CONTINUOUS FERMENTATION AND KINETIC EXPERIMENTS FOR THE CONVERSION OF CRUDE GLYCEROL DERIVED FROM SECOND-GENERATION BIODIESEL INTO 1,3 PROPANEDIOL AND BUTYRIC ACID

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ABSTRACT

This study investigated the performance of different mixed microbial cultures (MMC) able to ferment crude glycerol generated from animal fat-based biodiesel to produce 1,3 propanediol (1,3 PDO) and butyric acid, under non-sterile conditions. Eight different continuous flow stirred-tank reactors (CSTR) were set up with different inoculum types and growth media. The distribution of metabolic products under variable operating conditions was determined. All MMC were characterized from a kinetic point of view and overall stoichiometric reactions were constructed. Changes in the microbial communities were monitored by means of Next Generation Sequencing (NGS). Maximum substrate degradation rate reached approximately 110 g/L/d of glycerol (with a productivity of 38 g/L/d and 11 g/L/d for 1,3 PDO and butyric acid, respectively), obtained with an hydraulic retention time of 12 h and 60 g/L feed. The maximum feed concentration reached almost 90 g/L, leading though to an incomplete substrate degradation.

Keywords: Crude glycerol, CSTR, Fermentation, Mixed microbial cultures, 1,3 propanediol, butyric acid.

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1. INTRODUCTION

In the last decade, extensive growth of the biodiesel industry resulted in a glycerol surplus production and a significant decrease in crude glycerol prices [1], causing problems not only to the glycerol-producing and-refining industries, but also to the economic viability of the biodiesel industry itself [2,3]. In fact, while high purity glycerol is an important industrial feedstock, crude glycerol derived from biodiesel production possesses very low value (oscillating between 0 and 240 $/ton [4]), due to impurities such as methanol, heavy metals, soaps, etc. [5,6]. Moreover, it has been estimated that the projected volume production of crude glycerol over the next years will exceed the present commercial demand for purified glycerol [7], with an increasing EU biodiesel production capacity and a global production of glycerol from biodiesel that has exceeded 2 million tons [4,8]. As a consequence, chemical purification of such contaminants is becoming too costly, especially for small/medium-sized industries [9]. Thus, the development of new routes and efficient (in terms of productivity, yield and titer) as well as low-cost processes to convert crude glycerol into higher value products is expected to add value to the production of biodiesel and help the development of biorefineries.

Clearly, conversion of glycerol can be obtained by different physico-chemical and biological methods. Bioconversion of crude glycerol into biofuels and green chemicals may have several advantages, such as no need of energy-intensive pretreatment or purification, low nutrient requirements and co-production of H_2/biogas and other biofuels, which can be used as an energy source. A major challenge in the fermentation of low-grade crude glycerol, however, is to obtain microbial strains tolerant to undesirable inhibitory components, such as salts and organic solvents that are present in crude glycerol [10]. So far, most fermentation processes have been using pure or

refined glycerol as feedstock, while crude glycerol obtained from biodiesel industry is still relatively less investigated [11]. On the other hand, some studies have shown that using open mixed microbial cultures (MMC) in bioprocesses is a promising alternative approach, exploring the available diversity in nature [12], also in the case of glycerol conversion [13] . This is particularly advantageous if industrial waste feedstock, containing compounds of undefined composition, are used [10].

Glycerol bioconversion can lead to numerous value-added chemicals. 1,3 PDO is probably the most studied fermentation product from glycerol, with several patents and industrial plants already installed [14–17]. It represents a promising chemical for many synthetic reactions, particularly when used as a monomer for the synthesis of polytrimethylene terephthalate (PTT) polyesters [18]. Because of the environmental benefits and use of a renewable feedstock, the biotechnological synthesis of 1,3 PDO appears to be an attractive alternative to chemical synthesis [19].

On the other hand, very few studies have directly addressed the conversion of glycerol into butyric acid, which has many applications in food, pharmaceutical and chemical industries [20]. So far, biological butyric acid production has been mainly investigated using sugar-rich feedstocks and wild or engineered microbial strains. Despite the high yields, pure culture sterilization requirements, in combination with the requirements for pre-treatment and enzymes addition (in case of lignocellulosic biomasses), have not allowed for cost-efficient biological production of butyric acid on an industrial scale yet [21]. Various feedstocks have been studied for butyric acid production by fermentation [22–28], however, although a few research studies have focused on hydrogen production from glycerol and reported butyric acid as one of the by-products [29,30], there is a lack of studies investigating butyric acid production from crude glycerol. In a previous study, however, the authors have selected several MMC able to grow on animal fat-derived glycerol and produce, together with 1,3 PDO, butyric acid at interesting yields. Production of butyric acid along with 1,3

PDO could be of high importance since butyrate could be utilized by enriched consortia for bioplastics production, thus exploiting the full potential of crude glycerol as carbon source [31]. Clearly, production of butyric acid at industrial scale is dominated by chemical synthesis from crude oil [21]. On the other hand, the use of MMC fermentation has the potential to substantially improve the economics of microbial butyric acid production. Nonetheless, there are still important challenges with respect to their application at industrial scale, since the stability of such processes depends not only on operating conditions but also microbial interactions [32]. Furthermore, most studies have been focusing on the use of batch or fed-batch operations, and only few have addressed continuous mode. The latter would have the advantage to increase productivity, with an important impact on the reactor size and capital investment, as well as facilitating operations from a control point. Noticeably, the development of an efficient purification strategy is also considered of highest importance for biotechnological applications. A fermentation broth containing mixture of multiple components, such as, water, residual glycerol, by-products, macromolecules, salts and residual medium makes the downstream processing a potentially difficult separation challenge [11,33,34]. Therefore, significant technological advances and innovative approaches are also needed for cost-efficient recovery and purification of the fermentation products. Selective conversion of butyric acid (and eventually other volatile fatty acids) to polyhydroxyalkanoates (PHA), while leaving 1,3 PDO intact in a subsequent step, would thus facilitate its recovery [31].

The overall goal of this study was to test different MMC in continuous mode and identify operational conditions able to reach stable fermentation in non-sterile conditions, using animal fat-derived crude glycerol from second-generation (2G) biodiesel. The application of MMC, besides the aforementioned advantages, was deemed necessary since the crude glycerol used in this study was derived from animal fat based biodiesel processing and was highly inhibitory for single microbial strains widely known as efficient glycerol consumers, e.g. *Clostridium pasteurianum*. In

more detail, we aimed at a) studying the distribution of metabolic products during mixed culture fermentation under variable operating conditions, and b) defining the conditions and operating parameters necessary to maintain a stable MMC, through kinetic and molecular characterization of the microbial population.

2. MATERIAL AND METHODS

2.1 Media composition

Two different growth media were tested in this study: a very simple Minimal Medium (MM), not containing any yeast extract, tryptone, nor mineral and vitamin solution, and a complete synthetic medium for anaerobes (containing salts, vitamins and trace elements, beside pH buffers), called BA. Unless differently stated, initial glycerol concentration was approximately 10 g/L (in terms of glycerol content of the crude glycerol), while in CSTR experiments the concentration ranged between 10 g/L and 12.88 g/L. Crude glycerol, provided by Daka Biodiesel (Denmark), was obtained from the transesterification of butchery waste (based on animal fat categories 1 and 2 according to the EU regulation numbers 1069/2009 and 142/2011). The main characteristics of this type of crude glycerol are presented in the supplementary material (Table S1).

2.1.1 Minimal Medium

MM contained, per liter of distilled water: 10 g of glycerol, 3.4 g of $K_2HPO_4 \cdot 3H_2O$, 1.3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2 \cdot 2H_2O$ and 5 mg $FeSO_4 \cdot 7H_2O$ [35]. For cultivation, medium was dispensed into 125mL serum bottles and sealed with butyl rubber stoppers. Subsequently it was flushed with nitrogen for 3 minutes and inoculated with 10% v/v inoculum, before being incubated at 37 °C with continuous stirring (150 rpm). Initial pH was 7.

2.1.2 BA Medium

120 BA medium was prepared from the following stock solutions (chemicals in g/1 of double distilled
121 water): (A) NH_4Cl, 100; NaCl, 10; $MgCl_2 \cdot 6H_20$, 10; $CaCl_2 \cdot 2H_20$, 5; (B) $K_2HPO_4 \cdot 3\ H_2O$, 200; (C)
122 trace metal and selenite solution: (D) $NaHCO_3$ 52 g/L; (E) vitamin mixture, according to [36] . To
123 974 ml of redistilled water, the following stock solutions were added: A, 10 ml; B, 2 ml; C, 1 ml; D,
124 50 ml; E, 1 ml [37].

125 **2.2 Inoculum**

126 Five different MMC were previously selected through enrichment of activated and anaerobic sludge
127 in batch and fed-batch. Different selection strategies were compared, using different growth media
128 (BA and MM) and transfer strategies: a “Kinetic Control” (KC), with transfers every 21h, and “End
129 of Fermentation” (EF), in which the inocula were transferred into fresh medium after 72h, when no
130 more fermentation gases were produced. Four different MMC were obtained from the activated
131 sludge, while only one stable MMC was obtained through the enrichment of heat-treated anaerobic
132 sludge. Activated sludge was collected from the wastewater treatment plant of Daka Biodiesel,
133 Denmark. Anaerobic sludge was collected from the Municipal Wastewater Treatment plant in
134 Lyngby (DK) [10].

135 **2.3 Inoculum storage and activation**

136 Inoculum samples were stored in the freezer at -18°C. Prior to use, the frozen mixed cultures were
137 transferred to the refrigerator at 4°C, for 2 hours, and then for an additional hour at room
138 temperature, before being inoculated. 125 mL serum vials were used for batch experimentation. 45
139 mL growth medium (either MM or BA medium) were flushed for 5 minutes with a mixture of 80%
140 N_2 and 20% CO_2, in order to obtain anaerobic conditions, prior to inoculation (adding 5 mL
141 inoculum), and incubated at 37°C, using an orbital shaker at 150 rpm. Gas and liquid samples were
142 collected regularly. Batches at 24 h fermentation were used as (pre-activated) inoculum for

continuous experiments. In all experiments, 10% v/v inoculum was used to start up the fermentation and all operations were performed under non-sterile conditions.

2.4 Continuous Experiments

Continuous experiments were run to test the stability of the selected MMC and identify the operating parameters able to secure a stable fermentation. A 3L Applikon 1030 fermenter (with a working volume of 1 L) equipped with an ez-controller was used for this purpose, testing different MMC, growth media (BA and MM), pH and Retention Time (RT, which is equal to both hydraulic and solid retention time) conditions. Biogas was measured through a Ritter MilliGas counter (Type MGC-1). pH was controlled through the addition of alkali (KOH 4 M) and the temperature was kept at 37 °C. The reactor was flushed for 20 minutes with a mixture of 80% N_2 and 20% CO_2 to obtain anaerobic conditions prior to inoculation (10 % v/v). The feed vessels were also flushed with 80% N_2 and 20% CO_2 to obtain anaerobic conditions and were changed every 2-3 days with fresh medium; they were stored in a fridge (4 - 6 °C) during the operation (because of the non-sterile conditions) to minimize external microbe growth. The outlet vessel was changed regularly as well and it was connected with a vessel filled with water to discharge pressure and to prevent air inlet (Figure 1). Experiments were continued for at least 6 retention times (with a variability of the main metabolites ≤ 25%) after steady state was reached.

Shapiro-Wilks normal probability test and T-test for comparison of two sets of values were performed using OriginPro v 9.0.0.

[insert Figure 1]

2.5 Kinetic experiments

In order to kinetically characterize the MMC, further experiments were conducted in batch mode, through the fitting of kinetic equations to the experimental data.10 mL fermentation broth from each reactor, at steady state, were used as inoculum in 300 ml serum vials, which were sealed with

rubber stoppers and aluminum crimps. The final working volume was 100 ml. Prior the inoculation, the vials were flushed for 5 minutes with a mixture of 80 % N_2 and 20 % CO_2 in order to obtain anaerobic conditions. All tests were performed in duplicates. Media composition was the same as reported in paragraph 2.1 (MM and BA), with the addition of K_2HPO_4/ KH_2PO_4 buffer (1.13 g/L, 12.72 g/L for BA and 1.46 g/L, 11.42 g/L for MM) in order to hinder pH drop during batch fermentation (with an initial pH of 6.5). Incubation was at 37°C using an orbital shaker at 150 rpm. Samples were collected every three hours in the exponential phase and progressively in larger time intervals. At each sampling, biomass, VFAs, alcohols and organic acids and hydrogen were measured as reported in paragraph 2.7. In order to describe substrate consumption and biomass growth, the equation of Monod kinetics was used (Eq.1).

$$\frac{dx}{dt} = \mu X \qquad \text{being} \qquad \mu = \frac{\mu max \cdot S}{Ks+S} \qquad \text{Eq.1}$$

Where dx/dt is the microbial growth rate, μ and μ_{max} is the specific growth rate and maximum specific growth rate of the microorganisms, respectively, S is the substrate concentration and K_s is the saturation constant. The maximum specific growth rate was calculated from the initial rates (where $dx/dt = \mu_{max} X$), based on the fact that the specific growth rate is constant and equal to the maximum specific growth rate at high substrate concentrations. The yields of the products ($Y_{p/s}$) were expressed as mass of product per mass of substrate consumed (glycerol). The productivity (P) was expressed as mass of products per volume per time.

2.6 Stoichiometric calculations

Stoichiometric calculations were based on product yields and calculation of the glycerol electron equivalents, partitioned between energy producing reactions (catabolism of glycerol to various products) and biomass synthesis [27]. The theoretical energy reaction was constructed, assuming glycerol as the sole electron donor in the experiments and calculating the fraction of electron

190 equivalents found in each of the products. The organic half-reactions used for the substrate
191 (glycerol) and products are shown below (Eq. 2- 10).
192
193 Hydrogen: $H^+ + e^- \rightarrow ½\ H_2$ Eq.2
194 Acetic acid: $2/8\ CO_2 + H^+ + e^- \rightarrow 1/8\ CH_3COOH + 2/8\ H_2O$ Eq.3
195 Butyric acid: $4/20\ CO_2 + H^+ + e^- \rightarrow 1/20\ CH_3CH_2CH_2COOH + 6/20\ H_2O$ Eq.4
196 Ethanol: $1/6\ CO_2 + H^+ + e^- \rightarrow 1/12\ CH_3CH_2OH + ¼\ H_2O$ Eq.5
197 1,3 Propanediol: $3/16CO_2 + H^+ + e^- \rightarrow 1/16\ OHCH_2CH_2CH_2OH + ¼\ H_2O$ Eq.6
198 Lactic acid: $¼\ CO_2 + H^+ + e^- \rightarrow 1/12\ C_2H_4OHCOOH + ¼\ H_2O$ Eq.7
199 Propionic acid: $3/14\ CO_2 + H^+ + e^- \rightarrow 1/14\ CH_3CH_2COOH + 4/14\ H_2O$ Eq.8
200 Glycerol: $1/14\ OHCH_2CH(OH)CH_2OH + 3/14\ H_2O \rightarrow 3/14\ CO_2 + H^+ + e^-$ Eq.9
201 Valeric acid: $5/26\ CO_2 + H^+ + e^- \rightarrow 1/26\ C_5H_{10}O_2 + 8/26\ H_2O$ Eq.10
202
203 The fraction of the electron donors' electron equivalents used for energy production (f_e) was
204 calculated from the difference between the product yields predicted by the theoretical energy
205 reaction and the actual measured yields, as reported in [27]. The fraction of the electron donors'
206 electron equivalents used for cell synthesis (f_s) was then calculated using the following equation
207 (Eq.11):
208 $f_s + f_e = 1$ Eq.11
209 Subsequently, the microbial cell synthesis reaction was constructed using the cell formation half-
210 reaction (Eq. 12), taking NH_4^+ as nitrogen source and $C_5H_7O_2N$ as empirical formula for microbial
211 cells, according to [38].
212 $1/5\ CO_2 + 1/20\ HCO_3^- + 1/20\ NH_4^+ + H^+ + e^- \rightarrow 1/20\ C_5H_7O_2N + 9/20\ H_2O$ Eq. 12

The overall stoichiometric reaction was finally constructed as the sum of the energy and cell synthesis reactions, multiplied by f_e and f_s, respectively, as described in [38] and the theoretical biomass production was calculated from the stoichiometry of the overall reaction. For a data consistency check, a carbon recovery (Rc) calculation was carried out at the end of the batch cultures as well as at each steady state, according to [39]. Substrate removal rate (R_{Gly}) during steady state was calculated according to the following equation (Eq. 13):

$$R_{Gly} = (Gly_0\text{-}Gly)\cdot D \qquad \text{Eq. 13}$$

Where D is the dilution rate (h^{-1}), Gly_0 is the glycerol amount in the feed and Gly the concentration of glycerol in the reactor at steady state.

2.7 Analytical Methods

Detection and quantification of glycerol, ethanol, 1,3 PDO and lactic acid were obtained with a HPLC equipped with a refractive index, while VFA were analyzed by a gas chromatograph equipped with a flame ionization detector, as previously reported [10]. Hydrogen content was measured by a TCD-GC, as described in [10].

Biomass was estimated through the determination of Total Suspended Solids (TSS), according to standard methods [40]. Absorbance of samples was measured every day at an optical density of 600 nm (OD600), after the correlation with TSS. Total soluble metabolites (TSM) yield was calculated as the ratio between g of TSM/ g of glycerol consumed (expressed as a percentage), and used as a relative comparison of the substrate conversion ability of the different samples (or stated differently, as an easy estimate of the glycerol acidification efficiency of each tested conditions).

2.8 Next generation sequencing (NGS)

DNA extraction and 16S amplicon sequencing were performed according to the procedures described previously, using an Illumina MiSeq System [10]. Main comparisons between samples

were done at the Order and Genus level. Sequencing reads have been deposited to the Sequence read archive of NCBI under the Bioprojects PRJNA352657 and PRJNA352658.

Multivariate data analysis was performed using Unscrambler X 10.1 software (by Camo). A Principal Component Analysis (PCA) (Jackson 2003) [41] was chosen as a tool to explore the data matrix obtained from the relative abundance of genera and of the main fermentation parameters.

3. RESULTS

Eight different operating conditions (including RT, growth medium and inoculum type) were tested in continuous mode, comparing the performance of different (previously) enriched MMC [10] in non-sterile conditions. A typical example of the trend of main fermentation products, obtained during the tests with enriched anaerobic and activated sludge, is shown in the supplementary material Figure S1 and S2.

3.1 Glycerol conversion during continuous mode experiments

3.1.1 Glycerol conversion using enriched anaerobic sludge

Two different growth media (BA and MM) and retention times (12h and 24h) were tested and the substrate conversion and main metabolites obtained during the steady state are shown in Table 1 and Figure 2a. Preliminary tests were run to verify the effect of different pH (reactor BA-12h), which resulted to be a key parameter in controlling the sulphate reducing bacteria (SRB) community, originated from anaerobic sludge. Interestingly, SRB were able to out-compete the other microorganisms when growing at pH ≈ 7 (initial anaerobic sludge contained a total of 19 genera of SRB, mainly belonging to *Desulfovibrio* and *Desulfofrigus*, and accounting for 1.19% of the total genera retrieved [10]), while there was no evident sulphide production or inhibition at pH = 5.5. For this reason, all the following continuous experiments were run at pH = 5.5.

[Insert Table 1]

During steady state, 1,3 PDO represented the main metabolite (ranging from 4.89 to 6.45 g/L), followed by butyric acid (1.44 – 2.26 g/L), propionic acid (0.33 – 0.88 g/L) and acetic acid (0.21 – 0.33 g/L). Glycerol was completely consumed in all cases. Notably, in MM-24h initial butyric acid production was higher than 1,3 PDO, reaching a maximum concentration of 5.23 g/L; however 1,3 PDO turned out to be the main metabolite during the steady state. Average TSM yield reached 65.23 ± 3.48 % (corresponding to 74.9% ± 6.4 % in terms of Cmol), with 1,3 PDO and butyric acid accounting for 87.50 % of the TSM (95.8% in terms of Cmol).

In general, use of BA medium seemed to favour a comparably more stable distribution of metabolites. Interestingly, the use of a shorter RT did not seem to reduce the process stability, at the same time allowing for an increased glycerol conversion rate. For this reason, an RT of 12h was chosen for the following experiments, using enriched activated sludge.

It is worth noting that MM led to a higher butyric acid (around 25.80% compared to 17.63% of BA) and lower 1,3 PDO production (especially at the lower RT tested), which might be related to the absence of specific minerals and vitamins in the minimal medium. The production of 1,3 PDO, for instance, is typically vitamin B_{12}-dependent (even though some exceptions were discovered recently [42]), and thus its absence could favour the oxidative pathway [43].

[Figure 2]

3.1.2 Glycerol conversion using enriched activated sludge

Crude glycerol conversion ability of four different inocula was tested in continuous mode, using previously enriched activated sludge [10]. In order to better compare the experiments among the different inocula, it was decided to use the same operating conditions for all four experiments (while in the case of anaerobic sludge only one stable inoculum was available). Based on the observations of the previous continuous mode tests with anaerobic sludge, the RT was set at 12h (more stable) and pH at 5.5; temperature was kept at 37°C. A RT of 24 h was also tested in the case

of MM-EF, showing comparable distribution of the main metabolites as in the 12h RT. Thus the operation at 12h was considered preferable, also due to a higher productivity.

The growth medium used for the continuous experiments was kept the same as the medium used for the enrichment (BA or MM) [10]. The substrate conversion and main metabolites obtained during the steady state are reported in Table 1 and Figure 2b, respectively. In most cases, distribution of 1,3 PDO and butyric acid were similar to those observed in the previous experiments with anaerobic sludge, with the 1,3 PDO showing a two-fold higher concentration compared to butyric acid. However, the initial phase of the CSTR with activated sludge showed a higher variability. BA-EF, in particular, showed a different distribution of metabolites in the first 20 days, with higher butyric acid production (reaching up to 5.74 g/L), but conformed to the other reactors after reaching steady state. Differently from all the other CSTR experiments, MM-EF was the only inoculum that did not reach complete substrate degradation, and was associated to the lowest biomass concentration, even after increasing the RT to 24h (which did not lead to an increase of biomass nor substrate degradation efficiency).

Similarly to the anaerobic sludge MMC, 1,3 PDO always represented the main metabolite during steady state (ranging from 2.70 to 4.40 g/L), followed by butyric (1.09 – 1.98 g/L), acetic (0.14 – 1.03 g/L) and propionic acid (0 – 0.63 g/L). Average TSM yield (62.57 ± 3.37 %) was comparable to the one obtained with enriched anaerobic sludge (corresponding to 78.1% ± 4.0 % in terms of Cmol). Similarly, 1,3 PDO and butyrate accounted for 83.92 % of the TSM (85.2% in terms of Cmol), however biomass was generally lower (especially in the case of MM experiments), with an average of 0.45 ± 0.20 g/L, compared to 0.76 ± 0.08 of anaerobic sludge.

3.2 Production rates

3.2.1 Characterization of productivity using enriched anaerobic sludge

As can be observed in Table 2, there was no clear difference of productivity between the MMC grown with MM and BA medium when working at 24 h RT, while BA operated at 12h RT clearly favoured 1,3 PDO production, reaching a maximum of 12.89 ± 0.81 g/L/d. On the other hand, MM clearly favoured butyric acid productivity, no matter the RT. Despite the formation of butyric acid, hydrogen detected was in general extremely low, possibly due to syntrophic mechanisms that can lead to hydrogen consumption [44], or to secondary fermentation (*sensu* Agler [45]) in which butyric acid production is not obtained directly from glycerol conversion but rather from the conversion of other metabolites, such as i.e. lactic and acetic acid [10]. Substrate degradation rate (R_{Gly}) reached 12.64 ± 0.18 g/L/d in the case of 24h RT and 25.71 ± 0.07 g/L/d with 12h RT.

3.2.2 Characterization of productivity using enriched activated sludge

During steady state, maximum and minimum 1,3 PDO production were both obtained with MM (maximum of 8.88 ± 0.43 was obtained with MM-KC), while there seemed to be little difference in terms of main metabolites among the two inocula selected on BA medium. As already mentioned, MM-EF represented a special case (it was the only inoculum that did not completely degrade the substrate), developing the lowest biomass concentration (see Table 1) consequently also leading to the lowest production rates. Gas production was very low with the exception of MM-EF, which reached the highest values of almost 1125 mL/L/d, with a hydrogen content of 53.85%, and an average productivity of almost 600 mL/L/d during the steady state, (see Table 2).

Substrate degradation rate (R_{Gly}) reached on average 20.72 ± 0.46 g/L/d, with the exception of MM-EF, which was run at 24h RT during steady state, due to its incomplete substrate degradation (thus obtaining an R_{Gly} of 7.48 g/L/d). Interestingly, despite the difference in initial substrate concentration used with anaerobic and activated sludge (about 12.5 g/L glycerol and 10.5 g/L respectively), there seemed to be no evident effect on butyric acid productivity (with an average of 2.82 ± 1.21 and 2.75 ± 1.36 g/L/d, respectively). In fact, the two-tail T-test (paired two samples for

means) showed a P-value of 0.945, while the Shapiro-Wilks test did not reject normality (decision level at 5%).

[Insert Table 2]

3.3 Production yields

3.3.1 Characterization of production yields ($Y_{p/s}$) using enriched anaerobic sludge

Maximum butyric acid production yield was reached in the initial (and less stable) phase of CSTR operation, with 0.40 g/g in MM-24h. However, the yields decreased to an average of 0.14 ± 0.03 g/g during steady state (Figure 3a). 1,3 PDO production yield, instead, stayed relatively stable throughout the whole fermentation (with an average yield of 0.43 ± 0.05 g/g during steady state): maximum yield obtained during the initial phase reached 0.52 g/g (corresponding to 0.6 mol/mol), which also corresponded to the maximum observed during steady state with BA-12h (see Figure 3a). This represents 83% of the theoretical maximum yield [46]. These results are comparable with those of a recent study, using mixed cultures with pure glycerol fermentation, which reported yields from 0.52 to 0.64 mol/mol over a wide pH range [47].

3.3.2 Characterization of production yields ($Y_{p/s}$) using enriched activated sludge

On average, production yields using enriched activated sludge were comparable to those obtained with anaerobic sludge, with a slightly higher butyric acid (0.16 ± 0.003 g/g) and a lower 1,3 PDO production yield (0.37 ± 0.009 g/g), as can be observed in Figure 3b. Moreover, similarly to the anaerobic sludge, maximum butyrate yield was reached in the initial phase of CSTR operation, suggesting that a disturbed/periodic fermentation [48] (rather than steady state) might be preferable for butyric acid production in CSTR. Ai BinLing and colleagues [49], who investigated butyric acid production using MMC, observed i.e. the highest butyric acid production in a disturbed system, using a semi-continuous fermentation with intermittent discharging of the culture broth and replenishment with fresh medium. Maximum yield was obtained with enriched activated sludge

BA-EF, with up to 0.44 g/g, before reaching steady state. Finally, the use of the BA medium favoured a higher butyric acid/PDO ratio, which was almost twice the one obtained with MM (0.60 and 0.32 respectively).

[Insert Figure 3]

3.4 Stoichiometric analysis

Stoichiometric equations representing the overall stoichiometric reactions for the eight different CSTR conditions during steady state are reported in Table 3. The inoculum origin (anaerobic sludge or activated sludge) did not seem to have a significant effect on f_e (and thus f_s), with an average of 0.88 ± 0.04 ($f_s = 0.12 \pm 0.04$) in the case of anaerobic sludge, and 0.86 ± 0.05 ($f_s = 0.15 \pm 0.04$), respectively. The two-tail T-test (paired two samples for means) showed a P-value of 0.617, while the Shapiro-Wilks test did not reject normality (decision level at 5%). This means that the fraction of the electron donors' electron equivalents used for energy production (f_e) and cell synthesis (f_s) in activated and anaerobic sludge inocula was comparable. However, in the case of activated sludge the ratio of the experimental biomass yield to the theoretical biomass yield given by the stoichiometric equation was higher when using BA compared to MM medium, which might imply that the maintenance energy requirements were higher with MM medium. This could be explained by the fact that MM did not provide vitamins and growth factors to the microbial cells, which had an effect to the energy available for synthesis of new cells. In the case of anaerobic sludge, on the other hand, experimental to theoretical biomass ratios are very close to 1 which implies that maintenance energy requirements can be considered negligible in this case.

[Insert Table 3]

Finally, average values of carbon recovery (Rc) confirmed that there was a good closure [50], with an average of 100.45 ± 1.33 % for activated sludge and 101.6 ± 1.53 % in the case of anaerobic

sludge fermentation. Moreover, there was a good correlation between measured and calculated biomass, with a $R^2 = 0.95$ for activated and 0.91 for anaerobic sludge (Fig. S3).

3.5 Metagenomic analysis

3.5.1 Molecular Characterization of the MMCs during CSTR operations using enriched anaerobic sludge

There was a relatively similar evolution of the microbial community among the four different operating conditions, probably also due to the fact that there was only one starting inoculum (in the case of enriched anaerobic sludge). This was also reflected in the stability of the fermentation process. Overall, there was a dominance of bacteria belonging to the phylum Firmicutes (60.4%) and Proteobacteria (32.5%). As can be observed in Figure 4a, BA medium showed a slight increase in Clostridiales over time (mainly with the genera *Clostridium*, *Blautia*, *Sporanaerobacter*, *Alkaliphilus*), while the Bacteroidales disappeared. This was associated with a higher 1,3 PDO production, which reached around 70-80% of the TSM (Figure 5a). MM medium, on the other hand, showed an increase of Enterobacteriales (genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Erwinia*) and especially Burkholderales (*Delftia*), while the Bacteroidales disappeared. *Clostridium* represented by far the main genus (with an average relative abundance of 44.81%), followed by *Blautia* (7.05%), *Enterobacter* (6.15%) and *Pseudomonas* (5.65%). It is worth noting that *C. butyricum*, which was the dominant species found in MM (but absent in the BA samples), is known to perform a B_{12}-independent glycerol-oxidative pathway leading to primarily butyric acid. This might have contributed to the higher butyric acid production in MM, which reached a maximum of 56.2% in MM-24h (Figure 5a). Moreover, the higher butyric acid production was also associated to a higher abundance of *Delftia*, which reached a maximum of 7.70% and 15.55% in MM-12h and MM-24h respectively, compared to 2.01 % and 2.48% of BA-12h and BA-24h. Interestingly, even

401 though butyric acid was the dominant metabolite in BA-24h (D20 and D53), 1,3 PDO become
402 dominant during the steady state also in this case.
403 [Insert Figure 4]
404 PCA was performed (taking into consideration the variability of relative abundance of microbial
405 genera, together with the relative abundance of the main metabolites distribution, expressed in %) in
406 order to further investigate the relationships between the microbial groups and the metabolic
407 pathways (Fig. 6). The analysis was performed considering both, MM and BA experiments, at the
408 end of fermentation, and showed a correlation between the genera *Blautia* and *Lactobacillus*
409 together with the higher production of 1,3 PDO. PCA also confirmed the correlation of butyric (and
410 succinic) acid with Unclassified genera (which might explain why it was so difficult to correlate
411 butyric acid production to any of the dominant genera), and partially also to *Citrobacter*,
412 *Lysinibacillus* and *Delftia*. Finally, the analysis also showed that there was a clear negative
413 correlation between the 1,3 PDO and butyric acid pathway. Similar results were obtained also in the
414 case of activated sludge.
415 *3.5.2 Molecular characterization of the MMCs during CSTR operations using enriched activated*
416 *sludge*
417 A more complex situation could be observed with activated sludge MMCs (compared to the
418 anaerobic sludge), during the whole fermentation process (Figure 4b). Nonetheless, similarly to the
419 anaerobic sludge, there was a dominance of bacteria belonging to the phylum Firmicutes (51.9%),
420 followed by Proteobacteria (34.4%). More in detail, Bacteroidales, together with Flavo- and
421 Sphingobacteriales tended to disappear in MM-KC, with an increase in Enterobacteriales (with the
422 genera *Klebsiella*, *Enterobacter* and *Erwinia*), Lactobacillales (with *Lactobacillus*) and
423 Pseudmonadales (with the genus *Pseudomonas*). In the case of MM-EF there was a reduction of
424 Enterobacteriales, with a concomitant increase in Clostridiales, which became dominant (89.4%).

BA-KC, on the other hand, showed to a drastic decrease of Clostridiales and an increase in Enterobacteriales (with increase of the genera *Enterobacter*, *Trabulsiella*, *Klebsiella*, *Citrobacter* and *Acinetobacter*) and Pseudomonadales (with *Pseudomonas* and *Stenotrophobacter*). In general, MM-KC and BA-KC seemed to favor Enterobacteriales and Pseudomonadales (compared to the EF). Finally, BA-EF showed a relatively more stable evolution, except for a descrease in Clostridiales (with *Clostridium* decreasing from 27.23% to 17.58%) and increase in Unclassified Operational Taxonomic Units (OTUs) (with an increase of unclassified genera from 8.28% to 33.27%) after 30 days; notably this was associated to an inversion of the main metabolites, with a decrease of butyric acid, thus making 1,3 PDO the dominant metabolite in BA-EF-D30 (with 45.41%; Figure 5b). *Blautia* decreased from initial 21.61% to 12.95%. All the other genera showed a very low relative abundance.

Overall, *Clostridium* represented the main genus (with an average relative abundance of 33.87%), followed by Unclassified genera (15.65%), *Escherichia*, (4.76%), *Enterobacter* (4.16%), *Blautia* (4.13%), *Lactobacillus* (3.86%) and *Pseudomonas* (3.5%).

[Insert Figure 5]

Despite some general trends that could be observed, it was not always possible to clearly associate the dominance of certain OTUs to the distribution of the main metabolites. This might be due to the fact that in MMCs cross-feeding mechanisms can lead to the consumption of certain metabolites [51] and production of new ones (also by non-dominant species, which can have a significant effect despite their low abundance [52]). Moreover, this might also imply that metagenomics analysis alone is probably not sufficient to comprehensively describe all microbial interactions and the effect on the distribution of metabolites, due for instance to functional redundancies in the microbial community. Thus, additional information would probably be necessary. Similar conclusions were found in recent studies, that highlighted how high-throughput sequencing on its own is probably not

sufficient to track temporal and special population dynamics, while a combination of high-throughput sequencing with quantitative PCR analysis to measure total bacterial abundance would be advisable [51]. Similarly, the study by Moscoviz and colleagues showed that no direct correlation could be found between main metabolites (i.e. 1,3 PDO) and specific families of bacteria [47]. Thus, the development of advanced models, such as microbial interaction networks, would be helpful in interpreting such connections and might also provide novel insight in bioreactor control [53].

3.6 Kinetic characterization of MMCs

After reaching steady state, the MMC were used as inoculum for kinetic experiments in batch. Typical trend of the cumulative hydrogen production, microbial growth and substrate degradation curve can be found in Figure S4. As can be seen in Table 4, the batch experiments showed comparable results to those obtained in continuous (paragraph 3.4) in terms of distribution of electron fraction (f_e and f_s) for anaerobic and activated sludge, with an f_e of 0.88 ± 0.02 and 0.84 ± 0.02, respectively. Also the biomass yield was in good agreement with the CSTR results, with 0.06 ± 0.02 g/g obtained with anaerobic sludge and 0.05 ± 0.01 g/g with activated sludge. On the other hand, differently from the continuous operations, the batch tests showed incomplete substrate degradation in 5 of the 8 batch experiments, with a residual glycerol concentration of about 3.36 ± 0.31 g/L in the three activated sludge MMC and 6.60 ± 2.15 g/L in the two anaerobic sludge MMC (see Table 4). Among the MMC with complete substrate degradation, maximum growth rate (μ_{max}) was highest in BA-12h (even though on average there was no significant difference between activated and anaerobic sludge, with a μ_{max} of 0.11 ± 0.05 h^{-1} and 0.08 ± 0.02 h^{-1} respectively; P-value = 0.538), thus making it a better candidate for low RT in CSTR operations. Moreover, ethanol turned out to be one of the main soluble metabolites (in good agreement with previous results in batch conditions [10]), while it was hardly detected in continuous operations, underlining how the

different operation modes can significantly influence the metabolic pathway, irrespectively of the inoculum origin. In anaerobic sludge the main metabolites were represented by 1,3 PDO (0.57 - 4.61 g/L) followed by ethanol (0.26 – 1.47 g/L), butyric acid (0.07 - 1.08 g/L), acetic acid (0.05 - 0.77 g/L) and hydrogen (7.55 – 110.0 mL). Similar distribution was observed in the case of activated sludge, with 1,3 PDO (1.20 -3.66 g/L) followed by ethanol (0.17 - 2.03 g/L), butyric acid (0.26 – 0.54 g/L), acetic acid (0.02 – 0.66 g/L) and hydrogen (52.28 – 179.7 mL).

[Insert Table 4]

Average carbon recovery (Rc) at the end of fermentation reached 98.30 ± 3.02 %. It is noticeable that the f_s values obtained in batch experiments (0.14 ± 0.03) were comparable with those obtained in their continuous counterparts (0.12 ± 0.04). In fact, the two-tail T-test (paired two samples for means) showed a P-value of 0.547, while the Shapiro-Wilks test did not reject normality (decision level at 5%). This means that the percentage of the carbon of the substrate that is directed towards metabolites was similar to that under continuous operating conditions.

3.7 Improved CSTR operations

Based on the results obtained from the kinetic characterization of the MMC, together with the productivity and yields of the steady states, BA-12h was chosen as the best candidate for further studies. In fact, besides having the highest μ_{max} among the MMC with complete substrate degradation, BA-12h also showed maximum yields and productivities for 1,3 PDO (while butyric acid, the second most abundant metabolite, showed comparably modest results at steady state in all cases). In this experimentation, the ability of the selected MMC to withstand increasing crude glycerol concentrations was tested, in order to verify the efficacy of the enrichment and enhance the viability of the process. In fact, even though the MMC adaptation allowed working with non-pretreated crude glycerol, tests were performed at a concentration of approximately 10 g/L, up to

that point. Moreover, based on μ_{max} obtained through the kinetic experiments, it was decided to test an HRT of 6 h, in order to further investigate the potential of the selected MMC.

It is worth noting that BA-12h was able to grow for four days on crude glycerol with feed concentrations up to almost 90 g/L, with an RT of 12h (see Figure 7a). This means that the reactor was fed at high substrate concentration for 8 RTs (which should be considered enough to establish a steady state), showing however a residual glycerol concentration of 46.5 g/L. High degradation efficiency was observed when using a feed up to 50-60 g/L (while pure strains tested were not able to grow even at 10 g/L, without glycerol pretreatment). After two days of feeding with 60 g/L, residual glycerol was about 3.75 g/L, with a conversion of more than 94%. Maximum productivities reached 37.8 g/L/d and 11.14 g/L/d for 1,3 PDO and butyric acid, respectively, together with a substrate degradation rate of 110.44 g/L/d of glycerol. This corresponded respectively to a 2.9-fold, 3.7-fold and to a 3.9-fold increase, compared to the initial results obtained with BA-12h (see Table 2). Moreover, the selected MMC was also able to efficiently grow with an HRT of 6 h, using a feed concentration of up to 35 g/L of glycerol (Fig 7b). However, when further increasing the feed concentration to 42 g/L, there was a cells loss (up to 30% of biomass) and only 18% of the substrate was converted (with a residual glycerol concentration of up to 34.26 g/L), thus suggesting the need for a fine control of operating parameters. Comparable results were obtained by Chatzifragkou and colleagues, who reached a maximum productivity of 45 g/L/d PDO (with an RT of 12,5 h), while finding non-negligible amounts of residual glycerol inside the chemostat [54].

Highest final concentrations of 1,3 PDO production (from non-GMO) reported in international literature are usually ranging between 30 and 80 g/L, using various strains (i.e. *Klebsiella pneumoniae*, *Clostridium butyricum*, etc.) and in some cases also mixed cultures, both in sterile and non-sterile experiments [54–57]. These results were typically obtained in batch/fed-batch conditions using vegetable oil derived glycerol. Furthermore, high PDO productivities were obtained in CSTR

experiments in sterile conditions, while butyric acid production was usually low. Papanikolaou and colleagues [39] for instance, reported a maximum PDO productivity of 130 g/L/d, using a *Clostridium butyricum* strain grown on industrial glycerol, while even higher productivities (from 105 g/L/d - 240 g/L/d) were obtained with pure glycerol [58,59].

It is worth noting that the use of different crude glycerol types as substrate will obviously lead to the presence of different contaminants. This can lead to growth-restrictive conditions for some microorganisms, depending on the origin of the feedstock [54] as well as transesterification process used to produce biodiesel. Content of unsaturated free-fatty acids (FFAs), i.e., were reported to have a noticeable negative effect on cell growth, requiring pretreatment with non-polar solvents to remove FFAs and fatty acids methyl esters (FAMEs) from crude glycerol, to allow microbial growth comparable to pure glycerol [57]. For this reason, some authors consider the results obtained with different raw glycerol stocks hardly comparable [60]. The crude glycerol utilized in the present study, for example, was obtained from the transesterification of butchery waste, which resulted to be a very challenging substrate. Non-adapted anaerobic sludge was quickly inhibited (while pure strain of *Clostridium pasteurianum* did not grow at all, unless hexane-pretreated glycerol was used [10]. On the other hand it would be highly desirable to valorize a residue coming from a 2G biodiesel, as recommended by the EU Renewable Energy Directive 2009/28/EC. Nonetheless, only extremely few studies investigated the use of this type of substrate so far [10,61,62]. For this reason it was considered of strategic importance to develop an adapted mixed culture able to grow on non-treated (2G) crude glycerol, in a stable fermentation process.

On the other hand, also the type of fermentation used can have a significant effect on the process performances. For industrial fermentation applications, for instance, fed-batch culture can be often preferred over batch or continuous culture, mainly owing to the higher product concentration and yield that can be achieved, as well as the flexibility of fermentation operation and reduced chance of

544 contamination (compared to continuous operations) [63]. This means that, while batch and fed-
545 batch processes (which last relatively short) can provide similar performance in sterile and non-
546 sterile conditions, it is much more challenging to keep such performances in non-sterile CSTR
547 processes (which are supposed to last much longer). In fact, the contamination risk of glycerol
548 degrading (non-extremophilic) bioprocess is known to increase as low-grade raw glycerol fraction,
549 more complex medium and/or continuous mode of fermentation are being used [60]. An example is
550 provided by the study of Chatzifragkou and colleagues [54], that tested the stability of a CSTR
551 process, using *Clostridium butyricum* under non sterile conditions: even though the system was able
552 to run at steady state for 16 days (corresponding to 23 retention times), a degeneration of the culture
553 was observed after 21 days, with biomass and PDO concentrations tending to decline, accordingly
554 with rise of residual glycerol inside the chemostat.
555 The present study confirmed the possibility to developing a stable and continuous conversion of a
556 highly inhibiting crude glycerol stream in non-sterile, MMC-based CSTR operated at a steady state
557 for up to 116 RT in the case of BA-12h (and 166 RT with MM-12h). Even though the results
558 achieved in the present work are lower than the highest ones reported in literature the process has
559 the potential to be optimized for higher productivities and products concentrations. Further research
560 could therefore target optimization of key parameters, in order to further enhance productivities and
561 substrate conversion rates, while avoiding washout of cells, when working with higher feed
562 concentrations coupled with low RTs.

563 ## 4. CONCLUSIONS

564 All reactors were able to reach steady state in the tested conditions with most of them exhibiting
565 highly efficient substrate degradation (98.29 - 100%). *Clostridium* represented the dominant genus,
566 however the different Mixed Microbial Cultures differed in terms of sub-dominant Operational
567 Taxonomic Units. 1,3 PDO was the main metabolite in steady state, followed by butyric acid

(which showed better results in non-steady state experiments). Further tests have shown that it was possible to grow the adapted MMC on animal fat derived crude glycerol with feed concentrations up to almost 90 g/L, with a substrate conversion of almost 50%. Maximum productivity was obtained with 60 g/L feed at 12h RT, and reached 37.8 g/L/d for 1,3 PDO and 11.14 g/L/d for butyric acid (corresponding to a 2.9-fold, 3.7-fold increase, compared to the initial results), together with a substrate degradation rate of 110.44 g/L/d, in non-sterile conditions.

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Figures Captions

Figure 1. Scheme of the reactor system used for CSTR experiments.

Figure 2. Percentages and distribution of the main fermentation products obtained during steady state, with enriched anaerobic sludge (a) and enriched activated sludge (b). Activated sludge was run at 12h RT. 1,3 PDO = 1,3 Propanediol; BuA = Butyric acid; PA = Proprionic acid; AA = Acetic acid; EtOH = Ethanol; SA = Succinic acid; VA = Valeric acid.

Figure 3. Production yield of the main metabolites recorded during steady state, using enriched anaerobic sludge (a) and enriched activated sludge (b). The latter was run at 12h RT. Yields < 0.02 g/g are not reported. 1,3 PDO = 1,3 Propanediol; BuA = Butyric acid; PA = Propionic acid; AA = Acetic acid.

Figure 4 Metagenomic classification of enriched anaerobic sludge (a) and enriched activate sludge (b) MMCs at different time intervals, represented at the Genus (left) and Order level (right). D0-D81 = day 0 – day 81 of operation (with D0 taken prior to inoculation).

Figure 5. Distribution of the main soluble metabolites (in %) measured at the same time interval of the metagenomics analysis, using anaerobic sludge (a) and activated sludge (b). Metabolites < 2% are not reported. D0-D81 = day 0 – day 81 of operation (with D0 taken after inoculation). 1,3 PDO = 1,3 Propanediol; BuA = Butyric acid; PA = Propionic acid; AA = Acetic acid; EtOH = Ethanol; SA = Succinic acid; VA = Valeric acid; LA = Lactic acid.

Figure 6. Principal component analysis for anaerobic sludge, taking into consideration the relative abundance of microbial genera and main metabolites. Samples were taken at the end of fermentation. Similar results were obtained also with activated sludge.

Figure 7. Distribution of main metabolites and substrate conversion, under increasing feed concentrations, at 12 RT (A) and 6 RT (B). Batch start-up was performed with 20 g/L glycerol concentration.

Tables Captions

Table 1. Substrate conversion and biomass obtained during steady state with different enriched inocula (standard deviation < 10%). Activated sludge was run at 12h RT.

Table 2. Consumption of substrate and production rates of the main metabolites (> 0.3 g/L/d) recorded during steady state, using different enriched inocula. Activated sludge MMCs (MM-KC, MM-EF, BA-KC and BA-EF) were run at 12h RT.

Table 3. Stoichiometric coefficients for the overall stoichiometric reactions for the CSTR operations at steady state.

Table 4. Stoichiometric coefficients for the overall stoichiometric reactions for the kinetic batch experiments.

Figures and Tables

Figure 1.

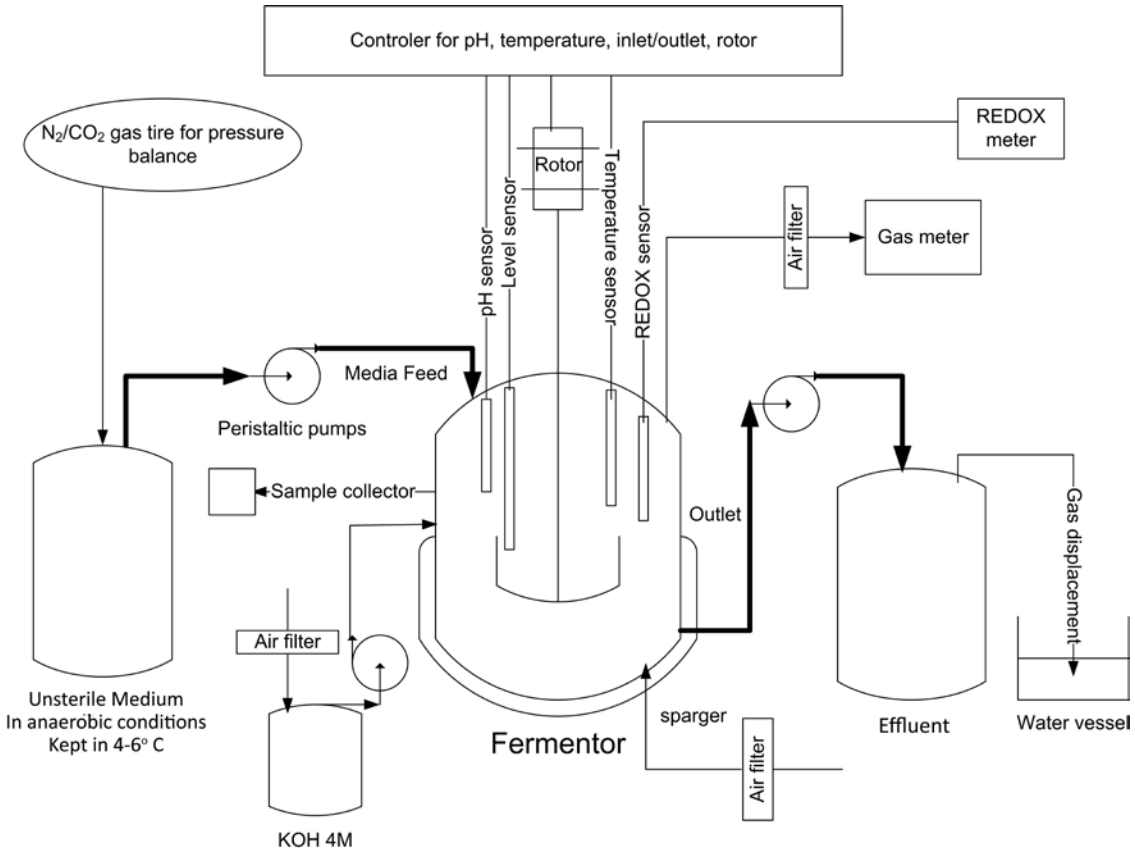


Figure 2.

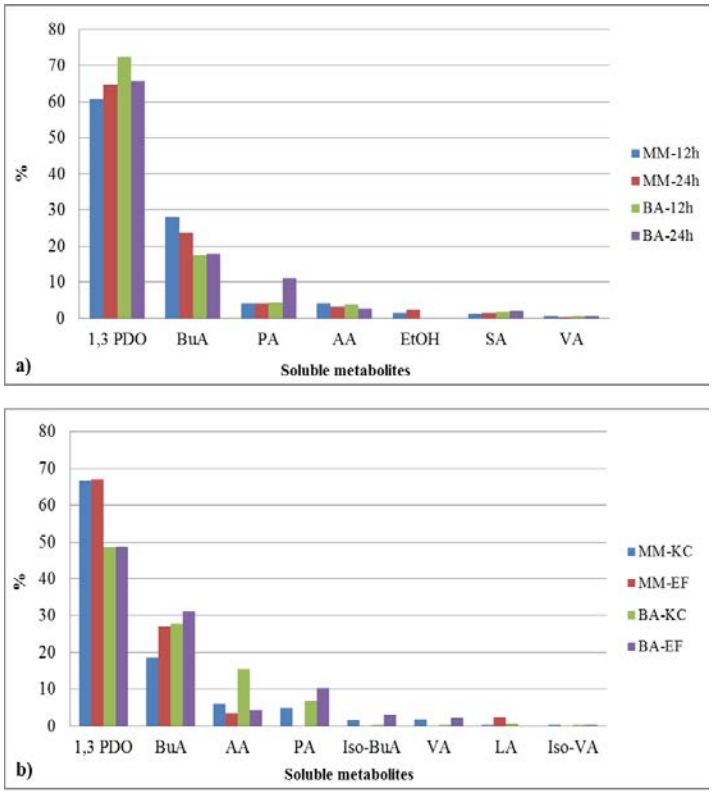
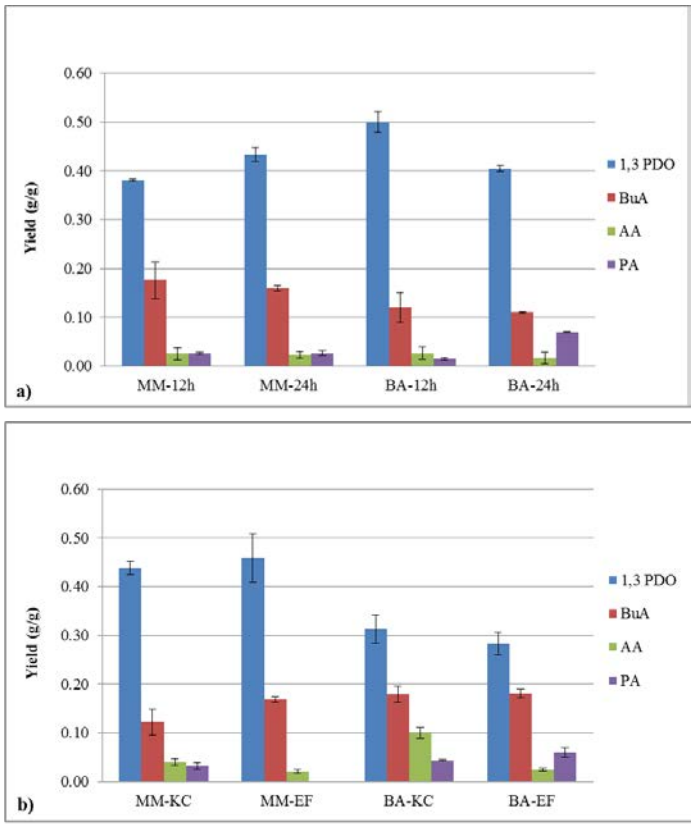

80
70
60
50
40
30
20
10
0
%
MM-12h
MM-24h
BA-12h
BA-24h
1,3 PDO
BuA
PA
AA
EtOH
SA
VA
a)
Soluble metabolites
MM-KC
MM-EF
BA-KC
BA-EF
Iso-BuA
LA
Iso-VA
b)

Figure 3


0.60
0.50
0.40
0.30
0.20
0.10
0.00
Yield (g/g)
1,3 PDO
BuA
AA
PA
MM-12h
MM-24h
BA-12h
BA-24h
a)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Yield (g/g)
1,3 PDO
BuA
AA
PA
MM-KC
MM-EF
BA-KC
BA-EF
b)

819 **Figure 4.**

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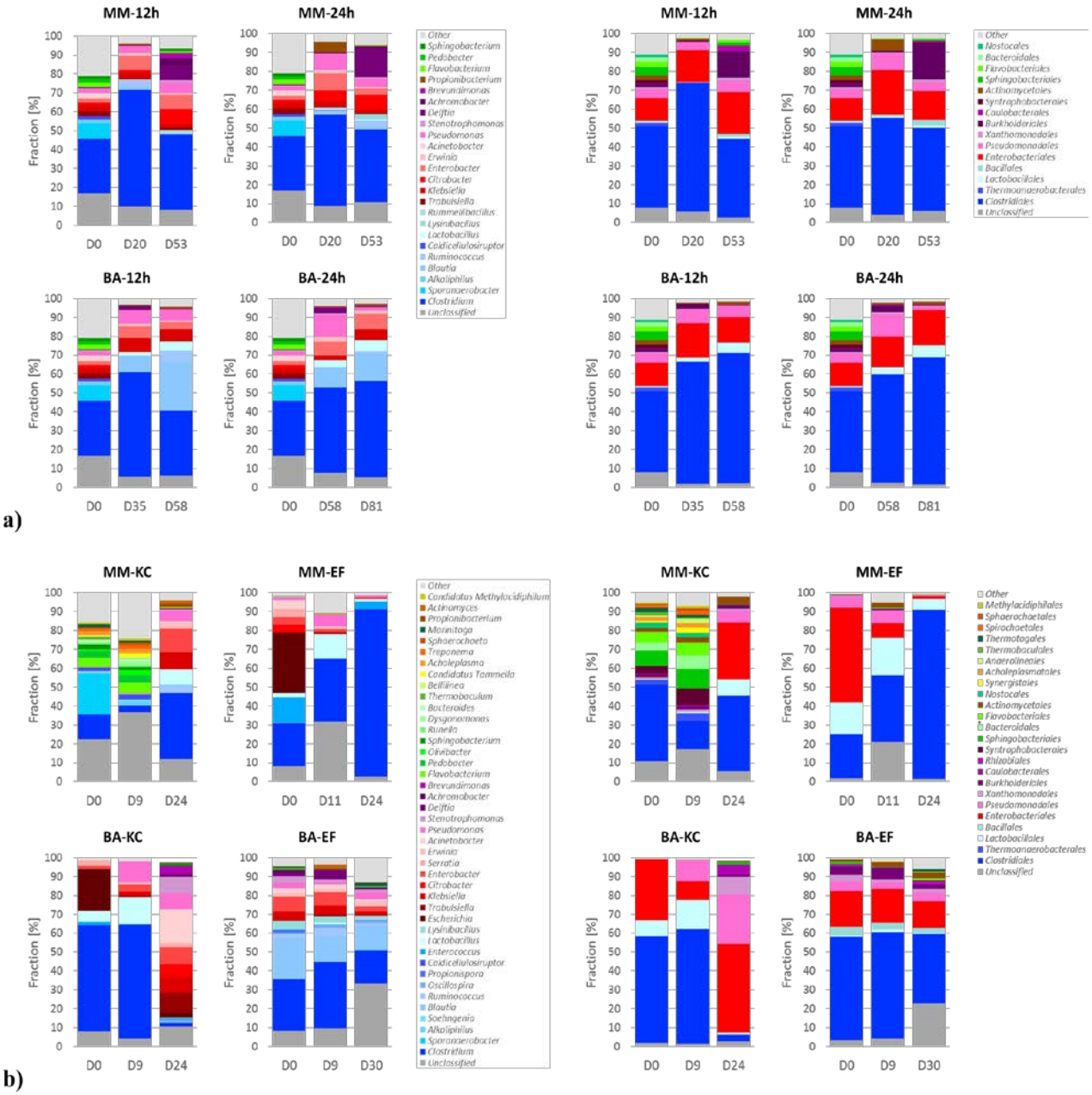

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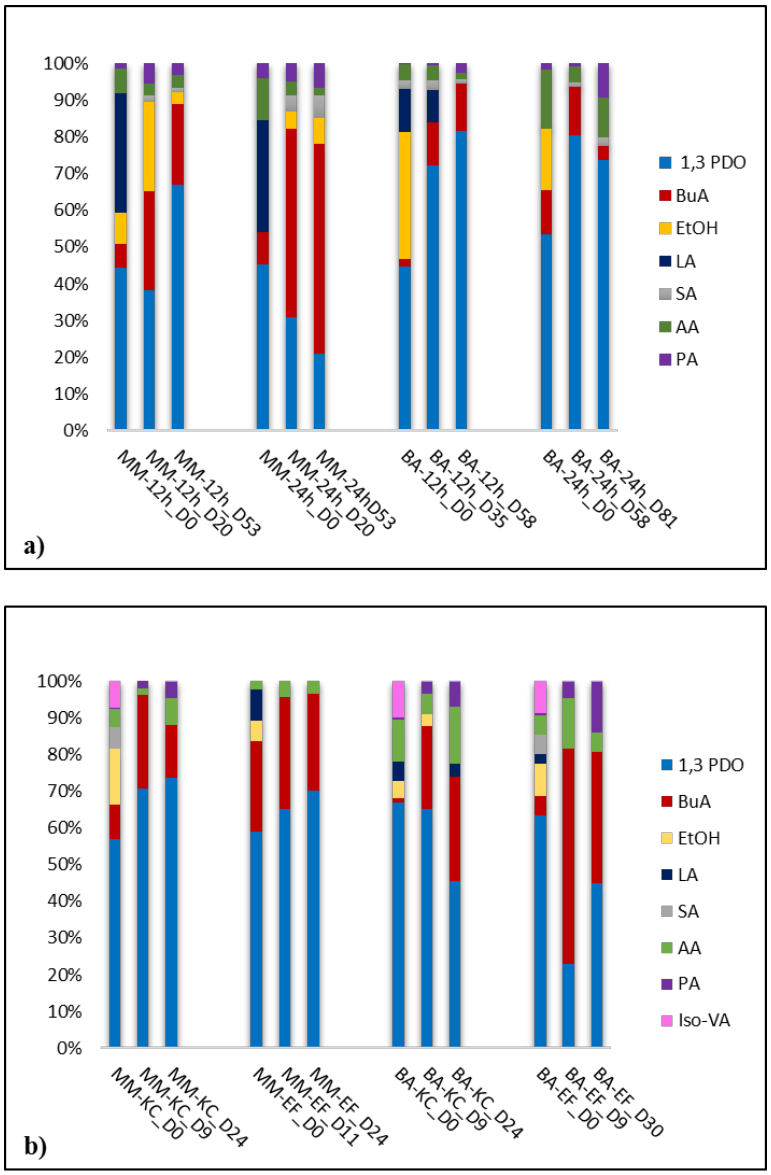
Figure 5.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1,3 PDO
BuA
EtOH
LA
SA
AA
PA
MM-12h_D0
MM-12h_D20
MM-12h_D53
MM-24h_D0
MM-24h_D20
MM-24hD53
BA-12h_D0
BA-12h_D35
BA-12h_D58
BA-24h_D0
BA-24h_D58
BA-24h_D81
a)
Iso-VA
MM-KC_D0
MM-KC_D9
MM-KC_D24
MM-EF_D0
MM-EF_D11
MM-EF_D24
BA-KC_D0
BA-KC_D9
BA-KC_D24
BA-EF_D0
BA-EF_D9
BA-EF_D30
b)

828 **Figure 6.**

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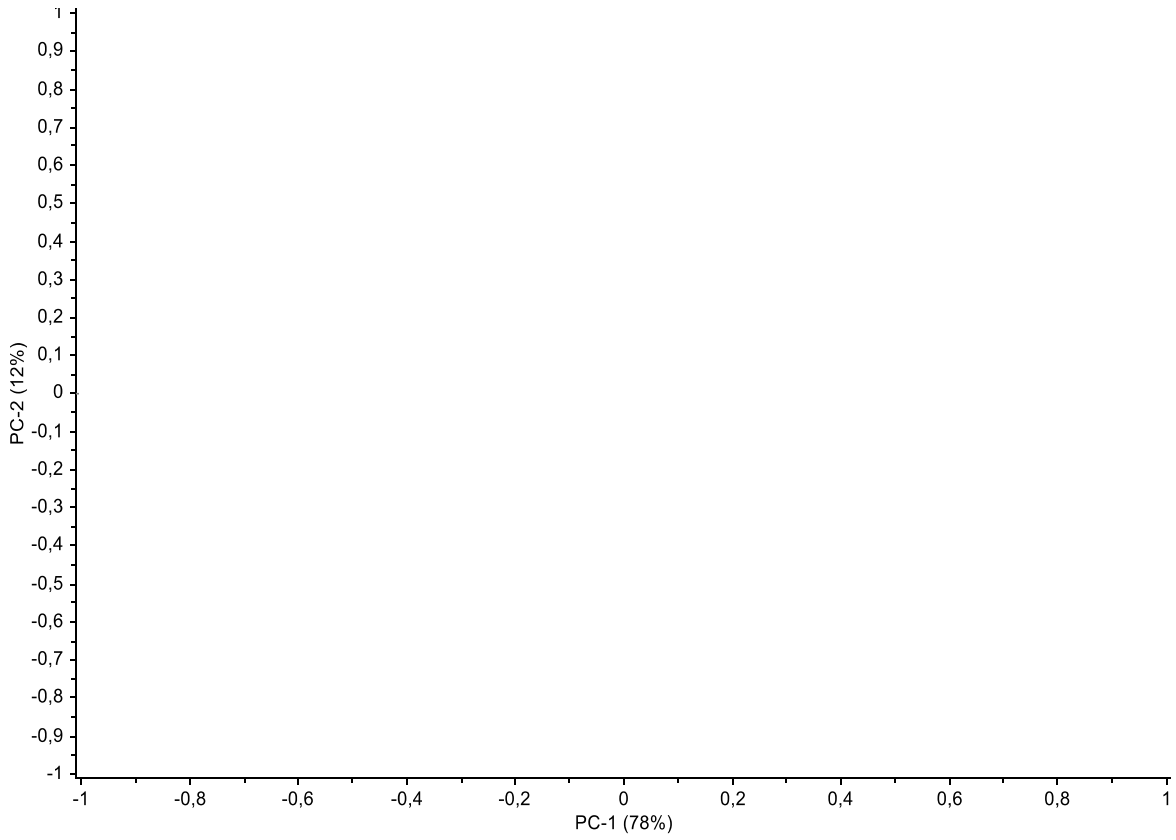

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832 **Figure 7.**

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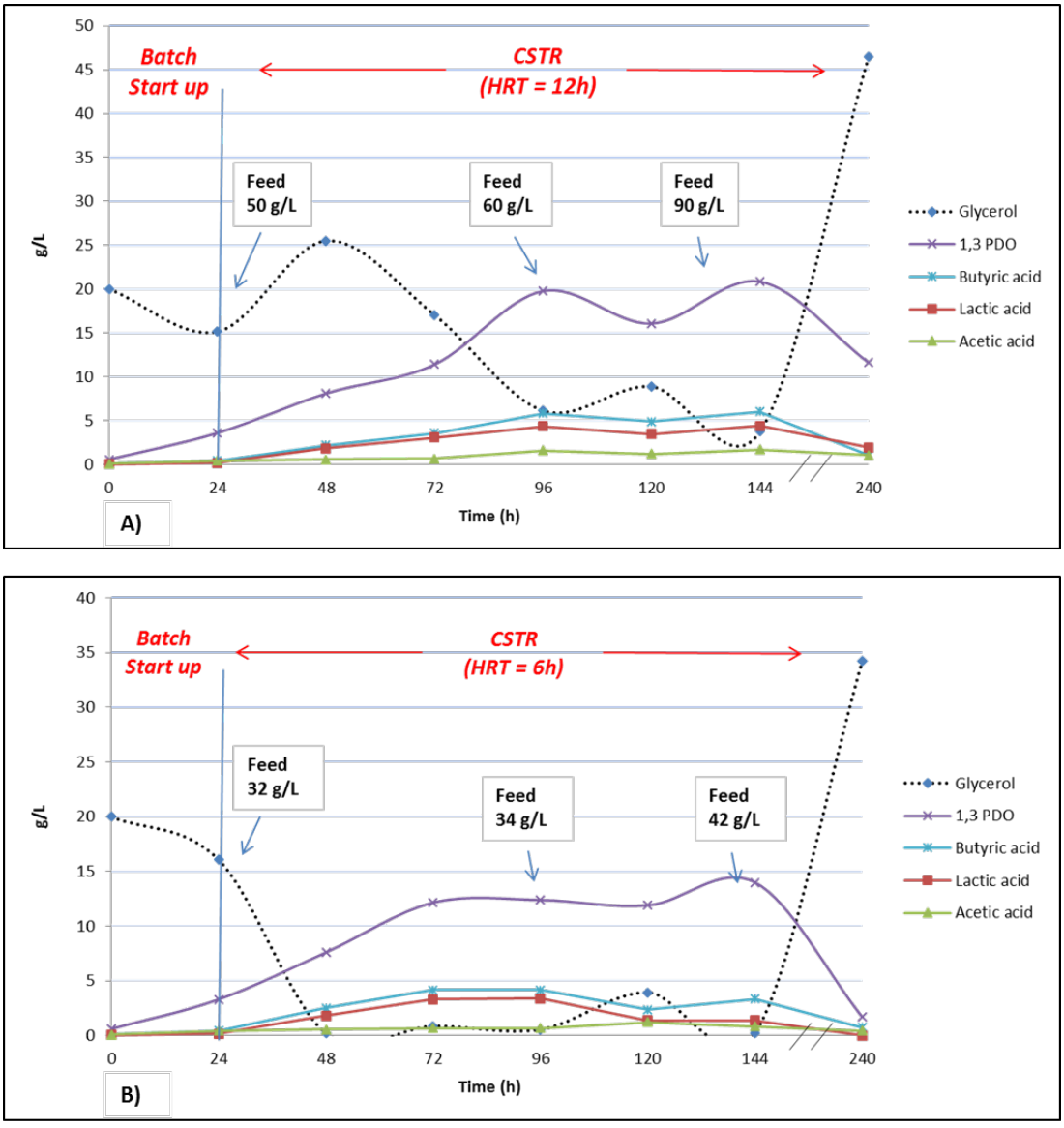

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836 **Table 1.**

Inoculum	**Gly cons.** g/L	**Residual Gly** g/L	**Substr. Degr.** %	**TSM** g/L	**TSM yield** %	**Biomass** g/L	**Y x/s** g/g
Anaerobic sludge							
MM-12h	12.83	0.05 ± 0.03	99.58	8.08	62.96	0.82 ± 0.10	0.064
MM-24h	12.51	0.21 ± 0.09	98.29	8.40	67.15	0.66 ± 0.06	0.053
BA-12h	12.88	0.00 ± 0.00	100.0	8.90	69.11	0.74 ± 0.09	0.057
BA-24h	12.76	0.12 ± 0.29	99.05	7.87	61.70	0.84 ± 0.36	0.066
Activated sludge							
MM-KC	10.13	0.14 ± 0.04	98.64	6.65	65.66	0.33 ± 0.04	0.033
MM-EF	6.46	3.66 ± 0.86	63.82	4.02	62.17	0.23 ± 0.03	0.036
BA-KC	10.39	0.01 ± 0.03	99.87	6.70	64.47	0.55 ± 0.02	0.053
BA-EF	10.60	0.08 ± 0.15	99.26	6.15	58.00	0.69 ± 0.06	0.065

837 Gly cons. = glycerol consumed; TSM = total soluble metabolites; Substr. Degr. = susbstrate degradation; Y x/s = biomass yield.

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Table 2.

Rates	*Anaerobic Sludge*				*Activated Sludge*			
	MM-12h	**MM-24h**	**BA-12h**	**BA-24h**	**MM-KC**	**MM-EF**	**BA-KC**	**BA-EF**
1,3 PDO *(g/L/d)*	9.78 ± 0.72	5.42 ± 0.51	12.89 ± 0.81	5.17 ± 0.37	8.88 ± 0.43	2.65 ± 0.25	6.50 ± 0.91	5.99 ± 0.65
Butyric acid *(g/L/d)*	4.51 ± 1.15	1.99 ± 0.28	3.09 ± 0.92	1.41 ± 0.16	2.48 ± 0.60	1.10 ± 0.16	3.72 ± 0.47	3.82 ± 0.30
Propionic acid *(g/L/d)*	0.66 ± 0.09	0.33 ± 0.20	0.74 ± 0.57	0.88 ± 0.17	0.80 ± 0.16	0.14 ± 0.04	2.06 ± 0.29	0.52 ± 0.08
Acetic acid *(g/L/d)*	0.65 ± 0.07	0.28 ± 0.08	0.67 ± 0.84	0.21 ± 0.03	0.65 ± 0.16	0.00 ± 0.00	0.90 ± 0.06	1.27 ± 0.25
Hydrogen *(mL/L/d)*	7.11 ± 5.40	0.26 ± 0.32	18.17 ± 12.95	6.97 ± 4.45	0.89 ± 0.11	594 ± 95.54	0.35 ± 0.03	51.15 ± 50.80
$\mathbf{R_{Gly}}$ (g/L/d)	20.28 ± 0.37	7.48 ± 0.69	20.78 ± 0.94	21.18 ± 0.58	25.66 ± 1.05	12.54 ± 1.37	25.77 ± 0.98	12.76 ± 1.29

RGly = Substrate degradation rate

Table 3.

per mol glycerol	Reactants				Products									Electron fractions		Rc
	Glycerol	**HCO_3^-**	**NH_4^+**	→	**H_2**	**BuA**	**AA**	**1,3 PDO**	**PA**	**EtOH**	**CO_2**	**H_2O**	**$C_5H_7O_2N$**	**f_e**	**f_s**	**%**
Anaerobic Sludge																
MM-12h	1.00	0.10	0.10		0.00	0.18	0.04	0.46	0.03	0.02	0.28	1.09	0.10	0.86	0.14	100.7
MM-24h	1.00	0.05	0.05		0.01	0.16	0.03	0.52	0.03	0.03	0.30	0.89	0.05	0.92	0.08	103.3
BA-12h	1.00	0.06	0.06		0.00	0.12	0.04	0.60	0.02	0.00	0.30	0.87	0.06	0.91	0.09	100.6
BA-24h	1.00	0.12	0.12		0.00	0.11	0.02	0.49	0.08	0.00	0.27	1.14	0.12	0.82	0.17	101.8
Activated Sludge																
MM-KC	1.00	0.07	0.07		0.00	0.13	0.06	0.53	0.04	-	0.27	0.96	0.07	0.90	0.10	102.4
MM-EF	1.00	0.10	0.10		0.00	0.17	0.03	0.55	0.00	-	0.42	0.62	0.10	0.91	0.09	100.0
BA-KC	1.00	0.12	0.12		0.00	0.19	0.15	0.38	0.05	-	0.20	1.15	0.12	0.84	0.15	99.4
BA-EF	1.00	0.13	0.13		0.02	0.19	0.04	0.34	0.07	-	1.29	0.25	0.13	0.81	0.19	100.5

Metabolites with values < 0.015 not reported. Activated sludge experiments were all run at 12 h RT. 1,3 PDO = 1,3 Propanediol; BuA = Butyric acid; PA = Propionic acid; AA = Acetic acid; EtOH = ethanol; C5H7O2N = empirical formula of biomass; Rc = Carbon recovery.

Table 4.

per mol glycerol	Reactants				Products									El. fractions		μ_{max}	Y x/s	Rc
	Glycerol	**HCO_3^-**	**NH_4^+**	→	**H_2**	**BuA**	**AA**	**1,3 PDO**	**LA**	**EtOH**	**$C_5H_7O_2N$**	**CO_2**	**H_2O**	**f_e**	**f_s**	(h^{-1})	(g/g)	%
Anaerobic Sludge																		
MM-12h *	1.00	0.09	0.09		0.03	0.22	0.01	0.37	0.02	0.100	0.09	0.32	1.09	0.86	0.13	0.08	0.06	96.1
MM-24h *	1.00	0.06	0.06		0.01	0.27	0.00	0.24	0.07	0.193	0.06	0.31	1.06	0.91	0.09	0.07	0.08	101.0
BA-12h	1.00	0.08	0.08		0.03	0.01	0.12	0.55	0.02	0.170	0.08	0.33	0.83	0.88	0.12	0.11	0.05	94.1
BA-24h	1.00	0.08	0.08		0.03	0.01	0.11	0.53	0.03	0.214	0.08	0.34	0.80	0.89	0.11	0.08	0.04	94.2
Activated Sludge																		
MM-KC *	1.00	0.13	0.13		0.55	0.08	0.01	0.33	0.03	0.238	0.13	0.56	0.82	0.82	0.18	0.06	0.04	100.0
MM-EF *	1.00	0.12	0.12		0.77	0.04	0.00	0.17	0.05	0.458	0.12	0.69	0.66	0.82	0.18	0.11	0.03	100.7
BA-KC	1.00	0.12	0.12		0.56	0.03	0.05	0.28	0.00	0.413	0.12	0.62	0.72	0.83	0.17	0.08	0.05	100.0
BA-EF *	1.00	0.09	0.09		0.26	0.05	0.15	0.54	0.04	0.072	0.09	0.36	0.78	0.87	0.13	0.17	0.05	100.5

* = incomplete substrate degradation; BuA = Butyric acid; AA = Acetic acid; 1,3 PDO = 1,3 Propanediol; LA = Lactic acid; EtOH = ethanol; ; $C_5H_7O_2N$ = empirical formula of biomass; Rc = Carbon recovery.

SUPPLEMENTARY MATERIALS

CONTINUOUS FERMENTATION AND KINETIC EXPERIMENTS FOR THE CONVERSION CRUDE GLYCEROL DERIVED FROM SECOND-GENERATION BIODIESEL INTO BUTYRATE AND 1,3 PROPANEDIOL

Cristiano Varrone [a*], Georgis Floriotis [a], Tonje M.B. Heggeset [b], Simone B. Le [2], Sidsel Markussen [b], Ioannis V. Skiadas [a], Hariklia N. Gavala [a]

[a] Technical University of Denmark, Department of Chemical and Biochemical Engineering, Lyngby, Denmark

[b] SINTEF, Materials and Chemistry, Biotechnology and Nanomedicine, Trondheim, Norway

Table S 1. Crude glycerol characteristics [8].

Content	Typical values
Raw Glycerine	75%
Fat	10%
Methanol	< 1%
Sulphur	1-2%
Moisture	10%
Ash	5%
Density	1,2-1,3 Kg/L
pH	1.5

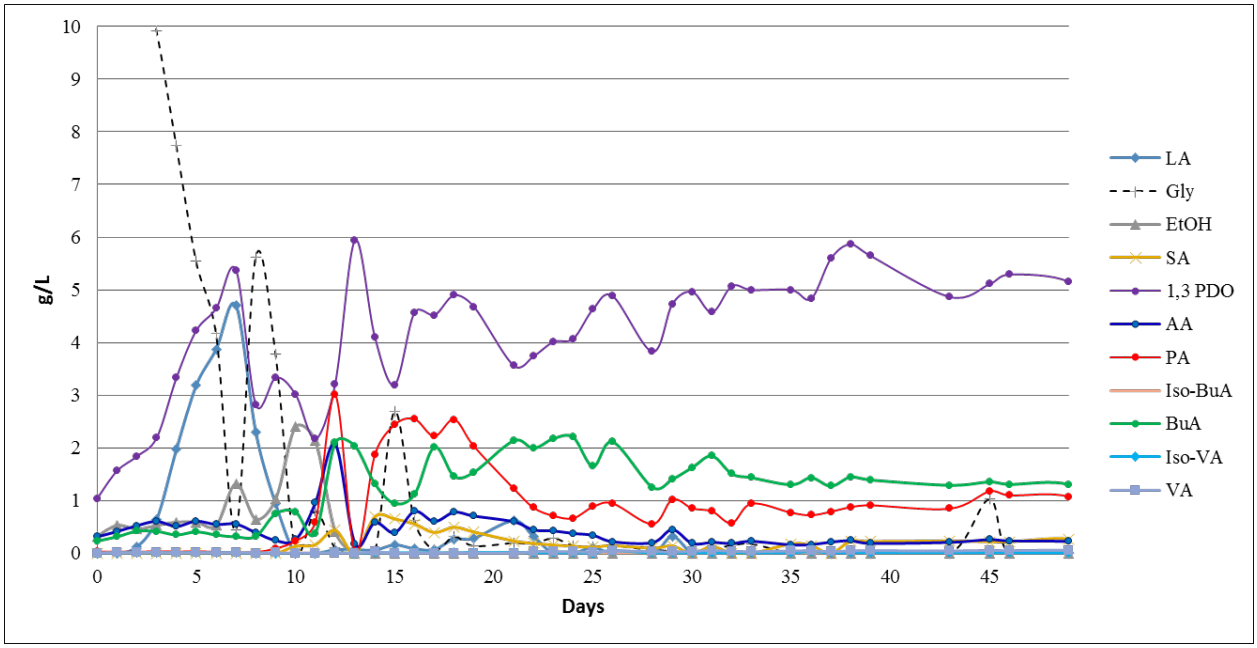

Figure S1. Typical distribution of main metabolites during CSTR experiments, using enriched anaerobic sludge with BA medium at HRT 24h (37 °C, pH 5.5).

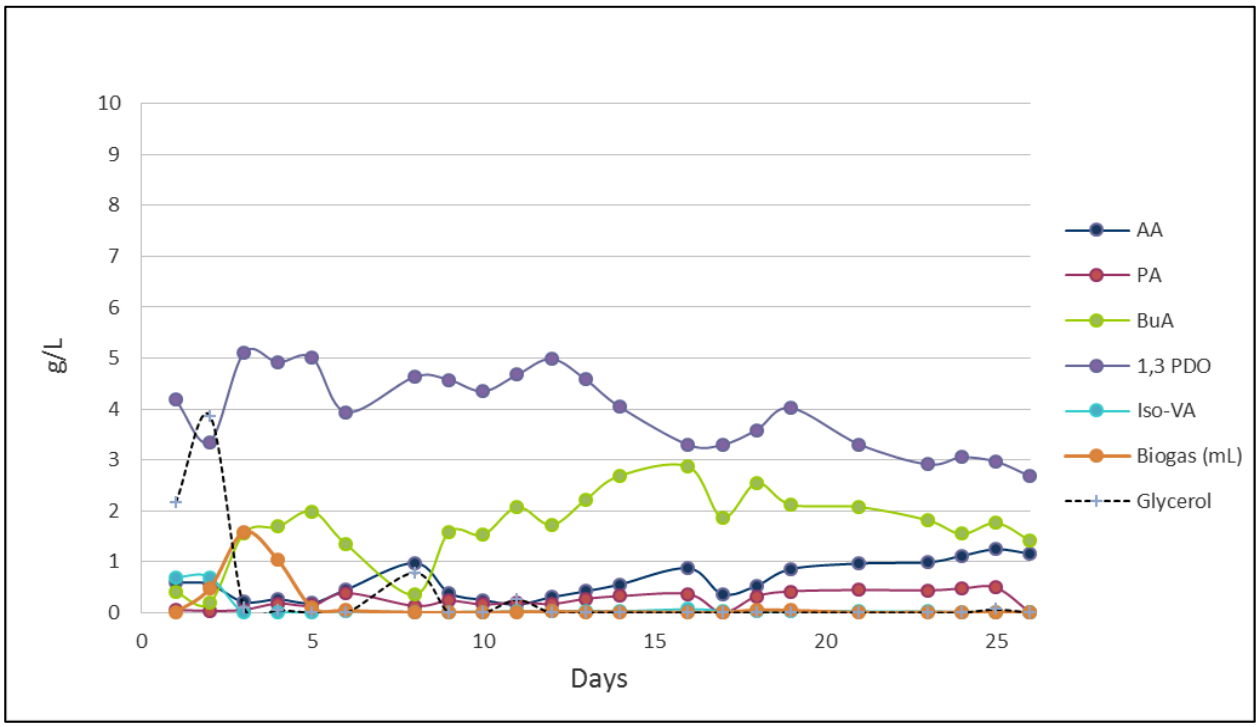

Figure S2. Distribution of main metabolites during CSTR experiments, using BA-KC (enriched activated sludge) at 12 h HRT (37 °C, pH 5.5).

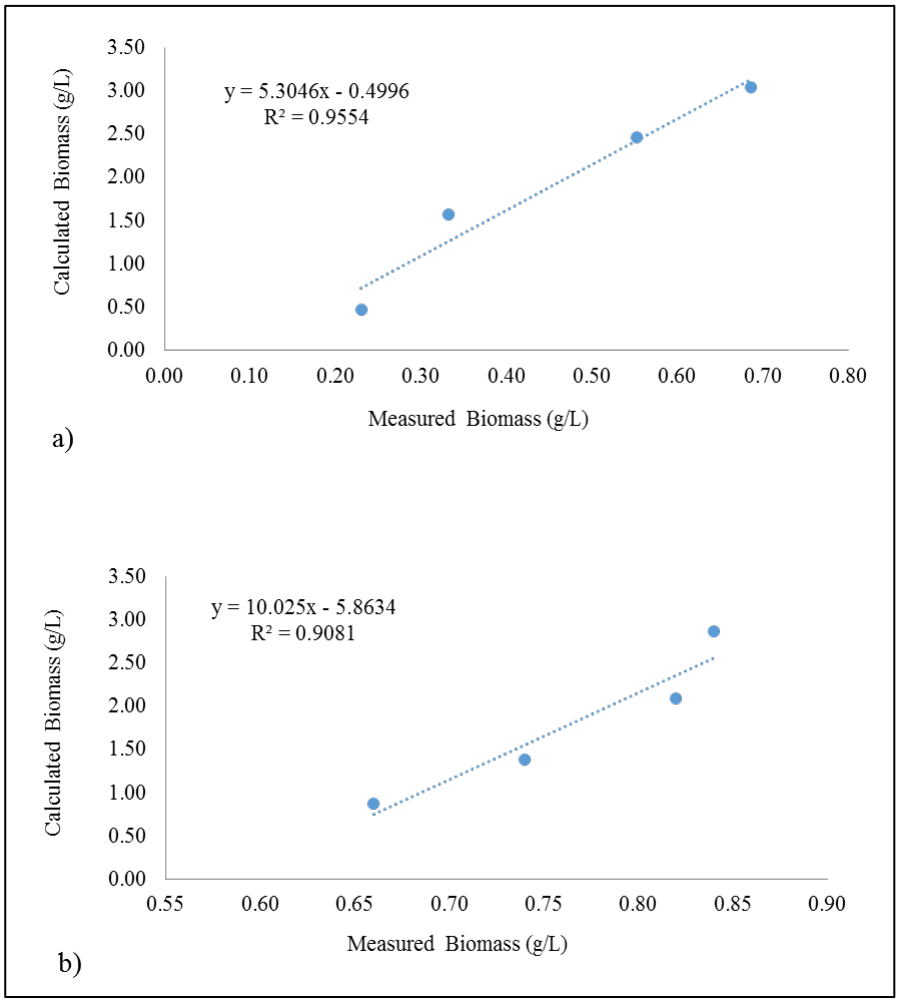

Figure S3. Correlation between measured and calculated biomass (g/L) of steady state CSTR experiments, with activated sludge (a) and anaerobic sludge experiments (b).

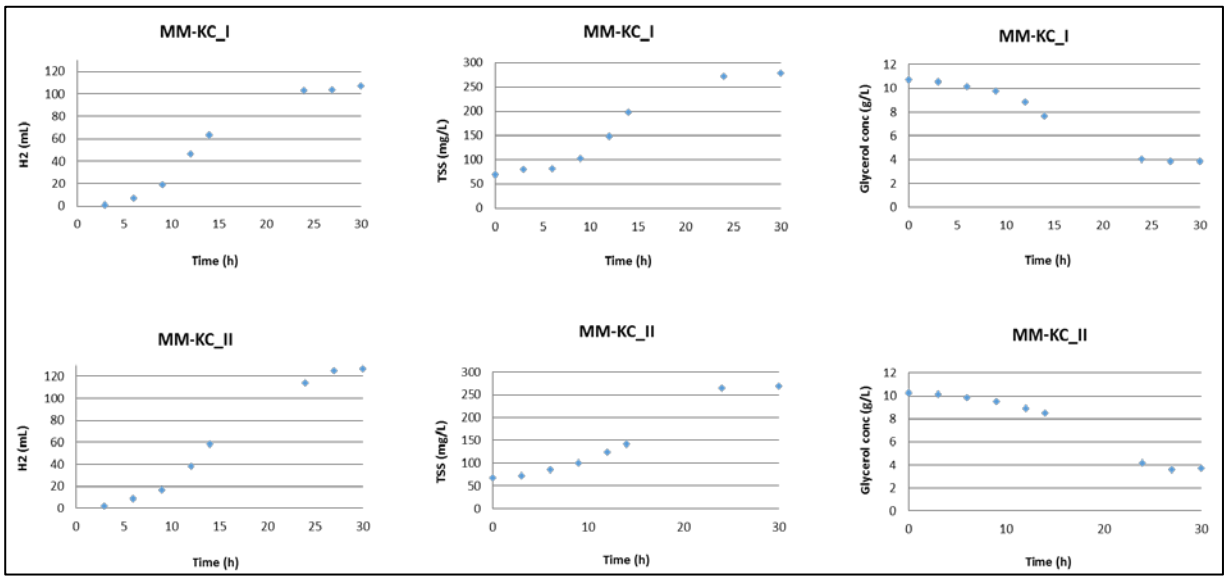

Figure S4. Example of kinetic experiments (here represented by MM-KC), showing (from left to right) the cumulative hydrogen production, microbial growth curve (as TSS) and substrate degradation, in two replicates (I and II).

Table S 2. Carbon balance of the CSTR experiments.

	Activated Sludge				*Anaerobic Sludge*			
Carbon balance	**MM-KC**	**MM-EF**	**BA-KC**	**BA-EF**	**MM-12h**	**MM-24h**	**BA-12h**	**BA-24h**
	Cmol	Cmol	Cmol	Cmol	Cmol	Cmol	Cmol	Cmol
Acetic acid	0.0266	0.0047	0.0687	0.0165	0.0218	0.0112	0.0223	0.0070
Butyric acid	0.1126	0.0504	0.1691	0.1666	0.2049	0.1100	0.1404	0.0640
Ethanol	0.0000	0.0000	0.0000	0.0133	0.0101	0.0113	0.0000	0.0000
Butanol	0.0000	0.0000	0.0486	0.0453	0.0000	0.0000	0.0000	0.0000
1.3 PDO	0.3499	0.1085	0.2563	0.2270	0.3856	0.2607	0.5084	0.2037
Lactic acid	0.0000	0.0033	0.0030	0.0133	0.0000	0.0000	0.0000	0.0000
Succinic acid	0.0000	0.0000	0.0000	0.0000	0.0011	0.0136	0.0049	0.0053
Propionic scid	0.0262	0.0000	0.0000	0.0000	0.0269	0.0165	0.0151	0.0270
Valerate acid	0.0116	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Isobutyric acid	0.0099	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C5H7O2N	0.0779	0.0235	0.1224	0.1505	0.1365	0.0472	0.0857	0.0846
CO2	0.0607	0.0245	0.0046	0.0341	0.0551	0.0428	0.0676	0.0316
Sum	0.6755	0.2149	0.6726	0.6667	0.8420	0.5133	0.8444	0.4232
Gly consumpt.	0.6599	0.2150	0.6769	0.6636	0.8359	0.4969	0.8394	0.4158
Rc (%)	102.4	100.0	99.4	100.5	100.7	103.3	100.6	101.8

C5H7O2N = biomass; Gly consumpt. = Glycerol consumption; Rc = carbon recovery.