kJ mol ⁻¹);
ΔG_r^I	Variation of Gibbs' free energy at initial experiment condition (kJ mol ⁻¹).

522 **8 Competing Interests**

523 The authors declare that they have no competing interests.

524 **9 Authors' Contribution**

525 All authors have planned the experiments. GM performed all the experiments, data treatment, statistics,
526 and mathematical modeling. GB treated and analysed all sequencing data. All authors have analysed
527 and discussed the results. GM have written the paper. All authors have read, reviewed, and approved
528 the final manuscript. Experiments were conducted in Montréal branch of National Research Council of
529 Canada from January to July of 2014.

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688

689 **13 One-Sentence Summary**

690 The inoculum pretreatment acid and thermal improved significantly the production of both ethanol and
691 butanol, through an anaerobic solventogenic process, using acetate and butyrate as carbon source and
692 H₂ as electron donor.