

## Diversity and metabolism of xylose and glucose fermenting microbial communities in sequencing batch or continuous culturing

Rombouts, Julius L.; Mos, Galvin; Weissbrodt, David G.; Kleerebezem, Robbert; Van Loosdrecht, Mark C.M.

**DOI**

[10.1093/femsec/fiy233](https://doi.org/10.1093/femsec/fiy233)

**Publication date**

2019

**Document Version**

Accepted author manuscript

**Published in**

FEMS Microbiology Ecology

**Citation (APA)**

Rombouts, J. L., Mos, G., Weissbrodt, D. G., Kleerebezem, R., & Van Loosdrecht, M. C. M. (2019). Diversity and metabolism of xylose and glucose fermenting microbial communities in sequencing batch or continuous culturing. *FEMS Microbiology Ecology*, 95(2), Article fiy233. <https://doi.org/10.1093/femsec/fiy233>

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1 **Diversity and metabolism of xylose and glucose fermenting**  
2 **microbial communities in sequencing batch or continuous**  
3 **culturing**

4  
5 Julius L. Rombouts\*, Galvin Mos, David G. Weissbrodt<sup>§</sup> and Robbert Kleerebezem<sup>§</sup>,  
6 Mark C.M. Van Loosdrecht<sup>§</sup>

7  
8 Delft University of technology, Department of Biotechnology, Van der Maasweg 9,  
9 2629 HZ Delft, the Netherlands.

10

11 \* Corresponding author, [julesrombouts@gmail.com](mailto:julesrombouts@gmail.com), +316 15654428

12 <sup>§</sup> Shared senior authorship

13

14

15 *Submission to FEMS Microbiology Ecology*

16

17

18 **Abstract**

19

20 A mechanistic understanding of microbial community establishment and product  
21 formation in open fermentative systems can aid the development of bioprocesses  
22 utilising organic waste. Kinetically, a single rate-limiting substrate is expected to  
23 result in one dominant species. Four enrichment cultures were operated to ferment  
24 either xylose or glucose in a sequencing batch reactor (SBR) or a continuous-flow

25 stirred tank reactor (CSTR) mode. The combination of 16S rRNA gene-based  
26 analysis and fluorescence *in situ* hybridization revealed no complete dominance of  
27 one species in the community. The glucose-fed and xylose-fed SBR enrichments  
28 were dominated >80% by one species. *Enterobacteriaceae* dominated the SBRs  
29 enrichments, with *Citrobacter freundii* dominant for xylose and *Enterobacter cloacae*  
30 for glucose. *Clostridium*, *Enterobacteriaceae* and *Lachnospiraceae* affiliates  
31 dominated the CSTRs enrichments. Independent of substrate, SBR communities  
32 displayed 2-3 times higher biomass specific rate of substrate uptake ( $q_s^{\max}$ ) and 50%  
33 lower biomass yield on ATP, to CSTR communities. Butyrate production was linked  
34 to dominance of *Clostridium* and low  $q_s^{\max}$  ( $1.06 \text{ Cmol}_s \text{ Cmol}_x^{-1} \text{ h}^{-1}$ ), while acetate and  
35 ethanol production was linked to dominance of *Enterobacteriaceae* and  
36 *Lachnospiraceae* and high  $q_s^{\max}$  ( $1.72 \text{ Cmol}_s \text{ Cmol}_x^{-1} \text{ h}^{-1}$  and higher). Overall, more  
37 diversity than expected through competition was observed, indicating mutualistic  
38 mechanisms might shape microbial diversity.

39

40 Keywords: Mixed culture fermentation – Bioreactor operation – Microbial diversity– r/K  
41 selection – Product spectrum – Kinetics

42

43

## 44 **Introduction**

45

46 The global aim of most societies to develop more circular economies (Ghisellini,  
47 Cialani and Ulgiati 2016) urges for a better use of organic waste as a resource. Until  
48 now, anaerobic digestion is the most common technology used to valorise this waste  
49 in the form of biogas. Several novel bio-based options that provide extra value to  
50 resource recovery are arising such as the production of polyhydroxyalkanoates

51 (Kleerebezem and van Loosdrecht 2007), alginate-like exopolymers (Lin *et al.* 2010),  
52 or medium chain length fatty acids (Spirito *et al.* 2014). The first step in these  
53 production routes consists of the conversion of polymeric carbohydrates into volatile  
54 fatty acids (VFAs) in a mixed-culture fermentative process (Marshall, LaBelle and  
55 May 2013). The alignment of VFA production to subsequent processing requires the  
56 identification of factors that drive product formation in microbial communities as  
57 function of process conditions. First attempts to describe steady-state patterns of  
58 mixed culture fermentation as function of an environmental parameter have provided  
59 incomplete insights in the product formation pathways established (Rodriguez *et al.*  
60 2006; González-Cabaleiro, Lema and Rodríguez 2015). Observed product spectra at  
61 neutral pH could not be simulated properly using these models oriented to ATP  
62 production maximisation, indicating incomplete model assumptions. To aid model-  
63 based developments there is a need for experimental studies giving a more  
64 comprehensive insight into fermentation of specific carbohydrates into VFAs.

65  
66 Xylose and glucose are the most abundant monomers found in lignocellulosic  
67 biomass (Anwar, Gulfraz and Irshad 2014). Fermentation of glucose or xylose can  
68 lead to different products, such as lactic acid, ethanol, hydrogen, and VFAs (Figure  
69 1). Xylose can be fermented through the pentose phosphate pathway (PPP) or the  
70 phosphoketolase pathway (PKP), resulting in a different stoichiometry. Using the  
71 PKP, 40% of the carbon is directly converted to acetate, while the remaining carbon  
72 enters into glycolysis. In PPP, all carbon is converted to intermediates for glycolysis,  
73 thereby bringing all carbon to pyruvate first (Figure 1). In the first part of glycolysis,  
74 one glucose is converted to pyruvate producing four electrons that can be transferred  
75 to NADH. If one acetate is produced, a net amount of one NADH is produced. These

76 electrons cannot be transferred from NADH to hydrogen, as NADH does not possess  
77 sufficient energy to drive this reaction (-320 mV and -414 mV for NADH and  
78 hydrogen respectively, Buckel and Thauer 2013). Hydrogen is produced through  
79 ferredoxin (-400 mV), which is produced when oxidising pyruvate to Acetyl-CoA  
80 (Figure 1). The NADH surplus is oxidised by other fermentative pathways, e.g.  
81 ethanol production, thereby stoichiometrically coupling acetate and ethanol  
82 formation. Recently, electron bifurcation has been proposed as a metabolic strategy  
83 in *Clostridium pasteurianum* (Buckel and Thauer 2013) used to conserve energy in  
84 fermentation by directly coupling acetate and butyrate formation (Li *et al.* 2008). This  
85 mechanism has been successfully incorporated in balancing of NADH of product  
86 spectra over a range of pH values (Regueira *et al.* 2018).

87  
88 Microbial enrichment cultures offer a powerful way of studying the establishment of a  
89 specific microbial niche (Beijerinck 1901), depending on the ecological conditions  
90 applied, such as pH, temperature, redox couple supplied, nutrients among others.  
91 Glucose fermentation has been relatively widely studied, including impacts of pH  
92 (Fang and Liu 2002; Temudo, Kleerebezem and van Loosdrecht 2007), temperature  
93 (Zoetemeyer *et al.* 1982), solid retention time (SRT) (Chunfeng *et al.* 2009), redox  
94 potential (Ren *et al.* 2007), inoculum type (Rafrafi *et al.* 2013), or hydrogen partial  
95 pressure (de Kok *et al.* 2013). Xylose is much less studied but its fermentation has  
96 been compared to glucose fermentation previously (Temudo *et al.* 2009).

97  
98 Most studies have been conducted in continuous-flow stirred tank reactors (CSTR),  
99 under which regime one substrate is continuously limiting (*i.e.*, operation at low  
100 residual concentration). In CSTR systems, affinity dictates the selection: organisms

101 establishing the lowest residual substrate concentration ( $C_s$ ) will dominate the  
102 enrichment (Kuenen 2014). Affinity is governed by both the maximum biomass  
103 specific growth rate ( $\mu^{\max}$ ) and the affinity constant for substrate ( $K_s$ ). Organisms  
104 competing for a substrate in a CSTR environment can, besides optimising their  $\mu^{\max}$ ,  
105 optimise their  $K_s$  value to actively take up the substrate and dominate the microbial  
106 community.

107

108 In a sequencing batch reactor (SBR) operation, substrate is supplied in a pulse,  
109 leading to a high concentration in the environment of the microorganisms during most  
110 of the time that substrate is taken up. Organisms with the highest  $\mu^{\max}$  will eventually  
111 dominate when substrate uptake is directly coupled to growth. The batch selective  
112 environment is traditionally used in microbiology to enrich and isolate organisms,  
113 using the shake-flask approach in combination with dilution series. Consequently,  
114 fast-growing microorganisms are overrepresented in databases of pure cultures  
115 (Prakash *et al.* 2013).

116

117 For both CSTR and SBR environments,  $\mu^{\max}$  is a selective force, which is a function  
118 of the biomass specific rate of substrate uptake ( $q_s^{\max}$ ), the biomass yield on  
119 substrate ( $Y_{x,s}$ ) and the maintenance rate on substrate ( $m_s$ ) (Pirt 1965). From a  
120 kinetic point of view, the microorganism with the highest competitive advantage in the  
121 environment will eventually outcompete the other microorganisms, which is either the  
122 highest  $\mu^{\max}$  (in SBR) or highest affinity (in CSTR) on glucose or xylose. Ultimately,  
123 we aim to investigate the hypothesis if limiting a single substrate in an enrichment  
124 culture leads to the enrichment of a single microbial species. From a competition  
125 point of view, one limiting substrate will select for the most competitive

126 microorganism. Given enough generations or SRTs, this microorganism will  
127 eventually dominate the enrichment culture.  
128  
129 Next to microbial competition on substrate, the different pathways for product  
130 formation are competing within microorganisms. Anabolism needs chemical energy in  
131 the form of ATP to synthesize biomass. Under similar anabolic efficiency, the  
132 catabolic pathway that yields more ATP per substrate ( $Y_{ATP,s}$ ) leads to the highest  
133  $Y_{x,s}$ . Harvested ATP can also be used for active substrate transport. Hereby,  
134 microorganisms lower their  $K_s$  and thereby create a lower  $C_s$  to sustain their selection  
135 in a CSTR environment. Fermentative microorganisms are known to choose between  
136 a high flux pathway (optimizing  $q_s^{max}$ ) or a high yield pathway (optimising  $Y_{ATP,s}$ ),  
137 which is best described by lactate versus acetate and ethanol formation in  
138 *Lactobacillus casei* (De Vries *et al.* 1970). Under CSTR cultivation, at high dilution  
139 rates lactate is formed and at low dilution rates acetate, ethanol and formate are  
140 formed. Lactate formation yields 2 ATP from 1 glucose, while acetate and ethanol  
141 yield 3 ATP from 1 glucose. Thus lactate production is linked to high  $q_s^{max}$ , while  
142 acetate and ethanol production is linked to high  $Y_{ATP,s}$ . Thus, a microorganism will  
143 preferentially involve a metabolic pathway that maximizes  $Y_{ATP,s}$  and/or  $q_s^{max}$  in a  
144 SBR environment and  $Y_{ATP,s}$ ,  $q_s^{max}$  and/or  $K_s$  in a CSTR environment.  
145 Here, we investigated whether SBR or CSTR environments fermenting either xylose  
146 or glucose enrich for an equal microbial community composition and result in  
147 equivalent metabolism and kinetics. Three environmental settings were applied to  
148 enrich for fermentative microorganisms: (1) a mineral medium with only glucose or  
149 xylose as carbon source for fermentation; (2) a combination of temperature, pH, and  
150 SRT to select mainly for primary fermentative microorganisms; and (3) suspended

151 cell cultures. The experimental set up was replicated from Temudo et al. (2009) for a  
152 direct comparison of results. The catabolic products,  $q_s^{\max}$ , and  $Y_{x,s}$  were measured  
153 for each enrichment in steady state in order to verify if a certain stoichiometry was  
154 linked to a certain metabolic strategy. In parallel, we analysed the microbial  
155 community compositions to test the microbial diversity hypothesis for enrichment on  
156 single substrates, and to link community structures to fermentative products and  
157 metabolic strategies.

158

## 159 **Materials and methods**

160

### 161 **Enrichment**

162

163 All enrichments were performed in 3-L jacketed bioreactors (Applikon, the  
164 Netherlands) with working volumes of 2 L. pH was maintained at  $8.0 \pm 0.1$  using  
165 NaOH at  $4 \text{ mol L}^{-1}$  and HCl at  $1 \text{ mol L}^{-1}$ . Temperature was maintained at  $30^\circ\text{C} \pm 0.1$   
166 using a E300 thermostat (Lauda, Germany). The cultures were stirred constantly at  
167 300 rpm. Anaerobic conditions were maintained by sparging the reactor with a flow of  
168  $576 \text{ mmol N}_2 \text{ h}^{-1}$  and off-gas was cooled to  $5^\circ\text{C}$  using a gas condenser. For the  
169 SBRs, a hydraulic retention time (HRT) of 8 h was maintained by removing 1 L of  
170 culture per cycle under a cycle time set to 4 h. For CSTRs, the HRT was directly  
171 linked to the dilution rate applied.

172

173 The synthetic cultivation medium was identical to the one used by Temudo et al.  
174 (2007) using 4 g of either xylose or glucose as carbon source per litre. The carbon  
175 source and the ammonium, phosphate and trace elements were fed separately from



176 12.5× concentrated stock solutions and diluted using N<sub>2</sub>-sparged demineralized  
177 water. Connected to the base pump was a pump supplying 3% (v:v) antifoam C  
178 (Sigma Aldrich, Germany), which ensured a flow of 3-5 mL h<sup>-1</sup> or 14-17 mL cycle<sup>-1</sup>.  
179 The glucose and xylose solutions were sterilized at 110°C for 20 min.

180

181 The inoculum was obtained from cow rumen through a butcher in Est, the  
182 Netherlands, and on the same day, transported to lab at room temperature and  
183 filtered on 200 µm and aliquoted in 50-mL portions, and frozen at -20°C using 10%  
184 glycerol. The seed biomass was then thawed on ice before adding 10 mL to the  
185 reactor to start each enrichment culture. When a full first batch was performed the  
186 CSTRs were set to continuous mode and the SBRs were set in cycle mode, gradually  
187 moving from 24-h to 12-h and 6-h in 3 days to the final desired 4-h cycles to maintain  
188 a HRT of 8 h. Steady state was assumed if during a period of at least 5 days no  
189 variation was in the product concentrations.

190

## 191 **Analytical methods**

192

193 Samples from the reactors were immediately filtered on 0.45 µm polyvinylidene  
194 fluoride membranes (Millipore, USA) and stored at -20°C until analysis. VFAs  
195 (formate to valerate), lactate, succinate, ethanol, glucose and xylose were analysed  
196 using high performance liquid chromatograph (HPLC) equipped with an Aminex HPX-  
197 87H column (BioRad, USA) maintained at 60 °C and coupled to ultraviolet (UV) and  
198 refraction index (RI) detectors (Waters, USA), using phosphoric acid at 0.01 mol L<sup>-1</sup>  
199 as eluent. For high butyrate concentrations above 1 mmol L<sup>-1</sup>, samples were  
200 analysed using gas chromatography (GC), since butyrate overlapped with ethanol on

201 the RI detector of the HPLC. GC was performed using a Chrompack 9001 (Agilent,  
202 USA) equipped with an injector maintained at 180°C, a fused-silica capillary column  
203 of 15 m × 0.53 mm HP-INNOWax (Agilent, USA) equilibrated at 80°C for alcohols  
204 with helium as carrier gas, and a flame ionization detector set at 200°C. Glycerol was  
205 detected using an enzymatic assay relying on glycerokinase, pyruvate kinase and L-  
206 lactate dehydrogenase, measuring NADH depletion at 340 nm (Megazyme, Ireland).

207

208 The off-gases were monitored on-line for H<sub>2</sub> and CO<sub>2</sub> by a connection to a NGA 2000  
209 MLT 1 Multicomponent analyser (Rosemount, USA). Data acquisition (base, H<sub>2</sub>, CO<sub>2</sub>)  
210 was made using a BBI systems MFCS/win 2.1 (Sartorius, Germany).

211

212 Biomass concentration was measured using a standard method which relies on  
213 centrifugation to separate the cells from the medium (APHA, 1998). This analysis  
214 was coupled to absorbance measurement at 660 nm to establish a correlation.

215 Absorbance values were used to calculate the biomass concentration during the  
216 batch experiments.

217

## 218 **Cycle analysis**

219

220 To characterise one cycle in SBR mode, one full cycle was sampled and product and  
221 biomass concentrations were measured in parallel to H<sub>2</sub> and CO<sub>2</sub> in the off-gas. In  
222 the CSTRs, one litre of volume was removed and one litre of medium was added to  
223 finally obtain a concentration of 4 g L<sup>-1</sup> of either xylose or glucose together with a  
224 stoichiometric amount of other nutrients. Sampling and off-gas analysis were carried  
225 out as in the SBRs.

226

227 **Microbial community analysis**

228

229 Genomic DNA was extracted using the Ultra Clean Soil DNA extraction kit (MOBIO  
230 laboratories, USA) following manufacturer's instructions, with the exception of heating  
231 the samples for 5 minutes at 65°C prior to bead beating. Highly molecular DNA was  
232 obtained (>10 kb) with a concentration of 10 ng  $\mu\text{L}^{-1}$  or higher. Extracted DNA was  
233 stored at -20°C until further use.

234

235 Analysis of 16S rRNA gene-based amplicon sequencing was conducted to get an  
236 overview of the predominant populations in the enrichments in time. The extracted  
237 DNA was sent for amplification and sequencing at a commercial company  
238 (Novogene, China). Amplification was achieved using the universal primer set 341f /  
239 806r targeting the V3-V4 region of the 16S rRNA gene (Table S1). All polymerase  
240 chain reactions (PCR) were carried out in 30  $\mu\text{L}$  reactions with 15  $\mu\text{L}$  of Phusion®  
241 High\_fidelity PCR Master Mix (New England Biolabs, USA), 0.2  $\mu\text{mol L}^{-1}$  of forward  
242 and reverse primers and 10 ng template DNA. Thermal cycling started with an initial  
243 denaturation at 98°C for 10 s, annealing at 50°C for 30 s and elongation at 72°C for  
244 60 s and ending with 72°C for 5 min. These pools of amplicon sequences were then  
245 sequenced using an IlluminaHiSeq2500 platform. The sequencing datasets were  
246 cleaned and trimmed according to Jia *et al.* (2016) and processed with Qiime  
247 (Caporaso *et al.* 2010) using UCLUST with a 97% stringency to yield operational  
248 taxonomic units (OTUs). OTUs were taxonomically classified using the RDP classifier  
249 (Wang *et al.* 2007) with 0.85 confidence interval against the Greengenes database  
250 release of August 2013 (DeSantis *et al.* 2006). Double check of OTUs identity factors

251 was then obtained by alignment against the NCBI RefSeq database using the basic  
252 alignment search tool for nucleotides (BLASTn) (Johnson *et al.* 2008).

253

254 Cloning-sequencing was conducted to obtain species level information. The near-  
255 complete 16S rRNA gene was amplified using the primers GM3f and GM4r (Table  
256 S1). The PCR products were purified using QIAquick PCR purification kit (QIAGEN,  
257 Germany), ligated, and transformed into competent *Escherichia coli* cells using the  
258 TOPO TA Cloning Kit (Invitrogen, USA). Transformed cells were plated on Luria-  
259 Bertani medium plates containing 50 µg kanamycin mL<sup>-1</sup>. After overnight incubation  
260 at 37°C, clones were randomly selected for amplification of the 16S insert into the  
261 PCR4-TOPO vector using the M13f and M13r primers (Table S1). Depending on the  
262 diversity of the sample, 8 to 55 clones were sequenced using Sanger sequencing  
263 (Baseclear, the Netherlands). The first and last 100 bp were removed using  
264 CodonCode aligner, as sequence quality was insufficient in these regions. Qiime  
265 processing was performed on the sequences as described above using a similarity  
266 criterium >99% which is defined to be the minimum similarity between species  
267 (Janda and Abbott 2007). BLASTn was used to retrieve the identity of each species,  
268 and BLAST results with the same species but a different strain were grouped  
269 together for phylogenetic resolution at species level. The closest relates strain was  
270 then used to retrieve genomic information. Sequences obtained are deposited under  
271 the BioProject accession number PRJNA505600 (raw merged amplicon reads) and  
272 MK185473 – MK185614 (1450 bp 16S genes) in the NCBI database. Cell fixation  
273 and fluorescence *in situ* hybridisation (FISH) were carried out as described by  
274 Johnson *et al.* (2009) using the probes listed in table S2, except that hybridization  
275 was carried out overnight. Additionally, DAPI staining was used to stain all microbial

276 cells by incubating the multi-wells microscopy slides of fixed cells with 10  $\mu\text{L}$  of a  
277 solution of 10 mg DAPI  $\text{mL}^{-1}$  per well for 15 min. The samples were analysed using  
278 an epifluorescence microscope (Axioplan 2, Zeiss, Germany). Digital images were  
279 acquired using a Zeiss MRM camera together with Zeiss imaging software  
280 (AxioVision version 4.7, Zeiss, Germany). The 1000x magnified images were  
281 improved by setting the 1x sharpening. Three images were taken at 400x and  
282 exported as TIFF and used for quantification of the cell surface using the QUIPS  
283 feature in Leica QWin V3 (Leica, Germany).

284

### 285 **Modelling of the cycle analysis**

286

287 To obtain the  $q_s^{\max}$  and  $\mu^{\max}$  for the CSTRs from the cycle analysis, a model was  
288 constructed. Herbert-Pirt relation for substrate uptake was simplified by neglecting  
289 maintenance, as maintenance is not measured and is assumed to be a small  
290 contribution compared to  $q_s^{\max}$ :

291

$$292 \quad \mu = Y_{xs} \cdot q_s \quad (1.1)$$

293

294 Monod kinetics were used to describe the growth rate as a function of the substrate  
295 concentration at a value of 0.1  $\text{mmol L}^{-1}$  of either xylose or glucose:

296

$$297 \quad \mu = \mu^{\max} \cdot \frac{C_s}{C_s + K_s} \quad (1.2)$$

298

299 The model estimated  $C_s$  and  $C_x$  by varying the biomass and substrate concentration  
300 at the start of the cycle analysis ( $C_{x,0}$ ,  $C_{s,0}$ ) and  $Y_{xs}$  and  $q_s^{\max}$  values giving the best

301 fit, and a boundary value of  $\mu$  is zero was applied when  $C_s$  was zero. The modelled  
302 values were then optimised to the measured data with a minimisation of the sum-  
303 squared error, using the non-linear solver in Microsoft Excel (2010).

304

### 305 **Analysis of on-line data collected from the bioreactors**

306

307 For SBRs, the  $\mu^{\max}$  was calculated per cycle using the recorded base dosage values.  
308 Microbial growth was directly correlated to the base consumption due to acid  
309 production in fermentation (Figure S3). A script was developed in Matlab (version  
310 2014, USA), further explained in the supplementary information (SI) section.

311

### 312 **COD and carbon balances**

313

314 During steady state carbon and chemical oxygen demand (COD) balances were set  
315 up using the elemental matrix given in table S4. COD and carbon balances were set  
316 up by multiplying the values in the table 9 with the in- and outgoing rates in the  
317 reactor, while the NADH, ATP and Gibbs energy balances were set up by multiplying  
318 the values in table 9 with the yield on glucose. Data reconciliation was used to obtain  
319 closed balances for H, C, O, N and charge using the method described by van der  
320 Heijden *et al.* (1994). These balances were used to calculate the Gibbs energy of  
321 dissipation.

322

323 Carbon and COD balances were set up for the cycle analyses by subtracting the  
324 amount of carbon or COD in the compounds measured at a time in the cycle from the  
325 measured available carbon or COD at the start of the cycle.

326

327 **Results**

328

329 **Xylose and glucose fermentation product spectra are similar in SBRs and**  
330 **different in CSTRs**

331

332 Four different enrichment reactors were operated and analysed for their main  
333 products in liquid and gas phase after steady-state was established; this was  
334 obtained after 20 SRTs for all enrichments. The glucose SBR exhibited the largest  
335 shift in product spectrum during the adaptation, as initially acetate and propionate  
336 were the dominant products which changed to acetate and ethanol as dominant  
337 products after 18 SRTs. The product spectrum in the xylose and glucose SBR  
338 enrichments was very similar, dominated by a catabolic reaction producing ethanol  
339 and acetate (Figure 2A), coupled with hydrogen and formate production (Figure 1).  
340 Regarding the by-products formed, the xylose SBR enrichment produced more  
341 succinate, while the glucose SBR enrichment produced more propionate and lactate.

342

343 The xylose CSTR enrichment also had a product spectrum dominated by acetate and  
344 ethanol (Figure 2B), coupled to the production of hydrogen and formate. In the  
345 glucose CSTR, butyrate was a dominant product, followed by acetate and ethanol  
346 (Figure 2B). Both these catabolic pathways were coupled with hydrogen and formate  
347 production. Regarding the by-products, similar to the SBRs, the glucose CSTR  
348 enrichment produced more propionate and lactate, while the xylose CSTR  
349 enrichment produced more succinate, with a significant yield of succinate production  
350 in this enrichment of  $0.09 \text{ Cmol Cmol}_S^{-1}$  succinate formed.

351

352 Summing up, the glucose SBR and the xylose SBR and CSTR enrichment displayed  
353 similar product spectra dominated by acetate and ethanol, while the glucose CSTR  
354 showed a mixed product spectrum of butyrate, acetate and ethanol. Glycerol was not  
355 detected in a significant amount in any of the enrichments. which was detected up to  
356 0.1 Cmol Cmol<sub>s</sub><sup>-1</sup> by Temudo et al. (2009).

357

### 358 **Carbon and COD balances were nearly closed in all enrichments**

359

360 For all enrichments the carbon and chemical oxygen demand (COD, *i.e.*, electron)  
361 balances could be closed from the measured products at 95% and 105%,  
362 respectively (Table S3). Only in the glucose SBR enrichment a significant amount of  
363 10% of carbon and COD could not be recovered in the outflows of the reactor. A  
364 characteristic peak at a retention time of 19.1 min was present on the HPLC UV  
365 channel for the glucose SBR which could not be identified but was confirmed to be  
366 neither 1,3-propanediol nor malate, fumarate, 2,3-butanediol, acetoin or  
367 hydroxyvalerate.

368

### 369 **No storage response or sequential fermentation during cycle analysis**

370

371 For all four enrichments a pulse experiment was performed, in which the substrate  
372 and products were measured in time and used to set up a carbon and COD balance  
373 over the cycle. A typical storage response would show COD “disappearing” during  
374 the initial fermentation phase until the substrate is depleted, while it reappears after  
375 substrate depletion as formed products. No such response was observed in both the



376 CSTR and SBR enrichments (Figure S2) and no sequential conversion of  
377 intermediate fermentation products was detected in the cycle analysis in SBRs  
378 (Figure S3).

379

### 380 **Fast kinetics for SBR enrichments and high biomass yield for CSTR** 381 **enrichment**

382

383 At steady state, the yield of biomass formation on substrate was determined in all  
384 four enrichments (Table 1). There was no significant difference in biomass yield  
385 between the glucose CSTR enrichment reported here and by Temudo *et al.* (2009).  
386 The xylose CSTR enrichment displayed a 43% lower biomass yield than the glucose  
387 CSTR, and a 25% lower value compared to the xylose CSTR enrichment reported by  
388 Temudo et al. 2009. The glucose SBR, the xylose SBR and the xylose CSTR  
389 enrichment showed similar biomass yield values.

390

391 Through analysis of the on-line fermentation data the  $\mu^{\max}$ -value for each  
392 fermentation cycle could be determined for the SBR enrichments (see SI, figure S5  
393 and S6). A cycle analysis in the CSTR enrichment cultures was used to estimate  
394  $q_s^{\max}$ . The actual  $q_s$ -value in the xylose CSTR enrichment was  $1.06 \text{ Cmol}_S \text{ Cmol}_X^{-1} \text{ h}^{-1}$   
395 <sup>1</sup>, which was 38% lower than the measured  $q_s^{\max}$ . The actual  $q_s^{\max}$ -value in the  
396 glucose CSTR enrichment was  $0.55 \text{ Cmol}_S \text{ Cmol}_X^{-1} \text{ h}^{-1}$  which was 48% lower than  
397 the maximal rate of glucose uptake. The xylose CSTR enrichment exhibited a 62%  
398 higher  $q_s^{\max}$ -value than the glucose CSTR enrichment. The  $q_s^{\max}$  value found for the  
399 xylose SBR enrichment was statistically significantly lower (33%) than for the glucose  
400 SBR enrichment (Table 1,  $p = 0.002$ ).

401

402 **Microbial community analyses highlighted higher diversity with xylose**

403

404 Amplicon sequencing of the V3-V4 region of the 16S rRNA gene was used to obtain  
405 a relative snapshot of the dynamics of the community over time. Then, FISH analysis  
406 with three different probes targeting the 16S rRNA of populations of the genus  
407 *Clostridium* and of the families of *Enterobacteriaceae* or *Lachnospiraceae* was used  
408 to analyse the microbial communities in the enrichments. Lastly, clone libraries were  
409 created of the full 16S gene to obtain species-level information of the communities.

410 Microbial diversity was evaluated by the abundance and number of families or genera  
411 present.

412

413 The xylose SBR enrichment was dominated by *Enterobacteriaceae* (Figure 3, Table  
414 2, figure S7) and a side population of *Lachnospiraceae* and *Clostridium* (Table 2).

415 The 16S amplicon sequencing revealed that the *Enterobacteriaceae* were dominated  
416 by *Citrobacter* species (Figure 3), which was confirmed to be *Citrobacter freundii*  
417 using the clone library (Figure 4).

418

419 The glucose SBR enrichment was dominated by *Enterobacteriaceae* (Figure 3, Table  
420 2, figure S7) with a side population of *Lachnospiraceae*. The 16S amplicon  
421 sequencing shows that the *Enterobacteriaceae* were dominated by *Enterobacter*  
422 species (Figure 3), which is confirmed to be *Enterobacter cloacae* by the clone library  
423 (Figure 4). Two other species also were confirmed using the clone library, *Raoultella*  
424 *ornithinolytica* and *Citrobacter freundii*. Thus, both SBR enrichments were dominated

425 by a single *Enterobacteriaceae* species, with side-populations of *Lachnospiraceae* in  
426 both SBRs, and *Clostridium* in the xylose SBR enrichment.

427

428 The glucose CSTR enrichment is dominated by *Clostridium* species (Figure 3, Table  
429 2, figure S7) with a side population of *Enterobacteriaceae* (Table 2). The 16S  
430 amplicon sequencing gave two main OTUs, an *Enterobacter* sp. and *Clostridium* sp.  
431 (Figure 3), which are confirmed to be *Clostridium intestinale* and *Raoultella*  
432 *ornithinolytica*.

433

434 The xylose CSTR enrichment is dominated by *Lachnospiraceae* and  
435 *Enterobacteriaceae* species (Figure 3, Table 2, figure S7). The 16S amplicon  
436 sequencing is dominated by a *Citrobacter* sp., while two OTUs from the  
437 *Lachnospiraceae* are present. The clone library reveals that the *Citrobacter* OTU  
438 corresponds to *Citrobacter freundii*, while only one of the *Lachnospiraceae* OTUs can  
439 be confirmed up to family level, as it only shows 96% sequence similarity with the  
440 closest cultivated relative *Lachnotalea glycerinii* (Table S6).

441

442 Summing up, it can be argued that the glucose SBR and CSTR enrichment showed a  
443 similar level of diversity, with a dominant species and a small side-population. The  
444 xylose SBR enrichment was more diverse than the glucose enrichments, as the side  
445 population contains both *Clostridium* and *Lachnospiraceae* species. In the xylose  
446 CSTR the largest diversity was observed, as here *Citrobacter freundii*, an  
447 uncultivated *Lachnospiraceae* species and a *Muricomes* population dominated.

448

449 **Discussion**

450

## 451 **Pathway analysis of the enrichments**

452

453 Under slightly alkaline and mesophilic conditions acetate and ethanol were the  
454 dominant products under SBR conditions, while butyrate formation occurred  
455 significantly under CSTR conditions. Compared to the work of Temudo *et al.* (2009)  
456 we observe a similar product spectrum in the glucose CSTR enrichment, though we  
457 observe more ethanol and less butyrate. The xylose CSTR enrichment is dominated  
458 by acetate and ethanol, while the enrichment of Temudo *et al.* (2009) had produced  
459 primarily butyrate and acetate. Acetate and ethanol have been shown as the  
460 dominant products at pH 7.9 and 30°C (Zoetemeyer, van den Heuvel and Cohen  
461 1982), while acetate and butyrate have been dominant products under at pH 7.0 and  
462 36°C (Fang and Liu 2002).

463

464 The rate of the supply of inert N<sub>2</sub> gas in the reactor broth was the only difference in  
465 experimental procedures between the present study and the work of Temudo *et al.*  
466 (2009). This could potentially change the hydrogen and carbon dioxide gas partial  
467 pressures. The impact of the gas flow rate on the fermentation pattern was  
468 investigated, in order to investigate if the gas flow rate could explain the differences  
469 in product spectrum observed. Little effect was found on all product yields and  
470 hydrogen partial pressure (Figure S1); thus, we expect no major impact of the gas  
471 flow rate. Furthermore, the glucose CSTR enrichment was duplicated and the  
472 resulting product spectrum of both enrichments was identical (Figure S1) which  
473 confirms the reproducibility of the enrichments.

474

475 A NADH balance was set up using the generalised metabolic network (Figure 1,  
476 Table S4), and the derivatives from the pyruvate to acetyl-CoA pathway were summed  
477 as a yield. The NADH balance of the four enrichments shows that the glucose CSTR  
478 has a small net producing NADH balance, whereas the two SBRs and the xylose  
479 CSTR have a small net NADH consuming balance. Minor discrepancies from the  
480 NADH-balance can possibly be explained by succinate production through an NADH  
481 producing pathway, such as through the oxidative branch of the TCA cycle.  
482 Assuming no net NADH consumption for succinate production would bring the two  
483 SBRs and the xylose CSTR to a closed NADH balance.

484

485 Comparable values for the acetyl-CoA derivatives and H<sub>2</sub>/formate production (Table 3)  
486 indicate that H<sub>2</sub>/formate production is directly coupled to pyruvate conversion to  
487 acetyl-CoA in the metabolic network as in Figure 1. Only for the xylose CSTR  
488 enrichment there is significantly less formate and H<sub>2</sub> found than acetyl-CoA derivatives,  
489 which suggest that H<sub>2</sub> and formate are consumed through homoacetogenesis as  
490 proposed by (Regueira *et al.* 2018).

491

492 The stoichiometric data argues for the PPP to be active in the xylose SBR, as acetate  
493 and ethanol are present in equimolar amounts and there is no excess of acetyl-CoA  
494 derivatives compared to formate/H<sub>2</sub>. If the PKP would have been active, more acetate  
495 compared to ethanol would have been expected and less acetyl-CoA derivatives  
496 compared to formate/H<sub>2</sub>. In *Clostridium acetobutylicum* the PKP has been  
497 significantly expressed under batch cultivation (Liu *et al.* 2012), but here the PPP is  
498 assumed to be the only pathway active under SBR conditions.

499

## 500 **Bioenergetics and the role of substrate uptake**

501

502 Using the metabolic network (Figure 1) the amount of ATP produced was estimated  
503 from the different catabolic products ( $Y_{ATP,s}$ ). Combining this yield with the biomass  
504 yield, the biomass yield on ATP ( $Y_{x,ATP}$ ) was calculated. The  $Y_{x,ATP}$  values for the  
505 xylose SBR and CSTR are very similar (Table 4), while the  $Y_{x,ATP}$  values for the  
506 glucose SBR and CSTR enrichments are higher (Table 4).  $Y_{x,ATP}$  values are  
507 confirmed by the dissipation energy, as the xylose SBR and CSTR enrichment show  
508 a similar value, while the value for the glucose SBR enrichment is higher and the  
509 highest value is reported for the glucose CSTR enrichment. This means the xylose  
510 enrichments have a considerably lower energetic efficiency than the glucose  
511 enrichments. The dissipation values obtained for glucose is in accordance with the  
512 average values for glucose ( $-236 \text{ kJ Cmol}_x^{-1}$ ), while that of xylose is considerably  
513 higher than according to the correlation function ( $-246 \text{ kJ Cmol}_x^{-1}$ ) (Heijnen, van  
514 Loosdrecht and Tijhuis 1992).

515

516 The higher dissipation in the xylose enrichments can be caused by the cost of  
517 transporting xylose over the cell membrane. Xylose can be taken up into the cell by  
518 two different mechanisms. XylE is an enzyme which uses the proton motive force to  
519 take up xylose from the surrounding medium, through the symport with one proton  
520 (Davis and Henderson 1987). When assuming a stoichiometry of  $2.67 \text{ mol H}^+$  per mol  
521 ATP used, this means xylose uptake XylE costs  $0.375 \text{ mol ATP}$  per mol xylose. A  
522 second method for active xylose uptake is via XylFGH, an ATP-binding cassette  
523 (ABC) transporter which uses the direct dephosphorylation of ATP to import xylose  
524 (Sumiya *et al.* 1995). XylE is known to be a low affinity transporter, while XylFGH is a

525 high affinity transporter (Sumiya *et al.* 1995). In *E. coli* it has been demonstrated that  
526 in batch conditions XylE plays a minor role in xylose uptake (Hasona *et al.* 2004).

527

528 The genome of the strain with the highest similarity was assessed for the presence of  
529 transporters. *Citrobacter freundii* strain P10159 dominant in the xylose SBR  
530 enrichment (Table S6) contains the XylE gene and not the analogues XylF, XylG or  
531 XylH (accession number CP012554.1) This argues for the nature of XylE as a high-  
532 rate xylose transport enzyme. A different *Citrobacter freundii* strain FDAARGOS  
533 (accession number CP026056.1) was populating the xylose CSTR, which contained  
534 neither XylE nor XylF, XylG or XylH. This suggests novel ABC transporters might be  
535 present in the xylose CSTR population.

536

537 Glucose uptake can be more energy efficient. The phosphotransferase system (PTS)  
538 is an uptake mechanism which couples the transfer of a phosphate group from PEP  
539 to glucose to transport glucose over the membrane, thus there is no net ATP cost for  
540 importing glucose as glucose-phosphate is directly produced. This complex is  
541 assumed to be active in both SBR and CSTR as this is observed to be the main  
542 transport system under glucose excess (Steinsiek and Bettenbrock 2012) and under  
543 substrate limitation (Babu *et al.* 2005). The *Enterobacter cloacae* strain AA4  
544 dominant in the glucose SBR enrichment and the *Clostridium intestinale* strain  
545 URNW dominant in the glucose CSTR enrichment both contain all five genes  
546 necessary to express the PTS complex in their genomes (accession number  
547 CP018785.1 and HM801879.1).

548

549 When incorporating this biochemical consideration for substrate uptake, the  $Y_{x,ATP}$   
550 value for xylose and glucose becomes similar (Table 4), while the 50% difference in  
551  $Y_{x,ATP}$  between SBR and CSTR enrichments remains.

552

### 553 **Xylose uptake is slower than glucose uptake in SBR**

554

555 When substrate is only used for growth and no storage products are formed, the  
556 competition in a SBR process is based on the  $\mu^{\max}$  of the competing microorganisms,  
557 which can be maximised through  $Y_{x,s}$  or  $q_s^{\max}$ . The SBR grown cultures described in  
558 this paper are optimized for  $q_s^{\max}$  (Table 1). The  $q_s^{\max}$  of the glucose SBR enrichment  
559 is 50% higher than the xylose SBR enrichment. The lower uptake rate for xylose can  
560 be explained by a kinetic bottleneck identified in the PPP. Gonzalez *et al.* (2017)  
561 have shown that in glycolysis *E. coli* metabolises glucose to fructose-6-phosphate at  
562 a rate of  $90 \text{ mmol g}_{DW}^{-1} \text{ h}^{-1}$ , while in the PPP rates to form fructose-6-phosphate did  
563 not exceed  $37 \text{ mmol g}_{DW}^{-1} \text{ h}^{-1}$ . The production of formate, acetate and ethanol  
564 exceeded these values for glucose, indicating the lower part of fermentation was not  
565 rate limiting.

566

### 567 **Acetate and ethanol production as a kinetic advantage**

568

569 The  $q_s^{\max}$  and  $\mu^{\max}$  for the CSTR grown glucose enrichment producing butyrate is  
570 significantly lower than the acetate and ethanol producing enrichment (Table 1 and  
571 Temudo *et al.* 2009). Furthermore, the xylose CSTR enrichment of Temudo *et al.*  
572 (2009) and the glucose CSTR enrichment performed here, showed a similar  $q_s^{\max}$ -  
573 value (Table 1) and both enrichments are producing a significant amount of butyrate.



574 On top of that, both SBRs produce dominantly acetate and ethanol, where  $q_s^{\max}$  is a  
575 more important competitive advantage than in CSTR conditions. The kinetic  
576 difference between butyrate forming and acetate and ethanol forming  
577 microorganisms is observed in pure cultures. The  $\mu^{\max}$  of *Clostridium tyrobutyricum*, a  
578 butyrate producer, is  $0.12 \text{ h}^{-1}$  (Liu and Yang 2006) and *Citrobacter* sp. CMC-1, an  
579 acetate and ethanol producer, is  $0.21 \text{ h}^{-1}$  (Mangayil, Santala and Karp 2011) grown  
580 under similar conditions. The fact that acetate and ethanol formation is related to  
581 higher  $\mu^{\max}$  is also indirectly shown by the study of Zoetemeyer *et al.* (1982), as a  $\mu$   
582 of  $0.25 \text{ h}^{-1}$  was applied here at pH 7.9 and  $30^\circ\text{C}$  obtaining a product spectrum of  
583 acetate and ethanol, while Temudo *et al.* (2009) and this study obtain also butyrate  
584 production at a  $\mu$  of  $0.13 \text{ h}^{-1}$ . This kinetic advantage seems to hold only for  
585 fermentations at pH higher than 6.25, as enrichments performed in CSTR mode at  
586 pH 5.5 above  $\mu^{\max}$  have demonstrated to systemically yield a product spectrum  
587 dominated by acetate, butyrate, and lactate (Rafrafi *et al.* 2013). This kinetic effect  
588 can be incorporated into model-based evaluation of mixed culture fermentations to  
589 improve the prediction of butyrate, acetate and ethanol production at neutral and  
590 alkaline pH.

591

### 592 **Butyrate production as an efficient pathway**

593

594 If acetate and ethanol production obtains a higher  $q_s^{\max}$  value than butyrate, and both  
595 pathways produce 3 mol ATP, there seems to be no advantage for butyrate  
596 production over acetate and ethanol production. Thermodynamically, butyrate  
597 formation yields more energy than acetate and ethanol production, ( $-264 \text{ kJ mol}^{-1}$  and  
598  $-226 \text{ kJ mol}^{-1}$  respectively). This energy is available in the step from crotonyl-CoA to

599 butyryl-CoA, which is calculated to be -50 kJ/mol (González-Cabaleiro, Lema and  
600 Rodríguez 2015). A direct conversion of this energy into a proton motive force has  
601 been rejected (Herrmann *et al.* 2008). Part of the energy can be conserved by  
602 coupling this energy to the transfer of the electrons from NADH to ferredoxin and  
603 then oxidizing ferredoxin with NAD<sup>+</sup> to generate a sodium motive force using the Rnf  
604 enzyme (Herrmann *et al.* 2008). Two of the six subunits of this complex are found in  
605 the genome of the *Clostridium intestinale* strain URNW, indicating the possibility of  
606 this mechanism being active in the glucose CSTR enrichment.

607

### 608 **Metabolic strategies in fermentation: r-organisms vs K-organisms**

609

610 The CSTR enrichments, when corrected for substrate uptake, show about 50%  
611 higher  $Y_{x,ATP}$  value than the SBR enrichments. The  $q_s^{max}$ -value on the other hand is  
612 2-3 times higher for the SBR enrichments compared to the CSTR enrichments.  
613 These observations correspond with the general microbial theory proposed on r- vs  
614 K-organisms (Andrews and Harris 1986). The r-organisms are more adapted to a  
615 substrate-abundant environment and display high  $q_s^{max}$  and  $\mu^{max}$  values. K-organisms  
616 are more adapted to crowded environment where substrate is limited and display  
617 high  $Y_{x,ATP}$  and  $K_s$  values. The reason r-organisms dissipate more energy than K-  
618 organisms in their metabolism may rely on the fact that at increasing growth rate  
619 more erroneous proteins are produced due to a higher error rate made during  
620 proofreading at higher speed (Yamane *et al.* 1977). Thus, more non-functional  
621 proteins are produced at higher growth rate. As protein production is estimated to  
622 cost >80% of the ATP to synthesise a cell (Hespell and Bryant 1979), larger error

623 rates will cause increased ATP cost per cell assuming a similar functioning protein  
624 content.

625

626 The community data shows that *Enterobacteriaceae* dominate the SBR  
627 environments, thus the *Citrobacter freundii* and *Enterobacter cloacae* species can be  
628 classified as r-organisms. *Enterobacteriaceae* species such as *E. coli* are well known  
629 to exhibit high growth rates in anaerobic environments with carbohydrates (De Vrije  
630 and Claassen 2003). *Clostridium* species on the other hand are often dominating in  
631 substrate-limited environments such as anaerobic digesters (Burrell *et al.* 2004),  
632 where the rate of hydrolysis of cellulose and hemicellulose is an order of magnitude  
633 lower than typical fermentation rates, creating a substrate-limited environment. In the  
634 glucose CSTR we observe a dominance of *Clostridium intestinale*, which fits with  
635 these observations.

636

### 637 **The microbial community composition and the effect of limiting a single** 638 **substrate**

639

640 First of all, it is noteworthy that the FISH imaging and the 16S rRNA gene amplicon  
641 sequencing data do not always correspond. In the glucose SBR, the dominance of  
642 *Enterobacteriaceae* on OTU-level is confirmed by the FISH analysis, but in the  
643 glucose CSTR enrichment the *Enterobacteriaceae* are observed to be a minor  
644 fraction on cell-level (FISH image), while 30% of the reads relate to  
645 *Enterobacteriaceae*. In the xylose CSTR a similar bias is observed, as 53% of the  
646 community is identified as *Lachnospiraceae* using FISH (Table 2), while only 15% of  
647 the reads relate to *Lachnospiraceae*. As we have corrected the data for copy

648 numbers, the bias is likely caused by DNA extraction and PCR biases, which are  
649 known to cause biases in amplicon sequencing data (Brooks *et al.* 2015). As  
650 proposed by Amann, Ludwig and Schleifer (1995), 16S rRNA gene sequencing and  
651 FISH analysis have to be used in parallel to obtain an accurate estimation of the  
652 microbial community structure, which is confirmed in the study here.

653

654 Here, populations of *Enterobacteriaceae*, *Lachnospiraceae* and *Clostridium*  
655 dominated the enrichments. *Clostridium* and *Enterobacteriaceae* populations have  
656 been reported in enrichments on mineral medium (Table 5), though for the first time  
657 *Lachnospiraceae* were enriched on xylose. We find that a significant presence of  
658 *Clostridium* was linked to butyrate production, as in the glucose CSTR, which is  
659 confirmed by other enrichment studies (Table 5). The butyryl-CoA dehydrogenase  
660 gene, which is responsible for the reduction of crotonyl-CoA to butyryl-CoA using  
661 NADH, is found in organisms in the *Clostridium* species, while neither in *Enterobacter*  
662 nor in *Citrobacter* species according to the NCBI Gene database.

663

664 The glucose enrichments seem to be dominated by a single species with one side  
665 populating family, which is *Enterobacter cloacae* in the glucose SBR and *Clostridium*  
666 *intestinale* in the glucose CSTR. It was expected that, when limiting a single  
667 substrate, one specialist will dominate the community after prolonged cultivation,  
668 displaying either the highest  $\mu^{\max}$  or the highest affinity. For the xylose enrichments,  
669 the communities are more diverse. In the xylose SBR, *Citrobacter freundii* dominated  
670 the culture, with a side-population of both, *Lachnospiraceae* and *Clostridium*. The  
671 xylose CSTR is populated by two *Lachnospiraceae* OTUs (Figure 3), one of which is  
672 confirmed to be an uncultivated *Lachnospiraceae* species (Table S6) next to a

673 population of *Citrobacter freundii*. Thus, xylose fermentation results in more microbial  
674 diversity than glucose fermentation.

675

676 All four enrichments are populated by more than one species, with stabilizing OTUs  
677 over time (Figure 3). This indicates that species have a reason to coexist in these  
678 single substrate limited systems. It is possible that mutualistic relationships between  
679 these species are present, *e.g.*, in the form of a B-vitamin exchange between species  
680 (Magnúsdóttir *et al.* 2015), as these communities are cultivated on mineral medium.  
681 Overall, it remains an important ecological question why in many cases rather diverse  
682 communities remain in very selective conditions with one limiting substrate.

683

684 Overall, this study aimed to show the impact of sequencing batch and continuous  
685 culturing on microbial communities fermenting lignocellulosic sugars such as xylose  
686 and glucose. Butyrate formation was linked to slow uptake rate, while acetate and  
687 ethanol formation was linked to high uptake rates. This kinetic effect can be taken  
688 into account in modelling efforts. In SBR, xylose was fermented 33% slower than  
689 glucose. SBR communities maximised their  $q_s^{\max}$ , while CSTR communities  
690 maximised their  $Y_{x,ATP}$ . SBR communities were dominated by r-strategists like  
691 *Citrobacter freundii* and *Enterobacter cloacae*, and the CSTR communities by K-  
692 organisms like *Clostridium intestinale* and *Lachnospiraceae* species. No significant  
693 storage of either xylose or glucose was observed in the SBR enrichments. The  
694 glucose enrichments confirmed the hypothesis that limitation of a single substrate  
695 leads to domination of a single species. The xylose enrichments displayed more  
696 microbial diversity, with the xylose CSTR up to three dominant populations.

697

698 **Acknowledgements**

699 The authors wish to thank Cor Ras and Max Zomerdijk for technical assistance in  
700 analytics, Ben Abbas for help with the clone libraries and sequencing, Lars Puiman  
701 for his help in improving the FISH hybridisation for Gram+ microorganisms and  
702 Rebecca Gonzalez-Cabaleiro at Newcastle University for discussion on metabolic  
703 strategies in fermentations. This work was supported by the Soenghen Institute for  
704 Anaerobic Microbiology (SIAM), SIAM gravitation grant, the Netherlands Organization  
705 for Scientific Research (024.002.002).

706

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847

848 Table 1:  $Y_{x,s}$  calculated on the basis of TSS/VSS measurements at steady state (n=3). For  
 849 the SBRs,  $\mu^{\max}$  was obtained from on-line fermentation data according to appendix VI. For  
 850 the CSTRs,  $q_s^{\max}$  was obtained from a substrate pulse experiment and subsequent fitting the  
 851 substrate concentration data, with  $R^2$  values of 0.97 and 0.92 for xylose and glucose  
 852 respectively. For the SBR  $\sigma_{q_s^{\max}}$  is calculated using error propagation and the covariance of  
 853 the  $\mu^{\max}$  and  $Y_{x,s}$  values. For the CSTRs  $\sigma_{q_s^{\max}}$  is calculated using error propagation and the  
 854 covariance of the  $C_s$  and  $C_x$  measurement, while  $\sigma_{\mu^{\max}}$  is calculated using error propagation  
 855 and the covariance of  $q_s^{\max}$  and  $Y_{x,s}$ .

	$Y_{x,s}$ [Cmol <sub>x</sub> Cmol <sub>s</sub> <sup>-1</sup> ]	$q_s^{\max}$ [Cmol <sub>s</sub> Cmol <sub>x</sub> <sup>-1</sup> h <sup>-1</sup> ]	$\mu^{\max}$ [h <sup>-1</sup> ]	Reference
Xylose SBR	0.12 ± 0.01	2.28 ± 0.10	0.28 ± 0.01	This study
Glucose SBR	0.13 ± 0.01	3.41 ± 0.24	0.45 ± 0.01	This study
Xylose CSTR	0.12 ± 0.01	1.72 ± 0.02	0.22 ± 0.01	This study
	0.16 ± 0.01	1.01	0.16	Temudo <i>et al.</i> (2009)
Glucose CSTR	0.21 ± 0.01	1.06 ± 0.02	0.22 ± 0.01	This study
	0.21 ± 0.01	NA	NA	Temudo <i>et al.</i> (2009)

856

857 Table 2: Result of the FISH quantification (n = 3), with percentages denoting relative  
 858 abundances calculated from the target-probe surface area compared to EUB338 probe  
 859 surface. Unidentified populations were calculated as the remaining percentage after  
 860 summing up the relative abundances of the known populations. The last column shows the  
 861 amount of surface probed by EUB338 compared to DAPI.

	<b>Chis150</b> vs. EUB338	<b>Lac435</b> vs. EUB338	<b>Ent183</b> vs. EUB338	<b>Unidentified</b> vs. EUB338	<b>EUB338</b> vs. DAPI
Xylose SBR	2% ± 2%	5% ± 1%	90% ± 3%	2%	96% ± 2%
Glucose SBR	ND	3% ± 2%	91% ± 3%	6%	100% ± 7%
Xylose CSTR	ND	53% ± 3%	44% ± 6%	3%	104% ± 14%
Glucose CSTR	89% ± 12%	ND	5% ± 0%	6%	89% ± 8%

862

863 Table 3: Net NADH balance calculated using table S4. Acetyl-CoA derivatives were calculated  
 864 from butyrate, acetate and ethanol production through the pyruvate to acetyl-CoA pathway  
 865 (Figure 1).

	Net NADH balance metabolism [mol <sub>NADH</sub> Cmol <sub>S</sub> <sup>-1</sup> ]	Acetyl-CoA derivates [mol Cmol <sub>S</sub> <sup>-1</sup> ]	Formate + H <sub>2</sub> [mol Cmol <sub>S</sub> <sup>-1</sup> ]
Xylose SBR	-0.03 ± 0.00	0.27 ± 0.00	0.26 ± 0.00
Glucose SBR	-0.03 ± 0.01	0.22 ± 0.00	0.23 ± 0.02
Xylose CSTR	-0.06 ± 0.01	0.27 ± 0.00	0.22 ± 0.01
Glucose CSTR	0.02 ± 0.01	0.24 ± 0.20	0.25 ± 0.01

866

867 Table 4:  $Y_{x,ATP}$  is calculated by assuming ATP formation per product (Table S4), for the  
 868 measured data and corrected for substrate uptake. Xylose uptake in the CSTR is assumed  
 869 by the XylFGH complex and the XylE complex in the SBR. Gibbs energy of dissipation is  
 870 calculated at 30°C and pH = 8 using the reconciled data.

	$Y_{xS}$ [Cmol <sub>X</sub> Cmol <sub>S</sub> <sup>-1</sup> ]	$Y_{ATP,S}$ [mol <sub>ATP</sub> Cmol <sub>S</sub> <sup>-1</sup> ]	$Y_{x,ATP}$ observed [g <sub>X</sub> mol <sup>-1</sup> ATP]	$Y_{x,ATP}$ corrected [g <sub>X</sub> mol <sup>-1</sup> ATP]	Gibbs energy of dissipation [kJ Cmol <sub>X</sub> <sup>-1</sup> ]
Xylose SBR	0.12 ± 0.01	0.42 ± 0.01	7.2	8.7	-378
Glucose SBR	0.13 ± 0.01	0.40 ± 0.01	8.2 <sup>1</sup>	8.2 <sup>1</sup>	-285
Xylose CSTR	0.12 ± 0.01	0.42 ± 0.01	6.8	12.8	-386
Glucose CSTR	0.21 ± 0.01	0.49 ± 0.03	13.4	13.4	-236

871 <sup>1</sup>Only 90% of glucose conversion is assumed here, as the COD and carbon balance only  
 872 close for 90%

873

874 Table 5: Reported predominant bacterial species for fermentative microbial communities  
 875 enriched on xylose or glucose as carbon sources in CSTR mode. Species were detected  
 876 using PCR and denaturing gradient gel electrophoresis or PCR and single strand  
 877 conformation polymorphism analysis

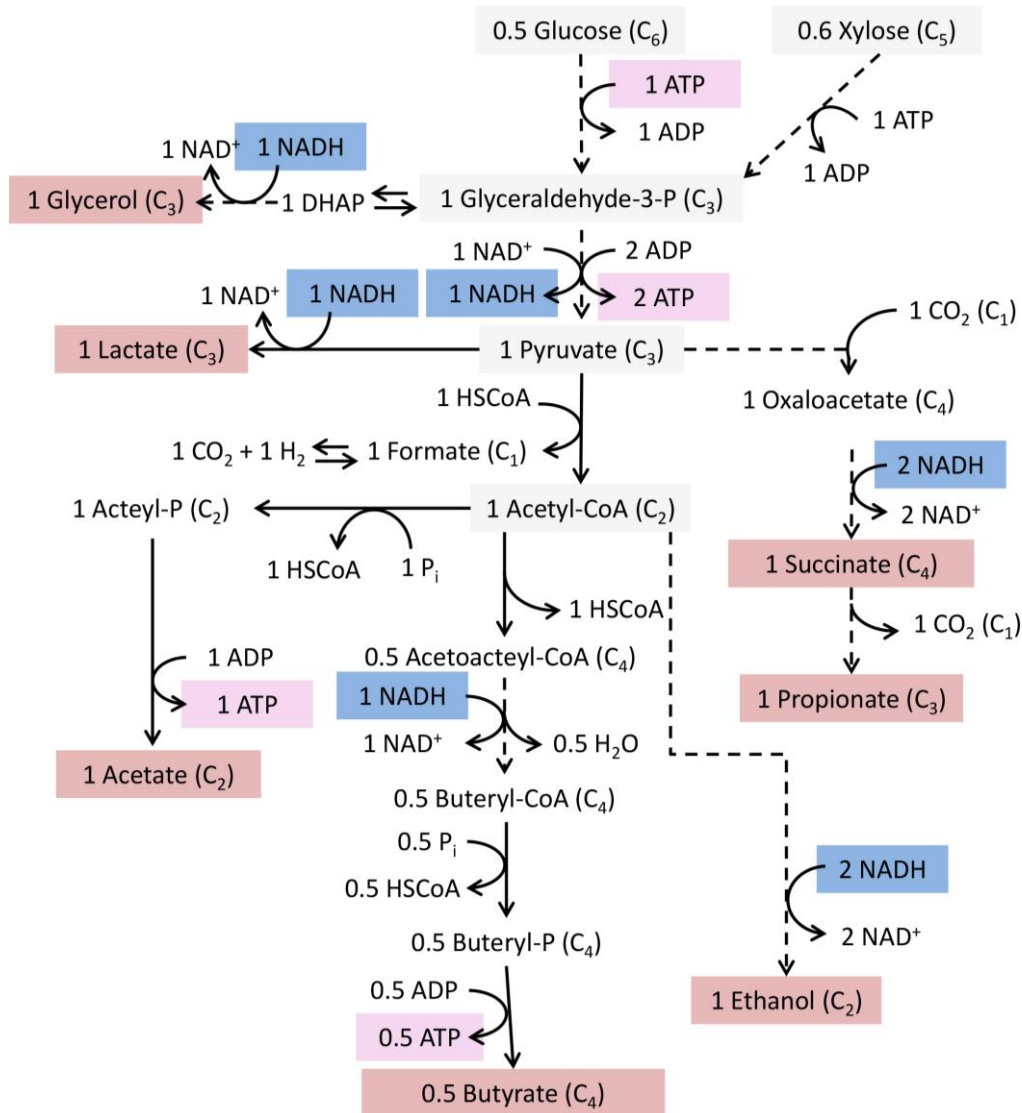
Substrate	Inoculum	T	pH range	Dominant carbon products	Organisms	Source
<b>Xylose</b>	Hot spring culture	45 °C	5.1	Acetate, butyrate	<i>Clostridium acetobutylicum Citrobacter freundii</i>	(Mäkinen, Nissilä and Puhakka 2012)
<b>Xylose</b>	Hot spring culture	37 °C	5.1	Acetate, butyrate, ethanol	<i>Clostridium acetobutylicum Clostridium tyrobutircum</i>	(Mäkinen, Nissilä and Puhakka 2012)
<b>Glucose</b>	Hot spring culture	37 °C	5.0	Acetate, butyrate	3 species of <i>Clostridium</i> 2 uncultured	(Karadag and Puhakka 2010)
<b>Glucose</b>	Activated sludge, cassava, rabbit droppings	37 °C	5.5	Butyrate, acetate, lactate*	<i>Clostridium pasteurianum, Clostridium beijerinckii, Lactobacillus paracasei</i>	(Rafrafi <i>et al.</i> 2013)
<b>Xylose 4 g/L</b>	Digestor sludge and acidification tank	30 °C	8.0	Acetate, butyrate	<i>Clostridium beijerinckii, Clostridium xylanovorans, Clostridium sp. CCUG</i>	(Temudo <i>et al.</i> 2008)
<b>Xylose</b>	Digestor	30 °C	8.0	Acetate,	<i>Citrobacter farmeri</i>	(Temudo <i>et</i>

<b>11 g/L</b>	sludge and acidification tank			butyrate, ethanol	<i>Clostridium intestinale</i> <i>Clostridium sp. CCUG</i>	<i>al.</i> 2008)
<b>Glucose</b>	Digestor sludge and acidification tank	30 °C	8.0	Acetate, butyrate, ethanol	<i>Clostridium quinii</i> **	(Temudo <i>et al.</i> 2008)

878 \* 50% of the COD coming out of the reactor was glucose

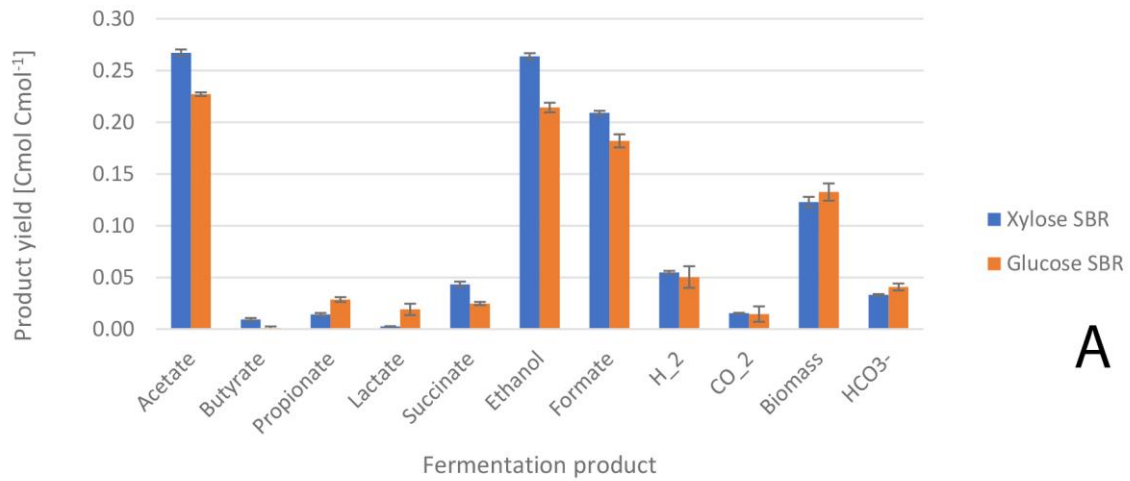
879 \*\* two other bands are visible which are not mentioned

880

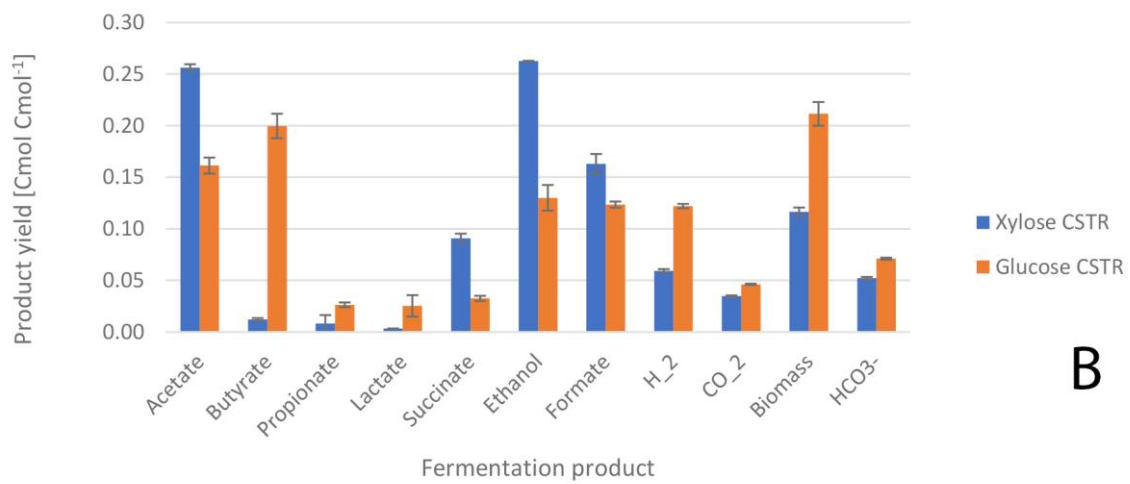


881  
 882 Figure 1: Intracellular metabolic network for xylose and glucose fermentations. Dashed lines  
 883 indicate lumped reactions, straight lines indicate single reactions. Xylose comes into the  
 884 glycolysis through the synthesis of 2 fructose-6-phosphate and 1 glyceraldehyde-3-  
 885 phosphate, through the PPP. The Emden-Meyerhof-Parnass pathway is used as this is the  
 886 common type of glycolysis encountered in energy limited anaerobes (Flamholz *et al.* 2013).  
 887 Figure is made on the basis of Madigan and Martinko (2006).  
 888





A



B

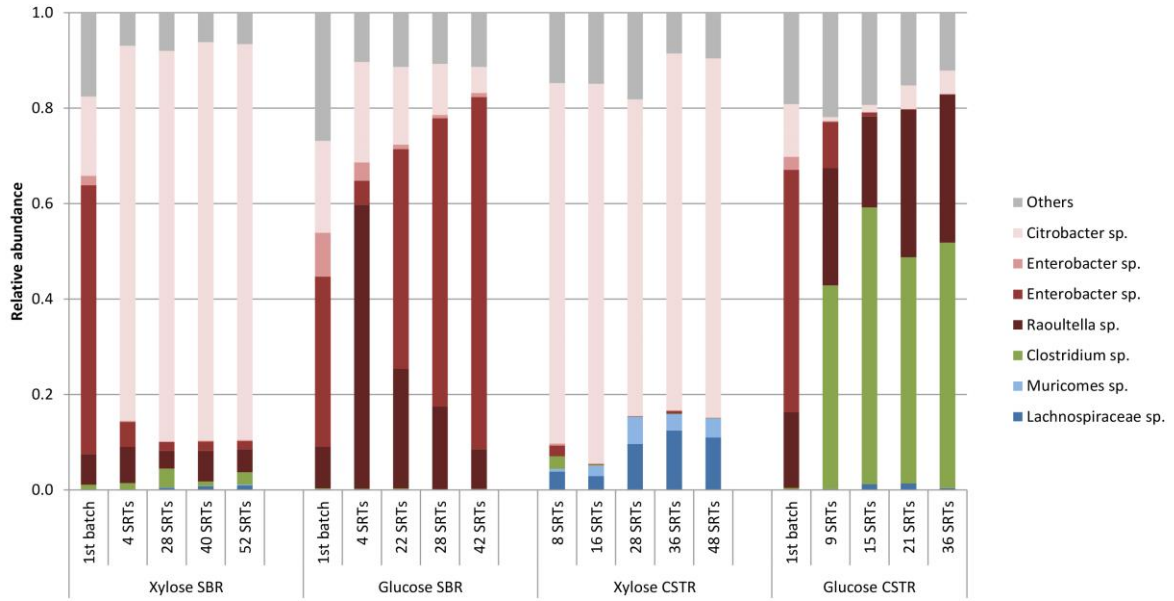
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890

891 Figure 2: Product spectra of mixed culture fermentations of SBRs (A) and CSTRs (B)

892 determined in steady state (n=3)

893



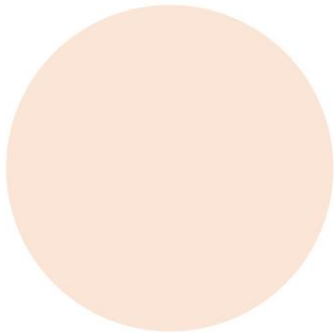
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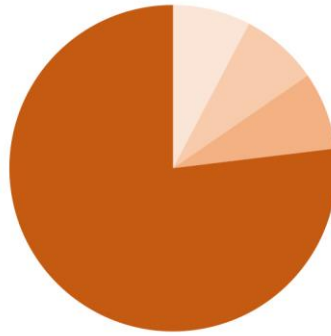
896 Figure 3: Overview of the amplicon results on the V3-V4 region of the 16S rRNA gene on  
 897 OTU level. All OTUs that contribute to <1% of the reads are grouped into the others fraction  
 898 (grey). In red OTUs belonging to the *Enterobacteriaceae* family are denoted, in green OTUs  
 899 belonging to the *Clostridiaceae* family and in blue OTUs belonging to the *Lachnospiraceae*  
 900 family. Closest related relatives found by BLAST used to characterize the OTU up to genus  
 901 level (Appendix V). OTUs matched at <97% are presented as species from a family.

902

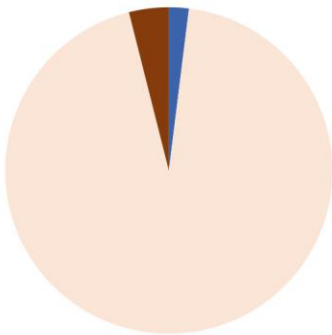
**Xylose SBR**



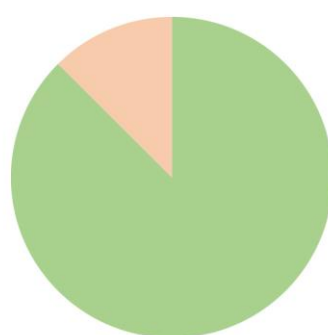
**Glucose SBR**



**Xylose CSTR**



**Glucose CSTR**



- Lachnospiraceae sp.
- Clostridium intestinale
- Citrobacter freundii
- Raoultella ornithinolytica
- Klebsiella sp. JT42
- Enterobacter cloacae
- Citrobacter pasteurii

903

904

905 Figure 4: Result of the clone library analysis in which strains that were found as closest

906 relative (Appendix VII) are grouped into species

907

908